Characterisation of neurodevelopmental disorder and ageing phenotypes in transgenic mouse and fruit fly models of *Neurexin1* dysfunction

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I declare that this thesis is my own work and has not been submitted in substantially the same form for a higher degree elsewhere.

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Abstract

Heterozygous (Hz) deletions in *Neurexin1 (NRXN1)*, which encodes a presynaptic cell adhesion protein, increase the risk of developing Schizophrenia (Sz) and Autistic Spectrum Disorder (ASD). In addition, there is accumulating evidence for the accelerated ageing hypothesis of Sz. Using a transgenic mouse model of *Neurexin1α* heterozygosity (*Nrxn1α* Hz mice) and a fly model overexpressing *Neurexin1 (Nrx-1)*, the current study aimed to investigate: (1) whether *Nrxn1α* heterozygosity causes Sz or ASD relevant phenotypes (behaviour and cognition) and endophenotypes (brain function) in mice, and whether these changes are temporally regulated across adult development, and (2) whether *Nrx-1* dysfunction affects life span and behavioural senescence using *Drosophila melanogaster* as a model organism.

It was found that *Nrxn1α* Hz mice show hyperlocomotor activity and increased anxiety-like behaviour in the Open Field (OF) test, deficits in recognition memory in the Novel Object Recognition Task (NORT), and defective reversal learning in a Cognitive Flexibility Task (CFT). In parallel, *Nrxn1α* Hz mice also show decreased cerebral metabolism in the frontal lobe and increased metabolism in dopaminergic and serotonergic system brain regions, as measured by ^{14}C-2-Deoxyglucose (^{14}C-2DG) functional brain imaging. Furthermore a selection of phenotypes and endophenotypes seen in *Nrxn1α* Hz mice are differentially regulated across adult development, including enhanced associative learning and increased cerebral metabolism in the Dorsal Raphé.

In *Drosophila melanogaster, Nrx-1* overexpression (in P{XP}Nrx^{d08766} flies) induced a small but significant life span extension and significant reductions in behavioural senescence as

measured by the Exploratory Walking (EW) assay. Furthermore P{XP}Nrx^{d08766} flies showed sex-dependant alterations in locomotor activity and sleep structure.

In conclusion, *Nrxn1α* heterozygosity in mice caused phenotypes and endophenotypes that are relevant to both Sz and ASD, including deficits in cognitive flexibility and hypofrontality. Some of these measures were differentially regulated across adult development. In addition, increased expression of *Nrx-1* reduced measures of ageing in the fly. Overall these data suggest that *Nrxn1α* Hz mice provide a useful translational model for drug discovery. In addition, some effects of *Neurexin* may be mediatiated through the modification of ageing processes, although further research is required to confirm this.

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Abbreviations

^{14}C-2-DG	^{14}C-2-Deoxyglucose
^{18}F-FDG	^{18}F-Fludeoxyglucose
ACC	Anterior Cingulate Cortex
ADHD	Attentional Deficit Hyperactivity Disorder
aPrl	anterior Prelimbic Cortex
ASD	Autistic Spectrum Disorder
BD	Bipolar Disorder
BPM	Behavioural Pattern Monitor
BTBR $T^{+}Itpr3^{tf}/J$	Black and Tan Brachyury, brachyury, inositol 1,4,5-triphosphate receptor 3; tufted
CACNA1C	*Calcium voltage-gated subunit alpha 1C*
CFT	Cognitive Flexibility Task
CNS	Central Nervous System
COS	Childhood Onset Schizophrenia
DISC1	*Disrupted In Schizophrenia 1*
DLO	Dorsolateral Orbital cortex
DLPFC	Dorsolateral Prefrontal Cortex
DLST	Dorsolateral Striatum
DNA	Deoxyribonucleic
DR	Dorsal Raphe
EDS	Extra-dimensional shift
EntoC	Entorhinal Cortex
EPM	Elevated Plus Maze
EW	Exploratory Walking
FDR	False Discovery Rate
FEP	First Episode Psychosis
Fmr1	*Fragile X Mental Retardation 1*
fMRI	functional Magnetic Resonance Imaging
FRA	Frontal Association Area
GRIN2A	*Glutamate ionotropic receptor NMDA type 2A*
GRINB	*Glutamate ionotropic receptor NMDA type 2B*
HDB	nucleus of the horizontal limb of the Diagonal Band of Broca
Hz	Heterozygous
IL	Infralimbic cortex
Ins	Insular cortex
KO	Knock out
LCGU	Local Cerebral Glucose Utilisation
LS	Lateral Septal Nucleus
MAM	Methylazoxymethanol acetate

MG	Medial Geniculate nucleus
mPrL	medial Prelimbic Cortex
MRI	Magnetic Resonance Imaging
MS	Medial Septal Nucleus
NaC	Nucleus Accumbens Core
NMDA	*N-methyl-D-aspartate*
NORT	Novel Object Recognition Task
Nrx-1	*Neurexin* (fly)
NRXN	*Neurexin* (human)
Nrxn	*Neurexin* (mouse)
NRXN1α	*Neurexin1α (human)*
Nrxn1α	*Neurexin1α (*mouse*)*
OD1	Odour Discrimination 1
OD2	Odour Discrimination 2
OD2R	Odour Discrimination 2 Reversal
OF	Open Field
OFC	Orbital Frontal Cortex
PC	Percentage Correct
PCP	Phencyclidine
PET	Positron-emission tomography
PFC	Prefrontal Cortex
Piri	Piriform cortex
RI	Recognition Index
RNA	Ribonucleic acid
RSC	Retrosplenial Cortex
Rt	Reticular thalamus
SHANK3	*SH3 and multiple ankyrin repeat domains 3*
SS	Stimunli Shift
Sz	Schizophrenia
TSC1	*Tuberous Sclerosis 1*
TTC	Trials to Criterion
UHR	Ultra High Risk of psychosis
VDB	nucleus of the vertical limb of the Diagonal Band of Broca
VL	Ventrolateral Thamalmus
VM	Ventromedial Thalamus
VTA	Ventral Tegmental Area
WCST	Wisconsin Card Sorting Task
Wt	Wild-type

Chapter 1 Introduction

1.1 Schizophrenia: Symptoms, disease progression, treatment and comorbidities

Symptoms

Schizophrenia (Sz) is a heterogeneous psychiatric disorder which can display symptoms of 3 main classes; positive, negative and cognitive symptoms (Van Os and Kapur, 2009). The positive, or psychotic, symptoms include hallucinations and delusions. The negative symptoms involve loss of a function such as lack of enthusiasm, lack of motivation and catatonia. Finally, the cognitive symptoms include deficits in working memory, reduced cognitive flexibility and a slower cognitive processing speed.

Disease progression

The age of onset for Sz symptoms tends be in the early 20's. However there are cases of Childhood Onset Schizophrenia (COS) (Russell, 1994; Spencer and Campbell, 1994; Bailly and De Chouly De Lenclave, 2004; Okkels *et al.*, 2013) and late onset, after the age of 40 years old (Howard *et al.*, 2000). It was previously thought that males had pronounced earlier age of onset of Sz symptoms compared to females (Hafner *et al.*, 1993), however more recently this age gap has be shown to be relatively small (around 1 year) (Naqvi *et al.*, 2005; Naqvi *et al.*, 2010; Eranti *et al.*, 2013). A growing body of evidence supports a prodromal phase which precedes the presentation of a full diagnosis of Sz. Patients in this 'Ultra High Risk' of psychosis (UHR) group present with symptoms that are sub-threshold for a full Sz diagnosis and around one third of them go on to develop Sz (Miller *et al.*, 2002; Yung *et al.*, 2003).

After first diagnosis of a psychotic episode (First Episode Psychosis (FEP)) around 25% of patients have symptoms that worsen over time, becoming a chronic disorder with multiple relapses of psychosis. By contrast around 25% have a good outcome at 1 year follow up (Van Os and Kapur, 2009).

Treatment

Current antipsychotic drugs only treat positive symptoms of Sz, and are not effective for every patient (Gardner and Bostwick, 2012). Antipsychotic drugs are based on the classic dopamine hypothesis of Sz and are categorised into typical and atypical classes. The typical antipsychotics (Chlorpromazine, Haloperidol, Pimozide) are D_2 receptor antagonists which have extra pyramidal (akinesia, akathisia and tardive dyskinesia) (Meltzer, 2013) and other side effects, such as hyperprolactinemia which causes galactorrhoea in both males and females (Bushe *et al.*, 2008). Such serious side effects led to the development of newer atypical antipsychotics (Clozapine, Olanzapine, Risperidone), which also have affinity at the D_2 receptor (Kapur and Mamo, 2003), but have additional therapeutic mechanisms including $5HT_{2A}$ and D_4 receptor antagonism (Meltzer, 2013). Unlike the typical antipsychotics, the atypicals produce less extra pyramidal side effects. However they are associated with increased risk of metabolic side effects including diabetes and weight gain, and agranulosis (in the case of Clozapine) (Meltzer, 2013). Furthermore, both classes also cause sedation, interference with sex life and problems with blood pressure (Muench and Hamer, 2010).

All symptom domains of Sz impact on quality of life in patients (Savilla *et al.*, 2008; Tomotake, 2011; Kitchen *et al.*, 2012; Kurtz *et al.*, 2012; Ojeda, Sanchez*, et al.*, 2012; Millier *et al.*, 2014). Although current antipsychotics can treat the positive symptoms, their side effects are severe which can lead to decreased quality of life for patients and increased non-

compliance in taking the medication (Dibonaventura *et al.*, 2012). Therefore there is a clear need for more research to understand the mechanisms underlying Sz, enabling new treatments to be developed in order to improve outcome for patients.

Co-morbidities

Sz patients often have co-morbidities including sleep disturbances, diabetes, obesity and metabolic syndrome. Sleep disturbances occur in 30-80% of Sz patients depending on symptom severity (Tandon *et al.*, 1992; Anderson and Bradley, 2013; Klingaman *et al.*, 2015), and include problems falling asleep (sleep onset latency), maintaining sleep (reduction of total sleep) and wakening frequently after falling asleep (sleep fragmentation) (Monti and Monti, 2004; 2005; Monti *et al.*, 2013; Chan *et al.*, 2016; Monti *et al.*, 2016). Research suggests that sleep disturbances are not dependent on antipsychotic medication or disease stage, since both antipsychotic medication naïve and treated patients, UHR patients, FEP patients and chronic Sz patients experience sleep disturbances (Monti *et al.*, 2013; Chan *et al.*, 2016; Monti *et al.*, 2016). In fact, some antipsychotic medications have been found to improve aspects of sleep, whereas others have been found to exacerbate sleep disruption in patients (Monti *et al.*, 2013; Chan *et al.*, 2016; Monti *et al.*, 2016). Circadian rhythms are also thought to be disrupted in Sz patients, which likely contribute to problems in the sleep-wake cycle (Monti *et al.*, 2013). Many of the neurotransmitter systems that are disrupted in Sz such as dopamine, glutamate and serotonin, are also involved in circadian sleep-wake cycle, which may contribute to sleep symptoms of Sz (Monti and Monti, 2005; Monti *et al.*, 2013). Furthermore, the interplay between dopamine dysregulation and sleep disruption may contribute to the manifestation of psychotic symptoms (Yates, 2016).

More recently there has been a growing concern about the general health of Sz patients who are often obese and are more likely to suffer from cardiovascular (Brink *et al.*, 2017; Correll *et al.*, 2017; Westman *et al.*, 2018) and metabolic health issues (Godin *et al.*, 2015; Kucerova *et al.*, 2015; Nyboe *et al.*, 2015), including diabetes (Bai *et al.*, 2013). This may be due to shared genetic risk factors for Sz and metabolic disease (Foley *et al.*, 2015), the contribution of sleep disturbances (Iftikhar *et al.*, 2015), unhealthy life style (De Leon and Diaz, 2005) or the use of antipsychotic medication (Ma *et al.*, 2014; Wani *et al.*, 2015), rather than the diagnosis itself. However changes in blood glucose and insulin resistance can be seen in FEP patients prior to administration of antipsychotic medication (Greenhalgh *et al.*, 2017).

Sz patients have a severely reduced life span (approximately 20 years less than the general population) compared to people who don't have a Sz diagnosis (Laursen *et al.*, 2012; Laursen *et al.*, 2014; Lee *et al.*, 2018). This is probably caused by a number of interlinking health issues including metabolic illness (Godin *et al.*, 2015), diabetes, poor diet, lack of exercise, smoking, excessive alcohol consumption (Laursen *et al.*, 2012), administration of antipsychotics (Kiviniemi *et al.*, 2013) and inflammation (Miller *et al.*, 2014). There is also emerging evidence for accelerated ageing in Sz (reviewed in section 1.13) which could contribute to reduced life span, however more research is needed in this area before any conclusions can be drawn.

1.2 Autistic Spectrum Disorder: Symptoms and Co-morbidities

Autistic Spectrum Disorder (ASD) incorporates a set of neurodevelopmental disorders with a spectrum of symptom severity which can range from mild to severe. Thus there are high

functioning individuals with ASD such as those with Asperger's syndrome (Tarazi *et al.*, 2015), and those with more severe symptoms resulting in low functioning, for instance Autism in individuals with Fragile X Syndrome (Niu *et al.*, 2017). There are similarities and differences between Sz and ASD. Like Sz, ASD is a heterogeneous developmental disorder. However unlike Sz, the prevalence of ASD is 4 times more common in males than females, and the age of symptom onset is much younger, at 1-3 years old. The core symptoms of ASD include social deficits such as problems with communication, social interaction and forming relationships. Other core symptoms include stereotyped or repetitive behaviours, and restricted interests and hobbies (First, 2013). In addition to the core symptoms of ASD, such as deficits in social cognition (Senju, 2013), ASD patients also have cognitive deficits that are shared with Sz. These include slowed cognitive processing speed (Oliveras-Rentas *et al.*, 2012; Hedvall *et al.*, 2013), deficits in working memory (Andersen *et al.*, 2015) and deficits in cognitive flexibility (Westwood *et al.*, 2016).

ASD has a number comorbidities, many of which have also been found in Sz, including sleep disturbances (Souders *et al.*, 2009; Baker and Richdale, 2015), obesity (Broder-Fingert *et al.*, 2014; Hill, A. P. *et al.*, 2015), epilepsy (Tuchman *et al.*, 2010; Maski *et al.*, 2011; Tuchman and Cuccaro, 2011), anxiety disorder and depression (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012). The incidence of sleep disturbance in ASD children is 25-55% higher than in typically developing children (Couturier *et al.*, 2005; Richdale and Schreck, 2009; Souders *et al.*, 2009; Malow *et al.*, 2016; Mazzone *et al.*, 2018), and the types of sleep disturbances found in ASD include insomnia and problems with sleep maintenance, in both children (Souders *et al.*, 2009) and adults (Baker and Richdale, 2015). Epilepsy is another comorbidity of ASD (Tuchman *et al.*, 2010;

Maski *et al.*, 2011; Tuchman and Cuccaro, 2011) that has also been found to be increased in Sz (Hyde and Weinberger, 1997; Cascella *et al.*, 2009; Hufner *et al.*, 2015), and anxiety disorder and depression are commonly found in both ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012) and Sz (Siris, 2000; Tibbo *et al.*, 2003; Braga *et al.*, 2004; Braga *et al.*, 2013; Young *et al.*, 2013; Temmingh and Stein, 2015; An Der Heiden *et al.*, 2016; Upthegrove *et al.*, 2017). By contrast, Attention Deficit Hyperactivity Disorder (ADHD) is prevalent in ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Mahajan *et al.*, 2012) but is not commonly associated with Sz. Patients with ASD also have a shortened life expectancy (Shavelle and Strauss, 1998; Shavelle *et al.*, 2001; Pickett *et al.*, 2006) of around 3 years from the age of 65 years old, however compared to Sz patients who have a shorted life span of 20 years, this is not as severe. There is currently no prescribed treatment for ASD, thus more research is needed to understand the disorder and develop treatments.

1.3 Risk factors for Sz and ASD

The exact cause of Sz and ASD is unknown, however a number of genetic and environmental risk factors have been highlighted, some of which are common to both disorders. There is a strong genetic component for both Sz and ASD, since the heritability is over 80% in both disorders (Cardno *et al.*, 1999; Taniai *et al.*, 2008).

Environmental risk factors

Environmental risk factors that have been found to increase the risk of Sz include birth complications, childhood trauma, urban living (Brown, 2011), migration (Cantor-Graae and Selten, 2005), famine (Susser and Lin, 1992; St Clair *et al.*, 2005) and cannabis use (Gage *et*

al., 2016). Environmental risk factors that increase the risk of developing ASD include increased maternal age (Sandin *et al.*, 2012), maternal migration (Dealberto, 2011), and prenatal exposure to antidepressants (Rai *et al.*, 2013), valproic acid (Christensen *et al.*, 2013) and other chemicals (Roberts *et al.*, 2007; Volk *et al.*, 2013).

Shared environmental risk factors for Sz and ASD include increased paternal age at birth (Gardener *et al.*, 2009; Hultman *et al.*, 2010; Miller *et al.*, 2011; Lampi *et al.*, 2013), and some prenatal complications including prenatal infection (Atladóttir *et al.*, 2010; Brown, 2011), gestational diabetes and maternal bleeding (Cannon *et al.*, 2002; Gardener *et al.*, 2009).

Genetic risk factors

In addition to environmental risk factors there are also genetic risk factors for Sz and ASD, some of which are again shared by both disorders. Genetic factors contributing to these disorders range from common low penetrance mutations, such as Single Nucleotide Polymorphisms (SNPs), or rare mutations in specific genes with high penetrance. Both rare and common variants in multiple genes have been identified as risk factors for the development of both ASD and Sz, supporting a shared genetic aetiology between these disorders. Examples of rare variants that are common to both disorders include mutations in *Disrupted in Schizophrenia 1 (DISC1)* (Song *et al.*, 2008; Zheng *et al.*, 2011), *SH3 and multiple ankyrin repeat domains 3* (*SHANK3*) (Gauthier *et al.*, 2010; Leblond *et al.*, 2014), *Glutamate ionotropic receptor NMDA type subunit 2A* and *2B* (*GRIN2A* and *GRIN2B*) (Tarabeux *et al.*, 2011) and *Calcium voltage-gated subunit alpha 1C* (*CACNA1C*) (Psychiatric Consortium, 2014; Li, J. *et al.*, 2015).

Examining Copy Number Variations (CNVs) has also identified chromosomal regions that confer risk for both disorders. CNVs are chromosomal sequences that are either repeated (duplicated) or deleted. CNVs can occur in Low Copy Repeat (LCR) regions, which are repeating sequences in the genome that have occurred through evolution (Stankiewicz and Lupski, 2002). CNVs can occur in this way during genetic recombination between LCRs that are homologous or adjacent to each other, through a process called non-allelic homologous recombination. This type of CNV accounts for some of the established shared genetic risk factors for Sz and ASD identified to date (Kirov, 2015). CNVs which have shared risk for both Sz and ASD include 2p16.3 (*Neurexin1* (*NRXN1))*, 1q21.1, 13q13.3 and 16p11.2 (Consortium, 2008; Mefford *et al.*, 2008; Stefansson *et al.*, 2008; Mccarthy *et al.*, 2009). Deletions at 2p16.3 (*NRXN1*), a gene which encodes a presynaptic cell-adhesion protein, are associated with an increased risk of developing both Sz and ASD. Interestingly, CNV type at 16p11.2 may be an important factor for determining risk of developing Sz or ASD, since duplications appear to be more strongly associated with Sz, while deletions appear to be more strongly associated with ASD (Marshall *et al.*, 2008; Weiss *et al.*, 2008; Mccarthy *et al.*, 2009; Walsh and Bracken, 2011; Chang *et al.*, 2017). Strengthening the role of altered *Neurexin-Neuroligin* signaling in these disorders, mutations in *Neuroligin* (a postsynaptic binding partner of *Neurexin*) have also independently been associated with ASD and Sz. For example variations in *Neuroligin3 and 4 (NLGN3, NLGN4)* have been found in ASD (Jamain *et al.*, 2003) and variations in *Neuroligin2* (*NLGN2*) have been found in Sz (Sun *et al.*, 2011).

A range of genetic syndromes have also been identified which involve mutations in specific genes, and dramatically increase the risk of developing ASD. These include *Fragile X Mental Retardation 1 (FMR1)*, *Tuberous Sclerosis 1 (TSC1)* (Geschwind and State, 2015), Rett

syndrome (*MECP2 gene*) (Chahrour *et al.*, 2016) and Phelan-McDermid syndrome (*SHANK3 gene*) (Uchino and Waga, 2013).

There is no single gene mutation nor insult from the environment that causes, or is specific to Sz or ASD. Furthermore Sz and ASD patients are likely to carry more than one of these genetic risk factors (Mitchell and Porteous, 2011), which may be associated with a common neurobiological pathway. It is becoming increasingly important to consider the two types of risk factor together. For example an environmental insult may coincide with an important developmental time point in gene expression in the brain which may be faulty, or may in fact cause altered gene expression, contributing to the development of the pathology and eventually to the clinical symptoms of Sz or ASD.

1.4 *NRXN1*: a shared genetic risk factor for Sz and ASD

The focus of this thesis is *Neurexin1* (*NRXN)* which is a shared genetic risk factor for both Sz and ASD, and has also been found in cases of intellectual disability (Friedman *et al.*, 2006; Guilmatre *et al.*, 2009). These mutations mainly consist of heterozygous exon deletions of *NRXN1,* that presumably result in altered expression of the *Neurexin1α* (*NRXN1α*) isoform. The mutation was first discovered in Sz by Kirov *et al.*, who found a heterozygous deletion which removed the promotor and exon 1 of *NRXN1* (2p16.3). This deletion was found in two Sz siblings and their unaffected mother in a Bulgarian sample of 93 patients and 372 controls (Kirov *et al.*, 2008). Walsh *et al.* also found heterozygous deletions eliminating exons in *NRXN1* in monozygotic twins with COS, but not in unaffected controls, using a sample of 233 patients and 268 controls (Walsh *et al.*, 2008). Furthermore Vrijenhoek *et al.* found 3 exonic deletions in *NRXN1* in 752 Sz patients, without finding any in 706 controls in

The Netherlands (however one intronic *NRXN1* deletion was identified in controls) (Vrijenhoek *et al.*, 2008). These findings were supported by a much larger scale study which examined 2,977 Sz patients and 33,746 controls in 7 European countries for CNVs in *NRXN1* (Rujescu *et al.*, 2009), which included patients from the Vrijenhoek *et al.* study. They found that heterozygous deletions were more prevalent in the Sz cases (approx. 1 in 248 deletions and 1 in 1,486 duplications), as compared to controls (approx. 1 in 689 deletions and 1 in 11,249 duplications). Furthermore approx. 1 in 595 of the deletions found in patients and 1 in 6,749 in the control sample were affecting exons (Rujescu *et al.*, 2009). A further study by Kirov *et al.* (with a sample of 471 patients and 2792 controls in the UK) found deletions in *NRXN1* in approx. 1 in 471 patients and 1 in 931 controls both disrupting exons (Kirov, Grozeva*, et al.*, 2009). Another study using a sample size of 560 Sz patients and 547 controls in a Japanese population, found 1 intronic *NRXN1* deletion in patients and none in controls (Ikeda *et al.*, 2010). A further study found a deletion in *NRXN1α* and one in *NRXN1β* in a French sample of 236 patients with Sz or schizoaffective disorder (Guilmatre *et al.*, 2009).

Two large scale Genome Wide Association Studies (GWAS) in Sz patients and controls found deletions in *NRXN1*. The first study, using a sample of 3,391 patients and 3,181 controls with European ancestry, found deletions in *NRXN1* in approx. 1 in 848 patients and 1 in 1,060 controls. However when only deletion in exons of *NRXN1* were included approx. 1 in 1,130 were found in patients and none in controls (Consortium, 2008). The second GWAS study found deletions in exons of *NRXN1* in approx. 1 in 358 patients and none in controls, using a sample of 1073 patients and 1148 controls with European or African American ancestry (Need *et al.*, 2009).

The deletions in *NRXN1* from most of the studies previously mentioned were analysed together, with a > 100 Kb inclusion criteria, in another study by Kirov *et al.* They calculated that 0.19% of Sz patients had a deletion in *NRXN1* compared to 0.04% of controls which was highly significant (odds ratio=4.78). Prevalence of deletions (> 100 Kb) that disrupted exons was calculated to be 0.16% of Sz patients as compared to 0.02% of controls which was also highly significant (odds ratio=7.44) (Kirov, Rujescu*, et al.*, 2009). All of the studies reviewed by Kirov *et al.* (2009) found deletions in *NRXN1* (in both controls and Sz patients) in the longer *NRXN1α* isoform (Consortium, 2008; Kirov *et al.*, 2008; Kirov, Grozeva*, et al.*, 2009; Need *et al.*, 2009; Rujescu *et al.*, 2009) except for one found in COS patients in the *NRXN1β* isoform (Walsh *et al.*, 2008). Another study, which wasn't included in the review by Kirov *et al*., found a deletion in the *NRXN1β* isoform in a Sz patient (Guilmatre *et al.*, 2009). This suggests that the *NRXN1α* isoform plays a primary role in governing the risk of developing Sz.

Variations in *NRXN1* have also been found in individuals with ASD. The first cases were 2 missense mutations in exonic regions in the *NRXN1β* isoform. This was found in 4 ASD patients compared to 0 controls, using a counterbalanced Caucasian and African American sample of 203 ASD patients and 535 control subjects (Feng *et al.*, 2006). Later mutations were also found in the *NRXN1α* isoform in individuals with ASD. A heterozygous *de novo* deletion in the exons of *NRXN1α* was found in a 7 year old boy with ASD symptoms and other abnormalities including facial dysmorphism (Friedman *et al.*, 2006). Later more heterozygous deletions in exons of *NRXN1α* were found to be associated with ASD. These were found using microarray and GWAS studies in large samples of European, Middle Eastern, Turkish and Pakistani ancestry (Marshall *et al.*, 2008; Morrow *et al.*, 2008; Glessner

et al., 2009), including in 2 ASD siblings from a sample of 1181 families in North America and Europe (containing 2 or more ASD patients) (Szatmari *et al.*, 2007). Other mutations found included an insertion in an intronic region *NRXN1α,* and a translocation preceding *NRXN1α* in a sample of 200 American ASD patients (Kim *et al.*, 2008). Also a number of ultra-rare splice variants in *NRXN1α* were found in Caucasian ASD patients and controls (Yan *et al.*, 2008). 2 more heterozygous deletions in *NRXN1α,* affecting exons, were found in a sample of 260 unrelated ASD patients compared to no *NRXN1α* mutations in a sample of 236 controls (Guilmatre *et al.*, 2009). A number of variations in *NRXN1α*, including 3 deletions, were found in ASD patients and to a lesser extent in controls (Liu *et al.*, 2012). Finally variations in *NRXN2* and *NRXN3* have also been documented in ASD patients (Gauthier *et al.*, 2011; Vaags *et al.*, 2012), but are less common than variations in *NRXN1*.

Heterozygous deletions are present in both patient and control populations (Itsara *et al.*, 2009; Ching *et al.*, 2010). This suggests that the mutation alone does not bring about Sz or ASD, as is the case for many other Sz and ASD risk genes. Therefore other factors, genetic or environmental, must also contribute. Although it has not been confirmed in human tissue, it is predicted that the mutations result in the loss of *NRXN1α* transcript generation from the affected allele. Therefore it is assumed that this results in a decreased level of *NRXN1α* protein (due to promotor and early exon regions being eliminated), producing *NRXN1α* protein hypofunction in these individuals.

1.5 *Neurexin* genes, protein structure and isoforms

Humans and other mammals have three *Neurexin* genes (1, 2 and 3) which have two independent promotors producing α and β *Neurexin* transcripts. There is also a γ Neurexin

transcript that is alternatively spliced from β *Neurexin* transcripts (Sudhof, 2017). *Neurexin1* and *Neurexin2* were thought be specific to neurons of the brain and nervous system (Ushkaryov *et al.*, 1992), with *Neurexin3* being found in other tissues (Occhi *et al.*, 2002). However *Neurexin1α* has also been found in β cells of the pancreas (Mosedale *et al.*, 2012), and within the CNS has been found to be expressed by astrocytes (Zhang *et al.*, 2014; Gokce *et al.*, 2016). *Neurexins* are part of a family of cell-adhesion molecules (Peles *et al.*, 1997) and have widely been shown to have presynaptic localisation, however *Neurexin1β* has also been found postsynaptically in mice (Taniguchi *et al.*, 2007) and the single *Drosophila Neurexin1 (Nrx-1)* has been found postsynaptically in embryonic neuromuscular junctions (NMJs) (Chen *et al.*, 2010).

Alternative splicing occurs in all 3 *Neurexin* genes at 6 splice sites (Figure 1.1) and can produce thousands of *Neurexin* variants (Ullrich *et al.*, 1995; Sudhof, 2017). *Neurexins*, due to their vast splice variants, have been suggested to act as surface recognition molecules that define synapse type (Ushkaryov *et al.*, 1992; Sudhof, 2017). Splice variants are also expressed differentially dependent on brain region (Ullrich *et al.*, 1995; Schreiner *et al.*, 2014; Treutlein *et al.*, 2014). Alternative splicing of *Neurexin* genes differs between genes 1, 2, and 3 (Fuccillo *et al.*, 2015), and some splice sites have been found to be regulated by neuronal activity (Rozic-Kotliroff and Zisapel, 2007; Rozic *et al.*, 2011; Rozic *et al.*, 2013; Ding *et al.*, 2017).

NRXN1α is a large gene (1,108.4 Kb) which encodes a protein that contains 6 laminin/*Neurexin*/sex-hormone domains (LNS 1-6), 3 EGF-like repeats, a short cysteine-loop domain, a transmembrane region and a cytoplasmic sequence. β *Neurexins* are essentially

shortened α *Neurexins*, with α *Neurexins* having longer extracellular domains (Tabuchi and Sudhof, 2002) (Figure 1.1).

Neurexin is evolutionarily conserved across species, thus animal models can be used to investigate the functional importance of *NRXN1* in humans. Mice have 3 *Neurexin* genes that are highly similar to human (Tabuchi and Sudhof, 2002). *Drosophila* have a single *Neurexin* gene (*Nrx-1)* which has conserved C terminal sequences (PDZ binding motifs) to mammalian *Neurexin1α* and around 36% similarity overall to human *NRXN1α* (Li *et al.*, 2007). Although a protein named *NeurexinIV* was discovered before *Nrx-1* in *Drosophila,* and was proposed at first to be part of the *Neurexin* family (Baumgartner *et al.*, 1996), its domain arrangement in fact differs from that of the *Neurexin* family (Li *et al.*, 2007) (Figure 1.2).

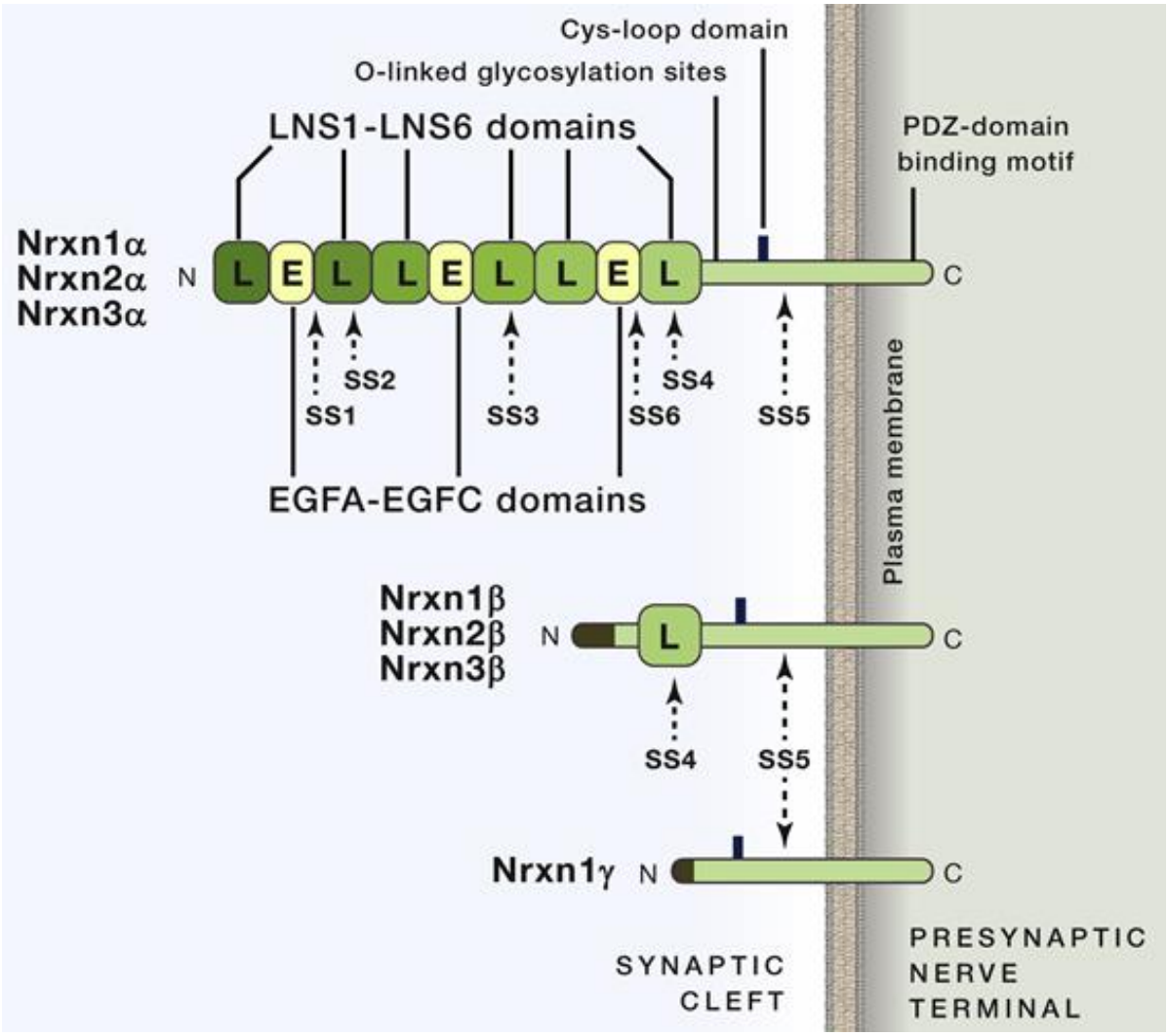

Figure 1.1 Structure, isofroms and splice sites of Neurexin proteins
LNS= Laminin/Neurexin/Sex-hormone, E= EGF-like domain, SS= Splice Site. Figure taken from Sudhof (2017).

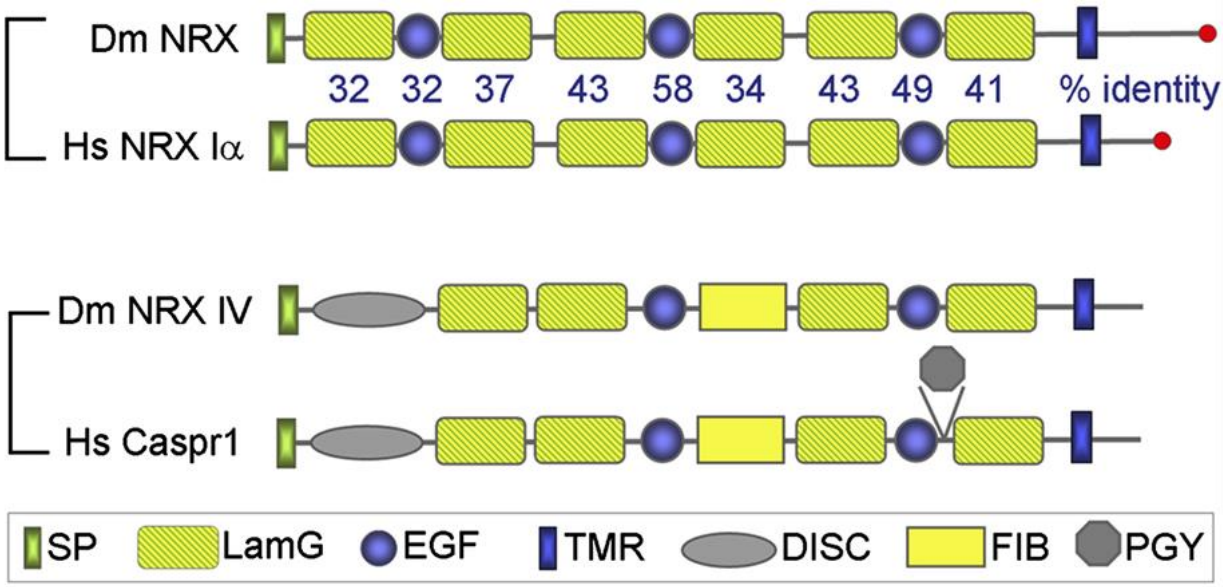

Figure 1.2 Drosophila Neurexin1 (Nrx-1), but not NeurexinIV, protein has a structure similar to human NRXN1α

The percent amino acid identity (overall 36%) between Drosophila Neurexin (DNRX or Nrx-1) and human Neurexin1α (NRXN1α) in specific domains is indicated. Dm=Drosophila melanogaster, Hs=Homosapien, NRX=Neurexin, NRX Iα=Neurexin1α, Caspr1=Contactin-associated protein 1, SP=signal peptide, LamG=laminin G domain, EGF=EGF repeat, DISC=discoidin-like domain, FIB=a region similar to fibrinogen, PGY=PGY repeat, TMR=transmembrane region. Figure taken from Li et al., (2007).

1.6 Functions of *Neurexins*

Since mutations in *NRXN1α* are associated with Sz and Autism, the focus here will mainly be on this isoform. α *Neurexins* have 3 known binding domains, LNS2, LNS6 and juxtamembranous sequences (Sudhof, 2017). These binding domains allow α-*Neurexins* to bind with a large and varied number of partners including, but not limited to, postsynaptic transmembrane proteins such as *Neuroligins* (Ichtchenko *et al.*, 1995; Ichtchenko *et al.*, 1996; Boucard *et al.*, 2005), Leucine-rich repeat transmembrane (LRRTM) proteins (De Wit *et al.*, 2009; Ko *et al.*, 2009), *GABA-A* receptors (Zhang *et al.*, 2010), latrophilins (Boucard *et al.*, 2012) and dystroglycan (Sugita *et al.*, 2001). α *Neurexins* also bind to proteins found in the synaptic cleft including, but not limited to, *Cerebellin1-3* (Cbln1-3) (Uemura *et al.*, 2010) and *Neurexophillins* (Missler *et al.*, 1998) (Figure 1.3). The diverse range of ligands which bind to *Neurexins* indicates that the *Neurexins* have a number of synaptic functions, many of which are probably yet to be discovered. Furthermore since there are 6 LNS binding sites on α *Neurexins*, and only 2 have known binding partners, it is likely that there are more undiscovered ligands. Many of the interactions with binding partners depend on alternative splicing of *Neurexins*, for instance the presence or absence of Splice Site 4 (SS4) (Ko *et al.*, 2009; Uemura *et al.*, 2010; Boucard *et al.*, 2012). Since *Neurexins* have so many ligands, they likely act in a complex molecular network that is constantly changing and is neuronal cell type specific (Sudhof, 2017).

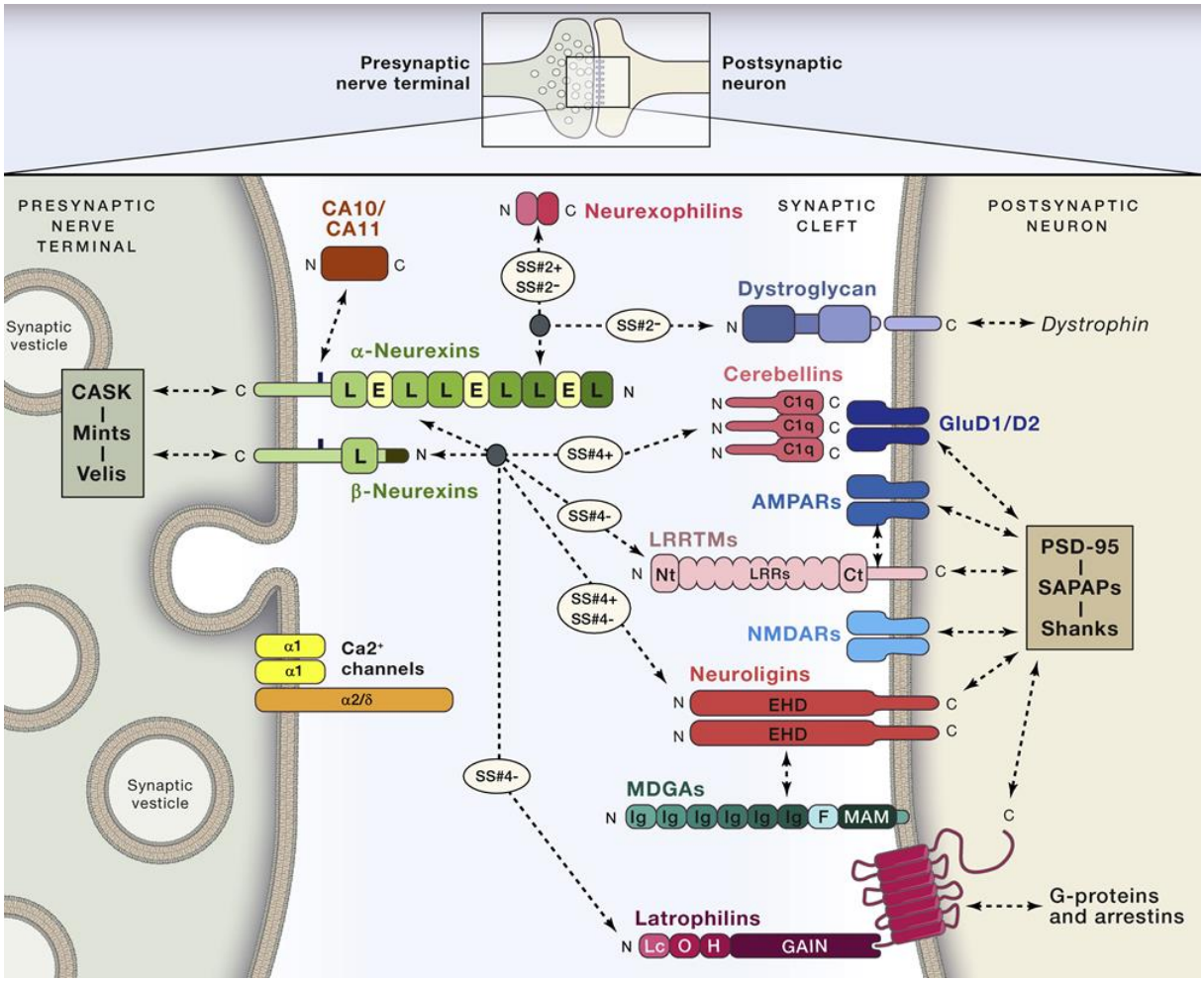

Figure 1.3 Ligand interactions of Neurexins

Schematic showing a selection of synaptic Neurexin ligand interactions. Requirements for Neurexin splice variants are indicated and possible competition between ligands are indicated by junctions marked with a grey circle. E=EGF-like domain, EHD=Esterase Homology Domain, L=LNS-domain, LRRs=Leucine-Rich Repeats, Nt and Ct, N- and C-terminal sequences surrounding LRRs, Ig=Ig-domain, F=fibronectin III domain, MAM=MAM-domain, Lc=lectin domain, O=olfactomedin-like domain, H=hormone-binding domain, GAIN=GAIN domain. Figure taken from Sudhof (2017).

Since *Neurexins* are part of the cell adhesion molecule family, their cell adhesion role is mediated in part via binding to *Neuroligin* (Song *et al.*, 1999), which plays a role in aligning pre and postsynaptic terminals during synapse formation (Scheiffele *et al.*, 2000; Dean *et al.*, 2003; Graf *et al.*, 2004). *Neurexins* bind to *Neuroligins* via the LNS6 domain, which is present on both α and β *Neurexins* (Figure 1.3) (Sudhof, 2017). Both α and β *Neurexins* can bind to *Neuroligins*, dependent on alternative splice variants of *Neurexins* (at Splice Site 4) and *Neuroligins* (at Splice Site B) (Boucard *et al.*, 2005; Chih *et al.*, 2006; Comoletti *et al.*, 2006). In addition to their role as cell adhesion molecules, the expanding body of research has uncovered a much more complex array of functions of *Neurexins*. Initial experiments in cells indicated that *Neurexins* were required for synapse formation and remodeling. Artificial synapse formation assays showed that expressing *Neurexin* on non-neuronal cells brought about postsynaptic specialisations in contacting neuronal cells (Graf *et al.*, 2004). Similarly, expression of *Neuroligin* in non-neuronal cells or the addition of soluble *Neuroligin* was sufficient to recruit *Neurexin* and induce presynaptic specialisations in neuronal cells (Scheiffele *et al.*, 2000; Dean *et al.*, 2003). Studies also showed that overexpression of *Neuroligins in vitro* increased synapse number (Scheiffele *et al.*, 2000; Chubykin *et al.*, 2007), whereas knockout (KO) of *Neuroligins* did not (Chanda *et al.*, 2017).

The idea that *Neurexin* is required for synapse formation became unfavorable when a study in α *Neurexin (Nrxn)* KO mice showed that deleting all 3 α *Neurexins* did not affect the ultrastructure of synapses. It did however cause lethality at postnatal day 1, and resulted in a decreased density of inhibitory but not excitatory synapses (Missler *et al.*, 2003). While the formation of synapses was mostly unaffected by the triple KO of α *Neurexins*, synaptic function was disrupted in both inhibitory and excitatory synapses. This was due to impaired

calcium entry into the presynaptic cell thus reducing neurotransmitter release from vesicles (Missler *et al.*, 2003). Also, a primary role for *Nrxn1α* in presynaptic calcium signalling was later found (Zhang *et al.*, 2005). In addition to these studies in mice, studies in *Drosophila* have shown that *Nrx-1* is required for synapse formation (Li *et al.*, 2007; Zeng *et al.*, 2007), which is likely due to *Drosophila* only having one *Neurexin* gene.

1.7 Previous studies in *Nrxn1α* knockout (KO) and Heterozygous (Hz) mice

The α *Neurexin* triple KO mice (Missler *et al.*, 2003) were created to investigate the effects of α-latrotoxin (present in black widow spider venom) in α *Neurexins*, which are receptors for the neurotoxin. The mutation present in these animals in *Nrxn1* removed the promotor and first exon of the *Nrxn1α* gene (Missler *et al.*, 2003), which creates a very similar deletion to those found in Sz and ASD patients as previously reviewed (Section 1.4). From these triple α *Neurexin* KO mice, *Nrxn1α* KO mice could be selectively generated and the effects of the single mutation investigated. Studies using the resulting *Nrxn1α* KO mice showed that they had reduced postsynaptic spontaneous currents in excitatory, but not inhibitory, neurons (Etherton *et al.*, 2009), which suggests an excitatory/inhibitory imbalance in these animals. These mice also showed altered behaviour such as reduced Pre-Pulse Inhibition (PPI) and altered nest building, maternal care and grooming. However showed no differences in spatial or working memory compared to Wild-type (Wt) mice (Etherton *et al.*, 2009; Grayton *et al.*, 2013). Furthermore, while one study found that locomotor activity in *Nrxn1α* KO mice was reduced and social behaviours were altered (Grayton *et al.*, 2013), an earlier study found no difference in these behaviours (Etherton *et al.*, 2009).

Since no homozygous deletions of *Nrxn1α* have been found in Sz or ASD individuals to date, it is arguably more translationally relevant to investigate heterozygous deletions in *Nrxn1α* in mice. Studies have shown that *Nrxn1α* Hz mice show no difference in nest building, grooming, anxiety-like behaviour, spatial memory or working memory (Laarakker *et al.*, 2012; Grayton *et al.*, 2013; Dachtler *et al.*, 2015). Studies also showed that *Nrxn1α* Hz mice have accelerated habituation on exposure to novel testing arenas (Laarakker *et al.*, 2012) and deficits in passive avoidance (Dachtler *et al.*, 2015). While one study found deficits in social recognition memory and no difference in locomotor activity (Dachtler *et al.*, 2015), others have found no difference in social recognition memory (Grayton *et al.*, 2013) and increased locomotor activity in male mice in response to a novel environment (Laarakker *et al.*, 2012). Studies have also shown that *Nrxn1α* Hz mice are able to discriminate between novel and familiar objects in a similar way to Wt mice (Laarakker *et al.*, 2012; Dachtler *et al.*, 2015). Taken together these findings suggest *Nrxn1α* Hz mice have subtle cognitive deficits and locomotor alterations, which are not robustly reported across studies. The subtlety of these effects on mouse behaviour is unsurprising, since deletions in *NRXN1α* are found in healthy controls as well as Sz and ASD individuals (Itsara *et al.*, 2009; Ching *et al.*, 2010).

1.8 Previous studies investigating altered *Neurexin* in flies

In addition to studies investigating *Nrxn1* dysfunction in mice, there have also been studies in flies. Complete KO of *Neurexin1* (*Nrx-1*) in *Drosophila melanogaster* causes a number of synaptic changes at larval glutamatergic neuromuscular junction. These include a decrease in number, changes in morphology, altered differentiation and defective neurotransmission (Li *et al.*, 2007). *Nrx-1* KO also causes reduced synapse number in the *Drosophila* larval brain (Zeng *et al.*, 2007). This differs from the effects of α *Neurexin* triple KO in mice, where

synapse morphology was unaltered and only inhibitory synapses showed a reduction in number (Missler *et al.*, 2003). The more severe effect on synapses observed in *Nrx-1* KO flies, suggests that *Nrx-1* plays a role in *Drosophila* synapse formation, and is likely due to flies only having a single *Neurexin* gene.

Nrx-1 has an effect on life span, since a *Nrx-1* KO fly strain was found to have reduced life span (Zeng *et al.*, 2007). *Nrx-1* also has effects on behaviour in *Drosophila,* since *Nrx-1* KO larvae were found to have reduced locomotor activity and reduced associative learning (Li *et al.*, 2007). Adult *Nrx-1* KO flies also have reduced associative learning and memory (Sun *et al.*, 2016) and have altered sleep structure (Larkin *et al.*, 2015; Tong *et al.*, 2016). Whether these effects are also present in *Nrx-1* Hz KO flies, which would model heterozygous deletions of *NRXN1α* in patients, remains to be determined. However *Nrx-1* hypomorph flies, which have decreased expression of *Nrx-1*, also have reduced associative learning as larvae (Zeng *et al.*, 2007) and adults (Sun *et al.*, 2016), and have altered sleep structure (Tong *et al.*, 2016).

1.9 Cognitive deficits in Sz

A number of widespread cognitive impairments are present in patients with Sz (Green *et al.*, 2004). These include deficits in aspects of executive functions including working memory (Lee and Park, 2005; Barch and Smith, 2008) and cognitive flexibility (Fioravanti *et al.*, 2005), and deficits in cognitive processing speed (Dickinson *et al.*, 2007), episodic memory (Ranganath *et al.*, 2008), attention (Orzack and Kornetsky, 1966), social cognition (Penn *et al.*, 2008; Van Hooren *et al.*, 2008) and language (Covington *et al.*, 2005). Many cognitive impairments in Sz are found in the Ultra High Risk of psychosis group (UHR) (Brewer *et al.*,

2006), First Episode Psychosis (FEP) (Bozikas and Andreou, 2011), chronic Sz (Mazhari and Moghadas Tabrizi, 2014) and relatives of Sz patients (Cella *et al.*, 2015). This provides evidence for cognitive deficits being a core component of the disorder, which are likely to be heritable, and are unlikely due to effects of antipsychotic medication. Deficits in cognition may be predictive of illness progression in the UHR group (Fusar-Poli *et al.*, 2012) and of overall functional outcome for patients (Nuechterlein *et al.*, 2011). Cognitive deficits are mostly found to be unrelated to positive/psychotic symptoms, but can be correlated with negative and disorganised symptom domains (O'leary *et al.*, 2000; Ventura *et al.*, 2010).

Many genetic risk factors for Sz and ASD have also been associated with cognitive deficits. For example, variations in *Disrupted in Schizophrenia 1 (DISC1)* increase the risk of developing Sz and ASD (Song *et al.*, 2008; Zheng *et al.*, 2011), and have been associated with poor performance of a spatial working memory task in healthy controls (Carless *et al.*, 2011). This demonstrates a link between variations in risk genes for the disorders and deficits in cognition, in individuals lacking a psychiatric diagnosis. Variations in *Calcium voltage-gated subunit alpha 1C* (*CACNA1C*) increase the risk of developing Sz and ASD (Psychiatric and Consortium, 2014; Li, J. *et al.*, 2015) and have also been associated with cognition. The rs2007044 allele (risk allele G) in *CACNA1C* has been associated with poor working memory in a large mixed Sz patient and healthy control sample (Cosgrove, Mothersill*, et al.*, 2017). Although this effect was mainly driven by the patients, a sub-study in healthy controls showed that the risk allele G was associated with poor functional connectivity in areas involved in working memory, such as the Dorsolateral Prefrontal Cortex (DLPFC) (Cosgrove, Mothersill*, et al.*, 2017). Another study found that the *CACNA1C*

risk allele G was associated with poorer performance on a logical memory task in Sz patients, while finding no effect of the allele in controls (Hori *et al.*, 2012). These studies suggest that the risk allele G is associated with reduced cognition in Sz patients (Hori *et al.*, 2012; Cosgrove, Mothersill*, et al.*, 2017). While the risk allele G appears not to be sufficient to cause cognitive deficits in controls, these individuals appear to have a functional brain endophenotype which may make them vulnerable to deficits in cognition (Cosgrove, Mothersill*, et al.*, 2017). Variations in *zinc finger binding protein 804A (ZNF804A)* and *microRNA 137 (MIR137)* increase the risk of developing Sz (Williams *et al.*, 2011; Psychiatric Consortium, 2014), and have also been found to affect cognition. The genetic variant rs1344706 in *ZNF804A* has been found to be associated with deficits in cognitive domains, including memory, in Sz patients but not control subjects (Walters *et al.*, 2010; Nicodemus *et al.*, 2014). Furthermore, increased *MIR137* polygene risk score has been found to be associated with poor attention and memory in both Sz patients and controls (Cosgrove, Harold*, et al.*, 2017). Thus providing further evidence that genes which increase the risk of developing Sz may also play a role in cognition.

Different cognitive domains can be tested in patients using various neuropsychological tasks. Although a number of cognitive domains are affected in Sz, deficits in two executive functions, working memory and cognitive flexibility, and deficits in cognitive processing speed will be the focus here.

Executive function is a collection of top-down cognitive abilities which are associated with the functioning of the frontal lobe of the brain. The low-order executive functions include working memory, cognitive flexibility and inhibition. The adequate functioning of these lower level executive functions, give rise to higher-order executive functions such as

reasoning, problem solving and planning (Diamond, 2013). Executive functions are cognitively taxing, and allow us to respond to an ever changing environment by changing our behaviour (Diamond, 2013). Many of the domains within the executive functions are affected in Sz.

Working memory is the temporary storage and manipulation of data required to successfully carry out many cognitive tasks and functions (Baddeley, 1992), and is classified as both an executive function and a type of memory. Working memory deficits are a core cognitive symptom in Sz (Lee and Park, 2005; Barch and Smith, 2008; Forbes *et al.*, 2009), and can be tested in patients using tasks such as the digit span task, the reverse digit span task (Conklin *et al.*, 2000) and the N-back task (Jacola *et al.*, 2014). Working memory deficits have been shown in the UHR (Kim *et al.*, 2011; Bang *et al.*, 2015), FEP (Gisselgard *et al.*, 2014; Olivier *et al.*, 2015), medication naïve (Fatouros-Bergman *et al.*, 2014) and chronic Sz patient groups (Mccleery *et al.*, 2014). Working memory abilities are highly heritable (estimated to be around 45%) (Ando *et al.*, 2001), and so it is unsurprising that many studies of unaffected relatives of Sz patients also have deficits in working memory (Park *et al.*, 1995; Conklin *et al.*, 2000; Myles-Worsley and Park, 2002; Conklin *et al.*, 2005; Barrantes-Vidal *et al.*, 2007; Horan *et al.*, 2008; Diwadkar *et al.*, 2011).

Cognitive flexibility is another executive function that is reduced in Sz. Cognitive flexibility is the ability to switch attention or task, to learn rules and apply them to new situations, and to know that rules have exceptions (Dajani and Uddin, 2015). In humans, cognitive flexibility can be tested using neuropsychological testing such as the Wisconsin Card Sorting Task (WCST), in which the participant must match cards based on a rule that changes as the task progresses (Milner, 1963). The WCST is a complex task which requires the participant to

maintain focused attention, inhibit and switch behavioural responses, and have sufficient working memory to enable effective problem solving and cognitive flexibility (Dajani and Uddin, 2015). Sz patients show deficits in this task due to increased perseverative responding, which means responding to a previous rule even though they are receiving negative feedback (Bellini *et al.*; Zanello *et al.*; Bellini *et al.*, 1991; Scarone *et al.*, 1993; Sullivan *et al.*, 1993; Battaglia *et al.*, 1994; Abbruzzese *et al.*, 1995; Haut *et al.*, 1996; Dieci *et al.*, 1997; Stratta *et al.*, 1997; Glahn *et al.*, 2000; Egan *et al.*, 2001; Gooding and Tallent, 2002; Cavallaro *et al.*, 2003; Altshuler *et al.*, 2004; Josman and Katz, 2006; Zanello *et al.*, 2006). This deficit may relate to symptom type, as a sub-group of Sz patients with paranoid symptoms were found to make more perseverative errors than a non-paranoid symptom sub-group in the WCST (Abbruzzese *et al.*, 1996). Increased perseverative errors in the WCST is found throughout disease progression in Sz, including in the UHR group (Ohmuro *et al.*, 2018), at FEP (Ilonen *et al.*, 2000; Rybakowski and Borkowska, 2002; Huang *et al.*, 2016) and in chronic Sz patients (Braff *et al.*, 1991; Ragland *et al.*, 1996). While some studies have shown that first-degree relatives of Sz patients have deficits in the WCST (Laurent *et al.*, 2001; Li, 2004) others have not (Scarone *et al.*, 1993; Stratta *et al.*, 1997; Szöke *et al.*, 2006). WCST deficits have also been found in the offspring of Sz patients (Wolf *et al.*, 2002; Ozan *et al.*, 2010). A meta-analysis assessing executive function in first-degree relatives of Sz patients, found deficits in a number of tasks including the WCST, although the effect size was small (Szoke *et al.*, 2005). This suggests that there is a genetic component to deficits in the WCST, and that deficits are not specific to a Sz diagnosis. This is due to unaffected first-degree relatives of Sz patients also showing deficits in this task, however these deficits are less severe (Szoke *et al.*, 2005). It has been shown that perseverative errors in the WCST were reduced at 3 year follow up, compared to baseline in FEP (Chang *et al.*, 2014). This may

be due to antipsychotic medication or other factors such as repeated experience causing enhanced performance of the task, however since no longitudinal control group was included in the study this cannot be determined. Regressive errors in the WCST are when a participant starts to show correct responding to a new rule, but then returns to responding to the old rule. One study noted that Sz patients also make more regressive errors, as well as perseverative errors, as compared to control subjects. However this was not found to be specific to Sz, as the effect was also found in bipolar disorder patients (Hill, S. K. *et al.*, 2015). By contrast another study reported that Sz patients make a similar number of non-perseverative errors compared to controls (Li, 2004). Regressive errors are not as widely measured/reported compared to perseverative errors, in studies using the WCST. Therefore, further characterisation is required before conclusions about regressive errors in Sz can be drawn. Some studies have suggested that deficits in working memory may underlie reduced performance of the WCST in Sz (Gold *et al.*, 1997; Glahn *et al.*, 2000; Hartman *et al.*, 2003; Waltz, 2017), whereas another study found this not to be the case (Stratta *et al.*, 1997).

Cognitive processing speed can be defined as the number of correct responses that can be made in a given time period during a task (Salthouse, 1996; Knowles *et al.*, 2015), and can be tested in humans using a number of tasks such as the digit symbol coding task (Dickinson *et al.*, 2007) and The Trail Making Test Part A (Nuechterlein *et al.*, 2011). Slowed cognitive processing speed is a prominent cognitive deficit in Sz (Dickinson *et al.*, 2007; Woolard *et al.*, 2010; Karbasforoushan *et al.*, 2015), and has been found in UHR (Bang *et al.*, 2015), FEP (Nuechterlein *et al.*, 2011), antipsychotic naïve FEP (Andersen, R. *et al.*, 2013; Fatouros-Bergman *et al.*, 2014) and chronic Sz patients (Sanchez *et al.*, 2009; Mazhari and Moghadas

Tabrizi, 2014). As reduced cognitive processing speed is present in the UHR group and antipsychotic medication free patients, it is unlikely to be a side effect of antipsychotic medication. Moreover, slowed cognitive processing speed is present in first-degree relatives of patients with Sz (Dickinson *et al.*, 2007), is predictive of UHR individuals that will convert to a full Sz diagnosis (Bang *et al.*, 2015), and is also indicative of functional outcome in Sz (Sanchez *et al.*, 2009; Nuechterlein *et al.*, 2011). Thus slowed cognitive processing speed is thought to be a core feature of Sz, which may underlie other impairments in cognition such as deficits in working memory (Brebion *et al.*, 2014) and other aspects of executive function (Ojeda, Peña*, et al.*, 2012). However, since slowed cognitive processing speed is found in unaffected first-degree relatives of Sz patients, this suggests that there is a genetic component to this cognitive domain, and that deficits are not specific to a Sz diagnosis.

1.10 Cognitive deficits common to both Sz and ASD

There are a number of cognitive deficits associated with ASD. In addition to cognitive deficits considered as core symptoms of the disorder, such as deficits in social cognition and language, there are also deficits in other domains such as autobiographical memory (Crane *et al.*, 2013), inhibition, cognitive flexibility (Hill, 2004), working memory (Wang *et al.*, 2017), and cognitive processing speed (Hedvall *et al.*, 2013; Haigh *et al.*, 2018). The latter 3 domains are also affected in Sz, as previously described, and will be reviewed in the context of ASD in this section. It is important to note that nearly all studies of cognitive ability in ASD include individuals that are high functioning only, as low functioning individuals are generally unable to understand how to complete neuropsychological tasks (Velez-Coto *et al.*, 2017). This means that the following studies can only be interpreted in terms of high functioning individuals with ASD, and cannot be generalised to low functioning individuals.

As previously outlined, working memory and cognitive flexibility are executive functions. A number of studies have found visual and spatial working memory deficits in high functioning ASD individuals, including children (Landa and Goldberg, 2005; Andersen, P. N. *et al.*, 2013; Andersen *et al.*, 2015; Brenner *et al.*, 2015), adolescents (Goldberg *et al.*, 2002; Luna *et al.*, 2002; Luna *et al.*, 2007; Steele *et al.*, 2007; Chien *et al.*, 2016), adults (Minshew *et al.*, 1999; Crane *et al.*, 2013) and aged adults (Geurts and Vissers, 2012). By contrast, some studies have shown that ASD patients have intact working memory (Ozonoff and Strayer, 2001; Williams *et al.*, 2005). Working memory deficits found in ASD may depend on the task used. For example studies have shown mixed findings for whether verbal working memory is intact (Williams *et al.*, 2005) or deficient (Bennetto *et al.*, 1996) in ASD. While working memory assessed using the N-back task (non-verbal) has been found to be intact in ASD (Ozonoff and Strayer, 2001; Koshino *et al.*, 2005; Rahko *et al.*, 2016), this may require the compensatory activation of different brain areas and networks as compared to controls (Koshino *et al.*, 2005; Rahko *et al.*, 2016). One study performed a direct comparison of three working memory tasks; the digit span, digit symbol and advanced trail making task in ASD patients. They found that while ASD patients had deficits in the digit symbol task, they performed as well as controls on the other two tasks (Nakahachi *et al.*, 2006). The authors suggest that complex working memory tasks may reveal deficits in ASD, but that these are due to problems with other cognitive domains and not working memory per se. Studies in first-degree relatives of ASD patients found that while parents show deficits in spatial (Hughes *et al.*, 1997; Koczat *et al.*, 2002) and verbal working memory (Gokcen *et al.*, 2009), siblings do not show deficits in spatial working memory (Hughes *et al.*, 1999). In summary, working memory deficits in ASD are dependent on the task used and, unlike in Sz, may not be a core cognitive symptom of the disorder.

Deficits in cognitive flexibility have been observed in high functioning individuals with ASD, in a number of tasks including set shifting (Shafritz *et al.*, 2008) and the WCST (Westwood *et al.*, 2016). However some studies have found no deficit in cognitive flexibility in ASD (Schmitz *et al.*, 2006; Robinson *et al.*, 2009; Taylor *et al.*, 2012). In the WCST, increased perseverative errors underlie the cognitive flexibility deficit seen in ASD, which is similar to the deficit found in Sz patients. This includes findings from children, adolescents and adults with ASD (Tsuchiya *et al.*, 2005; South *et al.*, 2007; Sumiyoshi *et al.*, 2011; Yasuda *et al.*, 2014; Yeung *et al.*, 2016). By contrast, one study did not find increased perseverative errors in WCST in ASD, but found deficits in other parameters of the task (Kaland *et al.*, 2008). A recent meta-analysis found that ASD patients show increased perseverative errors in the WCST as compared to controls, an effect that was consistent in both children and adults (Westwood *et al.*, 2016). Furthermore, one study found that in children with ASD, girls show more perseverative errors and complete fewer categories than boys (Memari *et al.*, 2013). Cognitive inflexibility in ASD has been found to be correlated with restrictive interests and repetitive behaviours, which are part of the defining symptoms of the disorder (Lopez *et al.*, 2005; D'cruz *et al.*, 2013; Mostert-Kerckhoffs *et al.*, 2015). Since many neuropsychological tests of executive function are delivered by an experimenter in person, and deficits in social cognition are a core feature of ASD, this could affect the performance of ASD individuals. However this does not seem to be the case, as results were found to be similar when using computer vs experimenter delivered cognitive flexibility tasks (Williams and Jarrold, 2013).

Findings from studies testing cognitive flexibility in first-degree relatives of ASD patients have been mixed. While one study found cognitive flexibility to be intact in parents and siblings of ASD patients performing Intra-Dimensional/Extra-Dimensional (ID/ED) set shifting

(Wong *et al.*, 2006), another study found deficits in this task in siblings (Hughes *et al.*, 1999). Similarly, while some studies have found no deficits in parents of ASD patients performing the WCST (Bolte and Poustka, 2006), others have shown deficits in this task in parents (Hughes *et al.*, 1997) and siblings (Sumiyoshi *et al.*, 2011) of ASD patients. Therefore while deficits in cognitive flexibility are found in ASD, it seems further investigation is required to determine whether first-degree relatives of ASD patients also have deficits in cognitive flexibility.

Processing speed has been be measured in ASD patients using tasks such as the Processing Speed Index (PSI) on the Wechsler Intelligence Scale (WISC) (Holdnack *et al.*, 2011; Mouga *et al.*, 2016) or the Trail Making Task (Nakahachi *et al.*, 2006; Han and Chan, 2017), which have both adult and child versions. A number of studies have found that ASD patients, like Sz patients, show slowed cognitive processing speed including both children (Mayes and Calhoun, 2007; Roberts *et al.*, 2011; Oliveras-Rentas *et al.*, 2012; Hedvall *et al.*, 2013; Travers *et al.*, 2014; Mouga *et al.*, 2016; Li *et al.*, 2017) and adults (Spek *et al.*, 2008; Lazar *et al.*, 2014; Travers *et al.*, 2014). Cognitive processing speed deficits may be related to symptom severity in ASD. One study found that cognitive processing speed was reduced in relatively low functioning children, and was not altered in relatively high functioning children, compared to controls (Han and Chan, 2017). Furthermore another study suggested that slowed processing speed in ASD is due to reduced motor functioning (Kenworthy *et al.*, 2013). They found that ASD patients performed at similar levels to controls in a processing speed task that had lower motor demands, but showed a deficit when motor demands of the task increased. There may also be sex specific differences in processing speed in children with ASD, as it was found that females have a faster processing speed than males (Koyama

et al., 2009). Interestingly studies in first-degree relatives of patients with ASD do not show slowed processing speed, including findings from parents (Bolte and Poustka, 2006) and siblings (Pilowsky *et al.*, 2007). This suggests that reduced cognitive processing speed may be a good predictor of symptoms in ASD.

1.11 Altered brain structure, function and connectivity in Sz

The most prominent structural feature of a chronic Sz brain is enlarged ventricles, as compared to non-Sz age matched controls (Johnstone *et al.*, 1976; Wright *et al.*, 2000; Horga *et al.*, 2011; Haijma *et al.*, 2013). It is also generally agreed that Sz patients have decreased grey matter in the frontal and temporal lobes, hippocampus, amygdala and insular cortex (systematic meta-analysis of structural MRI studies) (Shepherd *et al.*, 2012). Many of these changes, including ventricular enlargement and loss of grey matter in the frontal lobe, can also be seen in the UHR group (Vita *et al.*, 2012; Ziermans *et al.*, 2012; Cannon *et al.*, 2015; Chung *et al.*, 2017).

In terms of functional brain changes in Sz, many studies have shown that patients with both early and chronic Sz show decreased activation in the frontal lobe (hypofrontality), during resting-state (Ingvar and Franzen, 1974; Buchsbaum *et al.*, 1982; Farkas *et al.*, 1984; Wolkin *et al.*, 1985; Weinberger *et al.*, 1986) and task conditions (Andreasen *et al.*, 1992; Steinberg *et al.*, 1996; Hazlett *et al.*, 2000; Hazlett and Buchsbaum, 2001; Mitelman *et al.*, 2018). Although some studies did not find this effect during resting-state (Mathew *et al.*, 1982; Gur *et al.*, 1983; Sheppard *et al.*, 1983; Parellada *et al.*, 1994) or under task conditions (Parellada *et al.*, 1994; Callicott *et al.*, 2000; Hazlett *et al.*, 2000; Walter *et al.*, 2003; Schneider *et al.*, 2007), the general consensus from meta-analyses is that hypofrontality is a characteristic of

the Sz brain during resting-state (Hill *et al.*, 2004) and task (Davidson and Heinrichs, 2003; Glahn *et al.*, 2005) conditions. As well as the functional brain changes in the frontal cortex in Sz patients, meta-analyses have shown altered activation in a number or other areas such as the temporal lobe (Achim and Lepage, 2005; Li *et al.*, 2010; Mwansisya *et al.*, 2017), parietal lobe (Minzenberg *et al.*, 2009) and thalamus (Minzenberg *et al.*, 2009). The direction of the change in these areas appears to be dependent on rest vs task conditions, and dependent on task type under task conditions.

Since there are functional brain changes in Sz, it is unsurprising that there are also alterations of functional connectivity (how different brain areas are functionally connected to each other). Chronic Sz patients tend to have a more 'disconnected' brain (Liu *et al.*, 2008; Lynall *et al.*, 2010; Van Den Heuvel and Fornito, 2014), with frontal cortex (Rotarska-Jagiela *et al.*, 2010; Camchong *et al.*, 2011; Cole *et al.*, 2011; Woodward *et al.*, 2011), thalamic (Tomasi and Volkow, 2014) and fronto-hippocampal (Godsil *et al.*, 2013) pathways being affected. This reduced connectivity is thought to occur before or during the prodromal phase of this disorder, as UHR and FEP individuals also have a more disconnected brain (Benetti *et al.*, 2009; Dauvermann *et al.*, 2013; Schmidt *et al.*, 2014). Therefore it seems unlikely that this is caused by antipsychotic drugs. However, this is not always the case since one study found that patients in the early phases of the illness had hyperconnectivity in the prefrontal cortex (Anticevic *et al.*, 2015). The Default Mode Network (DMN), a network activated during cognitive rest, has also been shown to be abnormal in Sz. This includes abnormal deactivation during task and altered functional connectivity (Pomarol-Clotet *et al.*, 2008; Hu *et al.*, 2017), including hyperconnectivity (Whitfield-Gabrieli *et al.*, 2009; Li, M. *et*

al., 2015) of the DMN in Sz patients. DMN hyperconnectivity has also been found in first-degree relatives of Sz patients (Whitfield-Gabrieli *et al.*, 2009).

Some genetic risk factors for the development of Sz (*CACNA1C, DISC1, ZNF804A, MIR137*) and ASD (*CACNA1C, DISC1*) (Song *et al.*, 2008; Williams *et al.*, 2011; Zheng *et al.*, 2011; Psychiatric and Consortium, 2014; Li, J. *et al.*, 2015) have been found to be associated with altered brain function. For example, the risk variant rs1006737 in *CACNA1C* was associated with altered activation and connectivity in brain regions such as the prefrontal cortex and hippocampus, in Sz patients and healthy controls during episodic and working memory tasks (Erk *et al.*, 2014; Paulus *et al.*, 2014; Zhang *et al.*, 2018). Also, risk variants of *DISC1* have been found to alter brain activity in a number of areas, including the cuneus, cingulate cortex and parietal lobe in healthy controls, Sz patients and bipolar disorder patients (Chakirova *et al.*, 2011). The genetic variant rs1344706 in *zinc finger binding protein 804A (ZNF804A)* has been found to be associated with altered activation in brain areas such as the Dorsolateral Prefrontal Cortex (DLPFC), in healthy controls (Esslinger *et al.*, 2011). Similarly a variant in *microRNA 137 (MIR137)* (risk allele TT) has been found to be associated with disrupted functional connectivity between the DLPFC and the hippocampus in healthy controls (Liu *et al.*, 2014). Furthermore the risk allele TT has been associated with higher activation in the DLPFC, compared to GG/GT alleles, during a working memory task in both Sz patients and controls (Van Erp *et al.*, 2014). Finally, increased *MIR137* polygene risk score was found to increase brain activity in temporal, cingulate and thalamic regions in healthy controls, as working memory load increased during task based fMRI (Cosgrove, Harold*, et al.*, 2017). Collectively these studies provide evidence that genes which increase the risk of

developing Sz and ASD may also play a role in brain function, as altered brain function has been associated with variations in some of these risk genes in healthy controls.

1.12 Altered brain structure, function and connectivity in ASD and comparison to Sz

There are both structural and functional brain changes associated with ASD. Changes in brain structure in a vast number of areas has been observed in ASD (Stigler *et al.*, 2011), including increased brain volume in a number of cerebral brain regions (Palmen *et al.*, 2005). One study found increased total brain volume in children with ASD aged 2-4 years old but not aged 5-16 years old (Courchesne *et al.*, 2001), suggesting changes in volume arise from early brain overgrowth which slows after 4 years old. Differences in grey matter volume in ASD have been found in a number of areas including the frontal lobe. Decreases in frontal lobe volume have been found in children with ASD (Mcalonan *et al.*, 2005; Hardan *et al.*, 2006), while increases have been found in adolescents and adults with ASD (Waiter *et al.*, 2004; Hardan *et al.*, 2006; Hazlett *et al.*, 2006). By contrast, enlargements in the frontal lobe have also been found in children with ASD (Carper *et al.*, 2002; Brun *et al.*, 2009). Reduced grey matter volume in the frontal lobe is found in Sz (Shepherd *et al.*, 2012) and while the same region is implicated in ASD, the direction of change appears to be dependent on age (Waiter *et al.*, 2004; Hardan *et al.*, 2006). Changes in white matter have also been observed in ASD in a number of areas (Stigler *et al.*, 2011), including reduced white matter volume in the corpus callosum (Stanfield *et al.*, 2008; Frazier and Hardan, 2009).

A number of brain areas have been shown to have altered activation in ASD under task and rest conditions. These include functional changes in brain areas associated with social

cognition, such as decreased activation in the fusiform face area (FFA) during a face discrimination task (Schultz *et al.*, 2000). Task based fMRI studies including spatial working memory, inhibition and response monitoring tasks have found decreased activation in the anterior cingulate cortex (ACC), posterior cingulate cortex (PCC), DLPFC and parietal regions, in adults with ASD compared to controls (Luna *et al.*, 2002; Kana *et al.*, 2007; Thakkar *et al.*, 2008). Similarly decreased activation in the DLPFC and ACC have been found in adolescents with ASD during a visuospatial mental rotation task (Silk *et al.*, 2006). During rest conditions, children with ASD have been found to have decreased activation in the frontal and parietal lobe (Ohnishi *et al.*, 2000; Zilbovicius *et al.*, 2000). Some of the brain regions which show altered activation in ASD patients are also implicated in Sz. Frontal lobe hypofunction is commonly found in Sz, and has also observed in ASD individuals under task (Luna *et al.*, 2002; Silk *et al.*, 2006; Kana *et al.*, 2007; Mitelman *et al.*, 2018) and rest (Ohnishi *et al.*, 2000) conditions. Also, activation of the temporal and parietal lobes has been found to be decreased in ASD during task based functional imaging (Zilbovicius *et al.*, 2000; Kana *et al.*, 2007). These brain areas also show altered activation in Sz, however the direction of change appears to be dependent on the task used (Achim and Lepage, 2005; Minzenberg *et al.*, 2009; Li *et al.*, 2010; Mwansisya *et al.*, 2017).

In general findings from brain functional connectivity studies in ASD have found that long range pathways show hypoconnectivity, and short range pathways show hyperconnectivity (Rane *et al.*, 2015). Evidence for reduced functional connectivity in frontal-posterior pathways is reported in ASD. This includes both task based (Just *et al.*, 2007; Koshino *et al.*, 2008; Kana *et al.*, 2009; Schipul *et al.*, 2012) and resting-state (Cherkassky *et al.*, 2006; Abrams *et al.*, 2013; Di Martino *et al.*, 2013) functional brain imaging studies. Other regions

shown to have reduced functional connectivity in ASD include amygdala, frontal, temporal and ACC regions under task conditions (Agam *et al.*, 2010; Monk *et al.*, 2010). By contrast hyperconnectivity has also been observed in ASD including frontal, temporal, and amygdala areas during task (Shih *et al.*, 2010; Murphy *et al.*, 2012) and in areas such as the ACC and temporal lobe during rest conditions (Monk *et al.*, 2009). Altered activity in DMN has been observed in ASD, including failure to deactivate the network during task conditions (Kennedy *et al.*, 2006; Kennedy and Courchesne, 2008). Some findings of altered connectivity in ASD are similar to those found in Sz, for example hypoconnectivity in frontal regions and altered DMN activation (Pomarol-Clotet *et al.*, 2008; Lynall *et al.*, 2010; Hu *et al.*, 2017).

Recently a small number of structural and functional brain imaging studies have directly compared ASD, Sz, and healthy control subjects. One study investigated both grey and white matter across the whole brain, and found that the same brain areas were affected in both ASD and Sz (including frontal and temporal regions) but the effects were opposite in direction. ASD patients had increased grey matter and decreased white matter, and Sz patients had decreased grey matter and increased white matter, compared to controls (Mitelman *et al.*, 2017). Functional brain imaging studies comparing these groups have generally found that while the cognitive profile of ASD and Sz patients appear similar, the underlying brain activity differs between the two disorders. One study found that during a social judgement task activation in amygdala, cerebellar, fusiform and temporal regions was significantly increased in Schizotypal personality disorder compared to ASD, and activation of these areas in the healthy control group lay between the two patient groups (Stanfield *et al.*, 2017). Another study compared Sz, ASD and healthy subjects using PET imaging during a

serial verbal learning task (Mitelman *et al.*, 2018). They found that, in relation to controls, the Sz and ASD groups showed both convergent and divergent effects on function in a number of brain areas, whilst showing no difference in performance of the task. For instance, in relation to controls, both Sz and ASD groups had decreased frontal lobe and increased hippocampal metabolic rate. However, in the anterior cingulate and hypothalamus metabolic rate was higher than controls in ASD, and lower than controls in Sz. Finally, another study compared these three groups using resting-state connectivity fMRI and machine learning. They again identified both convergent and divergent features in brain networks between the two disorders using graph theory, including in the DMN, salience network and motor network (Mastrovito *et al.*, 2018).

In summary, a number of structural, functional and connectivity brain changes are found in ASD, some of which affect the same brain regions that are implicated in Sz. However these brain changes do not always occur in the same direction in the two disorders, which is supported by studies directly comparing ASD and Sz patient groups. Furthermore brain imaging research in ASD is underdeveloped compared to that of Sz. Therefore further research is required to draw strong conclusions about changes in brain structure, function and connectivity in ASD, and how these changes compare to those seen in Sz.

1.13 Schizophrenia: a disorder of accelerated ageing?

Both Sz and ASD are regarded as neurodevelopmental disorders, due to a vast literature supporting the disruption of developmental processes in both disorders. Since the emergence of symptoms in Sz comes later in life than those in ASD, it has been suggested that developmental dysfunction, which may be common to both disorders, is more severe in

ASD than in Sz, and that compensatory mechanisms may occur in early life in Sz which prevent or postpone the emergence of symptoms (Birnbaum and Weinberger, 2017). However there are multiple lines of evidence that although are weak individually, when combined point towards the potential contribution of accelerated ageing in Sz, following an initial developmental pathology.

Neurodevelopmental theory of Sz

Support for the neurodevelopmental model of Sz comes from a variety of studies including, but not limited to, risk factors for Sz (Brown, 2011), abnormalities in childhood development (Done *et al.*, 1994; Baum and Walker, 1995; Hollis, 1995; Cannon and Murray, 1998; Rutter *et al.*, 2006), evidence for a prodromal phase in Sz (Agius *et al.*, 2010; Chung and Cannon, 2015), unaffected sibling studies (Egan *et al.*, 2001; Gogtay *et al.*, 2007; Gogtay and Rapoport, 2008), the synaptic pruning model (Keshavan *et al.*, 1994; Mcglashan and Hoffman, 2000) and multiple risk genes for Sz being involved in brain development (Birnbaum and Weinberger, 2017).

Many of the environmental risk factors that have been highlighted for Sz are insults that occur in early life. Prenatal infection, pre-natal famine, birth complications and childhood trauma (Brown, 2011) could cause abnormal development in an individual, and are established risk factors for Sz. However, other risk factors such as migration (Cantor-Graae and Selten, 2005) and cannabis use (Gage *et al.*, 2016), usually occur later in life, yet are still associated with increased risk for the disorder. Similarly many environmental risk factors for ASD, which is a neurodevelopmental disorder, occur early in life (reviewed in section 1.3) (Roberts *et al.*, 2007; Sandin *et al.*, 2012; Christensen *et al.*, 2013; Rai *et al.*, 2013; Volk *et al.*, 2013).

It has been found that individuals that later develop Sz have often shown delayed childhood milestones, including delayed speech or motor skills, which provides further support for the neurodevelopmental model of the disorder (Done *et al.*, 1994; Hollis, 1995). Other childhood indications include underperformance in childhood school tests and neuropsychological testing, and social abnormalities (Done *et al.*, 1994; Jones *et al.*, 1994; Baum and Walker, 1995; Cannon and Murray, 1998; Davidson *et al.*, 1999; Rossi *et al.*, 2000; Cannon *et al.*, 2002; Fuller *et al.*, 2002; Reichenberg *et al.*, 2010). In particular deficits in working memory and processing speed, that are core cognitive deficits in Sz (Lee and Park, 2005; Dickinson *et al.*, 2007; Barch and Smith, 2008; Forbes *et al.*, 2009; Woolard *et al.*, 2010; Karbasforoushan *et al.*, 2015), were found to be present in children who later developed Sz symptoms (Reichenberg *et al.*, 2010). These cognitive findings in children were not observed in individuals who later went on to develop major depression disorder. However, both the environmental insults discussed and many of the possible tell-tale signs in childhood are not specific to Sz, and can also precede diagnosis of other psychiatric disorders.

Neuroimaging studies into COS, UHR individuals and unaffected siblings of Sz patients provide further support for the neurodevelopmental theory. Studies into COS and UHR individuals have shown that brain changes associated with Sz are present in both these groups (Gogtay and Rapoport, 2008; Chung and Cannon, 2015; Ordonez *et al.*, 2016; Chung *et al.*, 2017), suggesting that these changes may occur in the developing brain, prior to the diagnosis. A meta-analysis of neuroimaging studies in unaffected siblings of adult-onset Sz patients, found that they too showed ventricle enlargement and reduced hippocampal volume (Boos *et al.*, 2007). Also, siblings of COS patients can have childhood frontal and

temporal deficits in grey matter volume, that seem to balance out during adolescence (Gogtay *et al.*, 2007). It has been suggested that there is perhaps a genetic component that causes grey matter differences in childhood (first hit), which can be overcome in healthy siblings during adolescence, to give them resistance against secondary genetic or environmental insults (second hit) which then drives a pathway towards Sz symptoms in the affected sibling (Rapoport *et al.*, 2012). Support for this comes from evidence that genetic risk factors for Sz impact on brain structure (Gurung and Prata, 2015).

The synaptic pruning model of Sz also supports the idea of a neurodevelopmental cause of the disorder. It suggests that something has gone wrong with brain maturation processes during adolescence, whereby synaptic connections in the brain are remodeled, removed or reorganized (Feinberg, 1982; Rakic *et al.*, 1986; Zecevic *et al.*, 1989; Huttenlocher and Dabholkar, 1997; Giedd *et al.*, 1999), which would roughly coincide with age of Sz onset (Keshavan *et al.*, 1994). It is thought that, during this developmental time point, excitatory synapses that are pruned (Rakic *et al.*, 1986; Zecevic *et al.*, 1989) creating a balance between excitatory and inhibitory synapses in the adult brain (Selemon and Zecevic, 2015). If this does not occur in the correct way it could lead to an imbalance in excitatory and inhibitory synapses, which has been implicated in Sz (Lewis *et al.*, 2005; Gao and Penzes, 2015; Selten *et al.*, 2018). However a recent study has questioned the synaptic pruning hypothesis. The study found that the number of large spines in layer 3 neurons in the auditory cortex did not differ between Sz patients and controls at post mortem, but there were less new or small spines in Sz patients (Macdonald *et al.*, 2017). This suggests that the deficit may be in synapse generation rather than over-pruning.

Studies using post-mortem brain tissue have found that many genetic variations, in both coding and non-coding DNA, that are associated with increased risk of developing Sz are expressed prenatally and in placental tissue, but not postnatally or in adult brain (Birnbaum and Weinberger, 2017), providing further evidence for a developmental origin of Sz.

Accelerated ageing hypothesis

There is evidence that supports accelerated ageing both inside (neuroimaging studies) (Borgwardt *et al.*, 2009) and outside (oxidative stress and telomere length studies) of the brain (Kao *et al.*, 2008; Dietrich-Muszalska and Olas, 2009; Fernandez-Egea *et al.*, 2009; Jorgensen *et al.*, 2013) in Sz patients. The accelerated ageing hypothesis links with many characteristics of Sz. For instance, the often progressive nature of Sz symptoms leading to a chronic disorder, brain changes and cognitive deficits in Sz are similar to normal ageing, the increase in prevalence of age-related disease (e.g. diabetes) in Sz (Bai *et al.*, 2013), and the shortened life span of Sz patients (Laursen *et al.*, 2012; Laursen *et al.*, 2014). Recently it has been suggested that Sz is a disorder of segmental ageing. This means only certain aspects (pathways or tissues) undergo accelerated ageing, rather than a ubiquitous (all tissues) acceleration of ageing (Kirkpatrick and Kennedy, 2018). Since Sz is such a heterogeneous disorder it has been suggested that there may be a subgroup within the Sz population that show segmental ageing, and this may explain the mixed findings of the various measures of ageing in Sz patients (Kirkpatrick and Kennedy, 2018).

Support for the accelerated ageing hypothesis in Sz: Accelerated brain ageing

It has been proposed that processes of brain ageing are accelerated around the time of symptom onset and that this then slows over time. This suggests there is only a relatively

short period of time when there is accelerated brain ageing, and that the first episode/onset period seems to be more important than the age of the person (Borgwardt *et al.*, 2009). This may confound findings in post mortem studies as the brain of an aged person with Sz would look similar to an age matched control. This would be due to accelerated ageing not occurring at a constant rate and slowing/normalising over time (Borgwardt *et al.*, 2009). Unlike neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, the brains of Sz patients do not have biological markers of cell death or protein plaques or tangles. Nor does the Sz brain have a strict pattern of atrophy, which may explain the more varied psychiatric symptoms that present in Sz, compared to neurodegenerative diseases, and the greater variation in symptoms from patient to patient.

For many sufferers of Sz, symptoms worsen over time and it becomes a chronic disorder (Van Os and Kapur, 2009). This suggests that there may also be a progressive biological mechanism which underlies worsening symptoms. Longitudinal structural brain imaging studies in Sz patients have shown ventricular enlargement and volume reductions in areas such frontal lobe, temporal lobe and hippocampus. These brain changes have been observed within a few years of first episode diagnosis, and can be associated with poor outcome and symptom severity (Lieberman *et al.*, 2001; Mitelman *et al.*, 2007; Nakamura *et al.*, 2007). However these findings are also confounded by administration of antipsychotic medication.

Parallels between Sz and normal brain ageing

Similarities have been drawn between the brain of chronic Sz patients and the aged brain of non-schizophrenic individuals, in terms of structural and functional changes. In normal ageing, there is both global and region specific shrinkage of cortical grey and white matter

(Lockhart and Decarli, 2014), found using structural imaging studies. Decarli *et al.* found that in terms of regional volume decrease, the frontal lobe volume reduced the most with age (12% between 30-90 years of age) (Decarli *et al.*, 2005). This frontal volume reduction is thought to play a part in age-related cognitive decline (Salthouse, 2012; Harada *et al.*, 2013), since the region is associated with cognition and executive function. Other areas of reduced volume with age, in normal (non-Sz) brains, included the temporal lobe, parietal lobe and hippocampus (Decarli *et al.*, 2005). These findings, along with frontal lobe reduction, were supported by other cross sectional and longitudinal studies (Jernigan *et al.*, 1991; Jernigan *et al.*, 2001; Du *et al.*, 2006; Pfefferbaum *et al.*, 2013; Taylor, J. L. *et al.*, 2014). Similar to the effects of normal ageing on the brain, the brains of individuals with chronic Sz show reduced global volume/ventricular enlargement (Johnstone *et al.*, 1976; Wright *et al.*, 2000; Horga *et al.*, 2011; Haijma *et al.*, 2013) and region specific grey matter reduction in frontal, temporal and hippocampal regions (Shepherd *et al.*, 2012). These changes in grey matter are also prominent in prodromal Sz (Vita *et al.*, 2012; Ziermans *et al.*, 2012; Cannon *et al.*, 2015; Chung *et al.*, 2017), suggesting that structural brain changes in Sz that are similar to normal brain ageing may precede diagnosis. This is in line with Borgwardt *et al.*'s theory of accelerated brain ageing in Sz, as they suggested that the process is most severe around the time of symptom onset then declines thereafter (Borgwardt *et al.*, 2009).

There are also functional changes that occur in the normal ageing brain. In general, task based fMRI studies have found increased activation in the frontal and parietal brain regions with age in a number of cognitive tasks, which may be a compensatory mechanism to maintain function (Kennedy *et al.*, 2015; Rieck *et al.*, 2017). Since brain activation changes with age, it is unsurprising that connectivity undergoes changes also. Studies have shown

that the resting state networks in the brain (Default Mode Network) are more disconnected in old participants compared to young participants (Andrews-Hanna *et al.*, 2007; Wang *et al.*, 2010; Onoda *et al.*, 2012; Geerligs *et al.*, 2015). The frontal cortex in the Sz brain is thought to be functionally underactive (Davidson and Heinrichs, 2003; Hill *et al.*, 2004; Glahn *et al.*, 2005) which contrasts with the normal aged brain where this region is reported to be overactive (Li, H. J. *et al.*, 2015). However both normal (non-Sz) aged brains (Andrews-Hanna *et al.*, 2007; Wang *et al.*, 2010; Onoda *et al.*, 2012; Geerligs *et al.*, 2015) and chronic Sz brains (Benetti *et al.*, 2009; Schmidt *et al.*, 2014) are thought to have decreased connectivity, again drawing similarities between the two cases.

Some studies have systematically compared Sz patients with aged individuals in terms of brain structure and cognitive ability. Convit *et al.* systematically compared structural brain images of Sz patients (n=9) with young (n=9) and old (n=9) control subjects. The young participants were aged between 25-52 years old, and the old control group were aged between 58-76 years old. They found that Sz patients and the old control group had significantly smaller superior and orbital frontal gyri than young controls, but were not significantly different from each other (Convit *et al.*, 2001). Cognitive deficits in Sz can be likened to cognitive decline in normal ageing. For instance the most prominent cognitive deficit in Sz, slowed cognitive processing speed (Dickinson *et al.*, 2007; Woolard *et al.*, 2010; Karbasforoushan *et al.*, 2015), is also seen in healthy ageing (Salthouse, 1996). One study compared Sz patients to age-matched and elderly control participants. They found that elderly control participants actually performed better than Sz patients on a number of cognitive tasks, which involved processing speed and working memory (Loewenstein *et al.*, 2012).

Cross sectional studies of Sz patients across different ages can provide information about how brain changes that occur in Sz compare to normal ageing. A cross sectional study using a large sample size of over 150 Sz patients and controls aged 16-70 years old, found that not only did Sz patients have decreased total grey matter, decreased frontal grey and white matter and increased ventricular volume, but the Sz patients had a steeper decline in these changes when compared to controls (Hulshoff Pol *et al.*, 2002). A similar study found that Sz patients had steeper age-related decline in grey matter volume in the superior temporal cortex than controls (Nenadic *et al.*, 2012). They also found that when patients were divided into groups by symptom type, the paranoid group had a steeper age-related decline in grey matter in superior temporal cortex and inferior frontal gyri, whereas the negative symptom group showed little effect on age-related changes in superior temporal cortex. In contrast to these groups, the disorganised symptom group had higher age-related grey matter loss in the lateral cerebellum (Nenadic *et al.*, 2012). Another smaller cross sectional study of chronic never medicated Sz patients, aged 33-59 years old in rural China, showed increased age-related decline of frontal cortical thickness but slowed loss of parietal cortical thickness when compared to controls (Zhang, W. *et al.*, 2015). Another cross sectional study showed that Sz patients did not show increased age-related grey matter loss but did show increased age-related white matter loss, and also showed increased grey matter loss in the early stage of the disorder (Bose *et al.*, 2009). Further studies support progressive loss of brain white matter in Sz compared to controls (Mori *et al.*, 2007; Friedman *et al.*, 2008; Kochunov *et al.*, 2013; Skudlarski *et al.*, 2013). A recent cross sectional study with a large sample size found that grey matter volume loss was accelerated in Sz up to middle aged and white matter loss was accelerated after 35 years old as compared to controls (Cropley *et al.*, 2017). By contrast some studies have found no evidence for accelerated ageing in Sz in both grey

(Chiapponi *et al.*, 2013) and white (Voineskos *et al.*, 2010) matter. The findings from cross sectional structural brain imaging studies provide mixed information about progressive brain changes in Sz, although most generally agree that some form of brain volume reduction does occur over time compared to controls. Although cross sectional studies are useful, longitudinal studies provide a more powerful method of tracking progressive brain changes in Sz.

Longitudinal structural neuroimaging studies provide information on how the Sz brain changes over time, and how this compares to healthy controls. Longitudinal studies in patients in which the first brain scan was in FEP, with follow up scans between 8 months and 10 years, have shown progressive brain changes but with conflicting findings. Some studies found patients had progressive ventricular enlargement at 1 year follow up (Cahn *et al.*, 2002) and 10 year follow up (Delisi *et al.*, 2004), whereas others with follow up scans at 8 months (Puri *et al.*, 2001) and 1-2 years (Degreef *et al.*, 1991) found no significant change. In some studies, follow up patients were also found to have decreased volume in frontal and temporal cortex (Gur *et al.*, 1998; Ho *et al.*, 2003; Kasai *et al.*, 2003; Whitford *et al.*, 2006), whereas other studies found no change in temporal cortex (Delisi *et al.*, 2004).

The findings from longitudinal studies in patients which were first scanned in the chronic stage of Sz are more consistent, as they mostly agree that there is progressive ventricular enlargement at the 1-10 year follow up scans (Kemali *et al.*, 1989; Woods *et al.*, 1990; Davis *et al.*, 1998; Mathalon *et al.*, 2001; Saijo *et al.*, 2001; Thompson *et al.*, 2001; Vidal *et al.*, 2006). There have also been findings of decreased volume in frontal, temporal, thalamic and hippocampal brain areas (Jacobsen *et al.*, 1998; Mathalon *et al.*, 2001; Thompson *et al.*, 2001; Vidal *et al.*, 2006; Hulshoff Pol and Kahn, 2008). A meta-analysis analysis was

performed using 27 longitudinal volumetric MRI studies consisting of 928 Sz patients and 867 controls, with follow up scans between 1-10 years (Olabi *et al.*, 2011). It was found that Sz patients showed significantly greater loss of whole brain volume, frontal lobe volume, whole brain grey matter, and enlargement of lateral ventricles over time compared to controls. The findings from longitudinal studies in Sz patients, particularly in chronic patients, do not support Borgwardt *et al.'s* theory of accelerated brain ageing in Sz. Their theory postulates that accelarated brain ageing occurs at the highest rate around the time of symptom onset and slows over time (Borgwardt *et al.*, 2009). If this was the case there should be little evidence for progressive brain changes in longitudinal studies of patients which have baseline scans during the chronic phase of Sz, which is not the case. There is some evidence from longitudinal brain imaging studies that increased progressive loss of grey matter occurs in the early phase of Sz, and is not seen in chronic patients. However this is brain region specific and includes the fusiform gyrus and insular cortex (Takahashi and Suzuki, 2018). These findings may be in line with the theory of segmental, rather than ubiquitous, accelerated ageing in Sz (Kirkpatrick and Kennedy, 2018); whereby the rate of accelerated ageing may differ dependent on brain region.

Two studies have used machine learning to predict brain age and a diagnosis of Sz. Schnack *et al.* performed a longitudinal structural MRI study using a large sample size where subjects were scanned twice or more. The study utilised 341 Sz patients and 386 controls aged 16-67 at baseline. 378 of the subjects had 1 or more follow up scans at between 1-13 years. The findings of this study support the theory of accelerated ageing in Sz, since the model predicted the brain age of individuals with Sz to be around 3 years older than the actual age of the individual (Schnack *et al.*, 2016). The study also supported the notion that accelerated

ageing is highest at the time of symptom onset and soon after (Borgwardt *et al.*, 2009), which in this study was after 1 year (Schnack *et al.*, 2016). Another machine learning study predicted Sz patient brains to be 5 years older, and an UHR group to be nearly 2 years older, than their actual age (Koutsouleris *et al.*, 2014). The model was also able to predict negative and disorganised symptoms in Sz patients. However patients with major depression and bipolar disorder were also predicted to be 3-4 years older, suggesting that accelerated brain ageing may not be specific to Sz.

The effects of antipsychotic medication on brain structure cannot be ruled out, since Sz patients that are studied are often not antipsychotic free. Chronic exposure (8 weeks) to antipsychotic drugs in rats has been shown to cause a decrease in whole brain volume that is mainly driven by the decreased frontal lobe volume (Vernon *et al.*, 2011), however these brain changes have been shown to normalise following a period of antipsychotic drug withdrawal (8 weeks) (Vernon *et al.*, 2012). Furthermore there is evidence from non-human primate studies that antipsychotic medication causes decreased brain volume (Dorph-Petersen *et al.*, 2005; Konopaske *et al.*, 2007; Konopaske *et al.*, 2008). It has also been suggested that antipsychotic drugs may affect global (Fusar-Poli *et al.*, 2013; Veijola *et al.*, 2014; Vita *et al.*, 2015; Torres *et al.*, 2016) or regional (Smieskova *et al.*, 2009; Torres *et al.*, 2013) changes in brain volume in Sz patients, but this effect has not been agreed upon in the literature (Roiz-Santianez *et al.*, 2015; Lawrie, 2018).

In addition to antipsychotic medication there are a number of other potential confounding variables in neuroimaging studies that may account for the accelerated ageing-like brain changes seen in Sz. For example cannabis abuse (Rapp *et al.*, 2012; Malchow *et al.*, 2013) and unhealthy lifestyle e.g. obesity (Bischof and Park, 2015). There is also a need for better

matched control subjects who also have unhealthy lifestyles (involving poor diet and exercise and similar drug and alcohol use) which may then sufficiently control for these variables (Borgwardt *et al.*, 2009). There is some difficulty in following up experimental participants after long periods of time, which means some of the neuroimaging studies have relatively small sample sizes. This can mean that they lack sufficient statistical power, making generalisation of findings to the larger population of Sz patients difficult (Borgwardt *et al.*, 2009).

Support for the accelerated ageing hypothesis in Sz: oxidative stress and telomere length

Alongside neuroimaging studies, there are other experimental approaches that support the hypothesis of accelerated ageing in Sz. There are a number of studies which support the idea of peripheral accelerated ageing in Sz, in line with the oxidative stress theory of ageing. This theory suggests that ageing occurs due to an imbalance of reactive oxygen species and endogenous anti-oxidants. Thus a build-up of reactive oxygen species leads to cell damage, which characterises what we call ageing (Harman, 2009). A number of markers of oxidative stress can be measured. Some of these markers been found to be raised in Sz patients relative to age matched controls, in a variety of cells and tissues including blood cells and brain (Do *et al.*, 2000; Herken *et al.*, 2001; Yao *et al.*, 2004). In further support of this theory, it has been shown that patients given antioxidants in addition to antipsychotic medication had reduced clinical symptoms (Berk *et al.*, 2008). A number of molecules are known to be abundant in aged cells that have been found to be higher in Sz patients compared to healthy controls, including platelet carbonyl proteins (Dietrich-Muszalska and Olas, 2009), red blood cell thiobarbituric acid reactive species (Dietrich-Muszalska *et al.*, 2005) and urinary 8-oxo-

7,8-dihydro-2'-deoxyguanosine (Jorgensen *et al.*, 2013). However in similarity to neuroimaging studies, it is difficult to determine whether Sz as a disease causes increased oxidative stress or whether life style factors such as antipsychotic medication (Tsai *et al.*, 2013), obesity (Zhang, Y. *et al.*, 2015), smoking (Mesaros *et al.*, 2012) and recreational drug use (Lopez-Pedrajas *et al.*, 2015) are to blame.

Another potential marker of ageing is telomere shortening. Telomeres are structures which protect the ends of chromosomes and are shortened each time the cell divides, and so have been associated with ageing (Von Zglinicki and Martin-Ruiz, 2005; Mikhelson and Gamaley, 2012). There have been mixed findings from studies comparing leukocyte telomere length from blood samples in Sz patients and controls. Studies have reported shorter (Kao *et al.*, 2008; Yu *et al.*, 2008; Fernandez-Egea *et al.*, 2009; Kota *et al.*, 2015; Galletly *et al.*, 2017), longer (Nieratschker *et al.*, 2013) or the same length (Mansour *et al.*, 2011; Malaspina *et al.*, 2014) telomeres in Sz patients as compared to controls. These findings are unclear and again may be influenced by various life-style factors present in Sz patients. However a meta-analysis found Sz patients to have shorter leukocyte telomeres compared to controls, and suggested that a number of factors could contribute to this finding, including antipsychotic medication, oxidative state, metabolic syndrome, smoking, and paternal age (Polho *et al.*, 2015). The finding of shortened leukocyte telomeres in Sz is also supported by a more recent meta-analysis (Russo *et al.*, 2018) which found that Sz patients had shorter telomeres than controls in an under 50 years old group, but not in an over 50 years old group. The study, in contrast to the previous meta-analysis, also found that a diagnosis of Sz had a greater effect on telomere shortening than other factors such as smoking and alcohol consumption. Shortened leukocyte telomeres have also been found in UHR individuals

(Maurya *et al.*, 2017). A recent study also found that shortened telomeres in Sz was associated with an increase in an age-related inflammatory marker (CCL11), reduced grey matter volume and poor memory (Czepielewski *et al.*, 2018), which supports the accelerated ageing hypothesis. Furthermore another recent study found that although telomere length was decreased in patients compared to controls, there was no difference between patients and their unaffected siblings (Czepielewski *et al.*, 2016). This suggests that telomere length can be inherited and is not specific to a diagnosis of Sz. Shortened telomeres have also been found in brain tissue samples, specifically in white matter of the of the superior temporal gyrus of Sz patients compared to controls. However, this finding was not replicated in grey matter of the superior temporal gyrus, or in grey or white matter of the medial frontal gyrus (Van Mierlo *et al.*, 2017). This suggests that telomere shortening in Sz brain tissue is region and matter specific, which may provide support for the theory of segmental, rather than ubiquitous, accelerated ageing in Sz (Kirkpatrick and Kennedy, 2018). Finally, the reliability of telomere shortening as a biomarker of ageing has been questioned, and it had been suggested that more longitudinal studies are needed for accurate interpretation of findings using this measure (Mather *et al.*, 2011).

DNA methylation state from information gathered from over 350 promotors has been suggested to give a reliable indication of cellular ageing (epigenetic clock) (Horvath, 2013; Chen, B. H. *et al.*, 2016). DNA methylation findings from Sz patient brain tissue samples so far suggest that there is no evidence for accelerated ageing in the superior temporal gyrus (Mckinney *et al.*, 2017) or frontal cortex (Voisey *et al.*, 2017), since DNA methylation age strongly correlated with chronological age. However a number of issues with these studies were highlighted by Fernandez-Egea and Kirkpatrick. These issues include the different age

ranges of samples in control and Sz groups, and that markers from the epigenetic clock do not support accelerated ageing in known disorders of accelerated ageing, such as Hutchinson–Gilford syndrome (Fernandez-Egea and Kirkpatrick, 2017).

1.14 Aims

The aim of this project was to bring together two areas of Sz research; genetic risk factors for Sz, in particular *Neurexin1*, and the accelerated ageing hypothesis. The two main research questions were: (1) does *Neurexin1* heterozygosity, modelling mutations found in Sz and ASD, bring about Sz or ASD relevant phenotypes and endophenotypes in behaviour, cognition and brain function, and are these changes temporally regulated across adult development? and (2) Does *Neurexin1* dysfunction affect life span and behavioural senescence? To address these questions two different model organisms have been used, the mouse (*Mus musculus*) and the fruit fly (*Drosophila melanogaster*). The mouse model was used to investigate research question (1) and the fly model was used to investigate research question (2).

1.15 Experiments conducted in the mouse

The mouse model

The mouse model used had a heterozygous deletion in *Neurexin1α* (*Nrxn1α* Hz) removing the first exon of the *Nrxn1α* gene (Dachtler *et al.*, 2014), which had been generated from *α*-Neurexin triple KO mice (Missler *et al.*, 2003) (see Materials and Methods section 2.1.1 for details). Male and female *Nrxn1α* Hz and Wt mice were tested at 3, 6, 9, and 12 months old

in a between-groups design, to test how *Nrxn1α* heterozygosity affects behaviour, cognition and cerebral metabolism during adult development.

Experiment 1: The impact of *Neurexin1α* heterozygosity on locomotor activity and anxiety-like behaviour

The open field (OF) test was used to investigate response to a novel environment by measuring locomotor activity and anxiety-like behaviours. In this test the mouse was placed into a novel circular arena and video recorded for 15 minutes. Automated tracking software provided information on locomotor parameters such as distance moved, velocity and frequency of locomotor activity. Rodent locomotor hyperactivity has previously been used as a model of positive symptoms in Sz (Van Den Buuse, 2010), since subcortical hyperdopaminergia is linked to both positive symptoms in Sz patients (Uchida *et al.*, 2011; Harro, 2015) and hyperlocomotor activity in rodents (Creese and Iversen, 1975; Kelly *et al.*, 1975; Antoniou *et al.*, 1998). Therefore measuring locomotor activity can provide a proxy measure of dopamine hyperfunction, and potentially positive symptomatology, which cannot be directly assessed in animals. Furthermore dopaminergic dysfunction (Hellings *et al.*, 2017; Paval, 2017), and hyperactivity is also implicated in ASD, since ADHD is a common comorbidity of ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012; Antshel *et al.*, 2016).

The OF test also measured duration and number visits to the central zone providing an indication of anxiety-like behaviour. This is due to mice naturally spending more time in the outside of the OF arena compared to the inside (Thigmotaxis). Anxiolytic (anxiety reducing)

drugs have been shown to increase time spent in the central zone of the OF arena, whereas anxiogenic (anxiety inducing) drugs have been shown to reduce it (Simon *et al.*, 1994).

Experiment 2: The impact of *Neurexin1α* heterozygosity on learning and memory as measured by the Novel Object Recognition Task (NORT)

The NORT protocol used here tested short term recognition learning and memory. The protocol consisted of a one hour inter-trial interval between the acquisition phase, where two identical objects were presented to the mouse, and the test phase, where one object from the acquisition phase (familiar object) and one novel object was presented to the mouse. Mice naturally explore novel objects more than familiar objects (Frisch *et al.*, 2005; Kruk-Slomka *et al.*, 2014), and so if a mouse spends more time investigating the novel object, rather than the familiar object, this suggests it has formed a memory of the familiar object. Deficits in object recognition memory have been observed in both Sz (Danion *et al.*, 1999) and ASD patients (Blair *et al.*, 2002). A deficit in the NORT found in *Nrxn1α* Hz mice would suggest that *Nrxn1α* plays a role in influencing brain regions and circuits required for recognition memory.

Experiment 3: The impact of *Neurexin1α* heterozygosity on cognitive flexibility

The Cognitive Flexibility Task (CFT) was used in the current study as a rodent alternative to the WCST, and has been adapted from other rodent tasks of cognitive flexibility (Young *et al.*, 2010). Mice were required to dig in bowls of sand to retrieve sugar pellet rewards based on rules about odour and the location of the bowls. The CFT was a 4 phase task which required the functioning of different cognitive domains. This included associative learning,

rule generalisation, reversal learning and shifting attention. These cognitive domains are associated with different brain circuitry, and so this task was used to dissect whether these cognitive domains and the underlying circuitry are deficient in *Nrxn1α* Hz mice. Both Sz and ASD patients have difficulty with cognitive flexibility. Perseverative responding to an old rule despite receiving negative feedback, is seen in both disorders using tasks such as the WCST (reviewed in section 1.9 and 1.10). In ASD, cognitive rigidity forms part of the core symptoms of the disorder in the form of restrictive interests and repetitive behaviours (reviewed in section 1.2).

Experiment 4: The impact of *Neurexin1α* heterozygosity on cerebral metabolism

^{14}C-2-Deoxyglucose (^{14}C-2DG) functional brain imaging measures whole brain cerebral metabolism in the resting, rather than task based, brain state (Dawson *et al.*, 2013; Dawson *et al.*, 2015). ^{14}C-2DG is a radioactive analogue of glucose that is taken up into the brain. Therefore, the more metabolically active a brain area is, the more glucose it requires, and so the more ^{14}C-2DG accumulates in that region. This technique allowed detection of any differences in regional cerebral metabolism between *Nrxn1α* Hz and Wt mice. Functional changes have been highlighted in a number of brain regions in both Sz and ASD, some of which are common to both disorders (reviewed in Section 1.1 and 1.2). Any changes in cerebral metabolism were assessed for their translational relevance to those seen in Sz and ASD, and relevance to any behavioural changes found in the *Nrxn1α* Hz mice.

1.16 Experiments conducted in the fly (*Drosophila melanogaster*)

The fly model

To enable investigation of the effects of *Neurexin1* on ageing, in line with the accelerated ageing hypothesis of Sz, a *Drosophila melanogaster* model was used. The P{XP} Nrx^{d08766} mutant fly used had a homozygous p-element insertion into the single *Drosophila Neurexin1* gene *(Nrx-1)* altering expression of the gene. According to the published literature the P{XP}Nrx^{d08766} flies have reduced expression of *Nrx-1* evidenced by reduced *Nrx-1* protein levels (Zeng *et al.*, 2007; Tong *et al.*, 2016). This made the P{XP}Nrx^{d08766} fly a suitable candidate to model *Nrx-1* hypofunction, in relation to heterozygous deletions in *NRXN1α* which increase the risk of developing Sz and ASD.

Experiment 5: Characterising altered *Nrx-1* expression in the P{XP}Nrx^{d08766} fly

Following backcrossing of the P{XP}Nrx^{d08766} flies to a w^{1118} genetic background (experimental controls), *Nrx-1* expression was characterised by qPCR and western blotting to investigate the effect of the p-element insertion. Surprisingly, an increase in expression of *Nrx-1* at both the mRNA and protein level was found. Although this finding contrasted with those in the literature (Zeng *et al.*, 2007; Tong *et al.*, 2016) and meant the P{XP}Nrx^{d08766} flies no longer provided a suitable model for heterozygous deletions in *NRXN1α,* the flies were used to characterise the effects of *Nrx-1* hyperfunction on life span, behavioural senescence and sleep.

Experiment 6: The impact of *Nrx-1* hyperfunction on life span and behavioural senescence

Flies are relatively short lived compared to mice, making them a suitable model for testing effects on life span. Life span analysis was used to look for differences in P{XP}Nrx^{d08766} *Nrx-*

1 hypermorph flies compared to w^{1118} control flies, to investigate the effects of *Nrx-1* overexpression on ageing. However, life span analysis does not provide information about how flies function as they age. Therefore, Exploratory Walking (EW) behaviour was analysed in a cross sectional design across the life span, to test for differences in behavioural senescence in P{XP}Nrx^{d08766} flies. In this test flies are placed into a novel circular arena and video recorded for 15 minutes. Automated tracking software provides information on a number of locomotor parameters. Flies show a stereotypical pattern of behaviour in the EW assay. Young flies actively explore the edge of the arena (Soibam *et al.*, 2012) and spend little time in the centre, and old flies show a random walking pattern and spend more time in the centre (Ismail *et al.*, 2015) (Figure 1.4). Walking distance, walking velocity, walking duration, rotation frequency and frequency of visits to the central zone decrease with age, while duration of time spent in the central zone of the arena increases with age. The EW parameters provide information about both Central Nervous System (CNS) and neuromuscular ageing. Locomotor behaviour, which is controlled by the fly brain (Strauss *et al.*, 1992; Strauss and Heisenberg, 1993; Martin *et al.*, 1998; Strauss, 2002; Besson and Martin, 2005), provides an indirect measure of brain function and how it declines with age. However measuring locomotor behaviour is confounded by peripheral effects such as neuromuscular functioning, which also shows age-related decline. Duration and frequency of time spent in the central zone, and rotation frequency parameters are thought to be indicative of 'decision making' behaviours controlled by the fly brain (Strauss, 2002; Besson and Martin, 2005; Serway *et al.*, 2009; Ismail *et al.*, 2015), and are less confounded by peripheral effects. Investigating multiple EW parameters allows determination of whether effects are due to alterations in CNS ageing or neuromuscular ageing. Taken together, life span analysis and EW findings allow determination of whether the P{XP}Nrx^{d08766} flies are

long-lived, and whether this is accompanied by improved function during ageing. A previous study indicated that female flies show a steeper (earlier) age-related decline in spontaneous locomotor activity compared to male flies (Le Bourg and Minois, 1999). Furthermore sex differences in locomotor activity have also been found at young age whereby males show hyperlocomotor activity compared to female flies (Martin, 2004; Besson and Martin, 2005; Woods *et al.*, 2014). Therefore a secondary interest was to confirm sex differences in locomotor activity and its decline with age.

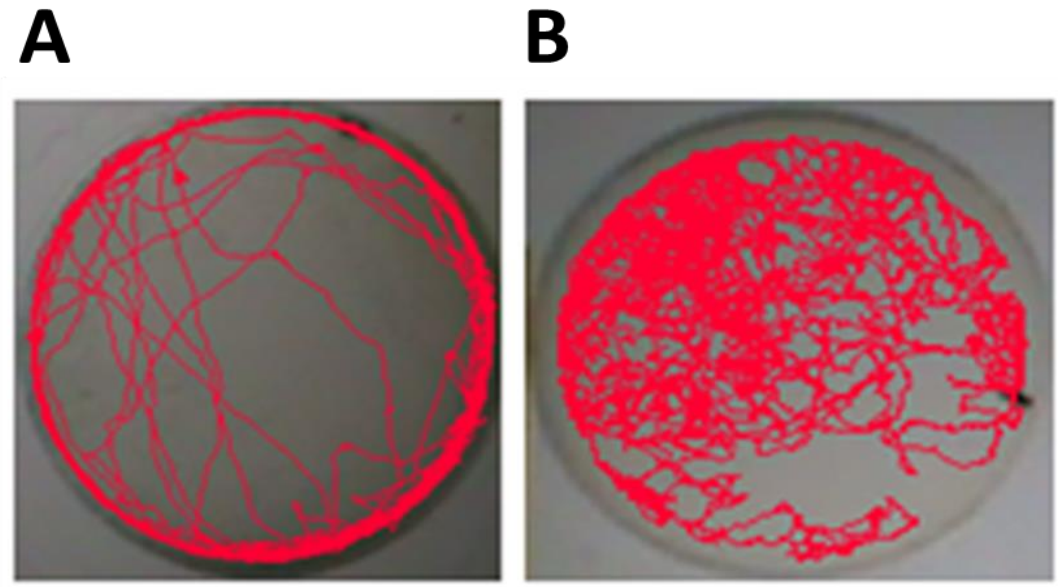

Figure 1.4 Flies spend more time in the central zone of the arena as they age
*Walking patterns of flies at 1 week old **(A)** and 7 weeks old **(B)** during the Exploratory Walking (EW) assay. Flies show an age-dependent stereotypical pattern of behaviour during the EW assay, whereby at young age they walk around the edge of the arena, spending little time in the centre **(A)**, and at old age they walk more randomly and spend more time in the centre **(B)**. Figure taken from Ismail et al. (2015).*

Experiment 7: Impact of *Nrx-1* hyperfunction on sleep structure across the life span

Sleep was analysed in flies using the Drosophila Activity Monitoring System (DAMS), which records fly activity over a number of days. Flies are considered asleep when they are inactive for >5 minutes (Shaw *et al.*, 2000). The neurobiology and regulation (homeostatic and circadian) of sleep is conserved in both the fly (*Drosophila melanogaster*) and mammals, thus making the fly a useful model organism to investigate sleep in humans (Hendricks *et al.*, 2000; Shaw *et al.*, 2000; Huber *et al.*, 2004; Sehgal and Mignot, 2011; Chakravarti *et al.*, 2017; Tomita *et al.*, 2017). However, unlike humans, flies have two peaks of activity in a 24 hour period with their highest levels of activity being at dusk and dawn (Dubowy and Sehgal, 2017). Flies also, unlike humans, sleep in multiple bouts with a duration usually <90 minutes (Andretic and Shaw, 2005). Therefore fly sleep is analysed in the light and dark phases separately, and both the structure and amount of sleep can be investigated using a number of parameters. These include, but are not limited to, total sleep, number of sleep bouts and sleep bout length (Andretic and Shaw, 2005). Male flies have increased total sleep, and sleep in less bouts that are of longer duration, than female flies (Koh *et al.*, 2006; Zimmerman *et al.*, 2012). As flies age their total sleep decreases and becomes more fragmented (bout number increases and bout length decreases) (Koh *et al.*, 2006; Zimmerman *et al.*, 2012; Metaxakis *et al.*, 2014), however age-related changes can vary dependent on light phase and fly genetic background.

Sleep was analysed in the P{XP}Nrx^{d08766} flies across the life span to provide a measure of brain function, since sleep is under the control of the fly brain. Although sleep structure does show some age-related changes, these changes are not as robust as the Exploratory

Walking (EW) parameters, and so this was a secondary use of this assay. Furthermore sleep is often reported to be disrupted in Sz and ASD (reviewed in Section 1.1 and 1.2). Performing sleep analysis enabled investigation of the impact of *Nrx-1* hypermorphism on (1) sleep structure, (2) age-related changes in sleep, and (3) whether any changes mirror those reported in ASD and Sz.

These experiments provided information on the impact of *Neurexin1* dysfunction on behaviour, cognition and cerebral metabolism in the mouse, and whether any phenotypes are similar to those seen in Sz and ASD. In addition, experiments in flies determined whether *Neurexin1* modulates ageing, behaviour senescence and sleep, and whether these related to changes present in Sz and ASD.

Chapter 2 Materials and Methods

2.1 Experiments conducted in the mouse

2.1.1 Mouse model

Mice that were Heterozygous (Hz) for *Neurexin1α (Nrxn1α)* were generated as described in Dachtler *et al.* (Dachtler *et al.*, 2014) as follows; B6;129-*Nrxn3*^{tm1Sud}/*Nrxn1*^{tm1Sud}/*Nrxn2*^{tm1Sud}/J mice (JAX #006377) males purchased from Jax Laboratory (*Nrxn1* Hz, *Nrxn2* Homozygous (Hm) and *Nrxn3* Wild-type (Wt)) were crossed with female C57BL/6NCrl strain mice (Charles River, Margate, United Kingdom) at the University of Leeds to give rise to mice that were either *Nrxn1α* Hz or *Nrxn2α* Hz. Male *Nrxn1α* Hz mice were crossed with Wt females through cousin mating. *Nrxn1α* Hz male and female mice received from the University of Leeds were mated to give rise to *Nrxn1α* Hz, *Nrxn1α* KO or Wt mice. *Nrxn1α* Hz males or females were crossed with C57BL/6NCrl (Charles River, Margate, United Kingdom) or Wt mice (through cousin mating) to produce *Nrxn1α* Hz and Wt litter-mate mice used as experimental animals in this study.

The generation of the B6; 129- *Nrxn3*^{tm1Sud}/*Nrxn1*^{tm1Sud}/*Nrxn2*^{tm1Sud}/J mice (JAX #006377) is described in the 006377 mouse strain data sheet on the Jax laboratory website (https://www.jax.org/strain/006377) and also in Missler *et al*. (Missler *et al.*, 2003). The murine *α-Neurexin* genes (1, 2 and 3) were mutated by targeted vectors (encoding *Nrxn1, Nrxn2* and *Nrxn3*) containing neomycin resistance genes which replaced the large first exons on each *α-Neurexin* gene. The construct was electroporated into 129/SV-derived embryonic stem cells and those which took up the construct correctly were injected into C57BL/6

blastocytes. Chimeric mice were then crossed with C57BL/6 mice and maintained on a mixed SV129/C57BL/6 background.

The majority of the experimental mice used in the current study (all the 6 and 12 month old group and some of the 3 and 9 month old groups) were the progeny of 3 sets of parents (1 pair and 2 trio's). A further 4 trios of breeders were used for the generation of the additional mice needed in the 3 and 9 month groups.

2.1.2 Genotyping

Mice were genotyped by the extraction of DNA from ear punches. Each ear punch was placed into a 1.5ml Eppendorf tube with 500µl of digestion buffer (50mM Tris, 100mM EDTA pH8, 100mM NaCl, 1% SDS, 1mg/Ml of Proteinase K (Sigma-Aldrich, UK) in distilled H_2O) which was incubated overnight at 37°C. Samples were vortexed and centrifuged (5 minutes, 13000rpm, room temperature) to pellet hairs and remaining matter. The supernatant was added to 300µl of isopropanol, inverted and allowed to stand for 15minutes to allow for DNA precipitation. Samples were centrifuged (10 minutes, 13000rpm, room temperature) to pellet DNA, isopropanol was removed and the pellet washed with 300µl of 70% ethanol. Samples were pulse centrifuged and all traces of ethanol were removed. 100µl TE buffer (10mM Tris, 1mM EDTA in molecular H_2O) was added to each sample and placed at 56°C for 60 minutes to allow the DNA to dissolve. The genotyping method was adapted from the JAX lab (https://www.jax.org/strain/006377#genotype) and used common forward primers 5' CTG ATG GTA CAG GGC AGT GGA CCA 3', Wt reverse primers 5' CGA GCC TCC CAA CAG CGG TGG CGG GA 3' and *Nrxn1α* KO reverse primers 5'GAG CGC GCG CGG CGG AGT TGT TGA C 3'. For each sample the total Polymerase Chain Reaction (PCR) volume was 10 µl and contained 5µl of Hotshot Diamond (Clent Life Science, Stourbridge, UK), 0.3µl reverse

primers, 0.3µl of forward primers, 3.4µl molecular H_2O and 1µl of DNA (diluted 1:10 in TE buffer). The PCR thermocycling sequence was as follows; 95°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 70°C for 30 seconds, 72°C for 30 seconds, and then 72°C for 2 minutes. PCR products were visualised by agarose gel electrophoresis, a band at 440bp indicated the Wt allele and a band at 390bp indicated the *Nrxn1α* KO allele. Wt mouse samples displayed a band at 440bp but not at 390bp, *Nrxn1α* Hz mouse samples displayed a band at both 440bp and 390bp.

2.1.3 Mouse housing conditions

All housing and experimental procedures were in compliance with the local ethical review panel of Lancaster University and the UK Home Office Animals Scientific Procedures Act 1986. Experimental mice were weaned at 3 weeks old and were single sex group housed up to a maximum of 6 mice per cage. Mice were kept at standard conditions (21°C, 45-65% humidity, 12:12h light/dark cycle with first light at 7am) in individually ventilated cages (I.V.Cs, Techniplast, UK). Cage enrichment included sizzle nest bedding, 1 x 4 cm wooden blocks and a dome refuge in each cage. Food (Teklad maintenance diet, Envigo) was provided *ad libitum* until 2 weeks before commencement of the Cognitive Flexibility Task (CFT) when mice began food restriction. Water was provided *ad libitum* throughout. 1-2 weeks before CFT commencement mice were singly housed to aid effective weight monitoring and individual calibration of food restriction. Animals remained on food restriction until they were sacrificed.

2.1.4 Experimental groups and protocol

Male and female *Nrxn1α* Hz and Wt mice were tested at four different age groups (3, 6, 9 and 12 months old) in a between-groups experimental design. Each mouse was tested in the Open Field (OF), Novel Object Recognition Task (NORT) and Cognitive Flexibility Task (CFT) respectively. At the end of behavioural testing mice underwent the ^{14}C-2-Deoxyglucose functional brain imaging protocol. The average age (weeks) of mice entering each part of the protocol is shown per group in Table 2.1.

2.1.5 Food Restriction

Animals were food restricted to motivate them to dig for food rewards in the CFT. Animals began food restriction and were singly housed at least 2 weeks prior to day 1 of the CFT and were food restricted to between 85-80% of their free feeding weight. Mice were weighed 5 times a week during food restriction. Food restriction began by giving each animal 3g of standard laboratory chow (Teklad maintenance diet, Envigo) which was then reduced until the animals reached 80% of their free feeding weight. This weight was then maintained throughout CFT testing. Once testing was complete animals were maintained at ~85% of their free feeding weight until they were sacrificed. The mean weights of each group prior to food restriction are shown in Table 2.1.

Group	Sex	Genotype	Free feeding weight (g)			OF age (weeks)			NORT age (weeks)			CFT age (weeks)			2DG age (weeks)		
			Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
3	All	All	25.3	4.5	40	11.9	0.4	40	12.1	0.4	40	14.4	1.0	37	14.8	0.8	20
3	F	All	21.6	1.3	21	12.0	0.4	21	12.2	0.4	21	14.4	0.9	18	14.7	0.7	10
3	F	Wt	21.4	1.5	9	11.8	0.4	9	12.0	0.4	9	14.5	1.0	9	15.0	0.9	5
3	F	*Nrxn1α* Hz	21.8	1.1	12	12.3	0.4	12	12.4	0.4	12	14.4	0.9	9	14.3	0.2	5
3	M	All	29.5	2.9	19	11.8	0.3	19	12.0	0.3	19	14.4	1.1	19	14.9	0.9	10
3	M	Wt	28.5	1.2	10	11.8	0.3	10	11.9	0.3	10	14.3	1.1	10	14.9	1.1	5
3	M	*Nrxn1α* Hz	30.4	3.6	9	11.9	0.2	9	12.0	0.2	9	14.4	1.2	9	15.0	0.9	5
6	All	All	32.6	6.8	36	24.0	0.2	36	24.1	0.2	36	27.1	1.4	33	28.0	1.4	20
6	F	All	27.7	4.5	19	24.0	0.2	19	24.2	0.2	19	26.9	1.6	16	28.1	1.5	10
6	F	Wt	29.2	4.6	11	24.1	0.1	11	24.2	0.1	11	26.7	1.6	8	28.1	1.6	5
6	F	*Nrxn1α* Hz	26.7	4.2	8	24.0	0.3	8	24.1	0.3	8	27.1	1.7	8	28.1	1.7	5
6	M	All	38.1	4.5	17	24.0	0.2	17	24.1	0.2	17	27.3	1.3	17	27.8	1.3	10
6	M	Wt	36.8	4.7	8	24.0	0.2	8	24.1	0.2	8	27.4	1.3	8	27.8	1.4	5
6	M	*Nrxn1α* Hz	39.4	4.1	9	23.9	0.2	9	24.1	0.2	9	27.3	1.4	9	27.8	1.4	5
9	All	All	39.9	7.3	38	37.9	0.6	38	38.0	0.6	38	39.4	0.9	37	40.7	1.3	20
9	F	All	37.5	7.7	19	38.0	0.8	19	38.2	0.8	19	39.4	0.9	18	40.7	1.3	10
9	F	Wt	35.8	5.7	10	38.1	1.2	10	38.2	1.2	10	39.4	1.0	10	40.7	1.4	5
9	F	*Nrxn1α* Hz	39.0	9.3	9	37.9	0.3	9	38.1	0.3	9	39.4	0.8	8	40.8	1.3	5
9	M	All	42.3	6.2	19	37.7	0.2	19	37.9	0.2	19	39.4	0.9	19	40.6	1.3	10
9	M	Wt	40.7	5.0	9	37.7	0.2	9	37.9	0.2	9	39.5	0.9	9	40.9	1.3	5
9	M	*Nrxn1α* Hz	44.0	7.2	10	37.7	0.2	10	37.9	0.2	10	39.2	1.0	10	40.3	1.4	5
12	All	All	43.4	8.0	36	51.5	0.4	36	51.6	0.4	36	54.4	1.6	35	54.6	1.1	20
12	F	All	39.6	7.9	18	51.4	0.4	18	51.6	0.4	18	54.3	1.4	18	54.3	0.6	10
12	F	Wt	36.1	5.3	9	51.7	0.2	9	51.8	0.2	9	54.4	1.5	9	54.4	0.7	5
12	F	*Nrxn1α* Hz	43.0	8.9	9	51.2	0.5	9	51.3	0.5	9	54.2	1.4	9	54.3	0.5	5
12	M	All	47.3	6.2	18	51.6	0.3	18	51.7	0.3	18	54.6	1.7	17	54.8	1.4	10
12	M	Wt	45.5	6.2	10	51.7	0.1	10	51.8	0.1	10	54.4	1.9	10	54.8	1.7	5
12	M	*Nrxn1α* Hz	48.6	6.2	8	51.5	0.4	8	51.6	0.4	8	54.8	1.7	7	54.7	1.2	5

Table 2.1 Average free feeding weight and age of mice entering each part of the experimental protocol

Details for each age group, sex and genotype are shown. F=female, M=male, Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous, g=grams, SD=standard deviation, OF=Open Field, NORT=Novel Object Recognition Task, CFT=Cognitive Flexibility Task, 2DG=^{14}C-2-Deoxyglucose functional brain imaging. Table includes Mean and SD of free feeding weight prior to food restriction and group sizes (n numbers) for animals which entered the experiment. The Mean and SD age of animals in each group and group sizes (n numbers) of animals which entered each part of the experimental protocol are shown. n numbers reported here may differ from those in the results section due to removal of outliers (outliers were removed by the protocol outlined in section 2.3.3).

2.1.6 Open Field (OF)

All mice were food restricted for at least 1 week prior to OF testing (in preparation for the subsequent CFT). On the day of testing animals were placed individually into empty holding cages and were habituated to the experiment room for 30 minutes. Mice were then placed individually into circular OF arenas made of white Perspex (diameter 38cm) and video recorded for 15 minutes using a webcam fixed to the ceiling. The experimental set up allowed 4 mice to be tested at the same time. Arenas were cleaned with 70% ethanol prior to the first run and between subsequent runs to remove any scent trails.

2.1.7 Novel Object Recognition Task (NORT)

Mice had previously undergone OF testing 1 day prior to NORT testing. On the day of NORT mice were placed individually into empty holding cages and were habituated to the experiment room for 30 minutes. Mice were then placed individually into the empty OF arena for a 5 minute habituation session. Mice were then quickly removed and two identical objects were placed into the arena (either 2 brown wooden cubes (3cm x 3cm x 3cm) or 2 blue wooden pyramids (5.5cm x 3cm x 2.5cm) (acquisition phase). Mice were then returned to the arena and allowed to explore the objects for a 10 minute acquisition phase, before being placed back into holding cages. After a 1 hour delay mice were returned to the arena for a further 10 minutes which this time contained one object from the previous exposure (familiar object) and one novel object (novel object), which comprised the test phase of the NORT. Arenas and objects were cleaned with 70% ethanol prior to the first run and between all subsequent runs. Mice were video recorded throughout the habituation, acquisition and test phases. Allocation of which object would be the familiar object and which would be the

novel object was randomised, as was the side (left or right) in which the novel object was placed during the test phase. All objects were presented 15cm from the edge of the arena.

2.1.8 Cognitive Flexibility Task (CFT)

Apparatus

The CFT apparatus (shown in Figure 2.1) consisted of a modified home cage (length 36cm, height 14cm, width 20cm) that was partitioned part way down, with two doors which slid in from the top creating 2 smaller choice areas (12cm x 10cm) and one large holding area (23cm x 20cm). There was also a large sliding door the width of both smaller areas to allow access to both sections at the same time. A piece of orange material was fixed to the outside of the right smaller section (experimenter's right, mouse's left) to create a visual distinction between the two otherwise identical areas. 3 modified translucent red pieces of Perspex created lids for each of the areas. Mice had access to water at all times from a small plastic bowl (2cm diameter, 1cm height) secured to the floor in the holding area. Glass bowls (5cm diameter, 3.5cm height) were used which contained sugar pellet food rewards (45mg Sucrose reward pellet, Test Diet) buried in media consisting of playground grade sand scented with different spices (Turmeric, Paprika, Cloves, Cinnamon, Nutmeg or Cumin, Schwartz spices). The media contained 0.1g of spice and 0.01g crushed reward/100g sand. The experimental set up included 2 CFT boxes to allow 2 mice to be tested simultaneously.

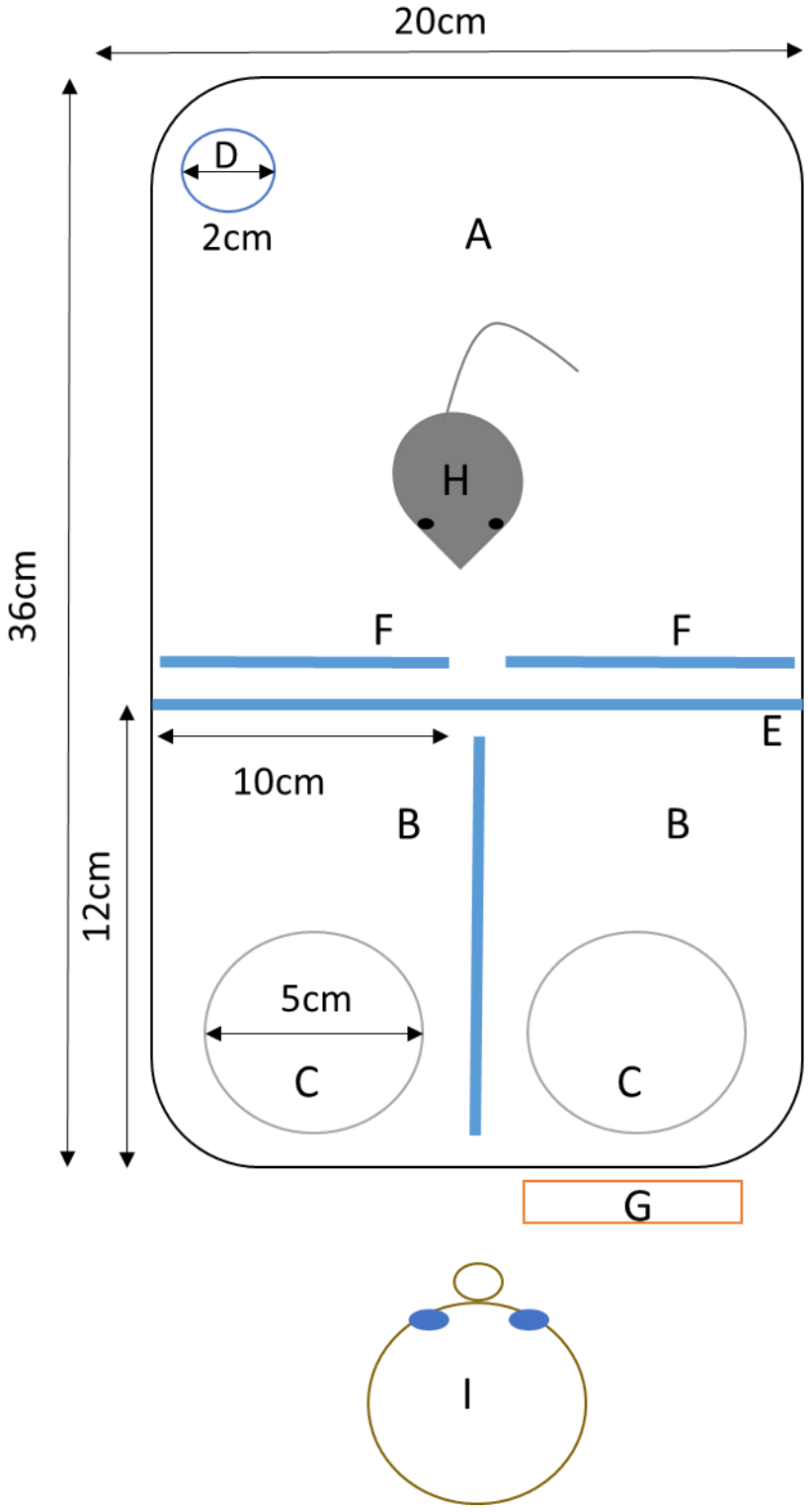

Figure 2.1 Diagram of CFT apparatus

A=holding area, B=choice area, C=glass bowl, D=water bowl, E=large sliding door to allow access to both choice areas at once, F=small sliding door to restrict access to one choice area following initiation of digging, G=orange material to create visual cue to discriminate between left and right sides, H=mouse, I=experimenter position. Diagram not drawn to scale.

Habituation to apparatus and reward

2-4 days prior to day 1 of CFT testing, singly housed mice were given glass bowls (5cm diameter, 3.5cm height) containing 5 sugar pellets (45mg Sucrose reward pellet, Test Diet) in their home cage. These bowls were identical to those used in the CFT and were given at the same time as normal feeding. It was noted that a small number of mice did not retrieve the food reward when presented in the home cage. Any mice that did not eat the sugar pellets from the bowls in the home cage after 1-2 days were put into empty holding cages (for 2-4 hours with *ad libitum* water) containing a maximum of 4 glass bowls in various orientations (upside down, sideways, right way up) containing a sugar pellet. Sugar pellets were also placed on the floor close to the bowls. This extra habituation encouraged the mice to go close to, and climb on, the bowls to gain the food rewards. All mice that underwent the extra habituation session subsequently ate sugar pellets from bowls placed in the home cage.

CFT protocol

The CFT was a 3 day protocol including 2 habituation sessions (day 1 and 2), a training discrimination test session (day 2) and the final test session (day 3) (Figure 2).

CFT day 1

Habituation session 1 – Habituation to apparatus and learning to dig

Mice had previously been habituated to the experiment room during OF and NORT testing. The purpose of the first session was to habituate the mice to the apparatus and to train them to dig in bowls of sand to retrieve a food reward. Mice were placed individually into the holding area of CFT box with the large sliding door in place. Two identical glass bowls

containing 1 sugar pellet were placed in each of the 2 smaller areas. Each trial began by a timer being pressed (which provided an acoustic cue) just before the removal of the large sliding door providing access to both bowls simultaneously. The mouse was then allowed to enter each choice area to retrieve the food rewards. Once the mouse had eaten the pellet and left a choice area, the smaller door was closed behind it. Once pellets were eaten from each bowl, and the second small door was closed, the trial terminated. This process was repeated for the second trial. For the third trial both bowls were filled (less than half way) with odourless sand and the mouse was required to retrieve the sugar pellet placed on top of the sand. For the subsequent trials the sugar pellet was placed in an indent in the sand, then trial by trial the pellet was gradually pushed further into the sand, until it was no longer visible (buried approx. 1cm below surface) and the mouse had to use its forepaws to dig in the sand to retrieve the reward. Once the mouse had successfully dug in both bowls to gain the food rewards for 2 or more trials, the training session was complete. This process took 8-12 trials in total to complete.

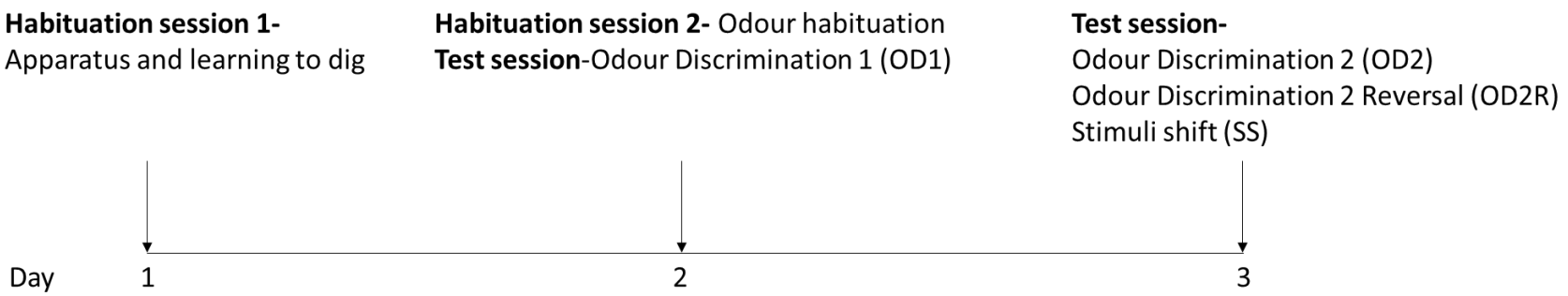

Figure 2.2 Timeline of the different sessions included in the 3 day CFT protocol

Day 1 consisted of a session habituating the mice to the apparatus and training them to dig in bowls of sand to retrieve food pellet rewards. Day 2 to consisted of one session habituating the mice to the all the odours to be encountered in the test sessions, and the first test session, Odour Discrimination 1 (OD1). Day 3 consisted of 3 test sessions: Odour Discrimination 2 (OD2), Odour Discrimination 2 Reversal (OD2R) and Stimuli Shift (SS).

CFT day 2

The purpose of the session on CFT day 2 was to habituate the mice to all the different odours that would be encountered in the CFT test sessions, and then to train them to discriminate between a pair of odours to gain a food reward.

Habituation session 2- Odour habituation

A total of 6 different odours (Turmeric, Paprika, Cloves, Cinnamon, Nutmeg, Cumin, Schwartz spices) were individually added to plain sand containing crushed sugar pellet rewards (0.1g of spice and 0.01g crushed reward/100g sand) and were presented in pairs (Figure 2.3) (one in each of the two bowls). The crushed reward was included in the digging media to prevent the mouse making choices based on being able to smell the food reward. The odour pairs for the 6 month group differed from that of the 3, 9, and 12 month old groups (Figure 2.3) as during testing it was observed that the mice found discriminating between Cloves and Nutmeg more difficult compared to the other odour pairs. In the first trial of the session, the mouse was required to dig in the two bowls (each containing a different odour from one of the odour pairs) to gain one sugar pellet reward from each bowl. In the second trial the same odour pair was presented again (in fresh bowls of sand), but this time the side of presentation (left or right) of each odour was reversed. For example, if in the first trial the Turmeric bowl appeared in the left hand side area (and the Paprika bowl in the right hand side area), then in the second trial the Turmeric bowl would appear on the right side (and the Paprika bowl on the left). The third trial and fourth trial followed this same format with a second odour pair, and the fifth and sixth trial followed the same format with a third odour pair. After 6 trials the mouse had encountered all the odours twice, once on the left side and once on the right side of the apparatus.

A

Set 1	Odour Pair
OD1	Cumin v Cinnamon
OD2/OD2R	Tumeric v Paprika
SS	Cloves v Nutmeg

Set 2	Odour Pair
OD1	Tumeric v Paprika
OD2/OD2R	Cloves v Nutmeg
SS	Cumin v Cinnamon

Set 3	Odour Pair
OD1	Cloves v Nutmeg
OD2/OD2R	Tumeric v Paprika
SS	Cumin v Cinnamon

Set 4	Odour Pair
OD1	Cumin v Cinnamon
OD2/OD2R	Cloves v Nutmeg
SS	Tumeric v Paprika

Set 5	Odour Pair
OD1	Tumeric v Paprika
OD2/OD2R	Cumin v Cinnamon
SS	Cloves v Nutmeg

Set 6	Odour Pair
OD1	Cloves v Nutmeg
OD2/OD2R	Cumin v Cinnamon
SS	Tumeric v Paprika

B

Set 1	Odour Pair
OD1	Cumin v Nutmeg
OD2/OD2R	Tumeric v Paprika
SS	Cloves v Cinnamon

Set 2	Odour Pair
OD1	Tumeric v Paprika
OD2/OD2R	Cloves v Cinnamon
SS	Cumin v Nutmeg

Set 3	Odour Pair
OD1	Cloves v Cinnamon
OD2/OD2R	Tumeric v Paprika
SS	Cumin v Nutmeg

Set 4	Odour Pair
OD1	Cumin v Nutmeg
OD2/OD2R	Cloves v Cinnamon
SS	Tumeric v Paprika

Set 5	Odour Pair
OD1	Tumeric v Paprika
OD2/OD2R	Cumin v Nutmeg
SS	Cloves v Cinnamon

Set 6	Odour Pair
OD1	Cloves v Cinnamon
OD2/OD2R	Cumin v Nutmeg
SS	Tumeric v Paprika

Figure 2.3 Odour pair allocation sets for each phase of the CFT

*Sets 1-6 were counterbalanced within each age group per genotype per sex using **(A)** for 6 month old mice and **(B)** for 3, 9 and 12 month old mice. OD1=Odour Discrimination 1, OD2=Odour Discrimination 2, OD2R=Odour discrimination 2 Reversal, SS=Stimuli Shift.*

Odour discrimination training - Odour Discrimination 1 (OD1)

After the 6 odour habituation trials the mouse immediately entered the Odour Discrimination 1 phase (OD1). In this phase the mice were required to learn that there was only one food reward to be gained from each trial, and that they could use odour to discriminate which bowl contained the reward. The odour pair used for the training discrimination phase was counterbalanced across experimental groups (male *Nrxn1α* Hz, male Wt, female *Nrxn1α* Hz and female Wt) within each age group (3, 6, 9 and 12 months old). The rewarded odour in each pair (odour 1 or odour 2) was randomised within each age group (3, 6, 9, 12 months old). The side of the apparatus in which the bowl containing the rewarded odour appeared on was pseudo-randomised, with a limit of 2 consecutive presentations on the same side. This was done using 2 different test sheets (Appendix A1) which determined which side the rewarded odour appeared for each individual trail. The use of the 2 different test sheets was randomised. In the first 4 trials the mouse was allowed to dig in both of the bowls (each containing one odour from the pair) to learn which odour was rewarded (4 'free' trials). When the mouse had retrieved the pellet and left a choice area, the smaller door was closed behind it. Once the mouse had visited both bowls and retrieved the sugar pellet from the bowl with the rewarded odour, both doors were closed and the trial ended. The latency to dig and whether the choice was correct or incorrect was recorded for each trial. A dig was defined as two digging movements with the forepaws, either two movements on the same forepaw or one movement from each forepaw. From the fifth trial onwards the mouse was only allowed to dig in one of the bowls, and as soon as the mouse dug in one of the bowls, the door was closed for the opposite area to deny access to the other bowl. Therefore the mouse was punished if an incorrect choice was

made as it was denied access to the correct bowl, thus could not gain a food reward for that trial. In the case of an incorrect choice there was no time limit on how long the mouse was allowed to dig for, to ensure that mice became aware that the unrewarded bowl contained no reward. Once the mouse voluntarily left the incorrect side, the small door was closed behind it and the trial ended. For all trials in the training discrimination phase there was a time limit of 3 minutes for digging initiation, after 3 minutes the timer was pressed (providing an acoustic cue), doors were closed and the trial ended. This was recorded as a 'time out' and next trial would be a repeat of this 'time out' trial. The training discrimination session ended once the mouse had made correct choices for 6 consecutive trials, which took on average ~17 trials for all mice in the study.

CFT day 3

Odour Discrimination 2 (OD2), Odour Discrimination 2 Reversal (OD2R) and Stimuli Shift (SS)

On CFT day 3 there were 3 phases: Odour Discrimination 2 (OD2), Odour Discrimination 2 Reversal (OD2R) and Stimuli Shift (SS). For all phases the odour pairs were counterbalanced across experimental groups (male *Nrxn1α* Hz, male Wt, female *Nrxn1α* Hz and female Wt) and the rewarded odour in the pair was randomised. The side of the apparatus in which the bowl containing the rewarded odour appeared on was pseudo-randomised, with a limit of 2 consecutive presentations on the same side. There were 4 'free' trials at the start of each phase, were the mouse was allowed to dig in both of the bowls on each trial without punishment. After 4 trials the punishment for incorrect choices (no access to the food reward) was introduced. The latency to the first dig and whether the choice was correct or incorrect was recorded for each trial. Once the mouse had made 6 consecutive correct

choices the phase was complete, and the mouse immediately moved onto the next phase. When 6 consecutive correct choices were made in the SS phase the CFT was complete.

OD2 was identical in format to OD1, however a different odour pair was used. In OD2R, the odour in the pair that was not previously rewarded, in OD2, became the rewarded odour (rule reversal). In the SS phase a different odour pair was used and this time the rewarded bowl was not determined by odour but instead by location (left and right side of the apparatus).

Testing sessions were terminated early if 6 or more consecutive 'time outs' occurred (usually due to the mouse falling asleep). If the OD1 session on CFT day 2 was terminated early, mice were still required to attempt OD2 the following day. However if sessions were terminated early from the OD2 phase and onwards no further data was collected. The sessions were also terminated early if the mouse failed to achieve 6 consecutive correct trials within 40 trials for OD1 and OD2, and within 60 trials for OD2R and SS.

2.1.9 ^{14}C-2-Deoxyglucose Functional Brain Imaging

Methods for measuring local cerebral glucose utilisation by ^{14}C-2-Deoxyglucose imaging are published in *Dawson et al.* (Dawson *et al.*, 2013). Mice were food restricted 4-5 hours prior to being dosed with 4.625 MBq/Kg ^{14}C-2-Deoxyglucose (Specific Activity = 55mCi/mmol, American Radiolabelled Chemicals, Inc) in physiological saline by *intraperitaneal (i.p)* injection (at a volume of 2.5mls/kg) at a steady rate over 10 seconds. Mice were then placed into empty holding cages with water available *ad libitum* for 45 minutes. Mice were then sacrificed by cervical dislocation and decapitated. A terminal blood sample was taken by torso inversion into weigh boats containing heparin, and blood glucose (mM/L) was measured directly from blood using an Accu-chek Aviva glucose monitor (Roche). Brains

were removed and snap frozen in cold isopentane (-40°C) and stored at -80°C until required. Blood samples were centrifuged to separate plasma and stored at -80°C until required. ^{14}C concentration was determined in blood plasma samples (10µl plasma in 1ml scintillation fluid) in triplicate using liquid scintillation analysis (Beckman). Frozen brains were sectioned coronally in 20 µm slices at -20°C in a cryostat. 3 consecutive slices were collected, thaw mounted onto coverslips and rapidly dried on a hot plate at 70°C. The next 3 consecutive slices were discarded, and this process was repeated throughout the whole brain. Coverslips containing brain slices and ^{14}C standards (40-1069 nCi g^{-1} tissue equivalents, American Radiolabelled Chemicals, Inc) were opposed to X-ray film (Kodak, Biomax MR) for 1 week to generate autoradiographic images. Films were then developed in accordance with manufacturer's instructions using an automated film developer (Konica Minolta, SRX-101A).

2.2 Experiments conducted in the Fly (*Drosophila melanogaster*)

2.2.1 Fly stocks and maintenance

P{XP}Nrx-1^{d08766} *Drosophila* were obtained from Bloomington Drosophila stock centre (Flybase ID FBti0042917). P{XP}Nrx-1^{d08766} flies contain a 7.3 Kb p-element inserted 198bp upstream of the translation initiation codon of the *Nrx-1* gene (Zeng *et al.*, 2007), which is reported to cause a decrease in *Nrx-1* protein (Zeng *et al.*, 2007; Tong *et al.*, 2016). P{XP}Nrx-1^{d08766} flies were backcrossed 5 times with white^{1118} (w^{1118}) control flies at 25-27°C. For the first backcross, virgin w^{1118} females were crossed with P{XP}Nrx-1^{d08766} males. Orange eyed virgin females were then selected from the first backcross and mated with w^{1118} males. The latter step was repeated 3 more times to give a total of 5 backcrosses to the control background. Pale orange eyed virgin females and males from backcross 5 were then mated together, to produce flies that were homozygous for the P{XP}Nrx-1^{d08766}

mutation. The homozygous progeny from this final cross had a dark orange eye colour and made up the P{XP}Nrx-1^{d08766} stocks used in the current study. The flies from these backcrosses were used for Cohort 1 and 2 life span experiments, the EW assay and sleep analysis. The flies from the first series of backcrosses were then backcrossed again prior to repeating the life span experiment for Cohort 3 and Cohort 4 and collecting fly tissue for investigation of *Nrx-1* expression by qPCR and Western blot. In the second round of backcrossing virgin w^{1118} females were crossed with P{XP}Nrx-1^{d08766} males from the stock maintained after the first series of backcrosses previously described. Orange eyed virgin females were then selected from the first backcross and mated with w^{1118} males. Pale orange eyed virgin females and males from backcross 2 were then mated together, to produce flies that were homozygous for the P{XP}Nrx-1^{d08766} mutation. The homozygous progeny from this final cross had a dark orange eye colour and made up the new backcrossed P{XP}Nrx-1^{d08766} stock. After backcrossing, all flies were maintained at 25°C constant humidity 12h:12h light/dark cycle on standard food (100g/L brewer's yeast (MP biomedicals), 50g/L sugar, 10g/L agar).

2.2.2 Experimental set up

All experimental flies were reared in the same way. Firstly flies from each of the 2 experimental groups (w^{1118}, P{XP}Nrx-1^{d08766}) were age matched by transferring them to new bottles and collecting eclosing adults. These adults were allowed to mate for 48 hours before being transferred to cages containing 1% agar red grape juice plates (to aid visual detection of fly eggs) with yeast paste to encourage egg laying. 24 hours later all caged flies were transferred onto fresh plates with a smaller amount of yeast paste. These steps synchronised the laying of eggs at a similar developmental stage. After a further 24 hours

eggs were collected from the plates by rinsing with 1x Phosphate-Buffered Saline (PBS) and pipetting 100µl eggs in solution into bottles containing standard food (100g/L brewer's yeast (MP biomedicals), 50g/L sugar, 10g/L agar), using a 200µl pipette a cut tip to create a larger diameter hole. These bottles were then maintained at 25°C for 10 days. Eclosing adults were transferred to fresh bottles and allowed to mate for 48 hours. Flies were then briefly anaesthetised with CO_2 and separated into single sex vials of 10 flies per vial containing standard food (100g/L brewer's yeast (MP biomedicals), 50g/L sugar, 10g/L agar). The vials were plugged using cotton wool. All flies were transferred to fresh vials 2-3 times a week. Three separate cohorts of flies for life span, Exploratory Walking (EW) and sleep analysis were reared and maintained in this way, and maintained as separate life span experiments. Different flies were sampled at each of the 7 time points for each of the behavioural analyses. Flies were raised in the same way as described above to repeat the life span experiment for Cohort 2, 3 and 4.

2.2.3 Tissue Collection

Whole flies were collected at 1 week old for use in qPCR and western blot. Flies from both genotypes and sexes were anaesthetised briefly using CO_2, placed into 1.5ml Eppendorf tubes (20 per tube) and snap frozen in liquid nitrogen. Flies were then stored at -80°C until required.

2.2.4 RNA Extraction and Preparation

Fly heads were separated from bodies by vigorous shaking in Eppendorf tubes immediately after removal from the -80°C freezer. 20 fly heads were used per RNA extraction and 6 extractions were carried per genotype and per sex (n=6). Fly heads were added to ribolyser

tubes (Cat # PFAW 1700-50-16, OPS Diagnostics) containing 500µl TRIzol (Cat #15596026, Invitrogen) on ice, then homogenised for 10 seconds using a FastPrep cell disrupter (MP Biomedical). A further 500µl of TRIzol was added to each tube and then tubes were then incubated for 5 minutes at room temperature. 200µl of chloroform was added to each tube and shaken vigorously for 15 seconds. Tubes were then incubated at room temperature for 3 minutes before centrifugation (12,000 rpm, 15 minutes, 4°C). The upper aqueous layer containing RNA was removed and added to fresh Eppendorf tubes. 500µl of isopropanol and 50µl of 3M Sodium acetate (NaOAc) was added to each tube and stored at -80°C for 4 hours. Tubes were then centrifuged (12,000 rpm, 15 minutes, 4°C). The supernatant was discarded and the pellet was washed in cold 70% ethanol and centrifuged (12,000 rpm, 10 minutes, 4°C). The washing and centrifugation step was repeated twice. Finally the supernatant was removed and the pellet allowed to air dry before being resuspended in 10µl of DEPC water. RNA samples were stored at -80°C until required. 2µg of RNA was treated to remove any DNA using RQ1 RNase-Free DNase kit (Cat #M6101, Promega) and converted to cDNA by Reverse Transcription reaction using SuperScript III First-Strand Synthesis System using the oligo(dT) method (Cat #18080051, Invitrogen).

2.2.5 qPCR

Primers for qPCR

Primers spanning an exon-intron boundary were designed to probe fly *Nrx-1* and Actin was used as a housekeeping gene to control for amount of cDNA in each sample. The primer sequences were as follows; *Nrx-1* F=ATCACCATCCGTGGAGATGT and R=CACCCGGATAACC ACTCG and Actin F=CACACCAAATCTTACAAAATGTGTGA and R=AATCCGGCCTTGCACATG.

Primer Efficiency Testing

Efficiency was tested for *Nrx-1* and Actin primers using a 1:2 serial dilution series of cDNA from a female w^{1118} fly head sample from a 1:2 to 1:256 dilution (starting concentration 66.6ng/µl). A total reaction volume of 10 µl for each of the two genes (*Nrx-1* and Actin) was used for each dilution of cDNA: 5µl SYBR Green JumpStart *Taq* ReadyMix (Cat #S4438, Sigma), 0.5µl F primer (10µM), 0.5µl R primer (10µM), 3µl DEPC H_2O, 1µl diluted cDNA sample. The PCR program was as follows: 1) 94°C for 3 minutes, 2) 94°C for 30 seconds, 3) 60°C for 30 seconds, 3) 70°C for 30 seconds, 4) Repeat steps 2-4 39 more times, 5) Melt curve ran at 65-95°C (0.5°C increments) for 5 seconds/step. Primer efficiency was calculated to be near 100% for both *Nrx-1* and Actin primers using Biorad CFX Manager Software v3.1, thus enabling the Delta-Delta Ct method to be used to calculate relative expression of *Nrx-1* in P{XP}Nrx-1^{d08766} flies (data not shown).

qPCR protocol

Six cDNA samples from fly heads were used per genotype and per sex (n=6). 33.3ng of cDNA was used in the PCR for each sample. A total reaction volume of 10 µl for each of the two genes (*Nrx-1* and Actin) was used for each sample consisting of: 5µl SYBR Green JumpStart *Taq* ReadyMix (Cat #S4438, Sigma), 0.5µl F primer (10µM), 0.5µl R primer (10µM), 3µl DEPC H_2O, 1µl cDNA. The PCR program was as follows: 1) 94°C for 3 minutes, 2) 94°C for 30 seconds, 3) 60°C for 30 seconds, 3) 70°C for 30 seconds, 4) Repeat steps 2-4 39 more times, 5) Melt curve ran at 65-95°C (0.5°C increments) for 5 seconds/step. Each sample was run in triplicate and samples were run across 2 plates which were counterbalanced for sex and for genotype.

2.2.6 Protein Extraction

Fly heads were separated from bodies by vigorous shaking in Eppendorf tubes immediately after removal from the -80°C freezer. 20 fly heads were used per protein extraction and 3 extractions were carried out per genotype and per sex (n=3). Fly heads were added to ribolyser tubes (Cat # PFAW 1700-50-16, OPS Diagnostics) containing 200µl of RIPA buffer (Tris 50nM, NaCl 150nM, Na Deoxycholate 0.5%, SDS 0.1%, IGEPAL CA-630 1% (Cat #I8896, Sigma), 10µl/ml phosphatase inhibitors (Cat # P5726, Sigma), 5µl/ml protease inhibitors (Cat #P8340, Sigma)) and homogenised using a FastPrep cell disrupter (MP Biomedical) for 10 seconds. Tubes were then centrifuged (10,000g, 10 minutes, 4°C) to pellet debris and the supernatant transferred to fresh tubes and stored at -20°C until required.

2.2.7 Western Blotting

Antibodies

The *Nrx-1* primary antibody was supplied by the Han Lab (Tong *et al.*, 2016) and was generated by published methods (Tian *et al.*, 2013). In brief, the extracellular region of *Nrx-1* (amino acid 1534-1690) encoded by LP14275 cDNA (Genbank Accession Number BT023898) was purified and injected into rabbits as an antigen. The antibody collected from rabbit serum was purified using an affinity column. A goat anti-rabbit IgG HRP-linked secondary antibody raised in goat (Cat #7074, Cell Signaling Technology) was used to allow visualisation of the *Nrx-1* band.

Western Blotting Protocol

Protein concentration was determined by Bradford assay. Biorad Stain-Free Technology (Colella *et al.*, 2012; Gilda and Gomes, 2013) was used to investigate relative quantity of

Nrx-1 between groups by normalising the *Nrx-1* band to total protein in each sample as a loading control. Protein from 3 extractions was used per genotype and per sex (n=3). For each sample, 20µg of protein in 10% β-mercaptoethanol in laemmli buffer (Biorad) (total volume of 15 µl) was incubated at 70°C for 30 minutes. Samples were then loaded into precast 4–20% Mini-PROTEAN TGX Stain-Free Protein Gels (Cat #4568096, Biorad) and run at 180V for 40 minutes in 1x TGS running buffer (Cat #1610732, Biorad). Gels were activated by UV light for 1 minute using the ChemiDoc XRS+ System (Biorad). Proteins were transferred from gels to nitrocellulose membranes using a Trans-Blot Turbo RTA Mini Nitrocellulose Transfer Kit (Cat #1704270, Biorad) on the Trans-Blot Turbo Transfer System (Biorad) for 7 minutes. Membranes were imaged using the ChemiDoc XRS+ System Stain-Free Technology (Biorad) for total protein. Membranes were placed in falcon tubes and blocked in 0.5% milk (milk powder, Marvel) in Tris-Buffered Saline with Tween (TBST) (Tris-Cl 50mM, NaCl 150mM, Tween 0.05%) for 1 hour at room temperature on a rocking roller, then incubated with *Nrx-1* primary antibody (1:500 in 0.5% milk in TBST) overnight at 4°C on a rocking roller. Membranes were washed 3 times for 15 minutes in 0.5% milk in TBST at room temperature on a rocking roller and then incubated with secondary anti-rabbit antibody (Cat #7074, Cell Signaling Technology; 1:2000 in 0.5% milk in TBST) for 1 hour at room temperature on a rocking roller. Membranes were washed 3 times for 15 minutes in 0.5% milk in TBST at room temperature on a rocking roller. Membranes were imaged using the ChemiDoc XRS+ System (Biorad). Western blotting was performed a total of 3 times for each sample.

2.2.8 Life Span

Dead and censored flies were recorded once a day 2-5 times a week upon being separated into single sex vials of 10 flies. Four life span experiments were performed in total, Cohort 1, 3 and 4 included males and females and Cohort 2 included males only, since most of the females died before single sex sorting due to moisture in the bottles they had been transferred into. Females entering the life span experiment from Cohort 2 appeared sickly and died much earlier than expected so were excluded from the experiment.

2.2.9 Exploratory Walking (EW)

EW flies were sampled from the appropriate life span cohort (as described in section 2.2.2). Flies were tested across the life span at 7 day intervals from 11-53 days old at 25°C between 8am-12pm, using different batches of flies for each time point (between groups design). On the test day flies were aspirated into circular Perspex arenas (4cm diameter/1cm height) with a 2% agar floor and allowed to habituate to their surroundings for 1 minute prior to video recording. The flies were recorded in the arena for 15 minutes (4 flies in separate arenas were recorded at the same time). Males and females were run alternately, with genotypes counterbalanced in each run. Where possible, n=16 per genotype and per sex were tested, however as flies aged this meant that lower fly numbers were available for testing at some of the later time points. Life span was recorded for all EW flies in parallel to those in the first life span experiment to check that they were ageing in the same way (as described in Section 2.2.8).

2.2.10 Sleep

Sleep analysis was undertaken in flies sampled from the appropriate life span cohort (as described in section 2.2.2) and were tested across the lifespan at weekly intervals from 11-53 days old, using different batches of flies for each time point (between groups design). On the test day flies were briefly anaesthetised with CO_2 and placed individually into glass tubes (65mm length, 3mm diameter, Trikinetics) containing standard food and plugged with cotton wool. Each tube was placed into one of four Drosophila Activity Monitoring Systems (DAMS), (Trikinetics) with counterbalancing of the groups across the four DAMS. The flies remained in the DAMS for 3-4 days at 25°C, constant humidity 12h: 12h light/dark cycle. During this time infrared beam crosses for each fly was recorded. Where possible n=16 per genotype and per sex were tested, however as flies aged this meant that lower fly numbers were available for testing at some of the later time points. Life span was recorded for all EW flies in parallel to those in the first life span experiment to check that they were ageing in the same way (as described in Section 2.2.8).

2.3 Data Analysis and Statistics

Mouse model

2.3.1 Open Field (OF)

OF videos were analysed in 1 minute time bins using tracking software (Ethovision XT v8.5, Noldus) which allowed for the arena to be divided into virtual central (15cm diameter) and outer zones. Parameters measured included walking distance, walking velocity, walking duration, walking frequency, and duration and frequency in the central zone of the arena. The effects of genotype, sex, age and time bin were tested by repeated measures ANOVAs in R. Pairwise t-test with False Discovery Rate (FDR) correction *post hoc* analysis was

performed for any significant interactions found by ANOVA, to test for significant differences between relevant groups. Significance was set at $p<0.05$.

2.3.2 Novel Object Recognition Task (NORT)

Both acquisition and test phase NORT videos were analysed in 1 minute time bins using Ethovision XT v8.5 which allowed for virtual object zones (encompassing the object itself) and object boundary zones (2cm distance around object) to be drawn around the objects and had a 3-point mouse detection feature (nose, centre, tail base). The duration of time exploring each of the two objects and the frequency of visits to the objects was then determined based on the nose point being in these zones.

The Recognition Index (RI) was calculated for both novel and familiar objects for minutes 2-6 of the test phase expressed as a percentage (%). Minutes 2-6 were used for RI analysis in the test phase as these were the time bins in which Wt mice showed the greatest distinction between the novel and familiar object across the timeframe of the testing phase. RI was calculated as; (duration + frequency of nose in object boundary zone/ duration + frequency of nose in object boundary zone for both objects added together) x 100. The effects of genotype, sex, age and object on RI were tested using repeated measures ANOVA. Pairwise t-test with FDR correction *post hoc* analysis was performed for any significant interactions found by ANOVA, to test for significant differences between the relevant groups. Significance was set at $p<0.05$.

To test whether object interaction levels were similar between Wt and *Nrxn1α* Hz mice, duration and frequency in object and object boundary zones (added together) were calculated separately for all 10 minutes of the acquisition phase, and minutes 2-6 of the test phase. The effects of genotype, sex and age on these measures of object interaction were

tested using ANOVA in R. Significance was set at $p<0.05$. Minutes 2-6 were used for the test phase as these time bins were used to calculate the RI.

2.3.3 Cognitive Flexibility Task (CFT)

For each phase of the task Trials to Criterion (TTC), Percentage of Correct trials (PC), correct trial latency and incorrect trial latency were calculated. Any outliers were removed using Mean± 1.96 x SD of the group (genotype and age group considered). The effect of genotype, sex and age was tested on each parameter by ANOVA in R with each phase of the CFT considered independently. To test the effect of genotype, sex, age and phase on TTC and PC, repeated measures ANOVAs were performed in R comparing OD2 vs OD2R and OD2R vs SS phases. Pairwise t-test with FDR correction *post hoc* analysis was performed for any significant interactions found by ANOVA, to test for significant differences between relevant groups. Significance was set at $p<0.05$.

For OD2R error type was investigated by calculating Regressive Errors (RE), Perseverative Errors (PE) and Correction Ratio (CR) after removing outliers on the basis of Mean± 1.96 x SD. RE were calculated by recording the number of correct trials performed by the mouse after a Punishment Induced Reorientation (PIR, making a correct choice in the trial following a punishment) for the OD2R phase. This excluded the first 4 'free' trials as no punishment was given for making an incorrect choice. The frequency of each of the 5 RE trial levels (0, 1, 2, 3 or 4 consecutive correct trials following a PIR) was recorded and divided by the total number of PIRs for OD2R phase to provide a proportion (Dawson *et al.*, 2012). This measure indicated the likelihood of 'regressing' to the incorrect choice at each trail level (1-5), while controlling for the total number of PIRs a mouse experienced for the OD2R phase. The proportion values at each trial level were not normally distributed and so were square

rooted to give the data normal distribution. The effects of genotype, sex, age and trial level (1-5) was tested using a repeated measures ANOVA in R. Significance was set at $p<0.05$.

The number of PEs was determined by measuring the number of incorrect choices made in 3 of the 'free' trials, trials 2-4, in OD2R. Since the data were not normally distributed, significant differences between genotype (Wt and *Nrxn1α* Hz) and group (3 month Wt, 3 month *Nrxn1α* Hz, 6 month Wt, 6 month *Nrxn1α* Hz, 9 month Wt, 9 month *Nrxn1α* Hz, 12 month Wt and 12 month *Nrxn1α* Hz) were tested separately using Kruskal-Wallis Chi-squared test in R. Significance was set at $p<0.05$.

The CR in OD2R was also calculated to provide another indication of PEs and the mouse's response to punishment. CR was calculated by counting the number of PIRs and dividing it by the total number of punishments (excluding the 4 'free' trials), indicating the likelihood of making a correct choice following a punishment. The effect of genotype, sex and age on CR was tested using ANOVA in R. Significance was set at $p<0.05$.

2.3.4 ^{14}C-2-Deoxyglucose (^{14}C-2-DG) functional brain imaging

The ^{14}C-2-Deoxyglucose functional brain imaging analysis is described in Dawson *et al.* (Dawson *et al.*, 2013). The autoradiographic images were analysed using computer-based image analysis (MCID/M5+). Local isotope concentration for each of the 49 Regions of Interest (RoI) were derived from the optical density of the autoradiographic image relative to the coexposed ^{14}C standard. The 49 regions of interest were measured with reference to a stereotaxic mouse brain atlas (Paxinos and Franklin, 2001). Each RoI was measured between 10-12 times per animal (in 6 brain slices in both brain hemispheres if sectioning quality allowed). Isotope (^{14}C) concentration was also measured for whole slices where RoI measures were taken, these were then averaged to provide a whole brain average for each

animal. The ^{14}C-2-DG uptake ratio (UR) was then calculated for each RoI by dividing them by the whole brain average for that animal. UR outliers were removed using Mean± 1.96 x SD per genotype, sex and age group. The effect of genotype, sex and age on the UR were tested by repeated measures ANOVA in each RoI, significance was set at $p<0.05$. Pairwise t-test with FDR correction *post hoc* analysis was performed for any significant interactions found by ANOVA, to test for significant differences between relevant groups. To validate ^{14}C-2-DG functional brain imageing method, the effect of genotype, sex and age on plasma ^{14}C-2-DG concentration, blood glucose and plasma ^{14}C-2-DG concentration: whole brain average ^{14}C-2-DG concentration ratio was tested by ANOVA, significance was set at $p<0.05$.

Fly model

2.3.5 qPCR

Cycle threshold (Ct) values were determined for each sample in triplicate. Individual replicates with Ct values of more than 0.5 difference to the other 2 replicates were excluded from analysis. Relative expression was calculated using the Delta-Delta Ct method and is as follows; average Ct values were taken for each sample for both Actin and *Nrx-1* primers. Samples were excluded if the Ct value for Actin was lower than for *Nrx-1*. For each sample the *Nrx-1* primer Ct value was subtracted from the Actin Ct value to provide a normalised Ct value. The normalised Ct value for a male control sample was then subtracted from the normalised Ct value of every other sample to provide a Ct difference value per sample. Fold change was then calculated for each sample using the formula $2^{-\text{Ct difference}}$. The effect of genotype and sex on Fold Ct change was then tested by ANOVA using R. Significance was set at $p<0.05$.

2.3.6 Western Blotting

For each sample *Nrx-1* abundance was normalised to total protein using Image Lab v 6.0 (Biorad) software to provide an Adjusted Volume (AV) value, which controlled for amount of protein loaded. Data from 2 blots containing all samples were used in the analysis. Data from one female P{XP}Nrx^{d08766} sample on one blot was removed as an outlier as there was a clear decrease in *Nrx-1* abundance compared to the same sample on other blots, due to a tear in the gel. The effect of genotype, sex and blot on AV was tested using data from both blots by repeated measures ANOVA in R. Significance was set at $p<0.05$.

2.3.7 Life Span

Life span data survival curves, plotting proportion of the population still alive for each recorded day, were analysed using Microsoft Excel for individual Cohorts. Differences in life span between groups (Cohort 1, 3, 4: w^{1118} male, w^{1118} female, P{XP}Nrx^{d08766} male, P{XP}Nrx^{d08766} female, Cohort 2: male w^{1118}, male P{XP}Nrx^{d08766}) was determined using log rank tests for individual Cohorts.

In addition to the statistical analysis of independent cohorts, survival analysis of the whole data set gained from all life span experimental cohorts was performed using a Cox proportional-hazards model (Cox, 1972). This approach allows for the regression of life span in relation to multiple predictor variables, in the case of this experiment fly genotype, sex and the experimental cohort. This analysis allowed determination of the impact of these different factors on the hazard rate and the relative hazard ratio, the likelihood of death at any particular point in time, between the different factors.

2.3.8 Exploratory Walking (EW)

The 15 minute videos were analysed using tracking software (Ethovision XT v8.5, Noldus) which allowed for the arena to be divided into virtual central (2cm diameter) and outer zones. Parameters measured included walking distance, walking velocity, walking duration, walking frequency, duration and frequency in the central zone of the arena, and rotation frequency. The effects of genotype (w^{1118} and $P\{XP\}Nrx^{d08766}$), sex (male and female) and age (11, 18, 25, 32, 39, 46, 53 days old) on EW parameters were tested by ANOVAs in R. Pairwise t-test with FDR correction *post hoc* analysis was performed for any significant interactions found by ANOVA, to test for significant differences between relevant groups. Significance was set at $p<0.05$.

2.3.8 Sleep

Any inactivity period (no beam crosses) lasting more than 5 minutes was counted as sleep (Shaw *et al.*, 2000). The DAMS data was analysed using BeFLY! Analysis Tools v7.23 (Ed Green) in Microsoft Excel (Liao *et al.*, 2017) for total sleep, mean sleep bout length and number of bouts of sleep, in light and dark phases separately. Data from the first 24 hours in the DAMS was excluded as habituation time. The effects of genotype (w^{1118} and $P\{XP\}Nrx^{d08766}$), sex (male and female) and age (11, 18, 25, 32, 39, 46, 53 days old) on sleep parameters were tested by ANOVAs in R. Pairwise t-test with FDR correction *post hoc* analysis was performed for any significant interactions found by ANOVA, to test for significant differences between relevant groups. Significance was set at $p<0.05$.

Chapter 3 *Nrxn1α* Hz mice show altered locomotor activity and anxiety-like behaviour in the Open Field (OF)

3.1 Introduction

The Open Field (OF) assay was used to test the response of the *Nrxn1α* Hz mice to a novel environment, by measuring locomotor activity (walking distance, velocity, duration and frequency) and anxiety-like behaviour (central zone duration and frequency of visits). Rodent locomotor hyperactivity can be used as a model of positive symptoms in Sz (Van Den Buuse, 2010), since subcortical hyperdopaminergia is linked to both positive symptoms in Sz patients (Uchida *et al.*, 2011; Harro, 2015) and hyperlocomotor activity in rodents (Creese and Iversen, 1975; Kelly *et al.*, 1975; Antoniou *et al.*, 1998). Thus measuring locomotor activity can provide a proxy measure of dopamine hyperfunction, and potentially positive symptomatology, which cannot be directly assessed in animals. In addition, mice naturally spend more time at the perimeter of the arena compared to the centre (Thigmotaxis) during the OF test. Increases or decreases in this behaviour provide indications of anxiety-like behaviour in mice.

3.2 Key findings

- ***Nrxn1α* Hz mice show increased initial freezing followed by hyperlocomotor activity**
- ***Nrxn1α* Hz mice show increased anxiety-like behaviour**
- **Locomotor activity and anxiety-like behaviour vary dependent on sex and age**

3.3 Results

3.3.1 *Nrxn1α* Hz mice show increased initial freezing followed by hyperlocomotor activity when exposed to a novel environment

Nrxn1α Hz mice showed reduced locomotor activity in comparison to Wt mice in the first minute of exposure to the novel environment. In this way significant time x genotype interactions for distance moved [$F_{(14, 2069)}$ =1.884, p=0.0238], moving velocity [$F_{(14, 1949)}$ =1.839, p=0.0286], moving duration [$F_{(14, 1949)}$ =2.074, p=0.0107] and not moving duration [$F_{(14, 1949)}$ =2.282, p=0.0043] were found. A trend towards a significant time x genotype interaction was also found for moving frequency [$F_{(14, 1949)}$ = 1.681, p=0.0530]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that *Nrxn1α* Hz mice showed significantly decreased distance moved (p=0.0496) and increased not moving duration (p=0.0328) in the first minute of the test when compared to Wt mice (Figure 3.1, 1A and 4A respectively).

During the first minute of the OF test, Wt mice showed high levels of locomotor activity that significantly reduced by the second minute in the arena (distance 1st minute v 2nd minute p=0.0018, velocity 1st minute v 2nd minute p<0.0001, moving duration 1st minute v 2nd minute p=0.0456, not moving duration 1st minute v 2nd minute p=0.0008) (Figure 3.1, 1A-4A). By contrast, *Nrxn1α* Hz mice did not show this response and locomotor activity was not altered significantly between the 1st and 2nd minute of the test for any locomotor parameters in these animals. In addition, these measures were not significantly different between *Nrxn1α* Hz mice and their Wt littermates during the second minute of the test. While locomotor activity continued to significantly decrease in Wt mice over the remaining time of the OF test, this decrease appeared to be attenuated in the *Nrxn1α* Hz mice. Thus

Nrxn1α Hz mice showed hyperlocomotor activity compared to Wt mice at later stages of the test. This was confirmed statistically by *post hoc* Pairwise t-test with FDR correction supporting significantly increased distance moved (8^{th} minute p=0.0246, 11^{th} minute p=0.0097), increased velocity (8^{th} minute p=0.0250 and 11^{th} minute p=0.0102), increased moving duration (8^{th} minute p=0.0068 and 11^{th} minute p=0.0091) and decreased not moving duration (8^{th} minute p=0.0067 and 11^{th} minute p=0.0090) (Figure 3.1, 1A-4A).

To further characterise the time-dependent nature of the altered locomotor activity present in *Nrxn1α* Hz mice, and supported by the observation of significant time x genotype interactions in this test, data from the 1^{st} minute and from minutes 2-15 were analysed separately (Figure A, panels B and C respectively). A significant main effect of genotype was found in data from both the first minute and for minutes 2-15 for distance moved (1^{st} minute: [$F_{(1, 130)}$ =7.102, p=0.0086], minute 2-15: [$F_{(1, 1819)}$ =24.813, p<0.0001]), velocity (1^{st} minute: [$F_{(1, 130)}$ =5.641, p=0.0190], minute 2-15: [$F_{(1, 1819)}$ =24.763, p<0.0001]), moving duration (1^{st} minute: [$F_{(1, 130)}$ =6.782, p=0.0103], minute 2-15: [$F_{(1, 1819)}$ =20.285, p<0.0001]) and not moving duration (1^{st} minute: [$F_{(1, 130)}$ =11.267, p=0.0010], minute 2-15: [$F_{(1, 1819)}$ =20.285, p<0.0001]), but not for moving frequency. This analysis further confirmed the hypolocomotor activity seen in *Nrxn1α* Hz mice during the first minute of the test with *Nrxn1α* Hz mice showing a significantly reduced distance moved (p=0.0070, main effect ANOVA), velocity (p=0.0178, main effect ANOVA) and moving duration (p=0.0098, main effect ANOVA), and significantly increased not moving duration (p<0.0001, main effect ANOVA) in comparison to Wt animals (Figure 3.1, panel B). By contrast, analysis of the data from 2-15 minutes in the arena supported significant hyperlocomotor activity in *Nrxn1α* Hz mice with significantly increased distance moved (p<0.0001, main effect ANOVA), velocity (p<0.0001, main effect ANOVA), moving duration (p<0.0001, main effect ANOVA) and

significantly decreased not moving duration ($p<0.0001$, main effect ANOVA) in comparison to Wt animals (Figure 3.1, panel C).

3.3.2 Age has little impact on changes in locomotor activity caused by *Nrxn1α* heterozygosity

Nrxn1α heterozygosity altered locomotor activity in mice in the OF, dependent on time, as previously described. It was found that locomotor activity varied with age in the four age groups of mice tested, however age did not impact on changes in locomotor activity caused by *Nrxn1α* heterozygosity, except for moving duration and not moving duration parameters at 9 months old only (data not shown). In this way no significant age x genotype interactions were found for any locomotor parameters in the first minute of testing. In addition, while significant age x genotype interactions were found for distance [$F_{(3, 1819)}= 8.781$, $p<0.0001$], velocity [$F_{(3, 1819)}= 8.783$, $p<0.0001$], moving duration [$F_{(3, 1819)}= 13.742$, $p<0.0001$], not moving duration [$F_{(3, 1819)=}$ 13.742, $p<0.0001$] and moving frequency [$F_{(3, 1819)}= 3.223$, $p=0.0218$] in minutes 2-15, evidence that age significantly impacts on changes in locomotor activity caused by *Nrxn1α* heterozygosity from *post hoc* testing was only found for moving duration and not moving duration parameters. *Post hoc* testing using Pairwise t-test with FDR correction showed that 9 month old *Nrxn1α* Hz mice had significantly higher moving duration ($p=0.0243$) and significantly lower not moving duration ($p=0.0230$) than 9 month old Wt mice in minutes 2-15 of OF testing (data not shown), while no significant differences were found at any other age group.

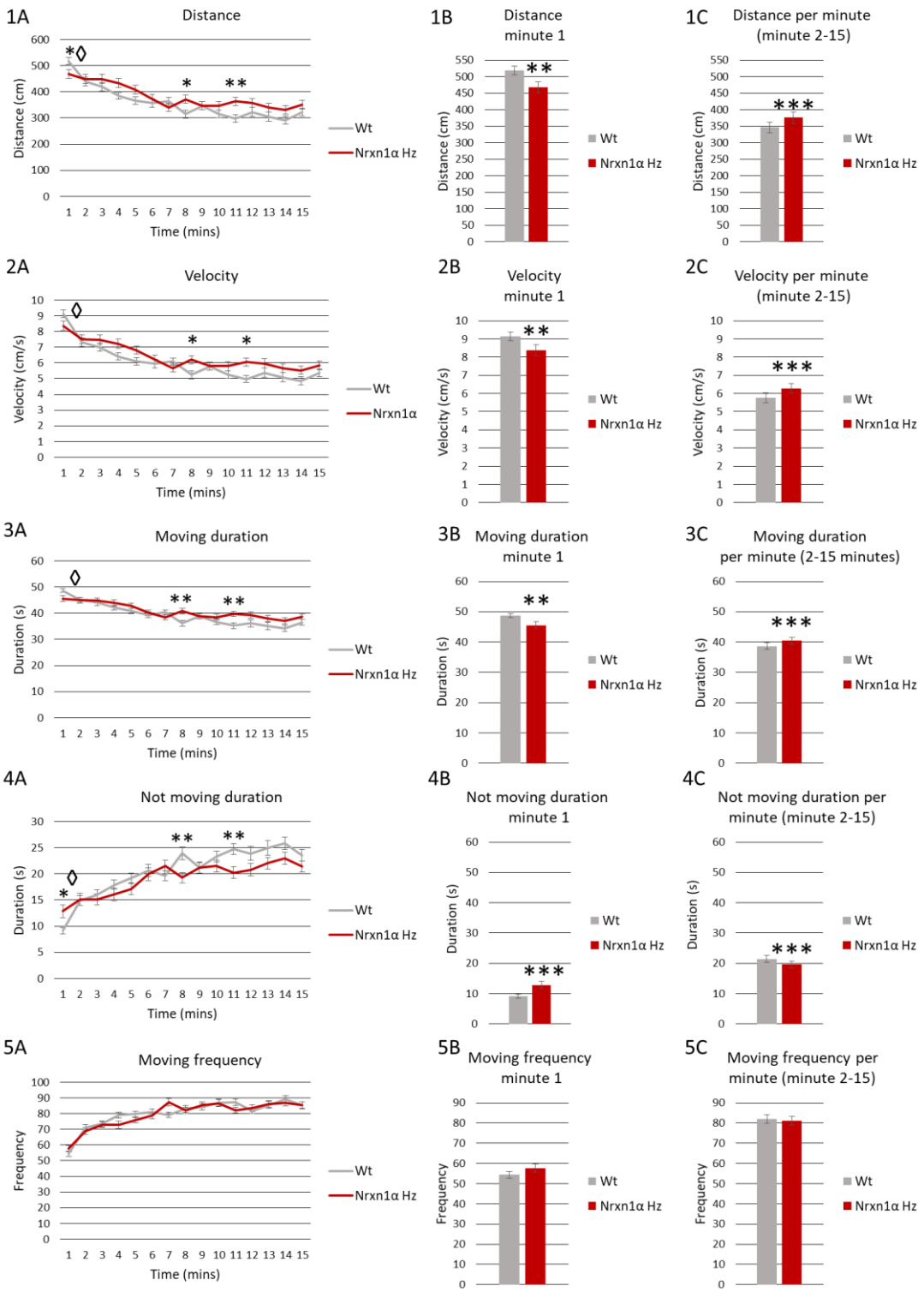

Figure 3.1 Nrxn1α Hz mice show increased initial freezing followed by hyperlocomotor activity when exposed to a novel environment *Data shown are mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. Measures of locomotor activity include* ***(1)*** *Distance,* ***(2)*** *Velocity,* ***(3)*** *Moving duration,* ***(4)*** *Not moving duration and* ***(5)*** *Moving frequency. Each measure is shown as* ***(column A)*** *per minute,* ***(column B)*** *averaged for minute 1 and* ***(column C)*** *averaged per minute for minutes 2-15. A significant time x genotype interaction was found for Distance [$F_{(14, 2069)}$ =1.884, p=0.0238], Velocity [$F_{(14, 1949)}$ =1.839, p=0.0286], Moving duration [$F_{(14, 1949)}$ =2.074, p=0.0107] and Not moving duration [$F_{(14, 1949)}$ =2.282, p=0.0043]. A significant main effect of genotype was found in data from both the first minute and for minutes 2-15 for Distance (1st minute: [$F_{(1, 130)}$ =7.102, p=0.0087], minute 2-15: [$F_{(1, 1819)}$ =24.813, p<0.0001]), Velocity (1st minute: [$F_{(1, 130)}$ =5.641, p=0.0190], minute 2-15: [$F_{(1, 1819)}$=24.763, p<0.0001]), Moving duration (1st minute: [$F_{(1, 130)}$ =6.782, p=0.0103], minute 2-15: [$F_{(1, 1819)}$ =20.285, p<0.0001]) and Not moving duration (1st minute: [$F_{(1, 130)}$ =11.267, p=0.0010], minute 2-15: [$F_{(1, 1819)}$ =20.285, p<0.0001]). Sample sizes were Wt n=72, Nrxn1α Hz n=74. ◊ denotes significant change in 2nd minute compared to 1st minute in Wt (Pairwise t-test with FDR correction). * denotes p<0.05, ** denotes p<0.01, *** denotes p<0.001 significant difference compared to Wt (Pairwise t-test with FDR correction in* ***column A*** *and main effect of genotype in ANOVA for* ***column B and C****).*

3.3.3 Locomotor activity varies with age and sex in a novel environment

Locomotor activity parameters varied dependent on age. For distance moved and moving frequency, mice of both sexes showed age-dependent differences (Figure 3.2 A and B respectively), however for movement velocity (Figure 3.3 A) and duration (Figure 3.3 B) male, but not female, mice showed age-dependent differences. Data from all 15 minutes of OF recording were used in the analysis of effects, as age did not significantly alter the changes in locomotor activity seen across time (while there were significant time x age interactions for locomotor parameters *post hoc* testing did not support the significance of these effects). A significant main effect of age was found for distance moved [$F_{(3, 1949)}$ =19.342, p<0.0001] and moving frequency [$F_{(3, 1949)}$ =21.748, p<0.0001]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that 6 month old mice moved significantly (or close to significantly) less distance than 3, 9, and 12 month old mice (3 v 6 p=0.0580, 9 v 6 p= 0.0230, 12 v 6 p=0.0370) (Figure 3.2 A). Furthermore 9 month old mice moved significantly less frequently (less stop-starts) than 3, 6 and 12 month old mice (3 v 9 p=0.0033, 6 v 9 p<0.0001, 12 v 9 p<0.0001). Furthermore 3 and 6 month old mice moved significantly less frequently than 12 month old mice (3 v 12 p<0.0001 and 6 v 12 p=0.0004) (Figure 3.2 B).

A significant sex x age interaction was found for velocity [$F_{(3, 1949)}$ =5.812, p=0.0006] and moving duration [$F_{(3, 1949)}$ =6.321, p=0.0003]. *Post hoc* testing also revealed that male mice showed significantly lower moving duration at 6 months old compared to 9 and 12 months old (6 v 9 p=0.0360, 6 v 12 p=0.0200) (Figure 3.3 A). Furthermore, velocity in 6 month old males was significantly decreased compared to 3, 9 and 12 month old males (3 v 6 p=0.0310, 9 v 6 p=0.0360, 12 v 6 p=0.0200) (Figure 3.3 B).

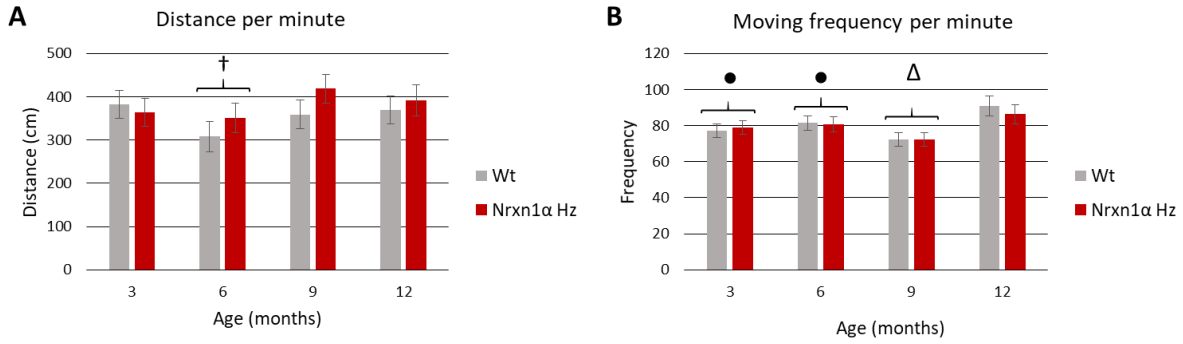

Figure 3.2 Distance moved and movement frequency varies with age

Data shown are mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. Measures of locomotor activity include ***(A)*** *Distance and* ***(B)*** *Movement frequency averaged per minute for the total 15 minutes of recording, plotted by genotype for each age group. A significant main effect of age was found for Distance [$F_{(3, 1949)}$ =19.342, p<0.0001] and Moving frequency [$F_{(3, 1949)}$ =21.748, p<0.0001]. Sample sizes were; Wt: 3 month n=21, 6 month n=15, 9 month n=19, 12 month n=17, Nrxn1α Hz: 3 month n=19, 6 month n=17, 9 month n=19, 12 month n=19. † denotes p<0.05 significant decrease compared to 9 month and 12 month old groups. Δ denotes p<0.05 significant decrease compared to 3 month, 6 month and 12 month old groups. ● denotes p<0.01 significant decrease compared to 12 month old group (Pairwise t-test with FDR correction).*

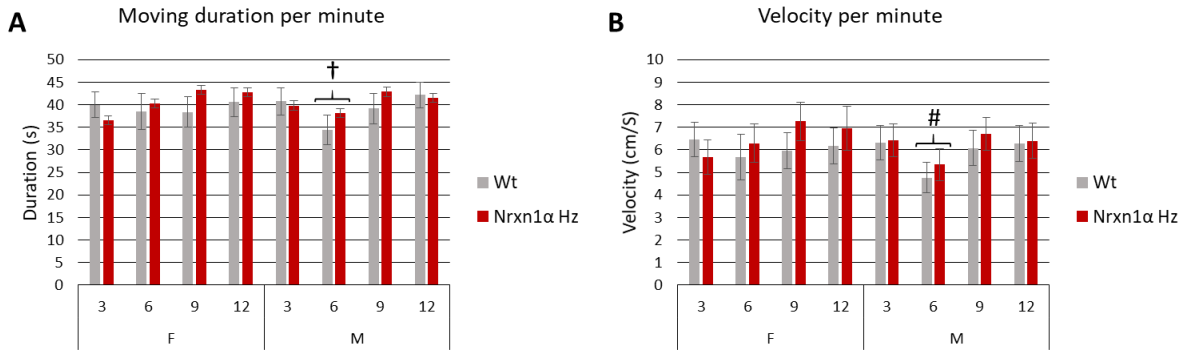

Figure 3.3 Movement duration and velocity varies with age in male but not female mice

Data shown are mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous, F=female, M=male, 3=3 months old, 6=6 months old, 9=9 months old, 12=12 months old. Measures of locomotor activity include ***(A)*** *Moving duration and* ***(B)*** *Velocity averaged per minute for the total 15 minutes of recording, plotted by genotype per age group and sex. A significant sex x age interaction was found for Moving duration [$F_{(3, 1949)}$ =6.321, p=0.0003] and Velocity [$F_{(3, 1949)}$ =5.812, p=0.0006]. Sample sizes were Wt; females: 3 month n=12, 6 month n=6, 9 month n=9, 12 month n=9, males: 3 month n=9, 6 month n=9, 9 month n=10, 12 month n=8, Nrxn1α Hz; females: 3 month n=9, 6 month n=9, 9 month n=10, 12 month n=9, males: 3 month n=10, 6 month n=8, 9 month n=9, 12 month n=10. † denotes p<0.05 significant decrease compared 9 month and 12 month old males. # denotes p<0.05 significant decrease compared to 3 month, 9 month and 12 month old males (Pairwise t-test with FDR correction).*

3.3.4 *Nrxn1α* Hz mice show increased anxiety-like behaviour in a novel environment

Anxiety-like behaviour was measured by creating a central zone in the arena during analysis and calculating duration of time spent inside the central zone and frequency of visits to the central zone. *Nrxn1α* Hz mice showed increased anxiety-like behaviour in the novel arena reflected by the decreased amount of time spent in the central zone (Figure 3.4 A). By contrast *Nrxn1α* Hz mice showed no difference in the number of central zone visits as compared to Wt mice (Figure 3.4 B). This altered behaviour in the *Nrxn1α* Hz mice was not significantly modified by age or sex (no significant age x genotype or sex x genotype interactions). As there was no significant time x genotype interaction for anxiety-like parameters (duration and frequency in the central zone of the arena) data from all 15 minutes of recording were included in the analysis. While significant age x genotype interactions were found for both duration [$F_{(3, 1949)} = 10.277$, $p<0.0001$] and frequency [$F_{(3, 1949)} = 11.384$, $p<0.0001$] in the central zone, *post hoc* testing revealed that there was no significant effect of genotype within each of the independent age groups. Therefore, a significant main effect of genotype [$F_{(1, 1949)} = 15.692$, $p<0.0001$] was accepted for duration in central zone but was not supported for frequency in the central zone (no significant main effect of genotype).

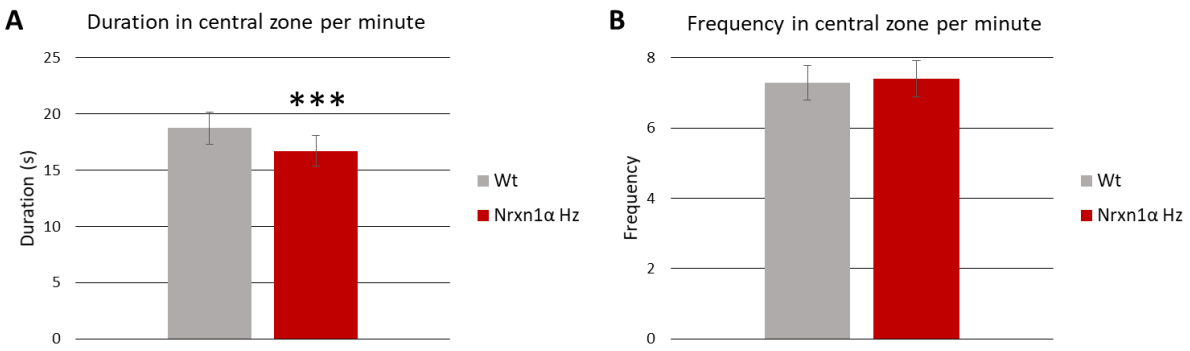

Figure 3.4 Nrxn1α Hz mice show increased anxiety-like behaviour in a novel arena

Data are mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. Anxiety-like measures include ***(A)*** *Duration and* ***(B)*** *Frequency in the central zone per minute averaged across all 15 minutes of recording. A significant main effect of genotype was found for duration in the central zone [$F_{(1, 1949)} = 15.692$, $p<0.0001$], but not for frequency in the central zone. Sample sizes were Wt n=72 and Nrxn1α Hz n=72. ***denotes p<0.0001 significant decrease compared to Wt (main effect ANOVA).*

3.3.5 Male and female mice show age-dependent changes in frequency of visits to the central zone

Male and female mice showed significantly increased frequency of visits to the central zone at 12 months old compared to younger ages (Figure 3.5 B). For females this effect was evident at 3 compared to 12 months old, while in males this effect was evident at 6 compared to 12 months old. A significant sex x age interaction was found for frequency of visits to the central zone [$F_{(3, 1949)} = 5.185$, $p=0.0014$] and duration in the central zone [$F_{(3, 1949)} = 11.384$, $p<0.0001$]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that 12 month old female mice had a significantly higher number of visits to the central zone than 3 month old female mice ($p=0.0460$), and male 12 month old mice had a significantly higher number of visits to the central zone than 6 month old male mice ($p=0.0310$) (Figure 3.5 B). Differences between age groups did not survive *post hoc* testing for duration in the central zone (Figure 3.5 A). There were also no significant differences between the two sexes at any age group, for frequency of visits to the central zone or duration in the central zone.

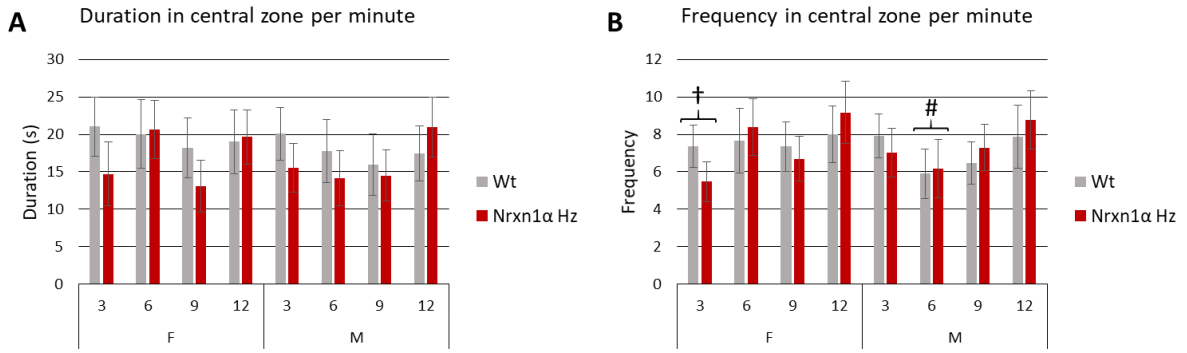

Figure 3.5 Male and female mice show age dependent changes in frequency of visits to the central zone
Data are mean ± SEM. F=female, M=male. Anxiety-like measures include ***(A)*** *Duration and* ***(B)*** *Frequency in the central zone per minute averaged across all 15 minutes of recording, plotted by genotype per age group and sex. A significant sex x age interaction was found for duration in the central zone [$F_{(3, 1949)}$ =11.384, p<0.0001] and frequency in the central zone [$F_{(3, 1949)}$ =5.185, p=0.0014]. Sample sizes were Wt; females: 3 month n=12, 6 month n=6, 9 month n=9, 12 month n=9, males: 3 month n=9, 6 month n=9, 9 month n=10, 12 month n=8, Nrxn1α Hz; females: 3 month n=9, 6 month n=9, 9 month n=10, 12 month n=9, males: 3 month n=10, 6 month n=8, 9 month n=9, 12 month n=10. † denotes p<0.05 significant decrease compared to 12 month old females. # denotes p<0.05 significant decrease compared to 12 month old males (Pairwise t-test with FDR correction).*

Open Field parameter	Impact of *Nrxn1α* heterozygosity	Modified by Sex?	Modified by Age?
Distance moved	↓ minute 1 ↑ minutes 2-15	-	-
Moving velocity	↓ minute 1 ↑ minutes 2-15	-	-
Moving duration	↓ minute 1 ↑ minutes 2-15	-	-
Moving frequency	-	-	-
Duration in central zone	↓	-	-
Frequency in central zone	-	-	-

Table 3.1 Summary table of the effects of Nrxn1α heterozygosity on locomotor activity and anxiety-like parameters in the OF

↑ denotes significant increase and ↓ denotes significant decrease in *Nrxn1α* Hz mice compared to Wt. – denotes no significant effect. The impact of *Nrxn1α* heterozygosity on locomotor or anxiety-like behaviours was not significantly modified by sex or age.

3.4 Discussion

There were three keys findings from application of the OF test in *Nrxn1α* Hz mice; (1) *Nrxn1α* Hz mice show increased initial freezing followed by hyperlocomotor activity when exposed to a novel environment, (2) *Nrxn1α* Hz mice show increased anxiety-like behaviour and (3) both locomotor activity and anxiety-like behaviour vary dependent on sex and age.

Nrxn1α Hz mice show increased initial freezing followed by hyperlocomotor activity when exposed to a novel environment

When exposed to a novel environment *Nrxn1α* Hz mice showed an increase in initial freezing behaviour as compared to Wt mice, evident in the first minute of the OF test. Wt mice showed higher locomotor activity in the first minute of the OF test, that was significantly reduced by the second minute, a response that was not seen in the *Nrxn1α* Hz mice (Figure 3.1 Panel A). During the remaining 14 minutes of the OF test *Nrxn1α* Hz mice showed increased locomotor activity as compared to Wt mice (Figure 3.1 Panel C), suggesting that the baseline level of locomotor activity is higher in *Nrxn1α* Hz mice following familiarisation with the OF arena. The initial response upon entering the arena of increased freezing in *Nrxn1α* Hz mice may suggest they are more fearful of novel environments than Wt mice, since freezing behaviour is a fear response in rodents (Ji and Maren, 2008). This suggestion would also be consistent with increased anxiety-like behaviour seen in *Nrxn1α* Hz mice (Figure 3.4 A).

Rodent locomotor hyperactivity has previously been used as a model of positive symptoms in Sz (Van Den Buuse, 2010), since sub cortical hyperdopaminergia is linked to both positive symptoms in Sz patients (Uchida *et al.*, 2011; Harro, 2015) and hyperlocomotor activity in rodents (Creese and Iversen, 1975; Kelly *et al.*, 1975; Zhou and Palmiter, 1995; Giros *et al.*,

1996a; Antoniou *et al.*, 1998). Therefore measuring locomotor activity can provide a proxy measure of dopamine hyperfunction, and potentially positive symptomology, which cannot be directly assessed in animals. Interestingly, some evidence suggests that Sz patients also show hyperlocomotor activity in novel environments (Perry *et al*., 2009). Dopaminergic dysfunction (Hellings *et al.*, 2017; Paval, 2017) and hyperactivity is also implicated in ASD, since ADHD is a common comorbidity of ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012; Antshel *et al.*, 2016). Patients with ADHD, another neurodevelopmental disorder, have been shown to have increased locomotor activity as part of the hyperactivity symptom domain of the disorder (Garcia Murillo *et al.*, 2015), and have also been found to have dopaminergic system dysfunction (Spencer *et al.*, 2005). Furthermore, there is also evidence for hyperactivity in children with deletions in *NRXN1α* (Ching *et al.*, 2010; Schaaf *et al.*, 2012). Since the *Nrxn1α* Hz mice in the current study show hyperlocomotor activity after the first minute of the OF test, this could indicate dopamine system dysfunction. However due to many other neurotransmitter systems being implicated in rodent locomotor activity (Viggiano, 2008), and the fact that the dopamine hypothesis does not fully explain positive symptoms in Sz (Moghaddam, 2003; Tamminga *et al.*, 2003; Moghaddam and Javitt, 2012; Laruelle, 2014), alterations in neurotransmitter systems other than the dopamine system could underlie the hyperlocomotor activity observed in the *Nrxn1α* Hz mice. The potential for dopaminergic alterations as a consequence of *Nrxn1α* heterozygosity certainly warrants further investigation, given the current findings and the central role for this neurotransmitter system in both Sz and ASD.

Although the OF test has mainly been used as an indicator of positive symptoms of Sz in animal models, tests of locomotor activity and exploration can also be back-translated to

human behaviour. This can be achieved using the behavioural pattern monitor (BPM), a novel arena containing objects, which is used to measure locomotor activity, exploratory behaviour and locomotor path patterns in both rodents and humans. In validation of the task, findings of hyperactivity and increased exploration of objects in mouse models of Bipolar Disorder (BD) in the BPM test, have been translational to findings in BD patients (Young *et al.*, 2016). A study using the BPM test in Sz patients showed that they were hyperactive in the later parts of the test (last 5 minutes) compared to control subjects (Perry *et al.*, 2009). Further analysis of these patients showed that they had significantly increased walking duration compared to control subjects (Perry *et al.*, 2010). These findings from the BPM test in Sz patients support the potential translation value of the locomotor alterations found in the OF in *Nrxn1α* Hz mice. Both Sz patients and *Nrxn1α* Hz mice showed hyperactivity in the later phases of the task and had increased movement duration, which suggests common brain circuitry may be affected in both cases even though the behavioural tasks are not exactly equivalent. Testing *Nrxn1α* Hz mice in the rodent BPM test would be of future interest to further test the translational value of this measure.

Other studies in *Nrxn1α* Hz mice did not analyse OF behaviour using 1 minute time bins (Laarakker *et al.*, 2012; Grayton *et al.*, 2013; Dachtler *et al.*, 2015), and so the initial reduction in locomotor activity that was identified in these animals in the current study, has not been reported. One study using *Nrxn1α* Hz mice found that male, but not female, mice showed hyperlocomotor activity during the first 5 minutes of exposure to a novel arena compared to Wt (Laarakker *et al.*, 2012). The current study found hyperlocomotor activity in both sexes after the first minute of OF testing, therefore the findings of Laarakker *et al.* only partially supports this. The ability to detect hyperlocomotor activity in female *Nrxn1α* Hz mice in the current study could be due to locomotor activity being measured across a longer

timeframe (15 minutes in the current study as compared to 3 x 5 minutes with a 60 minute inter-trial interval). However, in contrast to these findings, other studies using *Nrxn1α* Hz mice found no difference in locomotor activity as compared to Wt mice (Grayton *et al.*, 2013; Dachtler *et al.*, 2015). Interestingly a study in *Nrxn1α* KO mice have showed that females actually have hypolocomotor activity (Grayton *et al.*, 2013), in contrast to hyperlocomotor activity found here in *Nrxn1α* Hz mice, or no difference in locomotor activity found previously in *Nrxn1α* KO mice (Etherton *et al.*, 2009). This suggests there could be a complex gene-dosage relationship for the effects of *Nrxn1α* expression on locomotor activity. A study in *Nrxn1α* KO rats showed that both male and females displayed hyperlocomotor activity in the OF compared to Wt (Esclassan *et al.*, 2015), suggesting there may be species differences in the effects of *Nrxn1α* KO on locomotor activity. *Nrx-1* KO flies show reduced locomotor activity as larvae, however this is thought to be, in part, due to lost *Nrx-1* expression at the neuro-muscular junction (Li *et al.*, 2007). In support of findings of reduced locomotor activity in *Nrx-1* KO fly larvae, the current study found opposite effects of *Nrx-1* overexpression, since male adult flies showed hyperlocomotor activity (Section 8.4.2).

It is important to note that there were differences between studies in the OF tests performed, which may account for result discrepancies between previous studies and the current study for both locomotor activity and anxiety-like behavioural findings in the *Nrxn1α* mice. For instance Grayton *et al.* used a rectangular home cage set up that was novel upon entry, the duration of the test was 2 hours and the data was analysed in 10 minute time bins (Grayton *et al.*, 2013). Dachtler *et al.* used a square arena that the mouse remained in for 30 minutes and the data were analysed in 5 minute time bins (Dachtler *et al.*, 2015). Laarakker *et al.* used a rectangular empty home cage that the mice remained in for 3 x 5 minutes with

an inter-trial interval of 60 minutes (Laarakker *et al.*, 2012). Etherton *et al.* used a square arena with mice being exposed for 5 minutes on 4 consecutive days (Etherton *et al.*, 2009). By contrast, the current study used a novel circular white arena, with a test duration of 15 minutes and the data were analysed in 1 minute time bins. When comparing these studies to the current study, if prolonged or repeated exposure to the arena was performed only the initial response upon first entry to the novel arena was considered. As well as differences in OF protocols, smaller samples sizes were used in the previous studies compared to the current study which may not have had enough statistical power to detect small changes in locomotor activity or anxiety-like behaviour in *Nrxn1α* Hz or KO mice (Grayton *et al*. n=18-29, Laarakker *et al*. n=20, Dachtler *et al*. n=16-24, current study n=72-74 per genotype).

Other rodent models of Sz and ASD have been shown to have hyperlocomotor activity in a novel environment. These includes pharmacological models of Sz such as acute administration of amphetamine (modelling hyperdopaminergia (Howes and Kapur, 2009)) (Kuczenski *et al.*, 1995) and acute Phencyclidine (PCP) (modelling NMDA receptor hypofunction (Olney *et al.*, 1999)) (Gleason and Shannon, 1997) in rats, and both acute and sub-chronic administration of PCP in mice (Castane *et al.*, 2015). A number of genetic mouse models of Sz also show hyperlocomotor activity. These include Sz risk gene models such as *Neuroligin1 (Nlgn1)* Hz deletion mice (Karl *et al.*, 2007) (the trans-synaptic binding partner of *Nrxn1α*), mice expressing the dominant negative form of *Disrupted In Schizophrenia 1 (DISC1)* (Hikida *et al.*, 2007), and female *DISC1* D453G missense mutation mice (Dachtler *et al.*, 2016). In addition, hyperactivity is also found in *DAT* KO mice (Giros *et al.*, 1996b), a genetic model of hyperdopaminergia in Sz. Finally mouse models of maternal stress, which are of aetiological relevance to Sz (Koenig *et al.*, 2002; Khashan *et al.*, 2008) and ASD (Kinney

et al., 2008), have also been shown to induce hyperlocomotor activity in offspring (Matrisciano *et al.*, 2013; Bronson and Bale, 2014).

Various genetic mouse models of ASD have also been shown to demonstrate hyperlocomotor activity in a novel environment. These include the *Fragile X Mental Retardation 1* (*Fmr1*) KO mice modelling Fragile X syndrome, which is the leading genetic cause of autism (Coffee *et al.*, 2009), *eukaryotic translation initiation factor 4E transgenic (eIF4E Tg)* mice modelling a genetic risk factor for ASD (Neves-Pereira *et al.*, 2009), and the combined *eIF4E Tg*/*Fmr1* double mutant mice (Huynh *et al.*, 2015).

Summary 1

Nrxn1α Hz mice show initial increased freezing behaviour, reflected by initial hypolocomotion in the first minute, followed by hyperlocomotor activity in the OF. Increased freezing behaviour in the *Nrxn1α* Hz mice may be due to increased fear of the novel environment upon initial exposure, and may also relate to the increased anxiety-like behaviour seen in these animals (Section 3.6). Hyperlocomotor activity after the first minute of the OF in the *Nrxn1α* Hz mice could be due to alterations in specific brain areas or neurotransmitter systems, such as the dopaminergic system which is implicated in locomotor activity, Sz and ASD. However further research is required to confirm this. In support of hyperlocomotor activity after the first minute of testing in the *Nrxn1α* Hz mice, hyperactivity was also found in Sz patients at later time points in an activity test. Furthermore, although findings from the OF in previous studies of *Nrxn1α* Hz mice only partially support the findings of the current study, this may be due to differences in OF protocol, data analysis or sample sizes used. Finally, a number of rodent models of Sz and ASD, modelling various aspects of the disorders, show hyperlocomotor activity similar to

that found in the *Nrxn1α* Hz mice in the current study. This suggests that differing risk factors for these disorders converge on common biological pathways, which in turn leads to hyperlocomotor activity in these rodent models.

Nrxn1α Hz mice show increased anxiety-like behaviour

Nrxn1α Hz mice showed increased anxiety-like behaviour in the form of significantly decreased time spent in the central zone of the arena as compared to Wt mice (Figure 3.4 A). Anxiety involves complex circuitry involving emotion and fear which includes brain areas such as the amygdala, prefrontal cortex and insular cortex (Taylor and Whalen, 2015). Mice naturally spend more time in the outside of the OF arena compared to the inside (Thigmotaxis) which can be used as an index of anxiety. Anxiolytic (anxiety reducing) drugs have been shown to increase time spent in the central zone of the OF arena, whereas anxiogenic (anxiety inducing) drugs have been shown to reduce it (Simon *et al.*, 1994). A number of neurotransmitter systems have been implicated in anxiety-like behaviour in the OF. For example benzodiazepines, which potentiate GABAergic transmission at a subset of $GABA_A$ receptors, reduce anxiety-like behaviour in the OF (Simon *et al.*, 1994; Mohammad *et al.*, 2016), whereas KO of the serotonin transporter (SERT) in mice has been shown to increase anxiety-like behaviour in the OF (Kalueff *et al.*, 2007; Mohammad *et al.*, 2016). Therefore, brain regions and neurotransmitter systems involved in anxiety or fear circuitry could be altered in *Nrxn1α* Hz mice, resulting in increased anxiety-like behavioural measures. A significant reduction in the duration of time spent in the central zone of the arena may be due to hyperlocomotor activity in the *Nrxn1α* Hz mice. Significantly increased velocity in these mice (Figure 3.1 2C) would mean they enter and exit the central zone faster than Wt mice. However, increased anxiety-like behaviour in *Nrxn1α* Hz mice is further

supported by their increased freezing, a fear response in mice (Ji and Maren, 2008), upon first exposure (minute 1) to the OF arena (Figure 3.1 Panel A and B).

In contrast to the findings in the current study, three other studies testing *Nrxn1α* Hz mice found no difference in duration of time spent in the central zone of the OF arena compared to Wt mice (Laarakker *et al.*, 2012; Grayton *et al.*, 2013; Dachtler *et al.*, 2015). Studies in *Nrxn1α* KO mice have also shown no difference in duration of time spent in the central zone of the arena (Etherton *et al.*, 2009; Grayton *et al.*, 2013). Furthermore, *Nrxn1α* Hz mice have not been found to have altered anxiety-like behaviour in other tests of anxiety-like behaviour including the elevated plus maze (EPM) and the light/dark box (Grayton *et al.*, 2013; Dachtler *et al.*, 2015). However, male *Nrxn1α* KO mice have previously been found to have increased anxiety-like behaviour in the light/dark box test (Grayton *et al.*, 2013) but they were no different to Wt mice in the EPM (Etherton *et al.*, 2009; Grayton *et al.*, 2013). As explained in the previous section, there were a number of differences between studies including smaller sample sizes and protocol variations that could account for the contradiction in findings.

Anxiety is a common comorbidity of ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012; Vasa and Mazurek, 2015) and Sz (Siris, 2000; Tibbo *et al.*, 2003; Braga *et al.*, 2004; Braga *et al.*, 2013; Young *et al.*, 2013; Temmingh and Stein, 2015; An Der Heiden *et al.*, 2016; Upthegrove *et al.*, 2017). Increased anxiety-like behaviour has also been found in a number of animal models of relevance to Sz and ASD in the OF. These include rodent models of Sz such as the maternal immune activation (using Poly I:C) rat model (Gibney *et al.*, 2013) and female *DISC1* D453G missense mutation mice (Dachtler *et al.*, 2016). Increased anxiety-like behaviour was also

seen in female, but not male, *COMT* homozygous deletion mice in the light/dark box assay (Gogos *et al.*, 1998). Mouse models of genetic risk factors for ASD including mutations in *Nrxn2α* and mouse models of deletions at 16p11.2 (Kumar *et al.*, 2008; Weiss *et al.*, 2008; Fernandez *et al.*, 2010; Gauthier *et al.*, 2011; Mohrmann *et al.*, 2011) have also been shown to have increased anxiety-like behaviour in the OF (Dachtler *et al.*, 2014; Pucilowska *et al.*, 2015). Other genetic mouse models of ASD have shown increased anxiety-like behaviour in the EPM including a *Tuberous Sclerosis 2* (*Tsc2)* dominant negative mouse model of Tuberous Sclerosis (Ehninger and Silva, 2011), a disorder which commonly incorporates ASD (Bolton *et al.*, 2002), and the eIF4E Tg/*Fmr1* double mutant mouse, modelling two risk genes for ASD (Huynh *et al.*, 2015). Taken together, increased anxiety-like behaviour is found in a variety of rodent models of relevance to Sz and ASD, and in patients, suggesting that the different causes of Sz and ASD may converge on a common biological pathway to induce this phenotype.

Summary 2

The *Nrxn1α* Hz mice show increased anxiety-like behaviour in the OF which may be due to alterations in brain circuitry involved in fear and emotion. Previous studies in *Nrxn1α* Hz mice have found no differences in anxiety-like behaviour contrasting with the findings of the current study, however this may be due to differences in the OF protocol and sample sizes used. Anxiety is commonly found in both Sz and ASD, and anxiety-like behaviour has been shown to be increased in other rodent models of these disorders.

Locomotor activity and anxiety-like behaviour vary dependent on sex and age

Locomotor activity and anxiety-like behaviour in the OF showed variability dependent on sex and age (Figure 3.2 and 3.3). For locomotor parameters, 6 month old mice showed a reduction in distance moved compared to the other age groups, and 12 month old mice showed an increase in moving frequency compared to the other age groups, in both male and female mice (Figure 3.2 A and B respectively). There were also sex effects on locomotor activity that were modulated by age, including 6 month old male mice showing decreased moving duration and moving velocity compared to other age groups (Figure 3.3 A and B respectively). For anxiety-like behaviour, there was no evidence that age or sex impacted on duration of time spent in the central zone (Figure 3.5 A), however age did impact on the frequency of visits to the central zone within each sex (Figure 3.5 B). In this way, 3 month old female mice showed decreased frequency in the central zone compared to 12 month old females, and 6 month old males showed decreased frequency in the central zone compared to 12 month old males (Figure 3.5 B). The data support a complex trajectory of age-dependent effects on these measures of locomotor activity and anxiety-like behaviour, rather than a linear increase or decrease. This variability with age may reflect complex neurodevelopmental trajectories that occur during adult maturation, and these may be somewhat influenced by sex.

There are a limited number of studies that have compared different age groups of mice in the OF. One such study was carried out by Shoji *et al.* who used 1739 Wt littermate male mice of genetically modified mouse strains with a C57BL/6 background from multiple institutions in Japan (Shoji *et al.*, 2016). The mice were grouped into 4 age groups; 2-3 (n=340), 4-5 (n=135), 6-7 (n=54) and 8-12 (n=22) months old and tested in the OF. They

found that 2-3 month old mice showed significantly higher distance moved in the first 5 minutes of the OF test compared to 6-7 and 8-12 month old mice, and that 4-5 month old mice showed significantly increased distance moved compared to 8-12 month old mice. However when data were analysed across the whole OF test duration (120 minutes), no differences in distance moved between the different age groups were found. In the current study, only 6 month old mice showed significantly decreased distance moved compared to all other age groups, in the whole 15 minutes of the test. Since Shoji *et al.* do not report any other locomotor parameters that the current study used (moving duration, frequency or velocity), further comparisons between studies cannot be made for locomotor activity. However, for anxiety-like behavioural parameters both Shoji *et al.* and the current study found no difference in duration of time spent in the central zone of the arena between the different age groups of mice. The difference in findings between Shoji *et al.* and the current study for distance moved in the different age groups of mice could be due to a number of reasons. Firstly, the current study did not find a significant time x age interaction for distance moved and so data from all minutes of the OF recording was used, rather than looking in the first 5 minute time bin, as analysed in Shoji *et al.* Secondly the sample sizes in Shoji *et al.* were larger than those employed in the current study (current study: 3 month n=40, 6 month n=32, 9 month n=38 and 12 month n=36, Shoji *et al.* 2-3 month n=340, 4-5 month n=135, 6-7 month n=54 and 8-12 month n=22). Thirdly the age groups in Shoji *et al.* involved larger ranges in age than the current study, as groups included animals with an 8 week age range. Fourthly the mice used in the Shoji *et al.* study were not from the same colony and some had mixed C57BL/6 genetic backgrounds, whereas in the current study mice were from the same colony but included both Wt and *Nrxn1α* Hz genotypes. Finally, in Shoji *et al.* mice were raised in differing conditions since they were collected from different

institutions prior to behavioural testing, whereas all experimental animals in our study were born, raised and tested in the same animal unit. The locomotor activity parameters may be more sensitive to the differences between the two studies compared to anxiety-like behavioural parameters in the OF, since both the current study and Shoji *et al.* are in agreement that age did not affect duration of time spent in the central zone.

Two studies from Fahlstrom *et al.* compared male (Fahlstrom *et al.*, 2012) and female (Fahlstrom *et al.*, 2011) C57BL/6 mice separately at different ages. Fahlstrom *et al.* (2011) compared 3, 8, and 28 month old C57BL/6 female mice in the OF, and found that distance moved was significantly increased at 28 months old compared to the other age groups. There was also no difference in time spent in the central zone of the arena between age groups. This partially supports the findings in the current study where there was no difference in distance moved or duration of time spent in the central zone between 3 and 9 month old mice of both sexes. However male and female mice, rather than just female, and 9 month old mice, rather than 8 month old, were used in the current study which doesn't exactly align with the study by Fahlstrom *et al.* (2011). Fahlstrom *et al.* (2011) also analysed behaviour in much older mice (up to 28 months). Fahlstrom *et al.* (2012) compared 4, 22 and 28 month old C57BL/6 male mice in the OF test and found that distance moved, and duration and distance in the central zone all declined with age, suggesting that mice become less active and show more anxiety-like behaviour with age (Fahlstrom *et al.*, 2012). These changes are likely due to ageing since the 22 and 28 month old age groups in Fahlstrom *et al.* are considered to be aged mice, whereas the 12 month old groups in the current study and Shoji *et al.* are considered to be middle-aged.

Summary 3

The current study found that some locomotor activity and anxiety-like parameters varied with age. Other studies testing mice at different age groups partially support findings in the current study, but comparisons are confounded by a number of issues such as the sex of mice, sample sizes, differing age groups used and differing genetic backgrounds. There seems to be clear age-related effects on locomotor activity and anxiety-like behaviour in very old male and female mice (Fahlström *et al.*, 2011; Fahlstrom *et al.*, 2012), however since the current study used younger middle-aged mice these effects were not evident.

Future Experiments

The hyperactive phenotype observed in the *Nrxn1α* Hz mice could be further investigated to test if it is a specific response to a novel environment or whether they have a higher baseline level of locomotor activity compared to Wt mice. This could be done by either a repeated exposure to the OF arena (Laarakker *et al.*, 2012) to test if the hyperactive phenotype persisted after habituation, or could be done by monitoring activity in the home cage (Robinson and Riedel, 2014). Furthermore, the dopaminergic system could be investigated in these mice as a candidate mechanism underlying hyperlocomotor activity. This could be done using High Performance Liquid Chromatography (HPLC) to test for differences in levels of dopamine and metabolites in the dopamine synthesis pathway (Yao *et al.*, 2018) in *Nrxn1α* Hz mice. To allow for better translation between behaviour observed in the *Nrxn1α* Hz mice and behaviour in Sz patients the mice could be tested in the BPM, a task that has highly similar human and mouse versions, allowing results to be compared to those previously reported in Sz patients (Perry *et al.*, 2009; Perry *et al.*, 2010). Finally *Nrxn1α* Hz mice could be tested in other tasks that measure anxiety-like behaviour, such as

the EPM and the light/dark box, to further validate the findings of increased anxiety-like behaviour in these mice.

Conclusions

When exposed to a novel OF environment *Nrxn1α* Hz mice showed initial increased freezing followed by hyperlocomotor activity. *Nrxn1α* Hz mice also showed increased anxiety-like behaviour in the novel OF environment which may relate to anxiety present in both ASD and Sz. Although age did not modify the effect *Nrxn1α* heterozygosity on locomotor activity and anxiety-like behaviour, there was variability in these behaviours dependent on age.

Chapter 4 *Nrxn1α* heterozygosity, Sex and Age impact on performance in the Novel Object Recognition Task (NORT)

4.1 Introduction

The NORT protocol used tested short term recognition learning and memory. The protocol consisted of a one hour inter-trial interval between the acquisition phase, where two identical objects were presented to the mouse, and the test phase, where one object from the acquisition phase (familiar object) and one novel object was presented to the mouse. Mice naturally explore novel objects more than familiar objects (Frisch *et al.*, 2005; Kruk-Slomka *et al.*, 2014), and so if a mouse spends more time investigating the novel object, rather than the familiar object, this suggests it has formed a memory of the familiar object. Deficits in object recognition memory have been observed in both Sz (Danion *et al.*, 1999) and ASD patients (Blair *et al.*, 2002).

4.2 Key findings

- **_Nrxn1α_ heterozygosity causes a deficit in novel object recognition in female, but not male, mice in the initial analysis paradigm used**
- **Altering analysis parameters reveals sexual dimorphism in NORT behaviour**
- **Age impacts on novel object recognition**

4.3 Results

Nrxn1α heterozygosity causes a deficit in novel object recognition in female mice

In the Novel Object Recognition Task (NORT) female *Nrxn1α* Hz mice showed a deficit in novel object recognition after a 1 hour delay when compared to female Wt mice (Figure 4.1 A). Male mice of both genotypes did not show a novel object recognition response with the initial analysis parameters used in this study (Figure 4.1 B). A significant sex x age x object x genotype interaction was found for Recognition Index (RI) [$F_{(3,522)}$ =3.852, p= 0.0096]. To further investigate the sex dependent nature of this task, data were split by sex for further analysis. In female mice there was a significant object x genotype interaction [$F_{(1, 262)}$ =9.950, p=0.0018]. *Post hoc* testing using Pairwise t-test with FDR correction in Wt female mice revealed a significant increase in RI for the novel object as compared to the familiar object (p<0.0001). There was no significant difference in the RI for the novel and familiar object in female *Nrxn1α* Hz mice (Figure 4.1 A). In male mice, a significant age x object x genotype interaction was found was found [$F_{(3, 258)}$ =3.433, p=0.0176]. When male data were split by genotype and analysed further, a significant age x object interaction was found for *Nrxn1α* Hz mice [$F_{(3, 130)}$ =3.351, p=0.0211]. *Post hoc* testing using Pairwise t-test with FDR correction revealed no significant differences in the RI between the novel and familiar object of any age in male *Nrxn1α* Hz mice (Figure 4.1 B). No significant age x object interaction or significant effects of age or object were found for male Wt mice. These results support that there was no novel object recognition response in male mice for the analysis parameters used, as there was no significant difference in RI for novel vs familiar object in male Wt or *Nrxn1α* Hz mice.

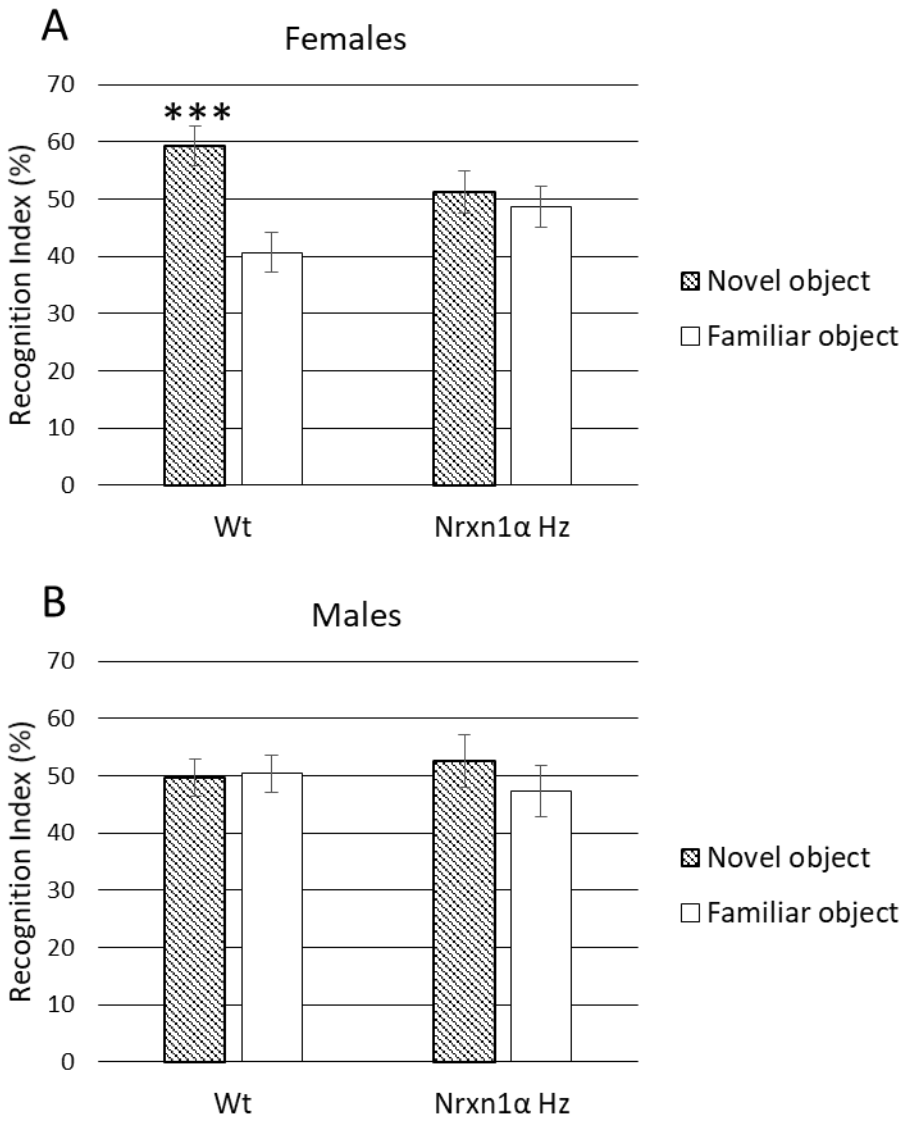

Figure 4.1 Nrxn1α heterozygosity causes a deficit in novel object recognition in female mice
*Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. Recognition Index (RI) for **(A)** female and **(B)** male mice for all age groups combined for 5 minute time bin of test phase of the NORT. RI combines measures of duration touching objects (seconds) and frequency of touching objects for a 5 minute time bin of the NORT test phase. A significant sex x age x object x genotype interaction was found for Recognition Index (RI) [$F_{(3,522)}$ =3.852, p= 0.0096] supporting differential effects between male and female mice. When data were split by sex a significant object x genotype interaction was found in female [$F_{(1, 262)}$ =9.950, p=0.0018] but not male mice. Sample sizes were females: Wt n=37, Nrxn1α Hz n=33, males: Wt n=34, Nrxn1α Hz n=35. *** denotes p<0.0001 significant difference compared to familiar object within genotype (Pairwise t-test with FDR correction).*

Nrxn1α Hz mice show no difference in object exploration during acquisition and test phases of the NORT

Nrxn1α Hz mice spent a similar amount of time and visited objects a similar number of times as compared to Wt mice during both the acquisition and test phases of the NORT (Figure 4.2). During both the 10 minute acquisition phase when mice explored 2 identical objects, and in the test phase when mice explored one familiar and one novel object (5 time bin), *Nrxn1α* Hz mice showed no difference in object interaction (in terms of either duration of time spent inside or number of visits to the object and object boundary zones) as compared to Wt mice (Figure 4.2 A and B). In this way no significant effect of genotype or significant age x genotype or sex x genotype interactions were found for duration or frequency exploring the objects in either the 10 minute acquisition phase or the 5 minute time bin of the test phase.

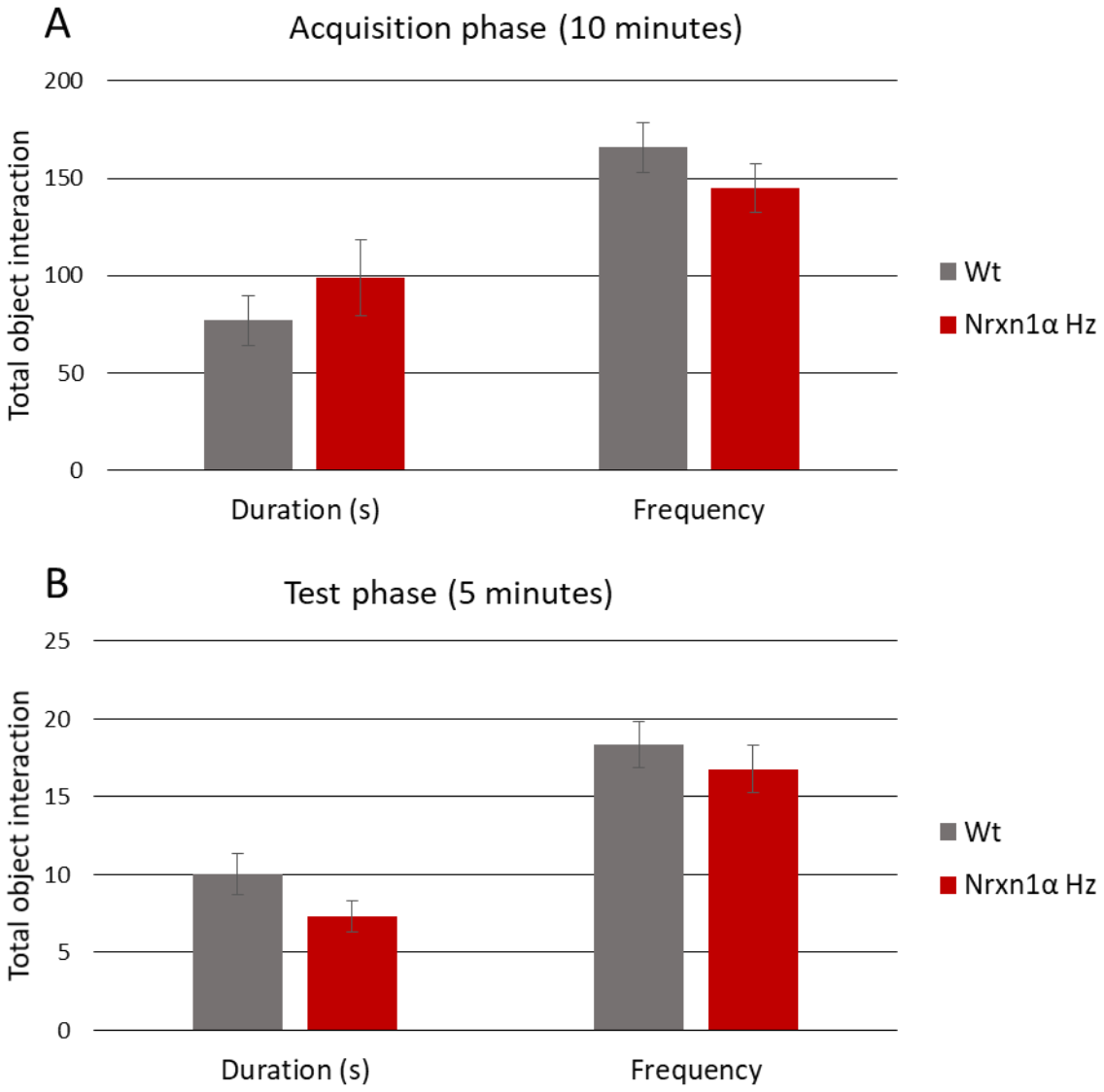

Figure 4.2 Nrxn1α Hz mice show no difference to Wt mice in object exploration during in either the acquisition or test phases of the NORT

Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. Total object interaction included total duration of time (seconds) spent interacting with objects or within object boundary zones and frequency of visits to objects or object boundary zones for ***(A)*** *acquisition phase (10 mins) and* ***(B)*** *1 hour delay test phase (5 mins). No significant main effect of genotype or significant interactions with genotype were found. Sample sizes were Wt n=74 and Nrxn1α Hz n=76.*

Sex and age impact on NORT performance

In the initial analysis paradigm used in the current study male mice showed no difference in RI for the novel vs familiar object (Figure 4.1 B) and this was not significantly influenced by age. By contrast, female Wt mice showed differences in the RI for the novel vs the familiar object that were significantly impacted on by age (Figure 4.3 A). In female mice, a significant age x object interaction was found [$F_{(3, 262)}$ =3.834, p=0.0108]. *Post hoc* testing using Pairwise t-test with FDR correction of data from both Wt and *Nrxn1α* Hz mice revealed that female mice showed a significant increase in RI for the novel object compared to the familiar object at 6 months old (p<0.0010) and a trend towards a significant difference at 12 months old (p=0.0826) but not at 3 or 9 months old (Data not shown). Since both genotypes were combined in this analysis and female *Nrxn1α* Hz mice showed a deficit in this task as previously described, data were split by genotype and Wt mice were analysed separately to further characterise the effect of age on object recognition in female mice. A significant age x object interaction was found for Wt female mice [$F_{(3, 546)}$ =5.457, p=0.0011] and *post hoc* testing using Pairwise t-test with FDR correction revealed the same pattern in the RI differences between novel and familiar object as when both genotypes were combined. Thus there was a significant difference in the RI of the novel object compared to the familiar object at 6 (p<0.0010) and 12 (p=0.0046) months old but not at 3 or 9 months old (Figure 4.3 A). Therefore age significantly impacted on the NORT performance in female mice.

No evidence that sex or age significantly altered parameters of object interaction during the acquisition phase of the task was found. However during the test phase of the task effects of sex and age were found on specific parameters (data not shown). In this way no

significant sex x age interaction or significant main effects of sex or age were found for total object interaction duration or frequency parameters in the 10 minute acquisition phase. Whereas in the NORT test phase (5 minute time bin) a significant main effect of sex [$F_{(1, 142)}$ =4.074, p=0.0454] was found for total object interaction duration, supporting males spending more time interacting with objects than females, and a significant main effect of age was found for total object interaction frequency [$F_{(1, 142)}$ =7.666, p=0.0064]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that 3 month old mice had a higher frequency of visits to the objects than 12 month old mice (p=0.0340).

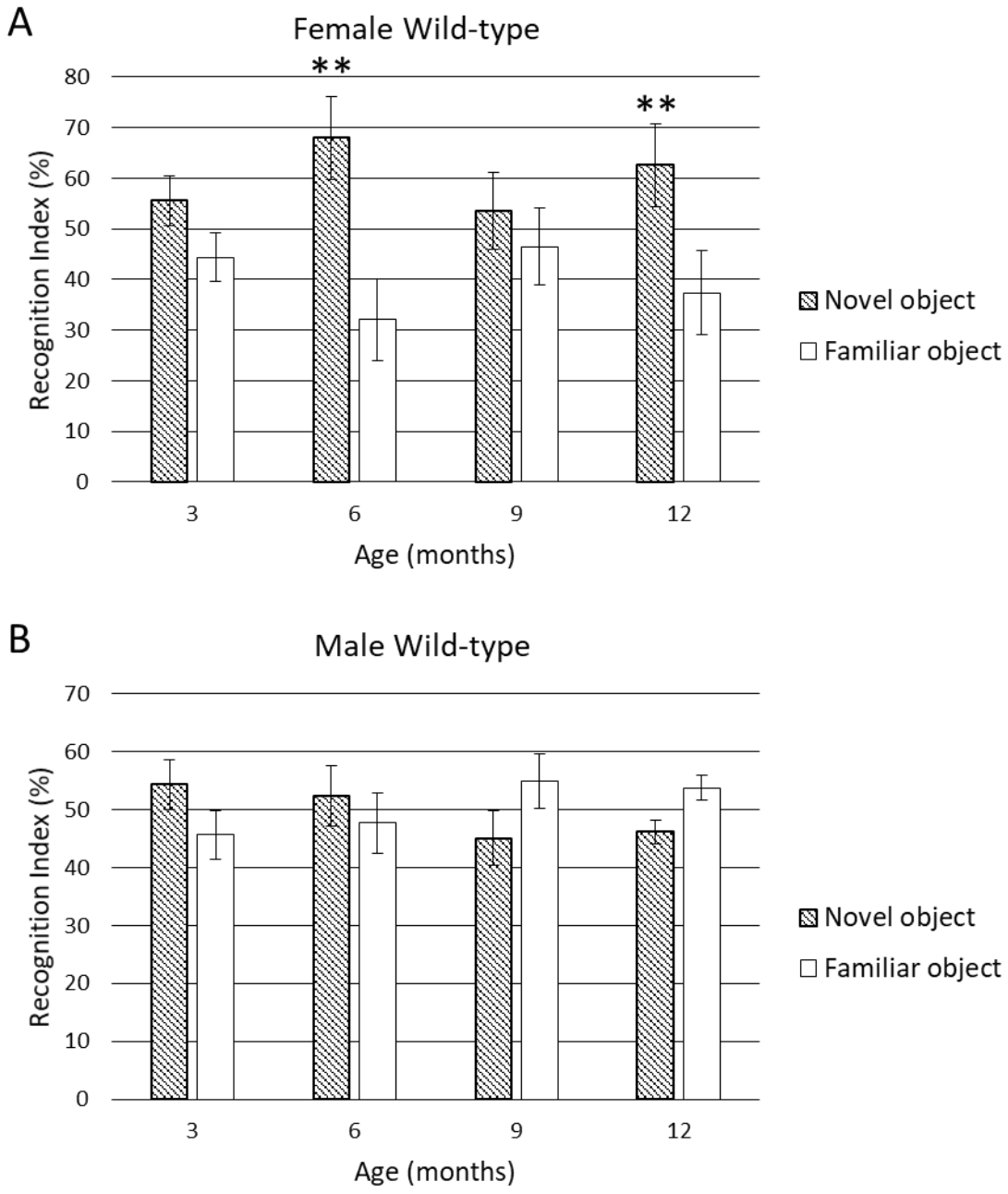

Figure 4.3 Age impacts on performance of the NORT in female, but not male, Wt mice
*Data shown are Mean ± SEM. Recognition Index (RI) for **(A)** female and **(B)** male Wild-type (Wt) mice. RI combines measures of duration touching objects (seconds) and frequency of touching objects for a 5 minute time bin of the NORT test phase. A significant object x age interaction was found in female [$F_{(3, 262)}$ =3.834, p=0.0108] but not male Wt mice. Sample sizes were; females: 3 month n=12, 6 month n=8, 9 month n=9, 12 month n=8, males: 3 month n=9, 6 month n=9, 9 month n=10, 12 month n=6. ** denotes p<0.01 significant increase compared to familiar object of same age group (Pairwise t-test with FDR correction).*

Adjusting the NORT analysis parameters to detect novel object differentiation in male mice

Alternative analysis parameters were used in order to detect a novel object differentiation response in Wt male mice (Figure 4.4). Instead of duration and frequency of the nose directly touching objects (which detected object differentiation in female wild-type mice), duration and frequency parameters of both the nose touching the object and the nose being present within object boundary zones (2cm zone around the object) were combined for the measure of object interaction. Again, the same 5 minute time bin as included in the previous analysis (minutes 2-6) of the NORT test phase was analysed. Using these adjusted parameters a novel object recognition differentiation could be detected in male Wt mice in the 3, 9 and 12 month old groups, but this was not present in any age group in male *Nrxn1α* Hz mice (Figure 4.4, A and B). However this novel object differentiation in male Wt mice was found to be a novel object preference in the 3 months old group but a novel object avoidance in the 9 and 12 month old groups (Figure 4.4, A). In female mice of both genotypes, novel object preference could be detected at 6 months old but not at any other age using the adjusted analysis parameters (Figure 4.4 C). This suggests that the nature of object exploration is different between the sexes, requiring slightly different parameters for the detection of novel object recognition. However, the findings from both sexes using the different analysis parameters support a deficit in novel object recognition in *Nrxn1α* Hz mice of both sexes.

A significant object x sex x age x genotype interaction was found for RI [$F_{(3, 374)}$ =4.972, p=0.0022] using the adjusted analysis parameters. When data were subsequently split by sex a significant object x age x genotype interaction was found [$F_{(3, 186)}$ =5.273, p=0.0016] in male but not female mice. When male data were split by genotype a significant object x age

interaction was found in Wt male mice [$F_{(3, 98)}$ =8.397, p<0.0001]. No significant object x age interaction or significant main effect of age or object were found in male *Nrxn1α* Hz mice (Figure 4.4 B). *Post hoc* testing using Pairwise t-test with FDR correction revealed that Wt male mice showed significantly increased RI for the novel object compared to the familiar object at 3 months old (p=0.0476), showed no difference at 6 months old and showed significantly decreased RI of the novel object compared to the familiar object at 9 (p=0.0149) and 12 (p<0.0001) months old (Figure 4.4 A).

Using the adjusted parameters, female mice showed no significant effect of genotype or significant interactions with genotype but did show a significant object x age interaction [$F_{(3, 186)}$ =2.964, p=0.0334]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that the RI for the novel object was significantly increased compared to the familiar object at 6 months old (p=0.021) but not at any other age in female mice (Figure 4.4 C).

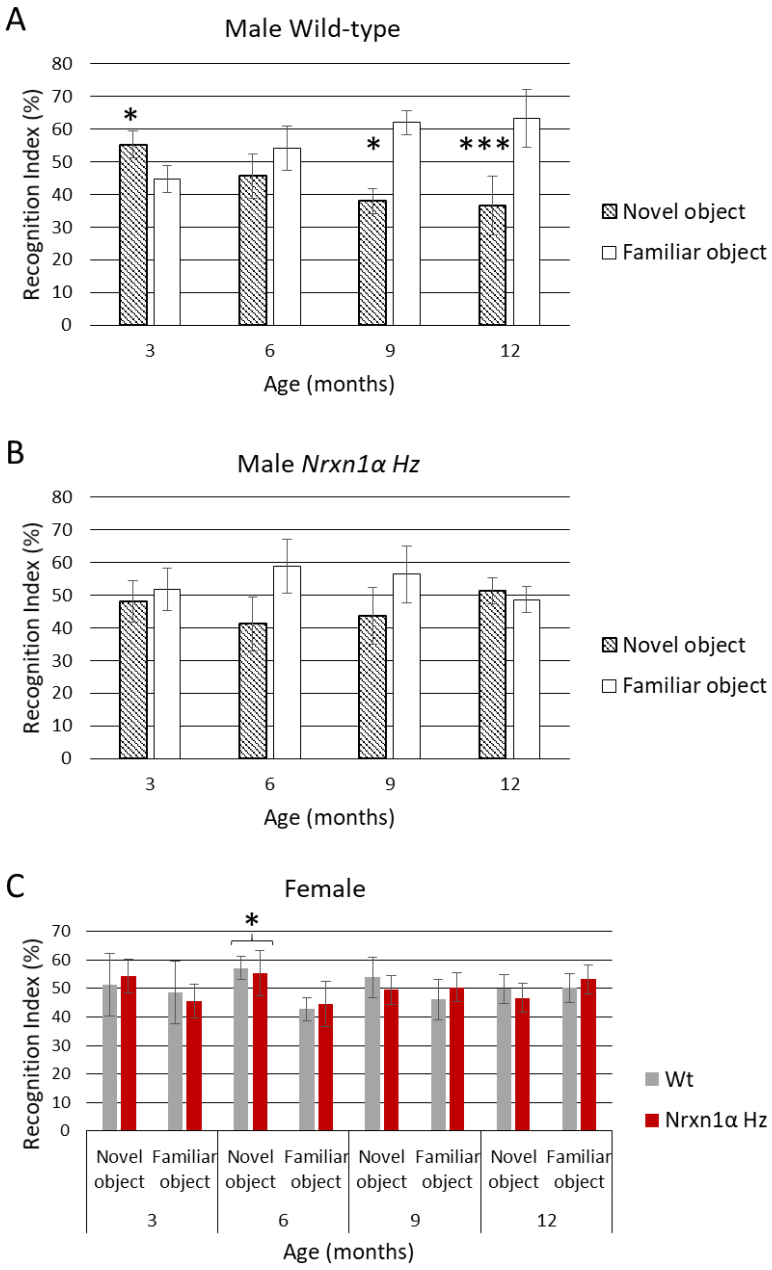

Figure 4.4 Adjusting the analysis parameters of the NORT allows detection of novel object differentiation in Wt male mice and female mice of both genotypes age dependently, but not male Nrxn1α Hz mice

Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=*Nrxn1α* Heterozygous. Recognition Index (RI) for **(A)** male Wt, **(B)** male *Nrxn1α* Hz and **(C)** female mice plotted by genotype. RI combines measures of duration (seconds) and frequency of nose point in object and object boundary zones for 5 minute time bin of the NORT test phase. A significant object x age interaction was found for Wt males [$F_{(3, 98)}$ =8.397, p<0.0001] and females (both genotypes combined) [$F_{(3, 186)}$ =2.964 4, p=0.0334] but not male *Nrxn1α* Hz mice. Sample sizes were; male Wt: 3 month n=9, 6 month n=7, 9 month n=5, 12 month n=6, male *Nrxn1α* Hz: 3 month n=8, 6 month n=5, 9 month n=4, 12 month n=7, female Wt: 3 month n=11, 6 month n=4, 9 month n=7, 12 month n=5, female *Nrxn1α* Hz: 3 month n=6, 6 month n=8, 9 month n=5, 12 month n=5. * and *** denotes, p<0.05 and p<0.001 respectively, significant difference compared to familiar object of same age group (Pairwise t-test with FDR correction).

Analysis parameters	Details	Effect of *Nrxn1α* heterozygosity?	Effect of Sex?	Modified by Age?
Initial	Interaction within object zone only	Deficit in females only independent of age	Novel object response detected in Wt females only	Novel object response in 6 and 12 months old Wt females only
Adjusted	Interaction within object zone and object boundary zone combined	Deficit in males only independent of age	Novel object response detected in Wt males and females (combined genotypes)	Novel object response in 3, 9 and 12 months old Wt males and 6 month old females (combined genotypes)

Table 4.1 Summary of findings using initial and adjusted analysis parameters in the NORT

4.4 Discussion

There were 3 keys findings from the NORT in this study (1) *Nrxn1α* heterozygosity caused a deficit in novel object recognition in female mice, (2) Wt female mice show a novel object recognition response that is influenced by age and (3) altering the analysis parameters uncovered a sex difference in NORT behaviour, a NORT deficit in male *Nrxn1α* Hz mice and effects of age on NORT in male Wt mice (Table 4.1).

Female *Nrxn1α* Hz mice show a deficit in the NORT

Nrxn1α heterozygosity caused a deficit in novel object preference behaviour after a 1 hour delay in female mice, evidenced by no significant difference in the RI for the novel vs the familiar object using the initial analysis parameters (Figure 4.1 A). By contrast female Wt mice showed significantly increased RI of the novel object compared to the familiar object (Figure 4.1 A). Since female *Nrxn1α* Hz mice explore both novel and familiar objects equally, this suggests that they do not recognise the familiar object that they were previously exposed to during the acquisition phase. As there was no difference in object exploration time in either the acquisition phase or test phase between *Nrxn1α* Hz and Wt mice (Figure 4.2 A and B), differences in the amount of object exploration cannot account for the deficit in novel object preference observed in female *Nrxn1α* Hz mice. This suggests female *Nrxn1α* Hz mice have a deficit in recognition learning and memory which could be due to dysfunctional learning, memory encoding (Winters and Bussey, 2005) or memory retrieval (Clark *et al.*, 2000). The protocol used in the current study used a 1 hour delay to test short term recognition learning and memory, which has been shown to involve a number of brain areas including the perirhinal cortex (Winters and Bussey, 2005), hippocampus (Hammond *et al.*, 2004), and nucleus accumbens (Sargolini *et al.*, 2003) in rodents. Therefore these

brain areas could be affected in the female *Nrxn1α* Hz mice. When the analysis parameters for NORT were adjusted, a deficit in NORT was also found in male *Nrxn1α* Hz mice (Figure 4.4 B) which suggests that brain areas such as the perirhinal cortex, hippocampus and nucleus accumbens could also be affected in male *Nrxn1α* Hz mice. Analysis of these regions was included in the functional brain imaging study (Chapter 6), however brain function in these regions was not found to be significantly altered in *Nrxn1α* Hz mice.

In contrast to the results in the current study another study, which used both male and female *Nrxn1α* Hz and *Nrxn1α* KO mice, showed no difference to Wt in performance in the NORT using a similar 1 hour delay protocol (Grayton *et al.*, 2013). However there were some differences between the two studies that could account for these contrasting observations. For instance, Grayton *et al.* only used 10 week old mice whereas the NORT deficit found in female *Nrxn1α* Hz mice in the current study incorporated mice from 3, 6, 9 and 12 month old groups. The current study also used a much larger number of animals than Grayton *et al.* (n=33-37 in the current study as compared to n=9-16 in Grayton *et al.*, per genotype per sex) providing greater statistical power. Wt, *Nrxn1α* Hz and *Nrxn1α* KO mice in the study by Grayton *et al.* showed a similar duration of time exploring the novel object as compared to the familiar object. If Grayton *et al.* had used the RI for novel vs familiar objects in their analysis, rather than comparing time spent with novel object between genotypes, they may have found that Wt mice did not show novel object preference in the test phase. Thus conclusions about the effect of *Nrxn1α* heterozygosity or KO on recognition learning and memory should be made with some caution in the Grayton *et al* study.

Other studies have found that *Nrxn1α* Hz and *Nrxn1α* KO mice, aged 8-16 weeks old, showed no deficit in the NORT using a 24 hour delay protocol which tests long-term rather

than short-term recognition learning and memory (Laarakker *et al.*, 2012; Grayton *et al.*, 2013; Dachtler *et al.*, 2015). The only study to report a difference between *Nrxn1α* Hz and Wt mice in a 24 hour delay NORT, was by Laarakker *et al.* They tested male mice only and found that while Wt mice showed a novel object recognition response in the first and last 5 minutes of testing (and when the two 5 minute time bins were combined), *Nrxn1α* Hz mice only showed a significant novel object recognition response in the first 5 minutes (and not in the last 5 minutes or when the two 5 minute time bins were combined). The authors suggested that this was due to an increased object discrimination capacity in *Nrxn1α* Hz mice, which meant it wasn't necessary for the mice to continue increased exploration of the novel object compared to the familiar object in the last 5 minutes of the test phase (Laarakker *et al.*, 2012).

Although Dachtler *et al.* did not find a deficit in long term novel object recognition memory with a 24 hour delay in *Nrxn1α* Hz mice, they did find a deficit in social recognition memory (Dachtler *et al.*, 2015). They found that *Nrxn1α* Hz mice did not show a difference in time exploring a novel conspecific mouse as compared to a familiar conspecific mouse immediately after (no delay) exposure to the 'familiar' mouse and an empty cage. This suggests that the *Nrxn1α* Hz mice have a deficit in short term social learning and memory which meant they did not recognise the familiar mouse, and so explored both familiar and novel conspecific mice equally. This is consistent with the deficit in short term object recognition learning and memory in *Nrxn1α* Hz mice found in the current study, since the social learning and memory test in Dachtler *et al.* involved short term memory (no delay from acquisition phase to test phase). Social recognition learning and memory was also tested by Grayton *et al.* in *Nrxn1α* Hz, *Nrxn1α* KO and Wt mice. However since the Wt mice in this study did not show increased time spent with a novel conspecific mouse compared to

a familiar conspecific mouse (novelty response), conclusions about whether social recognition learning and memory is affected in *Nrxn1α* Hz and *Nrxn1α* KO mice cannot be drawn (Grayton *et al.*, 2013). Overall the data in the current study combined with that of Dachtler *et al.* suggest that *Nrxn1α* Hz mice have deficits in short term recognition memory, which appear to be sex dependent for object recognition (current study) and observed in both sexes for social recognition (Dachtler *et al.*, 2015).

Deficits in object recognition memory have been observed in both Sz (Danion *et al.*, 1999) and ASD patients (Blair *et al.*, 2002). Many aspects of Sz and ASD can be modelled using rodents, a number of which have also been shown to have deficits in short term object recognition learning and memory. These include but are not limited to; pharmacological models of glutamate dysfunction, genetic models of susceptibility genes and developmental models involving prenatal insults. It was found that the sub-chronic PCP rat model, which models long term NMDA receptor hypofunction in Sz, had a deficit in the NORT with a 1 minute (Grayson *et al.*, 2007) and 1 hour delay (Vigano *et al.*, 2009; Pyndt Jorgensen *et al.*, 2015). Similarly rats and mice treated with MK-801, a non-competitive NMDA receptor antagonist, before the acquisition phase showed impaired performance in the NORT at a 1.5 hour delay (De Lima *et al.*, 2005; Nilsson *et al.*, 2007). Acute treatment with methamphetamine, which models hyperdopaminergia and psychotic symptoms (Howes and Kapur, 2009), caused a deficit in a 1 hour delay NORT 1 week after administration in rats (Herring *et al.*, 2008). A number of mouse models of susceptibility genes for Sz and ASD have shown impairment in short term novel object recognition. The catechol-O-methyl transferase (COMT) heterozygous deletion mice, which model dysfunctional dopamine metabolism in Sz, showed a deficit in the NORT in males and females after a 5 minute delay and in males after a 1 hour delay (Babovic *et al.*, 2008). Also Male *Neuregulin1* (*NRG1*)

transmembrane domain mutant heterozygous mice, a Sz susceptibility gene (Stefansson *et al.*, 2002; Li *et al.*, 2006; Ayalew *et al.*, 2012), also showed impairment in the NORT after a 10 minute delay (Duffy *et al.*, 2010). However no deficit in the NORT was seen with the 1 hour delay protocol in female *NRG1* transmembrane domain mutant heterozygous mice (Chesworth *et al.*, 2012). Male and female mice expressing a dominant negative mutation in *DISC1*, a genetic risk factor for Sz (Song *et al.*, 2008), showed a deficit in the NORT after a 1 hour delay if treated with poly (I:C) in utero (Ibi *et al.*, 2010). Poly (I:C) is a viral mimetic used to model prenatal infection, which is a known environmental risk factor for the development of Sz and ASD (Atladóttir *et al.*, 2010; Brown, 2011). Two mouse models of genetic risk factors for ASD that showed short term NORT deficits are the 16p11.2 heterozygous deletion mouse model which showed a deficit in the 1 hour (Yang *et al.*, 2015) and 3 hour (Pucilowska *et al.*, 2015), delay NORT and the *Shank3* homozygous KO mice which showed a deficit in the NORT after a 6 minute delay (Jaramillo *et al.*, 2016) and 1 hour delay (Yang *et al.*, 2012). Rodent models of prenatal insult associated with Sz and ASD that have also been found to have deficits in short term NORT. Prenatal exposure to valproic acid, known to increase the risk of ASD (Christensen *et al.*, 2013), causes a deficit in the NORT at 1 hour delay in mice (Takuma *et al.*, 2014). While mice exposed prenatally with poly (I:C) show a deficit in 1 hour delay NORT at 10 weeks old, no deficit is found at 5 weeks old (Ozawa *et al.*, 2006). Multiple lines of evidence, from patients and rodent models of Sz and ASD, support the possible convergence of both genetic and environmental risk factors on common pathways that can result in deficits in short term object recognition memory.

Female Wt mice show a novel object recognition response that is influenced by age

Using the initial analysis parameters, female Wt mice showed age dependent changes in novel object preference during adult development. This was evidenced by significantly increased RI for the novel object compared to the familiar object at 6 and 12 months old, but not at 3 or 9 months old. This suggests that Wt females were unable to recognise the familiar object at 3 or 9 months old, but were unimpaired at 6 and 12 months old. This could be due to developmental changes that occur during adult development from 3 to 12 months old which interfere with the mouse's ability to recognise objects. However other studies have shown that female C57BL6 mice show novel object preference at 1 hour delay at 3 month old (Li, Y. *et al.*, 2015) and at 1 min delay at 8 months old (Fahlström *et al.*, 2011). Since other studies have shown that female mice show novel object preference at the different age groups used in the current study, variability seen in this assay could be due to them behaving differently at the different ages. Changing the analysis parameters may enable a novel object response to be found in the 3 and 9 month old female Wt mice, but this may lose the response detected in 6 and 12 month old mice, thus making fair comparisons of NORT behaviour between the age groups difficult.

Other studies have shown that short term NORT performance declines with age when comparing 4-8 month old mice with 24-28 month old male mice using 1-3 min delays (Soontornniyomkij *et al.*, 2010; Ali *et al.*, 2011; Fahlstrom *et al.*, 2012; Soontornniyomkij *et al.*, 2012). However the current study did not use aged mice, as the oldest group was 12 months old, which would contribute to lack of any age-related decline in NORT performance.

Adjusting analysis parameters uncovers sexual dimorphism in NORT behaviour

Inferences about object recognition learning and memory cannot be made for male *Nrxn1α* Hz mice using the initial analysis parameters used in this study, since no novel object recognition response was seen in male Wt mice for comparison. Neither Wt nor *Nrxn1α* Hz male mice showed a significant difference in RI for the novel object compared to the familiar object during the 1 hour delay test phase (Figure 4.1 B). This may have suggested that all male mice, regardless of genotype, have a deficit in recognition learning, memory encoding or memory retrieval as compared to female mice. This is supported by a study which showed that C57BL/6 females display a stronger novel object preference than males (Bettis and Jacobs, 2012), however this was using a 24 hour delay protocol. An alternative explanation is that male mice explore the objects in a different way to female mice. When this idea was explored by changing the analysis parameters of the NORT test phase, by measuring object interaction in object boundary zones as well as the object zone, a novel object differentiation could be detected in male Wt mice (Figure 4.4 A). When the adjusted analysis parameters were used a deficit in NORT was found in male, but not female, *Nrxn1α* Hz mice (Figure 4.4 B and C). Also male Wt mice showed age-dependent effects on the novel object response, with significant differences in the RI for the novel vs the familiar object observed at all ages except 6 months old. The analysis parameters could be altered to optimise the response for 6 month old male Wt mice, but this may be at the expense of no longer detecting a novel object response in the other age groups making comparisons between groups difficult. Female mice (of both genotypes combined) only showed a novel object response at 6 months old and not at any other age when using the adjusted parameters, with a novel object preference detected in these animals. By contrast, male

mice showed a novel object preference or avoidance (Misslin *et al.*, 1982) dependent on age using these parameters. These results provide evidence that male and female mice behave differently in the NORT. Male Wt mice only showed a novel object response when the object boundary zone was included in the analysis (adjusted analysis parameters), whereas female Wt mice showed a novel object response in more age groups when only the object zone was included (initial analysis parameters). Another sex difference in NORT behaviour is highlighted with the adjusted analysis parameters in terms of which object the mice interact with more. Female mice showed a novel object preference response (significantly higher RI for the novel object vs the familiar object) in the 6 months old group, whereas male mice showed a familiar object preference or 'neophobic' (Misslin *et al.*, 1982) response (significantly higher RI for the familiar object vs the novel object) in the 9 and 12 months old groups, and showed a novel object preference response in the 3 months old group. Since the two sexes behave differently in NORT depending on analysis parameters used, it is difficult to detect optimum responses for both sexes and perform a fair comparison of their behaviour. This suggests that the current NORT protocol may not be the best test of recognition learning and memory when comparing across sex and age.

Future experiments and possible modifications of the NORT protocol

To further test recognition learning and memory a number of new experiments or modifications to the current NORT protocol could be carried out. Since a deficit in short term NOR was seen in *Nrxn1α* Hz mice, a short term social recognition memory task could be carried out to test if the deficit is generalised to social memory. This would involve a similar protocol to the NORT but instead of novel and familiar objects, novel and familiar conspecific mice would be used. Although short term social recognition memory has

previously been tested in *Nrxn1α* Hz mice (Grayton *et al.*, 2013; Dachtler *et al.*, 2015), mice were aged 4 months or younger and so *Nrxn1α* Hz mice of the older age groups used in the current study have not been tested. Previous studies using *Nrxn1α* Hz mice have shown that there was no deficit in long term object recognition learning and memory using a 24 hour delay NORT protocol (Laarakker *et al.*, 2012; Dachtler *et al.*, 2015). However larger sample sizes may have been needed to detect the deficit and these studies did not include mice older than 4 months old, therefore carrying out a 24 hour delay NORT protocol for *Nrxn1α* Hz mice aged 6, 9 and 12 months old (used in this study) and thus increasing the sample size could provide novel findings.

Further modifications to the current NORT protocol could be made to improve the novel object response in mice. Although the 5 minute time bin for NORT test phase analysis was selected for optimum novel object response in Wt animals, this could be investigated further as the optimum time bin may differ between male and female mice and the different age groups used. Also the lack of novel object response with the initial NORT analysis parameters used and the neophobic responses observed when using the adjusted analysis parameters in male mice could have been due to anxiety (Vogel-Ciernia and Wood, 2014). This could also be the case for the variable novel object response observed in female Wt mice using the initial analysis parameters. The current NORT protocol has a 15min habituation session the day before the NORT test day (Open Field) and a 5 minute habituation session on test day before the acquisition phase, whereby the animal is placed in the empty arena. To further reduce the possibility of anxiety the amount of habituation in the empty arena could be increased to 5 min per day for 6 days prior to NORT test day (Vogel-Ciernia and Wood, 2014). Reducing the light levels and adding bedding to the arena could also reduce anxiety (Vogel-Ciernia and Wood, 2014), however since a white floor is

required to contrast with the black colouring of the mice for good automated tracking, these conditions would need to be carefully considered. Alternatively a similar task to the NORT could be carried out using odours instead of objects, which is a more natural paradigm for mice, to test if a better measure of recognition memory could be acquired (Scott *et al.*, 2013).

Conclusions

Male and female *Nrxn1α* Hz mice showed a deficit in short term recognition learning and memory (dependent on analysis parameters used), which is supported by a deficit in short term social recognition learning found in *Nrxn1α* Hz mice of both sexes by others. Deficits in object recognition learning and memory have been found in Sz and ASD patients and in animal models, modelling certain aspects, of both disorders. This suggests that many factors associated with risk of developing these disorders converge on a common biological pathway which results in this type of cognitive deficit. Altering the NORT analysis parameters uncovered sexual dimorphism in NORT behaviour. This included a deficit in recognition learning and memory being detected in male, but not female, *Nrxn1α* Hz mice when using the adjusted analysis parameters. Furthermore there were variations in novel object recognition response dependent on age in both females using both analysis parameter variations, and in males in the adjusted analysis parameters. Taken together this suggests that the current NORT protocol may not be optimum for comparing object recognition learning and memory across sex and age. Modifications to the current NORT protocol could reduce the possibility of anxiety or improve detection of the novel object response per sex per age group, however an alternative task such as using novel odour recognition may provide a better alternative for comparing across sex and age.

Chapter 5 The impact of *Nrxn1α* heterozygosity, sex and age on cognitive flexibility

5.1 Introduction

Both Sz and ASD patients show deficits in cognitive flexibility (reviewed in section 1.9 and 1.10). The Cognitive Flexibility Task (CFT) was used in the current study as a rodent alternative to the Wisconsin Card Sorting Task (WCST), which tests cognitive flexibility in humans, and has been adapted from other rodent tasks of cognitive flexibility (Young *et al.*, 2010). Mice were required to dig in bowls of sand to retrieve sugar pellet rewards based on rules about odour and the location of the bowls. The CFT was a 4 phase task which required the functioning of different cognitive domains. This included associative learning, rule generalisation, reversal learning and shifting attention. These cognitive domains are associated with different brain circuitry, and so this task was used to dissect whether these cognitive domains and the underlying circuitry are deficient in *Nrxn1α* Hz mice.

5.2 The impact of *Nrxn1α* heterozygosity on cognitive flexibility

5.3 Key findings

- ***Nrxn1α* Hz mice show enhanced associative learning of a novel odour association dependent on age**
- ***Nrxn1α* Hz mice show a deficit in reversal learning**
- ***Nrxn1α* Hz mice show increased regressive, but not perseverative, errors during reversal learning**
- ***Nrxn1α* Hz mice have increased trial latencies**

5.4 Results

5.4.1 Associative learning of a novel odour-reward association (OD1)

Nrxn1α Hz mice show enhanced associative learning of a novel odour association at 3 and 9 months old

During the OD1 phase of the CFT mice are required to learn that one of the odours is rewarded and the other is not. This is the first time the mice have learned this form of discrimination. In this phase *Nrxn1α* Hz mice performed significantly better than Wt at 3 and 9 months old but not at the other ages (6 and 12 months) characterised (Figure 5.1 A and B). In addition, while Wt mice displayed significant variability in performance during OD1 across the different age groups tested, *Nrxn1α* Hz mice did not, showing a similar level of performance across all age groups (Figure 5.1 A).

A significant, or a close to significant, age x genotype interaction was found for trials to criterion (TTC) [$F_{(3, 85)} = 6.916$, $p<0.0010$] and percentage correct (PC) [$F_{(3, 89)} = 2.656$, $p=0.0533$]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that in OD1 *Nrxn1α* Hz mice showed significantly decreased TTC compared to Wt at 3 and 9 months old ($p=0.0002$, $p= 0.0206$ respectively), and significantly increased PC compared to Wt mice at 9 months old ($p=0.0410$) (Figure 5.1 A and B). When the effect of genotype on TTC was analysed across all age groups it was found to be significant [$F_{(1, 87)} =6.827$, $p=0.0106$] (Figure 5.1 A). However, this significant main effect of genotype cannot be accepted given the significant age x genotype interaction identified.

Nrxn1α Hz mice showed a consistent level of performance during OD1 across all 4 age groups. In this way, no significant difference in TTC or PC between *Nrxn1α* Hz mice at the

different age groups was found by *post hoc* testing (Figure 5.1 A and B). By contrast, Wt mice showed a varied performance during OD1 across the age groups in terms of TTC. At 9 months old, Wt mice showed significantly increased TTC compared to all other age groups of Wt mice (Wt: 9 v 3 $p=0.0052$, 9 v 6 $p<0.0001$, 9 v 12 $p<0.0001$, Pairwise t-test with FDR correction) (Figure 5.1 A). While PC at 9 months old also appeared to be lower in Wt mice, this was not confirmed statistically and there was no significant difference for PC across the different age groups (Figure 5.1 B).

Nrxn1α Hz mice show increased response latencies during associative learning of a novel odour discrimination

During OD1 *Nrxn1α* Hz mice showed enhanced performance in the task dependent on age, as described above. In addition, *Nrxn1α* Hz mice also showed significantly increased response latency in OD1 for both correct and incorrect trials as compared to Wt mice, an effect that was not significantly modified by age (no significant age x genotype interaction) (Figure 5.1 C and D). In this way, a significant effect of genotype was found for both correct [$F_{(1, 89)} = 9.475$, $p=0.0028$] and incorrect [$F_{(1, 82)} = 7.129$, $p=0.0091$] trial latency, revealing that *Nrxn1α* Hz mice took significantly longer to respond (initiate digging) as compared to Wt mice.

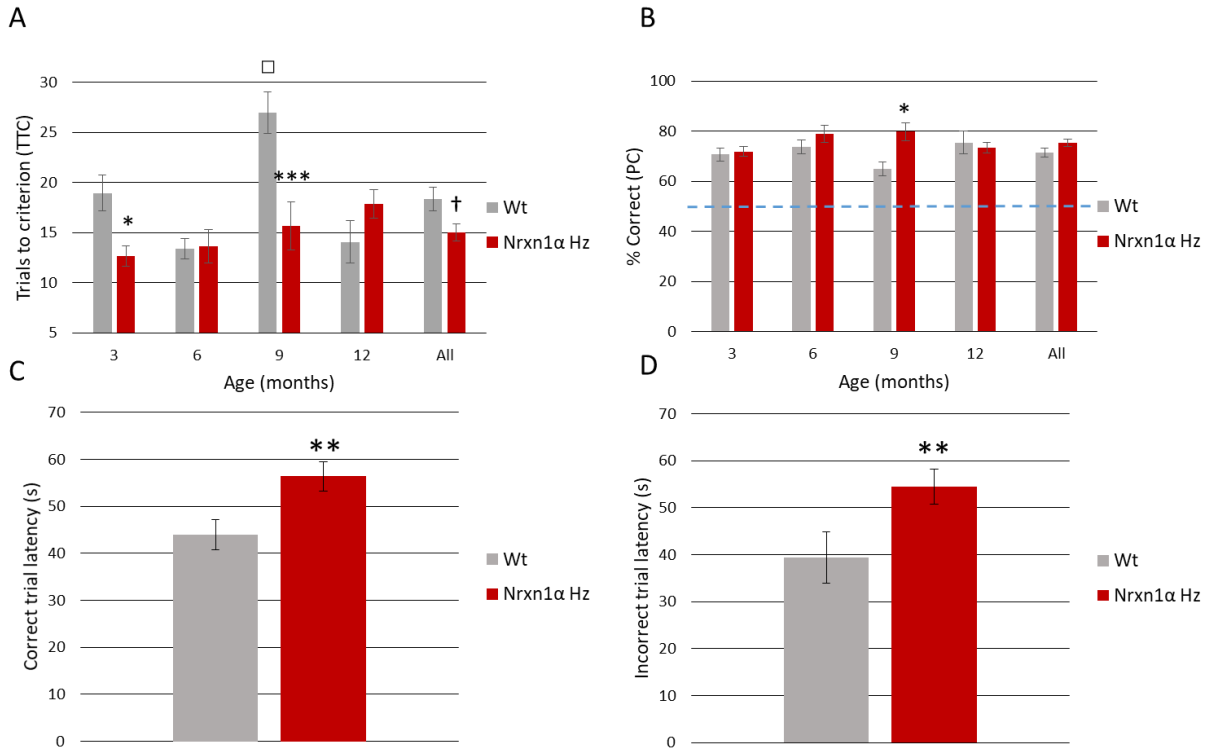

Figure 5.1 Nrxn1α Hz mice show age-dependent enhanced learning, reduced age-dependent variability in task performance and increased response latency across all ages, during Odour Discrimination 1 (OD1)

Data shown are mean ± SEM. Wt= Wild-type, Nrxn1α Hz = Nrxn1α Heterozygous. Measures of performance for associative learning include ***(A)*** *Trials to criterion (TTC) and* ***(B)*** *Percentage correct (PC) (per age group). Latency was measured for both* ***(C)*** *correct and* ***(D)*** *incorrect trials (all age groups combined). Significant or close to significant age x genotype interactions were found for TTC [$F_{(3, 85)}$ =6.916, p<0.001] and PC [$F_{(3, 89)}$ =2.656, p=0.0533]. A significant effect of genotype was found for correct [$F_{(1, 89)}$ = 9.475, p=0.0028] and incorrect [$F_{(1, 82)}$ = 7.129, p=0.0091] trial latency. Sample sizes were* ***(A)*** *Wt: 3 month n=17, 6 month n= 12, 9 month n=11, 12 month n=13, Nrxn1α Hz: 3 month n=16, 6 month n=9, 9 month n=13, 12 month n=14,* ***(B)*** *Wt: 3 month n=17, 6 month n=10, 9 month n=11, 12 month n=13, Nrxn1α Hz: 3 month n=15, 6 month n=9, 9 month n=12, 12 month n=14,* ***(C)*** *Wt n=52, Nrxn1α Hz n=53,* ***(D)*** *Wt n=50, Nrxn1α Hz n=48. Dotted line in* ***(B)*** *denotes level at which choices are made by chance (50%). * denotes p<0.05 and *** denotes p<0.001 significant difference compared to Wt within the same age group in* ***(A)*** *and* ***(B)*** *(t-test with FDR correction) and compared to Wt (all age groups) in* ***(C)*** *and* ***(D)*** *(main effect ANOVA). □ denotes p<0.01 significant increase compared to 3, 6 and 12 month old Wt groups (t-test with FDR correction). † denotes p<0.05 significant difference compared to Wt (all age groups) (main effect ANOVA).*

5.4.2 Associative learning with a second odour pair (OD2)

During discrimination of a second odour pair *Nrxn1α* Hz mice show increased response latency but do not differ from Wt mice in their ability to learn the discrimination

On the second day of the task mice are presented with a different odour pair to that presented in OD1 and they again must learn that only one of the odours in the pair is rewarded (OD2). During associative learning with the new odour pair, *Nrxn1α* Hz mice show no difference to Wt in the ability to learn this discrimination. In this way, no significant effect of genotype or age x genotype interactions were found for either the TTC or PC in this phase of the CFT (Figure 5.2 A and B). However, as seen in OD1, *Nrxn1α* Hz mice showed increased response latency for both correct and incorrect trials in OD2 (Figure 5.2 C and D). A significant effect of genotype was found for both correct [$F_{(1, 100)} = 13.961$, $p<0.0010$] and incorrect [$F_{(1, 89)} = 4.617$, $p=0.0344$] response latency.

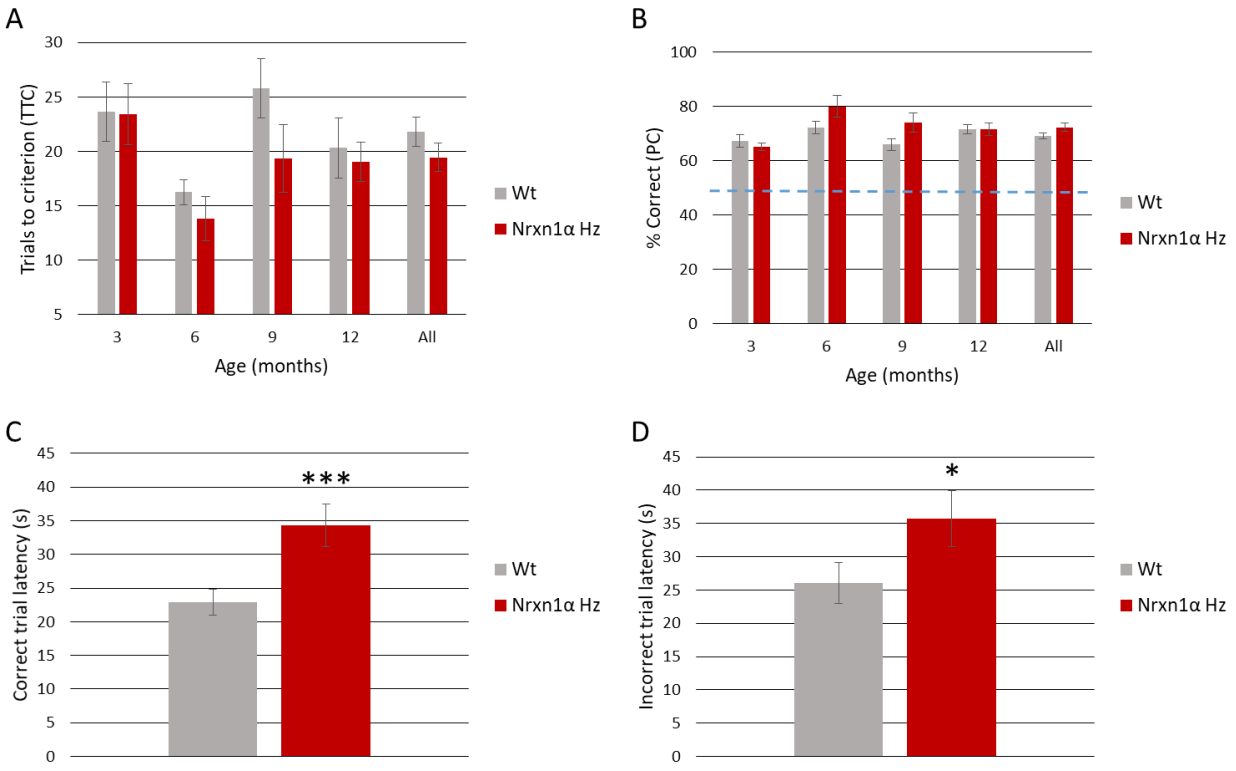

Figure 5.2. Nrxn1α Hz mice show no difference in their ability to learn the new odour discrimination but continue to show increased response latency as compared to Wt in Odour Discrimination 2 (OD2) *Data shown are mean ± SEM. Nrxn1α Hz =Nrxn1α Heterozygous, Wt =Wild-type. Measures of performance for associative learning include* ***(A)*** *Trials to criterion (TTC) and* ***(B)*** *Percentage correct (PC) (per age group). Latency was measured for both* ***(C)*** *correct and* ***(D)*** *incorrect trials (all age groups combined). A significant effect of genotype was found for correct [$F_{(1, 100)} = 13.961$, $p<0.0010$] and incorrect [$F_{(1, 89)} = 4.617$, $p=0.0344$] trial latency. Sample sizes were* ***(A)*** *Wt: 3 month n=16, 6 month n=13, 9 month n=14, 12 month n=13, Nrxn1α Hz: 3 month n=16, 6 month n=13, 9 month n=15, 12 month n=16,* ***(B)*** *Wt: 3 month n=17, 6 month n=12, 9 month n=15, 12 month n=16, Nrxn1α Hz: 3 month n=19, 6 month n=12, 9 month n=15, 12 month n=18,* ***(C)*** *Wt n=55, Nrxn1α Hz n=61,* ***(D)*** *Wt n=53, Nrxn1α Hz n=52. Dotted line in* ***(B)*** *denotes level at which choices are made by chance (50%). * denotes $p<0.05$ and *** denotes $p<0.001$ significant increase compared to Wt (main effect ANOVA).*

5.4.3 Odour discrimination reversal learning (OD2R)

The reversal phase (OD2R) is more cognitively challenging than the odour discrimination phase (OD2) for mice of both genotypes

The reversal phase (OD2R) was more challenging than the odour discrimination phase (OD2) for mice of both genotypes, evidenced by a significant decrease in PC during OD2R as compared to OD2 (Figure 5.2 B and 5.3 A). A significant phase x genotype interaction was found for PC [$F_{(1, 189)}$ =6.415, p=0.0121] and TTC [$F_{(1, 198)}$ =6.173, p=0.0138]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that both Wt and *Nrxn1α* Hz mice showed significantly decreased PC in OD2R as compared to OD2 (Wt p<0.001, *Nrxn1α* Hz p<0.0001). For TTC *Nrxn1α* Hz mice showed a significant increase in OD2R compared to OD2 (p=0.0016), however Wt mice did not.

Nrxn1α Hz mice show a deficit in reversal learning

Nrxn1α Hz mice showed a deficit in reversal learning (OD2R) as compared to Wt mice (Figure 5.3 A and B). This was evidenced by small but significant decrease in PC, across all age groups, in *Nrxn1α* Hz mice as compared to Wt controls. In this way a significant effect of genotype was found for PC [$F_{(1, 90)}$ = 6.063, p=0.0157], which revealed that *Nrxn1α* Hz mice showed a small decrease in PC (average ~58%) as compared to Wt mice (average ~62%) during reversal learning (Figure 5.3 A). No significant age x genotype interaction was found for PC. While TTC appeared to be greater in *Nrxn1α* Hz mice at 3, 6, and 12 months old, this effect was only found to be significant in 12 month old mice (Figure 5.3 B). For TTC, a significant age x genotype interaction was found [$F_{(3, 91)}$ =4.431, p=0.0059] in OD2R. *Post hoc* testing revealed that *Nrxn1α* Hz mice showed significantly increased TTC compared to Wt mice at 12 months old (p=0.0260). When the effect of genotype on TTC was analysed

across all age groups it was also found to be significant [$F_{(1, 91)}$ =4.544, p=0.0357], however cannot be accepted due to the significant age x genotype interaction for TTC. Overall these data support a deficit in reversal learning in *Nrxn1α* Hz mice.

Age effects on task performance were also found in Wt mice, as they showed increased TTC at 9 months old compared to other Wt age groups (Figure 5.3 B). A significant age x genotype interaction was found for TTC in OD2R [$F_{(3, 91)}$ =4.431, p=0.0059]. *Post hoc* testing revealed that Wt mice showed significantly higher TTC at 9 months as compared to Wt mice at 12 months old (p=0.0220), and a close to significant increase in TTC at 9 months old as compared to 3 months old (p=0.0660). By contrast, *Nrxn1α* Hz mice showed consistent TTC across the different age groups (Figure 5.3 A).

Nrxn1α Hz mice show increased response latency for incorrect trials but not correct trials during reversal learning

As well as showing a deficit in performance during reversal learning, *Nrxn1α* Hz mice also showed increased response latency for incorrect trials as compared to Wt mice during OD2R (Figure 5.3 D). Unlike the previous phases of the cognitive flexibility task, where *Nrxn1α* Hz mice showed increased latency for both correct and incorrect trials (Figure 5.1 C and D, and 5.2 C and D), only incorrect trials were affected during reversal learning. In this way a significant effect of genotype was found for incorrect trial latency [$F_{(1, 87)}$ = 15.938, p=0.0009] but not for correct trials.

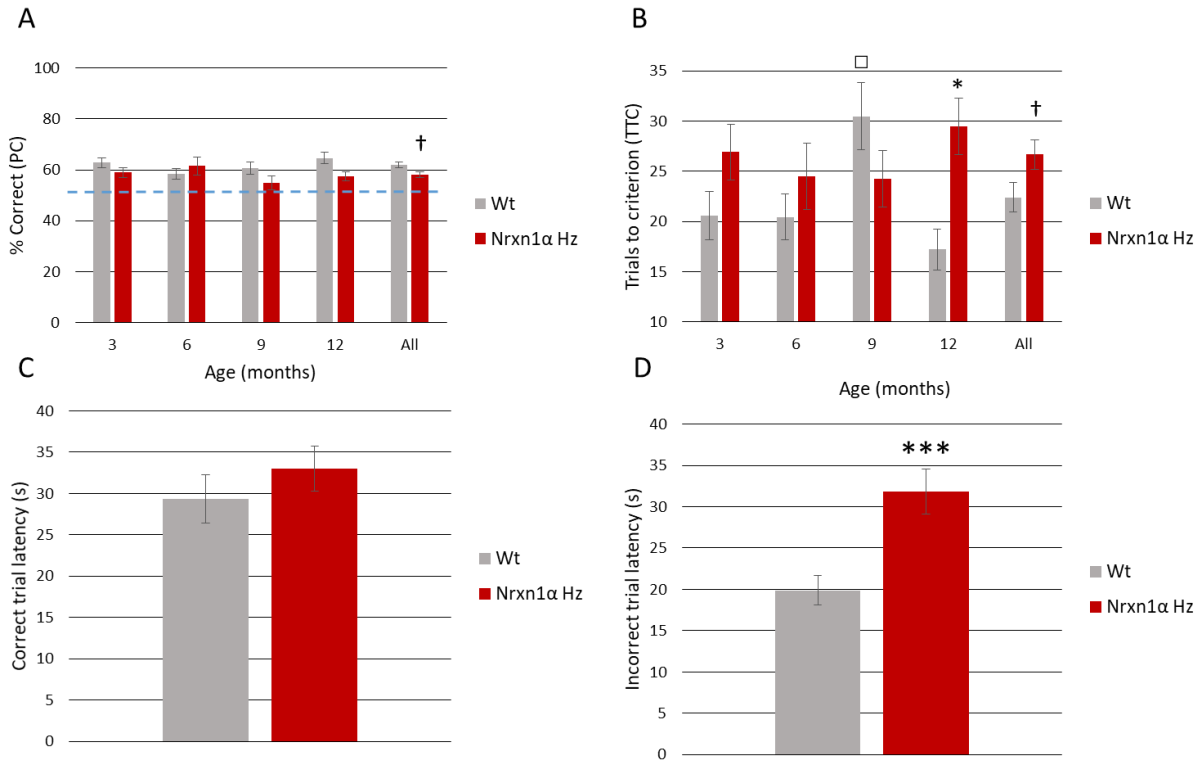

Figure 5.3 Nrxn1α Hz mice show impaired reversal learning and increased incorrect response latency in Odour Discrimination 2 (OD2R)

Data shown are mean ± SEM. Nrxn1α Hz = Nrxn1α Heterozygous, Wt= Wild-type. Measures of performance for reversal learning include ***(A)*** *Percentage correct (PC) and* ***(B)*** *Trials to criterion (TTC) (per age group). Latency was measured for both* ***(C)*** *correct and* ***(D)*** *incorrect trials (all age groups combined). A significant age x genotype interaction was found for TTC [$F_{(3, 91)}$ =4.431, p=0.0059]. A significant effect of genotype was found for PC [$F_{(1, 90)}$ = 6.063, p=0.0157], TTC [$F_{(1, 91)}$ =4.544, p=0.0357] and incorrect trial latency [$F_{(1, 87)}$ = 15.938, p<0.0010]. Sample sizes were* ***(A)*** *Wt: 3 month n=16, 6 month n=10, 9 month n=15, 12 month n=14, Nrxn1α Hz: 3 month n=17, 6 month n=8, 9 month n=12, 12 month n=14,* ***(B)*** *Wt: 3 month n=16, 6 month n= 9, 9 month n=15, 12 month n=14, Nrxn1α Hz: 3 month n=18, 6 month n=8, 9 month n=12, 12 month n=15,* ***(C)*** *Wt n=53, Nrxn1α Hz n=53,* ***(D)*** *Wt n=48, Nrxn1α Hz n=55. Dotted line in* ***(A)*** *denotes level at which choices are made by chance (50%). * denotes p<0.05 significant increase compared to 12 month old Wt (Pairwise t-test with FDR correction), *** denotes p<0.001 significant difference compared to Wt (main effect ANOVA), † denotes p<0.05 significant difference compared to Wt (main effect ANOVA), □ denotes p<0.05 significant increase compared to 12 month old Wt (Pairwise t-test with FDR correction).*

Increased regressive, but not perseverative, errors underlie the reversal learning deficit found in *Nrxn1α* Hz mice

To investigate the mechanism underlying the deficit in reversal learning seen in *Nrxn1α* Hz mice in OD2R, two different types of errors committed at this stage of the task were analysed. These were perseverative errors (PEs) and regressive errors (REs) (full details can be found in section 2.3.3). PEs were analysed in 2 ways, the first was the number of PEs committed in the 'free trials' (trial 2, 3 and 4), and the second was the correction ratio (CR) which indicates the likelihood of the mouse making a correct choice following punishment (withholding of reward) after committing an incorrect choice (from trial 5 onwards). REs were measured in the trials following a punishment induced reorientation of digging (i.e. when animals dig in the correct bowl in the trial following a punishment) in terms of the number of correct responses made before making an error again. As the criterion for completing each phase was set at 6 consecutive correct trials, animals can make 1-5 correct choices after a punishment induced reorientation of digging behaviour as part of this RE measurement. *Nrxn1α* Hz mice showed increased REs during reversal learning (Figure 5.4 A), but showed no difference in PEs (Figure 5.4 B) or response to punishment (Figure 5.4 C) as compared to Wt mice (Figure 5.4). In this way, a close to significant effect of genotype was found for REs [$F_{(1, 484)} = 3.673$, p=0.0559] showing that *Nrxn1α* Hz mice were more likely to make REs than Wt mice across all 5 trial levels. By contrast, *Nrxn1α Hz* mice showed a similar number of PEs compared to Wt mice during 'free trials' (trials 2-4) of OD2R (no significant effect of genotype). Furthermore, *Nrxn1α* Hz mice also showed no difference compared to Wt in CR during reversal learning (no significant effect of genotype), which indicates that *Nrxn1α* Hz mice had similar perseveration and a similar response to punishment to that seen in Wt mice.

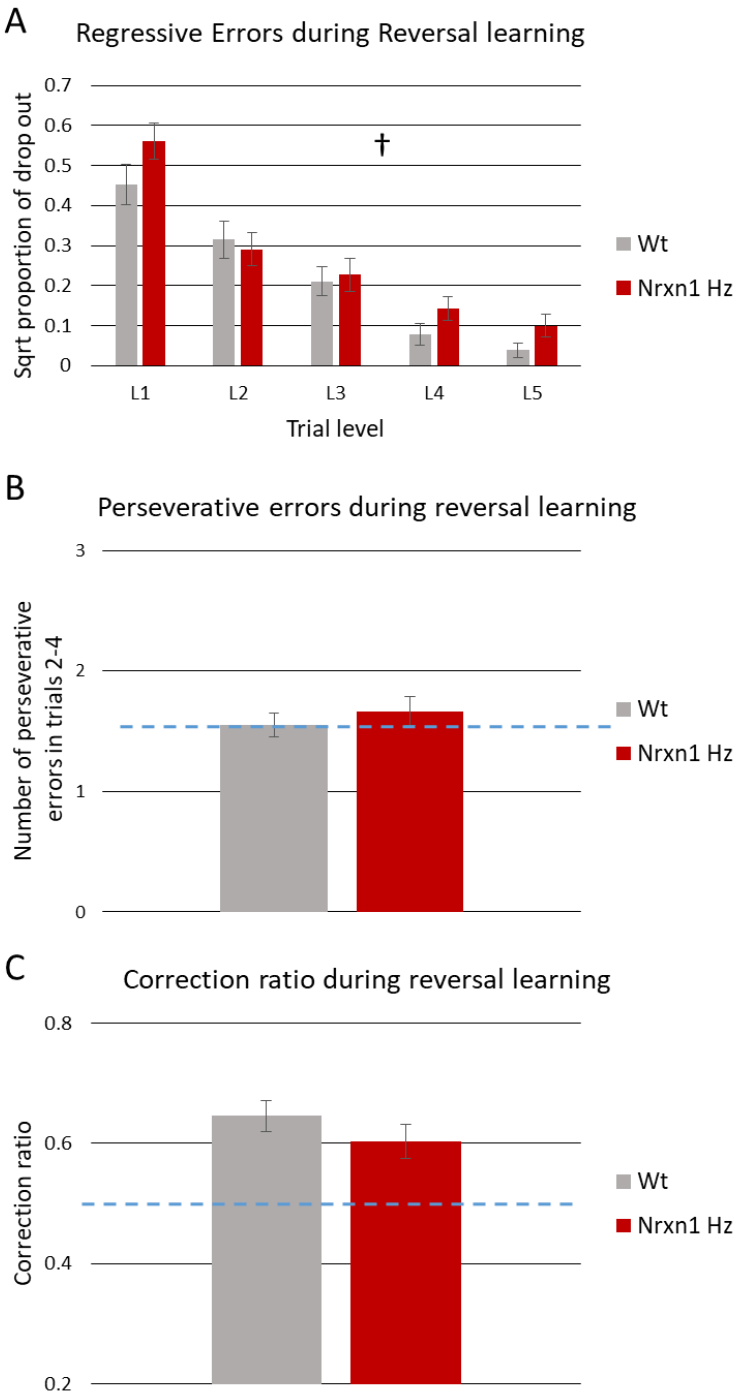

Figure 5.4 Increased regressive errors underlies the reversal learning deficit in Nrxn1α Hz mice
Data shown are mean ± SEM. Nrxn1α Hz = Nrxn1α Heterozygous, Wt= Wild-type. Types of errors measured during reversal learning (OD2R) include ***(A)*** *Regressive errors (REs) (proportion of drop out at each trial level following punishment induced reorientation (trial 5 onwards),* ***(B)*** *Number of perseverative errors (PEs) before punishment is introduced (data from 'free trials' 2, 3 and 4) and* ***(C)*** *Perseverative errors (PEs) following punishment using the correction ratio (CR) measure (likelihood of a correct choice being made after a punishment (trial 5 onwards). Sample sizes were* ***(A)*** *Wt n=58, Nrxn1α Hz n=55,* ***(B)*** *Wt n=58, Nrxn1α Hz n=54,* ***(C)*** *Wt n=47, Nrxn1α Hz n=49. Dotted line in* ***(A)*** *and* ***(B)*** *denotes level at which choices are made by chance (50%). † denotes a close to significant effect of genotype, Nrxn1α Hz increased compared to Wt [$F_{(1, 484)} = 3.673$, p=0.0559] (main effect, ANOVA).*

5.4.4 Shifting stimuli dimension (SS phase)

Shifting attention from the dimension of odour to location is unaffected in *Nrxn1α* Hz mice

By the SS stage of the CFT a large proportion of animals had stopped responding to the task, and so the number of animals reaching criteria (achieving 6 consecutive correct trials within 60 trials) was greatly reduced compared to the other test phases. However, the level of drop out at this stage of the task was similar between *Nrxn1α* Hz and Wt mice (Table 5.1). There was no difference between *Nrxn1α* Hz and Wt mice in their ability shift attention from the dimension of odour to location (left or right) in the SS phase (Figure 5.5 A and B) (no significant age x genotype interactions or significant effects of genotype for TTC or PC). There was also no difference between *Nrxn1α* Hz and Wt mice in latency for correct or incorrect trials during the SS phase of the task (Figure 5.5 C and D). In this way there was no significant effect of genotype for correct trial latency, and although there was a significant age x genotype interaction for incorrect trial latency [$F_{(3, 29)} = 3.315$, $p=0.0337$], *post hoc* testing revealed no significant genotype differences. To eliminate the possibility that animals reaching criteria in the SS phase hadn't formed a set for odour, any animals that completed the phase without receiving a punishment (n=6 Wt, n=6 *Nrxn1α* Hz) were removed and the data reanalysed. In line with the original analysis, no significant effect of genotype or genotype interactions were found for TTC or PC when the SS phase data was reanalysed.

Group	Task entry	OD1		OD2		OD2R		SS	
	#	#	%	#	%	#	%	#	%
Wt	**64**	**53**	**83**	**62**	**97**	**58**	**91**	**24**	**38**
F	**30**	**25**	**83**	**29**	**97**	**28**	**93**	**13**	**43**
3	8	8	100	8	100	8	100	3	38
6	7	5	71	6	86	6	86	1	14
9	6	5	83	6	100	6	100	4	67
12	9	7	78	9	100	8	89	5	56
M	**34**	**28**	**82**	**33**	**97**	**30**	**88**	**11**	**32**
3	9	9	100	9	100	9	100	6	67
6	8	6	75	8	100	5	63	1	13
9	10	7	70	9	90	9	90	2	20
12	7	6	86	7	100	7	100	2	29
Nrxn1α Hz	**69**	**54**	**78**	**66**	**96**	**55**	**80**	**25**	**36**
F	**33**	**26**	**79**	**32**	**97**	**28**	**85**	**13**	**39**
3	9	9	100	9	100	8	89	4	44
6	7	4	57	7	100	6	86	2	29
9	8	6	75	7	88	7	88	4	50
12	9	7	78	9	100	7	78	3	33
M	**36**	**28**	**78**	**34**	**94**	**27**	**75**	**12**	**33**
3	10	8	80	10	100	10	100	6	60
6	7	5	71	6	86	2	29	0	0
9	9	7	78	8	89	6	67	5	56
12	10	8	80	10	100	9	90	1	10

Table 5.1 Numbers and percentages of animals reaching criteria for each phase of the cognitive flexibility task (CFT)

Wt = Wild-type, Nrxn1α Hz = Nrxn1α Heterozygous, F= female, M=male, #= total number of animals reaching criteria for each phase, % = percentage of animals reaching criteria for each phase out of total animals that entered the task per group. Task phases included: OD1= Odour Discrimination 1, OD2= Odour Discrimination 2, OD2R= Odour Discrimination 2 Reversal, SS= Stimuli Shift.

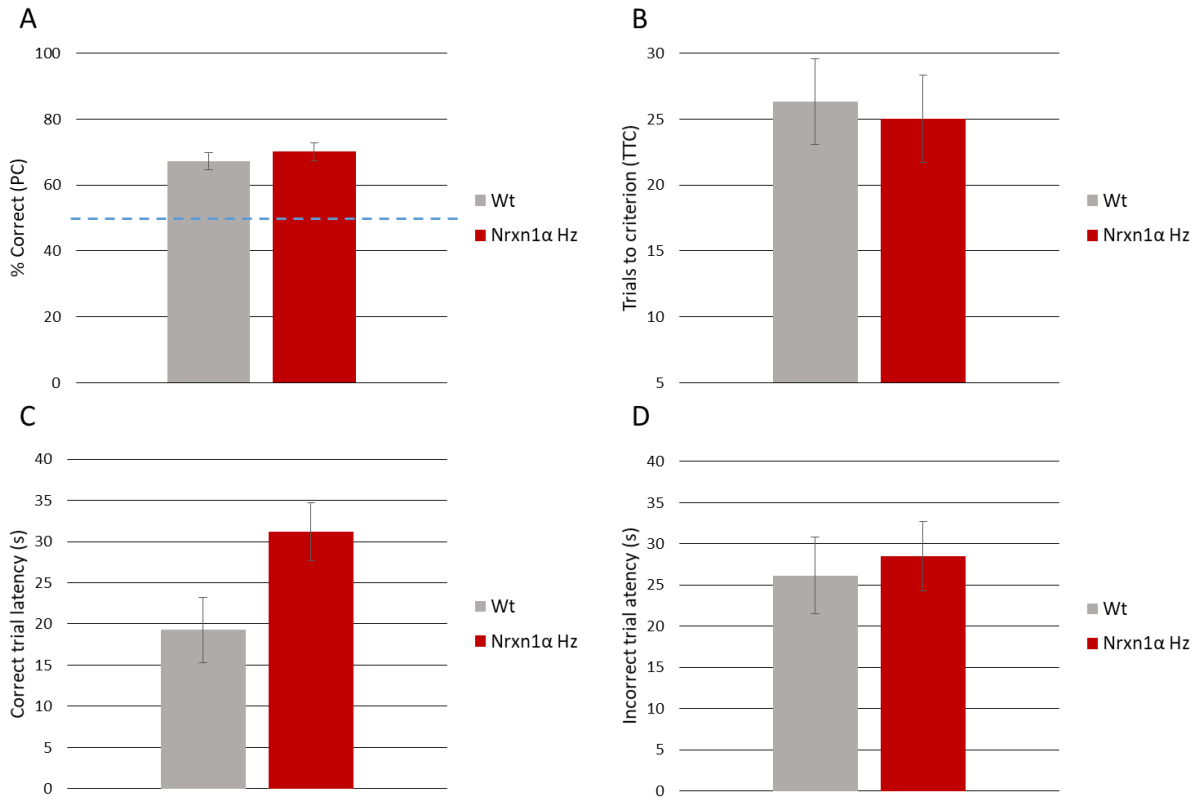

Figure 5.5 Nrxn1α Hz mice show no difference to Wt in their ability to complete the phase or in response latency during the Stimuli Shift (SS) phase

Data shown are mean ± SEM. Nrxn1α Hz= Nrxn1α Heterozygous, Wt= Wild-type. Measures of performance for stimuli shift learning include ***(A)*** *Percentage correct (PC) and* ***(B)*** *Trials to criterion (TTC) (all age groups combined). Latency was measured for both* ***(C)*** *correct and* ***(D)*** *incorrect trials (all age groups combined). Sample sizes were* ***(A)*** *Wt n=23, Nrxn1α Hz n=25,* ***(B)*** *Wt n=24, Nrxn1α Hz n=25,* ***(C)*** *Wt n=24, Nrxn1α Hz n=24,* ***(D)*** *Wt n=21, Nrxn1α Hz n=23. Dotted line in* ***(A)*** *denotes level at which choices are made by chance (50%).*

Task Phase	Parameter	Impact of *Nrxn1α* heterozygosity	Modified by Sex?	Modified by Age?
OD1	PC	↑	×	✓ (9 months old)
	TTC	↓	×	✓ (3 and 9 months old)
	CTL	↑	×	×
	ITL	↑	×	×
OD2	PC	-	-	-
	TTC	-	-	-
	CTL	↑	×	×
	ITL	↑	×	×
OD2R	PC	↓	×	×
	TTC	↑	×	✓ (12 months old)
	CTL	-	-	-
	ITL	↑	×	×
SS	PC	-	-	-
	TTC	-	-	-
	CTL	-	-	-
	ITL	-	-	-

Table 5.2 Summary table of the impact of Nrxn1α heterozygosity on parameters in the Cognitive Flexibility Task (CFT) by task phase

OD1=Odour Discrimination 1, OD2=Odour Discrimination 2, OD2R= Odour Discrimination 2 Reversal, SS=Stimuli Shift, PC=Percentage correct, TTC=Trials to criterion, CTL=Correct trial latency, ITL=Incorrect trial latency. ↑=significant increase, ↓=significant decrease, - = no significant effect, ×=effect not modified by sex/age, ✓= effect modified by age.

5.5 The impact of age and sex on performance of the Cognitive flexibility Task (CFT)

Key findings

- **Sex and age affect learning of a novel odour discrimination (OD1)**
- **6 month old mice show enhanced performance in the discrimination of a second odour pair (OD2)**
- **9 month old Wt mice show impaired reversal learning (OD2R)**
- **Age does not affect ability to shift attention from the stimulus dimension of odour to location (SS)**
- **Trial latency varies dependent on age and sex**

5.6 Results

5.6.1 Associative learning of a novel odour-reward association (OD1)

Performance in OD1 is influenced by age and sex

In OD1, 9 month old female mice had decreased performance compared to females from other age groups in terms of TTC but not PC. By contrast, male mice showed consistent levels of performance across all age groups for both TTC and PC parameters (Figure 5.6 A and B). In this way a significant sex x age interaction was found for TTC [$F_{(3, 87)} = 2.984$, p=0.0356] but not PC. *Post hoc* testing using Pairwise t-test with FDR correction revealed that 9 month old female mice took significantly more trials to reach criterion than 12 month old female mice in OD1 (p=0.0210). Female mice at 3, 6 and 12 months old had similar TTC

in OD1. There was no significant difference in TTC for the different age groups in male mice. There was no significant effect of age or significant interactions with age for PC in OD1.

Trial latencies vary with age in male, but not female, mice in OD1

Male mice showed increased trial latency at 9 months old as compared to other age groups for both correct and incorrect trials, an effect that was not seen in female mice (Figure 5.6 C and D). Significant, or close to significant, sex x age interactions were found for both correct [$F_{(3, 89)} = 2.586$, $p=0.0581$] and incorrect [$F_{(3, 82)} = 2.800$, $p=0.0451$] trial latency. *Post hoc* testing using Pairwise t-test with FDR correction revealed that at 9 months old, male mice had significantly lower correct trial latency than 6 ($p=0.0290$) and 12 month old ($p=0.0290$) male mice. For incorrect trial latency male mice had significantly, or trending towards significantly, increased latency at 6 months old as compared to 3 ($p<0.0001$), 9 ($p<0.0001$) and 12 ($p=0.0666$) month old mice. Also, male 12 month old mice had significantly higher incorrect trial latency than 3 ($p=0.0500$) and 9 ($p=0.0093$) month old mice. The only age group that showed a sex difference in incorrect trial latency was at 6 months old were male mice had significantly higher latency than female mice ($p=0.0300$). There was no evidence that trial latency varied significantly with age in female mice during OD1.

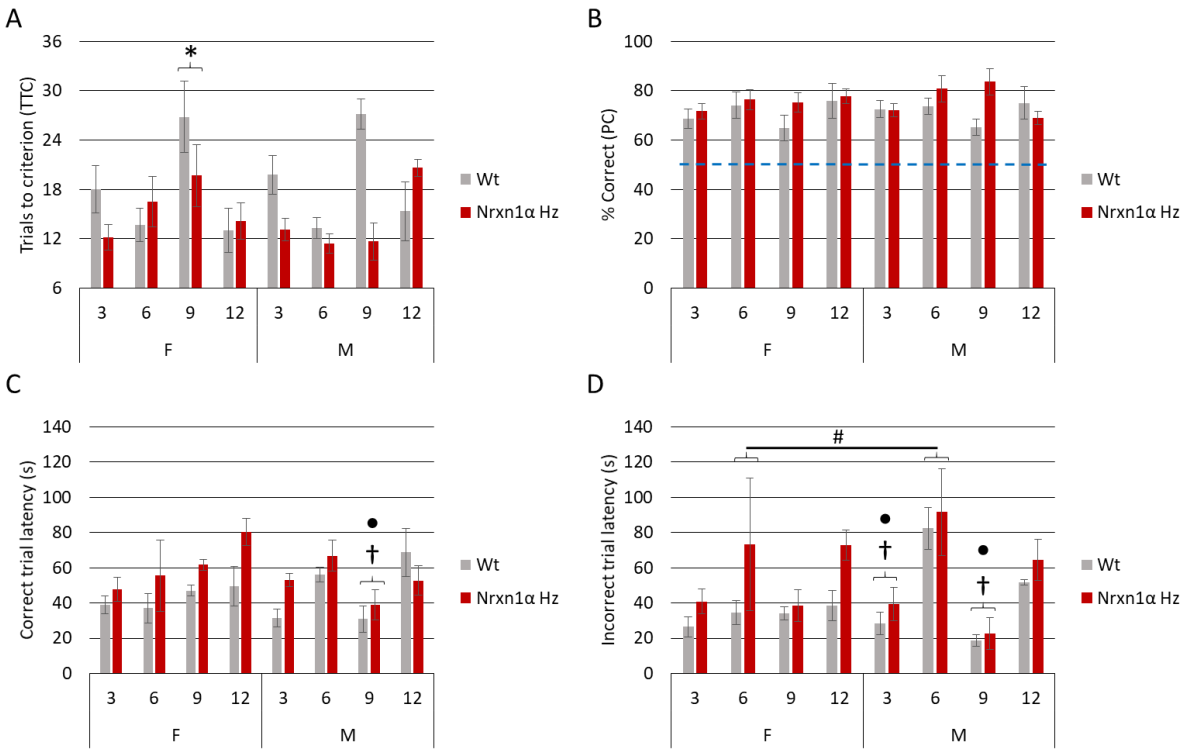

Figure 5.6 Performance and response latency varies dependent on age and sex in Odour Discrimination 1 (OD1)

Data shown are mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous, F=Female, M=Male, 3=3 month old, 6=6 month old, 9=9 month old, 12=12 month old. Measures of performance for associative learning include ***(A)*** *Trials to criterion (TTC) and* ***(B)*** *Percentage correct (PC). Latency was measured for both* ***(C)*** *correct and* ***(D)*** *incorrect trials. All graphs plotted by genotype per age group per sex. Significant or close to significant sex x age interactions were found for TTC [$F_{(3, 87)} = 2.984$, p=0.0356], correct trial latency [$F_{(3, 89)} = 2.586$, p=0.0581] and incorrect trial latency [$F_{(3, 82)} = 2.800$, p=0.0451]. Sample sizes were* ***(A)*** *Wt; F: 3 month n=8, 6 month n=3, 9 month n=5, 12 month n=7, M: 3 month n=9, 6 month n=7, 9 month n=6, 12 month n=6, Nrxn1α Hz; F: 3 month n=7, 6 month n=4, 9 month n=6, 12 month n=6, M: 3 month n=8, 6 month n=5, 9 month n=6, 12 month n=8,* ***(B)*** *Wt; F: 3 month n=8, 6 month n=5, 9 month n=5, 12 month n=7, M: 3 month n=9, 6 month n=7, 9 month n=6, 12 month n=6, Nrxn1α Hz; F: 3 month n=8, 6 month n=4, 9 month n=6, 12 month n=7, M: 3 month n=8, 6 month n=5, 9 month n=6, 12 month n=8,* ***(C)*** *Wt; F: 3 month n=7, 6 month n=5, 9 month n=5, 12 month n=7, M: 3 month n=8, 6 month n=4, 9 month n=6, 12 month n=7, Nrxn1α Hz; F: 3 month n=8, 6 month n=4, 9 month n=6, 12 month n=7, M: 3 month n=8, 6 month n=5, 9 month n=7, 12 month n=8,* ***(D)*** *Wt; F: 3 month n=8, 6 month n=5, 9 month n=5, 12 month n=6, M: 3 month n=8, 6 month n=7, 9 month n=5, 12 month n=4, Nrxn1α Hz; F: 3 month n=8, 6 month n=4, 9 month n=6, 12 month n=7, M: 3 month n=7, 6 month n=5, 9 month n=5, 12 month n=8. Dotted line in* ***(B)*** *denotes level at which choices are made by chance. * denotes p<0.050 significant increase compared to 12 month old females, ● denotes p<0.05 significant decrease compared to 12 month old males. † denotes p<0.05 significant decrease compared to 6 month old males, # denotes p<0.050 significant increase in males compared to females at 6 months old (Pairwise t-test with FDR correction).*

5.6.2 Associative learning with a second odour pair (OD2)

6 month old mice showed enhanced performance when compared to 3 and 9 month old mice when learning to discriminate a second odour pair

During OD2, 6 month old mice performed significantly better than 3 and 9 month old mice in terms of TTC and PC parameters (Figure 5.7 A and B). In this way a significant effect of age was found for both PC [F (3, 100) = 4.852, p=0.0034] and TTC [F (3, 108) = 4.503, p=0.0051]. *Post hoc* testing using Pairwise t-test with FDR correction, revealed that 6 month old mice showed significantly decreased TTC as compared to 3 (p=0.0110) and 9 (p=0.0220) month old mice. Furthermore, post-hoc testing also revealed that 6 month old mice showed significantly increased PC as compared to 3 month old mice (p=0.0015). There were no significant age x genotype or sex x age interactions for TTC or PC in OD2.

Trial response latency during OD2 is altered dependent on sex and age

As well as differences dependent on age for TTC and PC in OD2, there were also age dependent differences in trial latency. Furthermore, age dependent changes in correct, but not incorrect, trial latency were also significantly impacted on by sex (Figure 5.7 C and D). A significant sex x age interaction was found for correct trial latency [$F_{(3, 100)} = 2.738$, p=0.0474] and a significant effect of age was found for incorrect trial latency [$F_{(3, 89)} = 14.509$, p<0.0001]. *Post hoc* testing revealed that 6 month old male mice took significantly longer than all other age groups to make correct choices (3m vs 6m p=0.0001, 9m vs 6m p= 0.0001, 12m vs 6m p=0.0337). In female mice, 6 and 12 month old mice took significantly longer to make a correct choice than 3 month old mice (3m vs 6m p=0.0057, 3m vs 12m p=0.0014). For incorrect choices, 6 month old mice (pooled for sex as no significant sex x age

interaction was found) took significantly longer to make incorrect choices than 3 (p=0.0110) and 9 (p=0.0220) month old mice.

5.6.3 Odour discrimination reversal learning (OD2R)

The reversal phase (OD2R) is more cognitively challenging than the odour discrimination phase (OD2) across all age groups

Across all age groups tested, mice found the reversal phase (OD2R) more challenging than the odour discrimination phase (OD2), evidenced by significantly decreased PC and increased TTC in OD2R compared to OD2 (data not shown). In this way a significant phase x age interaction was found for PC [$F_{(3, 189)}$ =3.007, p=0.0316] but not for TTC. *Post hoc* testing using Pairwise t-test with FDR correction revealed that in all four age groups, mice showed significantly decreased PC in OD2R when compared to OD2 (3 month p=0.0379, 6 month p<0.0001, 9 month p<0.0001, 12 month p<0.0010). For TTC, a significant effect of phase was found [$F_{(1, 198)}$ =7.803, p=0.0057] revealing significantly higher TTC in OD2R as compared to OD2.

Trial latency during reversal learning varies dependent on age in male mice

Although performance parameters (PC and TTC) were not influenced by age or sex in OD2R (data not shown), trial latency was affected by age in male, but not female, mice in this phase of the task (Figure 5.8 A and B). Significant or close to significant sex x age interactions were found for both correct [$F_{(1, 90)}$ = 2.533, p=0.0620] and incorrect [$F_{(3, 87)}$ = 5.061, p=0.0028] trial latency in OD2R. *Post hoc* testing using Pairwise t-test with FDR correction revealed that in males, 6 and 12 month old mice took significantly longer than 3 and 9 month old mice to make both correct and incorrect choices (correct trial latency: 3m

vs 6m p=0.0119, 9m vs 6m p=0.0077, 3m vs 12m p= 0.0284, 9m vs 12m p= 0.0091, Incorrect trial latency: 3m vs 6m p= 0.0210, 9m vs 6m p= 0.0100, 3m vs 12m p= 0.0500, 9m vs 12m p=0.0210). There was also a trend towards 9 month old female mice taking significantly longer to make incorrect choices than 3 month old female mice (p=0.0620), but no significant evidence that age impacted on correct choice latency in female mice.

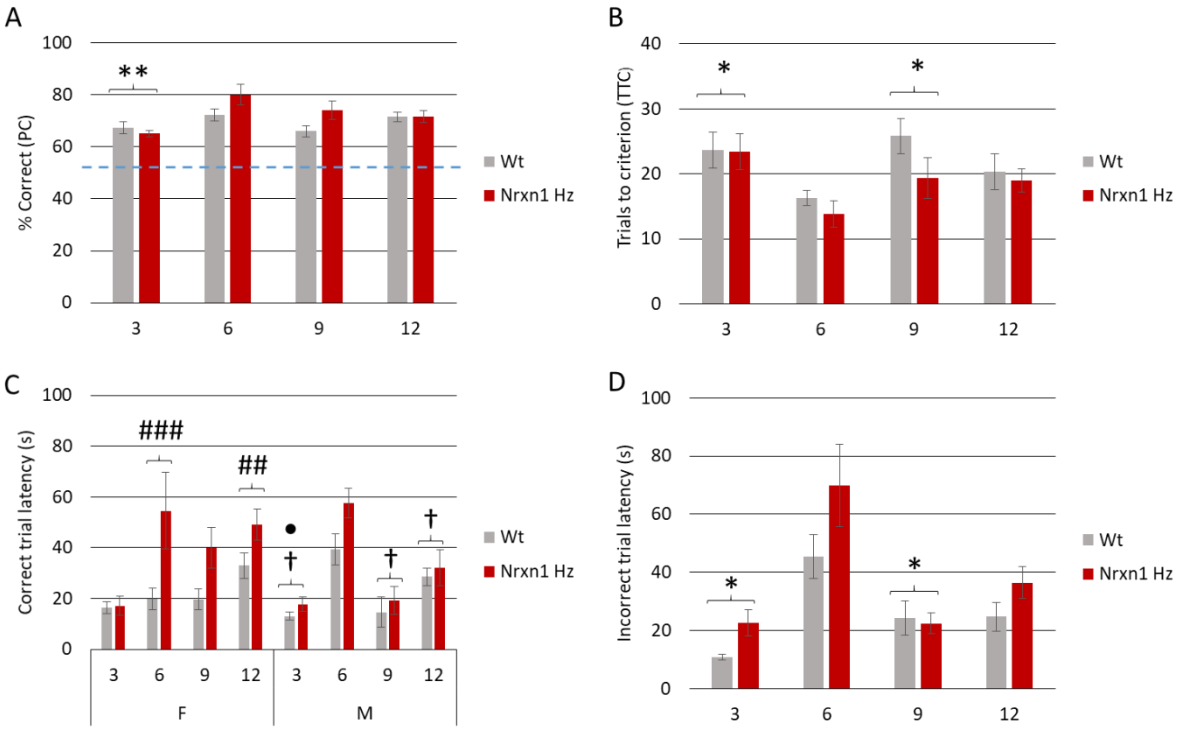

Figure 5.7 Performance and response latency are influenced by age and sex in Odour Discrimination 2 (OD2)

Data shown are mean ± SEM. Wt= Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous, F=Female, M=Male, 3=3 month old, 6=6 month old, 9=9 month old, 12=12 month old. Measures of performance for associative learning include ***(A)*** *Percentage correct (PC) and* ***(B)*** *Trials to criterion (TTC), plotted by genotype per age group. Latency was measured for both* ***(C)*** *correct and* ***(D)*** *incorrect trials plotted by genotype per group. Significant effects of age were found for PC [$F_{(3, 100)} = 4.852$, $p=0.0034$], TTC [$F_{(3, 108)} = 4.503$, $p=0.0051$] and incorrect trial latency [$F_{(3, 89)} = 14.509$, $p<0.0001$]. A significant sex x age interaction was found for correct trial latency [$F_{(3, 100)} = 2.738$, $p=0.0474$]. Sample sizes were* ***(A)*** *Wt; 3 month n=16, 6 month n=13, 9 month n=14, 12 month n=13, Nrxn1α Hz; 3 month n=16, 6 month n=13, 9 month n=15, 12 month n=16,* ***(B)*** *Wt; 3 month n=17, 6 month n=12, 9 month n=15, 12 month n=16, Nrxn1α Hz; 3 month n=19, 6 month n=12, 9 month n=15, 12 month n=18,* ***(C)*** *Wt; F: 3 month n=7, 6 month n=6, 9 month n=5, 12 month n=9, M: 3 month n=8, 6 month n=6, 9 month n=8, 12 month n=6, Nrxn1α Hz; F: 3 month n=8, 6 month n=7, 9 month n=5, 12 month n=8, M: 3 month n=9, 6 month n=6, 9 month n=8, 12 month n=10,* ***(D)*** *Wt; 3 month n=14, 6 month n=13, 9 month n=14, 12 month n=12, Nrxn1α Hz; 3 month n=16, 6 month n=10, 9 month n=11, 12 month n=15. Dotted line in* ***(A)*** *denotes level at which choices are made by chance (50%). * denotes $p<0.05$ and ** denotes $p<0.01$ significant difference compared to 6 month old group. ## denotes $p<0.01$ and ### denotes $p<0.001$ significant increase compared to 3 month old females. ● denotes $p<0.05$ significant decrease from 12 month old males. † denotes $p<0.05$ significant decrease from 6 month old males (Pairwise t-test with FDR correction).*

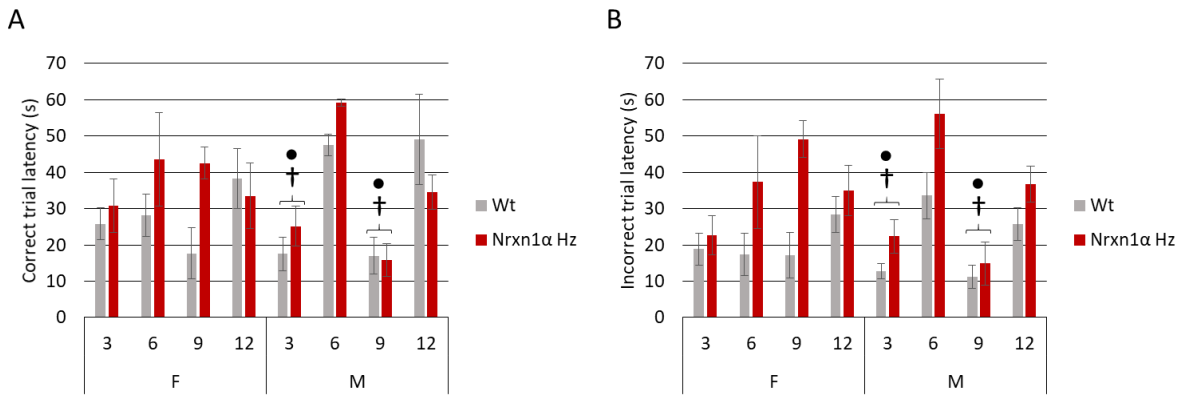

Figure 5.8 Trial latency during reversal learning (OD2R) varies dependent on age in male, but not in female, mice

Data shown are mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous F=Female, M=Male, 3=3 month old, 6= 6 month old, 9=9 month old, 12=12 month old. Latency was measured for both ***(A)*** *correct and* ***(B)*** *incorrect trials plotted by genotype per group. Significant sex x age interactions were found for correct [$F_{(1, 90)}$ = 2.533, p=0.0620] and incorrect [$F_{(3, 87)}$ = 5.061, p=0.0028] trial latency. Sample sizes were* ***(A)*** *Wt; F: 3 month n=7, 6 month n=6, 9 month n=5, 12 month n=8, M: 3 month n=8, 6 month n=4, 9 month n=8, 12 month n=7, Nrxn1α Hz; F: 3 month n=8, 6 month n=6, 9 month n=7, 12 month n=5, M: 3 month n=10, 6 month n=2, 9 month n=6, 12 month n=9,* ***(B)*** *Wt; F: 3 month n=6, 6 month n=5, 9 month n=5, 12 month n=7, M: 3 month n=8, 6 month n=4, 9 month n=7, 12 month n=6, Nrxn1α Hz; F: 3 month n=8, 6 month n=6, 9 month n=7, 12 month n=7, M: 3 month n=10, 6 month n=2, 9 month n=6, 12 month n=9. ● denotes p<0.05 significant decrease compared to 12 month old males. † denotes p<0.05 significant decrease compared to 6 month old males.*

5.6.4 Shifting stimuli dimension (SS)

Age does not affect ability to shift attention from the stimuli dimension of odour to location

There were no significant effects of age for the SS phase of the task. However it is important to note that a large proportion of animals did not reach criteria in this phase, and so sample sizes of certain age groups were relatively small (see Table 5.1). During the SS phase of the task female mice showed significantly increased latency (slower responses) for correct trials as compared to males (ANOVA main effect of sex [$F_{(1, 33)}$ =5.241, p=0.0286]) (data not shown). However, after removing 12 animals from the analysis that completed the SS phase without receiving a punishment (indicating they may have not formed a set for the odour discrimination phase), the sex effect for correct trial latency was no longer found.

5.7 Discussion

5.7.1 Effects of *Nrxn1α* heterozygosity on performance in the CFT

There were 3 main findings in the CFT for *Nrxn1α* Hz mice: (1) *Nrxn1α* Hz mice showed enhanced associative learning of a novel odour discrimination dependent on age, (2) *Nrxn1α* Hz mice showed a deficit in reversal learning that results from increased regressive, but not perseverative errors, and (3) *Nrxn1α* Hz mice had slower response latency during associative and reversal learning (Table 5.2).

5.7.2 *Nrxn1α* Hz mice show enhanced associative learning of a novel odour discrimination dependent on age

In the OD1 phase of the CFT *Nrxn1α* Hz mice showed enhanced associative learning of a novel odour discrimination as compared to Wt mice at 3 and 9 months old, evidenced by

significantly decreased TTC at 3 and 9 months old and increased PC at 9 months old (Figure 5.1 A and B). No differences in performance were found in *Nrxn1α* Hz mice in the second associative learning phase of the task (OD2) (Figure 5.2 A and B). The age-dependent nature of enhanced associative learning in *Nrxn1α* Hz mice could be caused by a disruption of developmentally regulated *Nrxn1α* expression levels in the brain during adult maturation. One study has previously characterised *Nrxn1* expression in the hippocampus and cerebral cortex of male mice at 10, 30, 50 and 80 weeks old (approx. 2.5, 7.5, 12.5 and 20 months old) (Kumar and Thakur, 2015). The authors found that *Nrxn1* expression is significantly increased at 30 weeks old (7.5 months), in both the hippocampus and cortex, in comparison to expression levels at 10 weeks (2.5 months) old. By contrast, expression was not significantly different from that seen at 10 weeks old when animals were 50 weeks old (12.5 months) and was found to be significantly decreased at 80 weeks old (20 months), in both hippocampus and cerebral cortex. This suggest that a complex pattern of developmentally regulated *Nrxn1* expression exists across the lifespan in the brains of adult Wt mice. The increase in *Nrxn1* expression seen after 10 weeks old (2.5 months) which then decreases and returns to similar levels at 50 weeks (12.5 months) old may affect associative learning in Wt mice. A suggestion supported by our observation that associative learning in OD1 is impacted in adult Wt animals as they age (Figure 5.1 A). One possibility is that heterozygous deletion of *Nrxn1α* may attenuate these developmentally regulated alterations in *Nrxn1α* expression, which might then contribute to the enhanced associative learning seen at specific ages, thus explaining the enhanced associative learning in 3 and 9 month old *Nrxn1α* Hz mice in comparison to Wt mice, that isn't seen in 12 month old mice. However this would not explain why 6 month old *Nrxn1α* Hz mice do not show enhanced associative learning in comparison to Wt animals of the same age.

Although no previous studies have tested mice with mutations in *Nrxn1α* in associative learning tasks, two studies have shown that they have enhanced learning in other learning paradigms. Laarakker *et al.* showed that male *Nrxn1α* Hz mice showed enhanced discrimination of the novel vs familiar object in a NORT. This was evidenced by *Nrxn1α* Hz mice showing a novel object response in the first 5 minutes of the task but not the last 5 minutes, suggesting that they discriminated between the objects faster than Wt mice, as Wt mice showed a novel object response in both time bins (Laarakker *et al.*, 2012). However a novel object response in the first 5 minute time bin and not in the second 5 minute time bin does not necessarily demonstrate enhanced object discrimination, for example it may be due to decreased object exploration time commonly seen at later time points in the NORT. In addition, these results contrast with findings in the current study (chapter 4) where a deficit in NORT was found in female and male *Nrxn1α* Hz mice (dependant on analysis parameters used). Another study has reported enhanced learning in *Nrxn1α* KO mice, in the form of enhanced motor learning on the rotarod as compared to Wt mice (Etherton *et al.*, 2009). Despite these observations it is unclear how enhanced novel object learning and motor learning might relate to the enhanced associative learning seen in mice heterozygous for *Nrxn1α* in the CFT.

In contrast to the finding in *Nrxn1α* Hz mice in the current study, studies in *Nrxn1α* KO rats showed that male, but not female, *Nrxn1α* KO rats had an impairment in spatial associative learning compared to Wt rats (Esclassan *et al.*, 2015). Similarly studies of *Nrx-1* hypofunction in *Drosophila* have shown deficits in associative learning in both larvae (Zeng *et al.*, 2007) and adult flies (Sun *et al.*, 2016). This suggests that brain circuitry involved in associative learning may be differentially affected by *Neurexin* heterozygosity/hypofunction in the

mouse, rat and fly or perhaps that the enhanced learning in the *Nrxn1α* Hz mice is task specific and cannot be generalised to other associative learning paradigms.

Research into ASD generally focuses on deficits in the disorder, however there is evidence for enhanced perceptual functioning in visual perception in low level cognitive tasks (Dakin and Frith, 2005; Mottron *et al.*, 2006; Simmons *et al.*, 2009). Enhanced performance in visual search tasks has also been shown in children (Shah and Frith, 1983; Plaisted *et al.*, 1998b; Kaldy *et al.*, 2011), adolescents (Joseph *et al.*, 2009; Chen *et al.*, 2012) and adults (Remington *et al.*, 2012) with ASD. Furthermore, one study showed that high functioning adults with ASD had enhanced discriminative ability when visual stimuli were novel, but not when they were familiar, compared to control subjects (Plaisted *et al.*, 1998a). This parallels the enhanced odour discrimination seen in the *Nrxn1α* Hz mice when the odour-reward discrimination was novel in the OD1 phase (Figure 5.1), but not following exposure to the discrimination rule in the OD2 phase (Figure 5.2). Although the cognitive tasks and stimuli type were not there same in the current study using mice and in Plaisted *et al.* investigating ASD patients, these observations may still be translationally relevant in terms of cognitive processes involved in learning to discriminate between stimuli for the first time compared to after pre-exposure to the stimuli/discrimination rule.

Alongside studies in ASD patients, studies in two mouse models of ASD have showed enhanced fear conditioning, which is a type of associative learning and memory. These include the Leucine rich repeat and fibronectin type III domain containing protein 2 (Lrfn2) KO mice (Morimura *et al.*, 2017), modelling dysfunction of a post synaptic adhesion protein associated with ASD (Voineagu *et al.*, 2011) and a mouse model of Timothy syndrome (TS) (Bader *et al.*, 2011), a syndrome which includes ASD (Napolitano *et al.*, 1993), which has a

heterozygous missense mutation in Cav1.2 L-type calcium channel. The Lrfn2 KO mice showed enhanced contextual fear conditioning (fear response to the conditioning environment) and enhanced discrimination between conditioned environment and a novel environment as compared to Wt mice (Morimura *et al.*, 2017). The mouse model of TS showed enhanced contextual and cued (fear response to the conditioned stimulus) fear conditioning (Bader *et al.*, 2011). These studies provide some support for enhanced associative learning observed in *Nrxn1α* Hz mice in the current study, however there are some important differences between associative learning in the CFT in the current study and fear conditioning used to test the Lrfn2 KO and TS mouse models. The CFT used appetitive associative learning that involved a 2-choice discrimination and required working memory to complete the 6 consecutive correct choices in the OD1 phase of the task, whereas in fear conditioning the associative learning is both aversive and Pavlovian (pairing of a conditioned stimulus with and unconditioned stimulus) and the memory component is more long term (1 hour or 1 day compared to seconds between trials in the CFT) for testing of learning and memory.

In summary, *Nrxn1α* Hz mice show enhanced associative learning in the OD1 phase at 3 and 9 months old which could be due to *Nrxn1α* heterozygosity attenuating the normal developmental expression patterns of *Nrxn1α* seen in Wt mice, bringing about varied associative learning ability across age in Wt mice. This certainly warrants further systematic characterisation. Some support for enhanced associative learning in *Nrxn1α* Hz mice comes from other studies using *Nrxn1α* mutant mice which report improvements in other learning paradigms compared to Wt mice. However studies of *Nrxn1α* heterozygosity/hypofunction in rats and flies have found impaired associative learning. The data also suggest that the enhanced performance of *Nrxn1α* Hz mice in the OD1 phase of the CFT may have

translational relevance to the enhanced perceptual learning of novel discrimination stimuli seen in ASD. Moreover, these observations parallel the enhanced associative learning in fear conditioning seen in other mouse models of ASD.

5.7.3 *Nrxn1α* Hz mice showed a deficit in reversal learning due to increased regressive errors

Nrxn1α Hz mice showed a deficit in reversal learning evidenced by decreased PC across all age groups and increased TTC at 12 months old (Figure 5.3 A and B). When error type was investigated it was found that *Nrxn1α* Hz mice make more regressive errors than Wt (Figure 5.4 A), whilst measures of perseverative errors are similar across the two genotypes (Figure 5.4 B and C). There seems to be a parameter specific and age dependent effect for increased TTC at 12 months old in *Nrxn1α* Hz mice, however there is also a significant main effect of genotype across all ages supporting increased TTC overall in *Nrxn1α* Hz mice. *Nrxn1α* Hz mice did not show any deficits in the SS phase of the task (Figure 5.5 A and B).

Studies of the effects of *Neurexin1* dysfunction on learning

No previous studies using *Nrxn1α* Hz or KO mice have tested reversal learning in any behavioural paradigm. However one study tested spatial reversal learning in *Nrxn1α* KO rats and found that male, but not female, rats had a deficit in this type of reversal learning, supporting the reversal learning deficit seen in *Nrxn1α* Hz mice. However, unlike findings from the current study, male *Nrxn1α* KO rats also had a deficit in initial spatial associative learning (Esclassan *et al.*, 2015), while we found no evidence for a deficit in associative learning in *Nrxn1α* Hz mice. The deficit in associative learning in *Nrxn1α* KO rats may also contribute to their deficit in reversal learning in the spatial testing paradigm, and this learning paradigm does not allow for characterisation of the error type underlying this

deficit. Characterising the performance of *Nrxn1α* KO and Hz rats in the CFT would be of future interest, particularly to determine if the behavioural alterations we have identified are maintained across these species.

ASD and Sz patients have deficits in cognitive flexibility

Both Sz and ASD patients show deficits in cognitive flexibility tasks such as the WCST, whereby individuals with both disorders show increased perseverative errors (Bellini *et al.*; Zanello *et al.*; Bellini *et al.*, 1991; Scarone *et al.*, 1993; Sullivan *et al.*, 1993; Battaglia *et al.*, 1994; Abbruzzese *et al.*, 1995; Haut *et al.*, 1996; Dieci *et al.*, 1997; Stratta *et al.*, 1997; Glahn *et al.*, 2000; Egan *et al.*, 2001; Gooding and Tallent, 2002; Cavallaro *et al.*, 2003; Altshuler *et al.*, 2004; Tsuchiya *et al.*, 2005; Josman and Katz, 2006; Zanello *et al.*, 2006; South *et al.*, 2007; Sumiyoshi *et al.*, 2011; Yasuda *et al.*, 2014; Yeung *et al.*, 2016). The reversal learning deficit seen the *Nrxn1α* Hz mice is translatable to deficits in cognitive flexibility in the WCST in Sz and ASD patients. However reversal learning is one type of cognitive flexibility and the deficits seen in patients tend to be in another form of cognitive flexibility, the dimensional set shifting aspect of the WCST. In addition, *Nrxn1α* Hz mice showed increased regressive errors during this reversal stage rather than perseverative errors which are commonly reported in patients. However, as well as deficits in the WCST, Sz patients have also been shown to have deficits in reversal learning, including probabilistic reversal learning (Waltz and Gold, 2007; Schlagenhauf *et al.*, 2014; Culbreth *et al.*, 2016) and reversal learning in the ID/ED set shifting tasks (Mckirdy *et al.*, 2009). Reversal learning deficits have also been observed in ASD during spatial reversal learning tasks (Coldren and Halloran, 2003) and in probabilistic reversal learning tasks (D'cruz *et al.*, 2013). This supports the potential translational relevance of the reversal learning deficit found in the *Nrxn1α* Hz mice in the

current study. Similar to our observations in *Nrxn1α* Hz mice, studies showing ASD patients to be impaired in reversal learning interestingly report that ASD patients are unimpaired in the initial associative learning phase, equivalent to OD1 in the current study (Coldren and Halloran, 2003; D'cruz *et al.*, 2013). By contrast studies reporting deficits in reversal learning in Sz patients found impairment in the initial learning phase of the task (Schlagenhauf *et al.*, 2014; Culbreth *et al.*, 2016), except for one study (Waltz and Gold, 2007). This suggests that the phenotype of unimpaired (or enhanced) associative learning and impaired reversal learning could be more translationally relevant to altered cognitive flexibility in ASD than Sz. The phenotype is further supported by domain specific enhanced learning seen in ASD patients and also in animal models of ASD, as previously discussed.

Regressive errors in cognitive flexibility tasks

Studies have also shown that Sz patients make more regressive, as well as perseverative, errors in the WCST (Franke *et al.*, 1992; Snitz *et al.*, 1999; Hill, S. K. *et al.*, 2015). Also increased regressive errors in the WCST appears not to be specific to Sz since patients with Bipolar Disorder (BD) also showed this behaviour (Hill, S. K. *et al.*, 2015). By contrast another study has reported that Sz patients make a similar number of non-perseverative errors to controls in the WCST (Li, 2004). Studies in ASD patients have shown that both perseverative and non-perseverative errors were increased compared to control subjects in the WCST (Shu *et al.*, 2001; Landry and Al-Taie, 2016). Another study found that during probabilistic reversal learning, increased regressive errors and not perseverative errors were found to underlie to reversal learning deficit in ASD (D'cruz *et al.*, 2013). Thus the increase in regressive errors identified in *Nrxn1α* Hz mice shows alignment with the types of errors reportedly committed by both Sz and ASD patients during reversal learning.

Some studies have suggested that deficits in working memory and slowed cognitive processing speed (described in section 1.9) may underlie reduced performance of WCST in Sz (Gold *et al.*, 1997; Glahn *et al.*, 2000; Hartman *et al.*, 2003; Waltz, 2017) whereas another study found that not to be the case (Stratta *et al.*, 1997). Deficits in working memory may contribute to the increase in regressive errors (non-perseverative), leading to the deficit in reversal learning found in *Nrxn1α* Hz mice. However one study found that when working memory load was reduced in the WCST, the number of both perseverative and non-perseverative errors was reduced in Sz patients (performance improved), but a deficit compared to controls subjects still remained (Hartman *et al.*, 2003). This suggests that while working memory deficits may contribute to performance in WCST, they appear not to have a specific effect on non-perseverative errors in Sz patients.

Other rodent models relevant to ASD and Sz also show reversal learning deficits

A number of animal models of Sz and ASD have shown deficits in reversal learning. However there are mixed findings in these models as to whether initial associative learning is intact or impaired and whether the number of regressive errors is increased, as seen in the *Nrxn1α* Hz mice in the current study. Acute treatment with amphetamine or PCP, modelling hyperdopaminergia or NMDA receptor hypofunction in Sz respectively, caused a reversal learning deficit in a lever pressing operant box task in female Lister Hooded rats (Idris *et al.*, 2005). This finding was replicated when investigating the effect of acute treatment of PCP on reversal learning in male Sprague Dawley rats (De Bruin *et al.*, 2013). However unlike *Nrxn1α* Hz mice, these models also showed a deficit in the initial associative learning phase prior to the reversal phase (Idris *et al.*, 2005; De Bruin *et al.*, 2013). By contrast another study of acute treatment of PCP in male Sprague Dawley rats revealed intact associative

learning but impaired reversal learning in a 2-choice odour discrimination digging task similar to the CFT used in the current study (Gastambide *et al.*, 2012), paralleling the findings in *Nrxn1α* Hz mice. In further support of findings in the current study, rats acutely treated with PCP were found to have increased regressive errors in the reversal learning phase (Gastambide *et al.*, 2012). However in contrast to the findings of the current study, the PCP treated rats also showed a deficit when there was a shift in the rewarded stimuli (Extra-dimensional Shift EDS), whereas *Nrxn1α* Hz mice in the current study had no deficit in the SS phase of the CFT (Gastambide *et al.*, 2012). It is important to note that the digging task protocols in Gastambide *et al.* and the current study were not identical. In the rat version there are more phases, multiple reversal phases and the EDS phase shifts from the stimuli of odour to digging medium or visa-versa (Gastambide *et al.*, 2012). The CFT in the current study has less phases in total, one reversal phase and the SS phase involves a shift in stimuli from odour to location. Prolonged PCP administration has also been shown to induce deficits in reversal learning. When female Lister Hooded rats were treated sub-chronically with PCP they were found to have intact associative learning but impaired reversal learning in a lever pressing operant box task (Abdul-Monim *et al.*, 2007; Idris *et al.*, 2010). Furthermore, in a 2-choice digging task similar to the CFT, male Lister Hooded rats treated sub-chronically with PCP were found to have impaired reversal learning on the last of 3 reversal phases only and, in support of findings from the current study, showed significantly increased regressive errors during reversal learning (Dawson *et al.*, 2012). However, treatment with sub-chronic PCP also impaired rats on the intra-dimensional/Extra-dimensional (ID/ED) shift ratio (Dawson *et al.*, 2012), which does not align with the preserved SS shift capabilities identified in *Nrxn1α* Hz mice.

Rat models of early life insults, a factor which may increase risk of developing Sz, have also shown impaired reversal learning. These include rats prenatally exposed to Methylazoxymethanol acetate on Embryo Day E17 (MAM) and isolation-reared rats, both of which model a number behavioural and neurophysiological aspects of Sz (Moore *et al.*, 2006; Fone and Porkess, 2008; Powell, 2010). In support of the current study MAM rats showed a deficit in reversal but not associative learning in a 2-choice digging task, but unlike the findings from the current study they also showed an EDS shift deficit and increased perseverative errors in the reversal phase (Gastambide *et al.*, 2012). Isolation-reared rats also showed a deficit in reversal learning but intact associative learning in a 2-choice digging task at 38 and 80 days old (Powell *et al.*, 2015).

A number of other mouse models relevant to ASD have also shown impaired reversal learning. The BTBR $T^{+}Itpr3^{tf}$/J (BTBR) mouse, originally bred for diabetes related research but found to display a number of ASD-like behaviours (Bolivar *et al.*, 2007; Moy *et al.*, 2007), showed intact associative learning, a deficit in reversal learning and increased regressive errors in a 2-choice spatial location task (Amodeo *et al.*, 2012), similar to the cognitive flexibility profile seen in *Nrxn1α* Hz mice. Interestingly, these phenotypes were only seen when the reversal learning paradigm was probabilistic with 80% accurate feedback on correct responses and not when feedback was 100% accurate for correct responses. Although the phenotypes in the BTBR mice are similar to the findings of the current study, they are only revealed in the BTBR mice when the task is more difficult, which suggests that the *Nrxn1α* Hz mice may be more severely impaired than the BTBR mice in reversal learning. When BTBR mice are prenatally exposed to valproic acid, a known risk factor for ASD (Christensen *et al.*, 2013), they show deficits in both associative learning and reversal

learning in a place learning task (Puscian *et al.*, 2014). By contrast C57BL/6 mice show intact associative learning and impaired reversal learning after prenatal exposure to valproic acid (Puscian *et al.*, 2014). This may suggest that when there is two predisposing factors thought to cause ASD like symptoms the cognitive impairment is more severe, since associative as well as reversal learning is impaired.

The *Fmr1* KO mouse which models Fragile X syndrome, a syndrome that includes ASD (Geschwind and State, 2015), has been shown to have intact initial learning but impaired reversal learning in the Morris Water Maze (Nolan and Lugo, 2018). Finally 16p11.2 deletion mice, which model a genetic risk factor for ASD (Weiss *et al.*, 2008), show an impairment in associative learning and reversal learning with increased perseverative errors in a touch screen operant box task (Yang *et al.*, 2015).

In summary, *Nrxn1α* Hz mice show deficits in reversal learning (OD2R phase) but intact associative learning (OD2 phase), or enhanced associative learning of a novel discrimination (OD1), in a 2-choice digging task. *Nrxn1α* Hz mice also show increased regressive, but not perseverative, errors during reversal learning. Both Sz and ASD patients have been shown to have deficits in cognitive flexibility tasks including the WCST and reversal learning tasks, and the reversal learning deficit seen in *Nrxn1α* Hz mice may have translational relevance to these deficits. By contrast to the current findings of increased regressive errors in *Nrxn1α* Hz mice, Sz and ASD patients tend to show increased perseverative errors during cognitive flexibility tasks, however there are studies showing that regressive errors are also increased in patients. Interestingly, a number of other rodent models of Sz and ASD also show reversal learning deficits, some of which also show intact associative learning and increased regressive errors during reversal learning, paralleling our observations in *Nrxn1α* Hz mice.

Deficits in working memory have been suggested to underlie deficits in cognitive flexibility in patients. *Nrxn1α* Hz mice could have dysfunctional working memory contributing to the increased in regressive errors during reversal learning, and this certainly warrants further systematic characterisation, given that studies in humans showing that poor working memory increases both perseverative and regressive errors.

5.7.4 *Nrxn1α* Hz mice have increased trial latency during associative and reversal learning

Nrxn1α Hz mice show increased latency to dig for both correct and incorrect trials during associative learning (OD1 and OD2) and for incorrect trials during reversal learning (OD2R) (Figure 5.1-5.3 C and D). However, *Nrxn1α* Hz mice show no difference in latency to initiate digging for correct trials in reversal learning (OD2R) and for both correct and incorrect trials during the stimuli shift (SS) phase as compared to Wt mice (Figure 5.3 C and 5.5 C and D).

Increased trial latency could suggest that the *Nrxn1α* Hz mice have slowed cognitive processing speed during associative and reversal learning, which causes an increase in time needed to make choices and to initiate digging during the task. Slowed cognitive processing speed is a key cognitive symptom in Sz (Dickinson *et al.*, 2007; Sanchez *et al.*, 2009; Woolard *et al.*, 2010; Nuechterlein *et al.*, 2011; Andersen, R. *et al.*, 2013; Fatouros-Bergman *et al.*, 2014; Mazhari and Moghadas Tabrizi, 2014; Karbasforoushan *et al.*, 2015) and has also been found in ASD patients (Mayes and Calhoun, 2007; Spek *et al.*, 2008; Roberts *et al.*, 2011; Oliveras-Rentas *et al.*, 2012; Hedvall *et al.*, 2013; Lazar *et al.*, 2014; Travers *et al.*, 2014; Mouga *et al.*, 2016; Li *et al.*, 2017). However, the suggestion that the increased latency seen in *Nrxn1α* Hz mice may be related to the slowed cognitive processing speed seen in ASD and Sz should be interpreted very cautiously. Factors other than slowed cognitive processing

speed could account for increased response latencies in *Nrxn1α* Hz mice. Differences in motivational factors, including appetitive motivation, could contribute to the increased time taken to initiate digging seen in *Nrxn1α* Hz mice and cannot currently be ruled out as part of our testing protocol employed in this study. Another factor which could affect time taken to initiate digging is whether or not the mouse visited the other bowl before initiating digging as this would take extra time. It is unkown if there is a genotype effect in regard to this. Furthermore the *Nrxn1α* Hz mice could move slower than the Wt mice increasing time taken to initiate digging, however since *Nrxn1α* Hz mice were found to be hyperactive in the OF test (Chapter 3) this may contradict this notion. However, OF tests locomotor activity in a novel environment whereas in the CFT the mice had been habituated to the testing box, so differences in locomotor activity between genotypes cannot be completely ruled out.

In summary, *Nrxn1α* Hz mice showed increased response latencies during associative and reversal learning in the CFT, which may be indicative of slowed cognitive processing speed. Slowed cognitive processing speed is a key cognitive feature in Sz cognitive symptoms and has also been found in a number of studies of ASD patients. However, other factors including reduced motivation or reduced locomotor activity could account for the increase in response latencies in *Nrxn1α* Hz mice in the CFT, and these cannot be ruled out. Further testing in other behavioural testing paradigms would need to be carried out to truly establish whether decreased processing speed or motivational factors contribute to increased response latency seen in *Nrxn1α* Hz mice.

5.7.5 *Nrxn1α* Hz mice show intact ability to shift from the stimulus dimension of odour to location

Although *Nrxn1α* Hz mice had a deficit in reversal learning, their ability to shift from the stimuli dimension of odour to location in predicting reward was found to be intact. This was evidenced by no significant difference in PC or TTC compared to Wt mice during the SS phase (Figure 5.5 A and B). This could suggest that the *Nrxn1α* Hz mice have a selective impairment in reversal learning (OD2R) or that the SS phase does not involve a true dimensional shift involving the prefrontal cortex, which may have lead to the SS phase not being as difficult for mice to complete as the OD2R phase. In support of the latter suggestion, both Wt and *Nrxn1α* Hz mice showed a significant increase in PC in SS when compared to OD2R. A significant phase x genotype interaction was found for PC [$F_{(1, 22)}$ =6.004, p=0.0157] across the two phases. In contrast to these observations, there was no significant effect of phase or phase x genotype interaction for TTC when comparing OD2R and SS phases. *Post hoc* testing using Pairwise t-test with FDR correction revealed that both Wt and *Nrxn1α* Hz mice had increased PC in the SS phase as compared to the OD2R phase (Wt OD2R vs SS p=0.0497, *Nrxn1α* Hz OD2R vs SS p<0.0001). This may suggest that the hippocampus, rather than the prefrontal cortex, was recruited during this phase or that mice prefer spatial cues over odour cues to guide their behaviour in this task. The latter could be confirmed by using a protocol which first trains the animals to dig for rewards based on location and then switches the stimuli dimension to odour to test for differences. Previous work has shown that the *Nrxn1α* Hz mice have intact spatial memory (Grayton *et al.*, 2013) which supports the notion that the SS phase may not have required normal functioning of the prefrontal cortex but instead the hippocampus.

5.7.6 Performance in the CFT varies dependant on age and sex of mice

Sex and age of mice caused CFT parameters to vary during associative (OD1 and OD2) and reversal learning (OD2R), but not during an attentional shift from the stimulus of odour to location (SS).

There was no clear linear relationship between age and the parameters measured (PC, TTC, correct/incorrect latency) in any phases of the CFT i.e. there were no step-wise improvements or impairments as age increased. Instead there was a more variable pattern of behaviour dependent on age group. In the associative learning phase of the CFT (OD1 and OD2) sex and age determined performance, dependent on the performance parameter being measured and the phase of the task. In OD1, 9 month old female mice showed reduced performance when compared to 12 month old females evidenced by significantly increased TTC. By contrast male mice had similar TTC values across all age groups (Figure 5.6 A). Also in OD1, 9 month old Wt mice showed impaired associative learning compared to all other age groups of Wt mice, evidence by significantly increased TTC (Figure 5.1 A). By contrast there were no significant effects of sex or age on PC during OD1 (Figure 5.1 B and 5.6 B). In OD2, 6 month old mice of both sexes showed improved associative learning compared to 3 and 9 month old mice, evidenced by a significant increase in PC when compared to 3 month old mice and a significant decrease in TTC compared to 3 and 9 month old mice (Figure 5.7 A and B). In OD2R, 9 month Wt mice showed impaired reversal learning compared to 12 month old Wt mice in terms of significantly increased TTC (Figure 5.3 B). There were no significant effects of sex or age on ability to shift attention from the stimuli dimension of odour to location (SS) (data not shown), in terms of TTC and PC. These data suggest that associative learning and reversal learning are influenced, in a complex manner,

by ageing in adult mice. This could be due to developmental processes occurring in the brain during mouse adult maturation.

A limited number of studies have characterised aspects of learning and cognition at different ages in adult mice. For example, one study found that at 6 months old, Wt mice took less TTC on the initial associative learning phase, reversal and extra-dimensional shift phase (shifting attention from one stimuli dimension to another) than at 14 months old, in a longitudinal attentional set shifting task (Zhuo *et al.*, 2007). This suggests that cognition is impaired with age in mice for this task. These differences were not seen when comparing 6 and 12 month old mice in the current study, since 6 and 12 month old mice showed similar TTC and PC throughout all testing phases. However the mice in the Zhuo *et al.* study were 2 months older than the oldest mice in the current study, which may explain the impairments seen in all phases of the set shifting task in old mice which were not seen in the current study. Furthermore Zhuo *et al.* used a within-subjects design and so previous exposure to the task may have interfered with learning at 14 months old compared to 6 months old.

Another study tested 4 age groups of male mice (2-3, 4-5, 6-7 and 8-12 months old) with C57BL/6 genetic background in contextual fear conditioning (fear of the conditioning environment) and cued fear conditioning (fear of the conditioned stimulus), which are types of associative learning (Shoji *et al.*, 2016). In contrast to the variability in associative learning with age found in the current study, Shoji *et al*. found that contextual and cued fear conditioning was reduced with age in a linear, stepwise manner. However there are a number of differences between the two studies, such as the current study used mice of both sexes in an appetitive 2-choice odour based task and Shoji *et al*. used only male mice in an aversive fear based task.

Trial latency during associative learning (OD1 and OD2) and reversal learning (OD2R) was impacted on by age mainly in male mice (Figure 5.6-5.7 C and D and 5.8 C and D), except for correct trials in OD2 in female mice (Figure 5.7 C). During OD1, OD2 and OD2R male 3 and 9 month old mice show similar latencies and male 6 and 12 month old mice show similar latencies (Figure 5.6-5.7 C and D and 5.8 C and D). At present it is difficult to determine the cause of this variation in trial latencies with age in male mice, it may be that response latency is sensitive to developmental trajectories in male, and not in female, mice. As previously discussed, changes in trial latency could relate to processing speed in mice. However other factors such as motivation or attention could play a role.

In summary, sex and age impact on TTC, PC and trial latency during associative learning (OD1 and OD2), and trial latency during reversal learning (OD2R), but do not impact on parameters in the stimuli shift (SS) phase of the CFT. There were no clear stepwise increases or decreases in these parameters as age of mice increased. This may be due to mice in the current study being middle-aged and so may not have been old enough to see age-related effects that may be become apparent at old age.

5.7.7 Future experiments

The findings from the CFT have indicated a number of themes that require further investigation in the *Nrxn1α* Hz mice. These include whether enhanced associative learning and impaired reversal learning in *Nrxn1α* Hz mice can be generalised to other types of learning tasks, whether *Nrxn1α* Hz mice have impaired working memory, potentially contributing to the deficit in reversal learning seen in the CFT, and whether *Nrxn1α* Hz mice have slowed cognitive processing speed, that could contribute to the increase in trial latency

seen in these animals during the CFT. *Nrxn1α* Hz mice showed enhanced associative learning when the association was novel (OD1) but not when it was familiar (OD2) and showed a deficit in reversal learning. Associative learning could be tested in other tasks to see if the enhancement is task specific or can be generalised to other paradigms. These could include touchscreen associative learning tasks (Marquardt *et al.*, 2014) or Pavlovian conditioning, such as fear conditioning (Shoji *et al.*, 2016). Other types of learning could also be tested such as spatial learning using the Morris Water Maze, Barnes maze or radial arm maze. It would be important to characterise initial learning rates in these tasks to test if they were enhanced in *Nrxn1α* Hz mice compared to Wt. *Nrxn1α* Hz mice could also be tested in spatial reversal learning tasks (Esclassan *et al.*, 2015) to see if the deficit in reversal learning in the CFT generalises to other types of reversal learning. Further investigation is required to reveal the cause of the reversal learning deficit seen in *Nrxn1α* Hz mice. The current study revealed there was an increase in regressive errors in *Nrxn1α* Hz mice compared to Wt, which although provides information about the reversal learning phenotype, does not uncover the mechanism causing the deficit. As suggested previously a working memory deficit in the *Nrxn1α* Hz mice could cause them to have a reversal learning deficit in the CFT, which is also commonly seen in patients with Sz (Lee and Park, 2005; Barch and Smith, 2008; Forbes *et al.*, 2009). Therefore testing the *Nrxn1α* Hz mice in a working memory based task such as the touch screen Trial-Unique delayed Nonmatching-to-Location (TUNL) task (Oomen *et al.*, 2013) or odour-span task (Young *et al.*, 2007) could be used to more specifically address this question. Finally, the current study found increased response latency in the *Nrxn1α* Hz mice which could be due to slowed cognitive processing speed. However in order to confirm this, other possible factors would need to be eliminated, for instance motivational factors. To test motivational factors *Nrxn1α* Hz mice could be tested

in a progressive ratio task whereby the mice would be required to make an increasing number of lever presses in an operant box, or nose touches in a touchscreen task (Heath *et al.*, 2015) in order to gain a defined quantity of food reward. *Nrxn1α* Hz mice could also be tested in the 5-choice serial reaction time task (Remmelink *et al.*, 2017), which can measure response latency and latency to collect food reward. This would allow the response time (processing speed) and food related motivation to be separated. If response latency was slow and reward collection was fast this could indicate slowed processing speed rather than a motivational cause. Furthermore *Nrxn1α* Hz mice could also be tested in the sucrose preference test (Liu *et al.*, 2018), to confirm whether they value food rewards in the same way as Wt mice, ruling out the possibility that *Nrxn1α* Hz mice may not find the sugar pellet food reward as rewarding as Wt mice do. Performing this variety of tests would provide more information about the underlying cause of the reversal learning deficit seen in *Nrxn1α* Hz mice.

With regards to the effect of age group on associative learning, other tests of associative learning could be performed to test if performance varied at the same age groups (9 and 6 months old). Since behaviour in the CFT did not alter in a linear manner as age increased for any of the parameters tested, the task is probably not suitable for measuring modifications to age-related changes in behaviour in middle-aged mice. Therefore other tests of learning would be required for this purpose. If a cognitive test was found that showed age-related changes in middle-aged mice this test could then be used to test whether *Nrxn1α* heterozygosity affected this behaviour at different ages.

5.7.8 Summary

Nrxn1α Hz mice showed enhanced associative learning when the association was novel, a deficit in reversal learning which was associated with increased regressive, but not perseverative errors, and slower response latencies during associative and reversal learning. Enhanced associative learning and impaired reversal learning in *Nrxn1α* Hz mice was age dependent. ASD patients and mouse models of ASD have also shown enhanced learning in laboratory tasks. Both Sz and ASD patients and animal models of the disorders have shown deficits in reversal learning, mainly associated with increased perseverative errors. However some studies in patients have also found increased regressive errors during reversal learning, and intact associative learning, more closely supporting findings in *Nrxn1α* Hz mice in the current study. Further studies are required to confirm whether the reversal learning deficit and slowed response latency in the CFT in *Nrxn1α* Hz mice is due to impaired working memory and slowed cognitive processing speed. Sex, genotype and age affected performance of the CFT, dependent on parameter and phase. No parameters showed step-wise increases or decreases as age of mice increased across the 4 age groups, suggesting that the CFT is not useful to measure modifiers of age-related changes in behaviour in middle-aged mice, and so another behavioural task would need to be tested.

Chapter 6 The impact of *Nrxn1α* heterozygosity, sex and age on cerebral metabolism

6.1 Introduction

^{14}C-2-Deoxyglucose (^{14}C-2DG) functional brain imaging measures whole brain cerebral metabolism (Dawson *et al.*, 2013; Dawson *et al.*, 2015). ^{14}C-2DG is a radioactive analogue of glucose that is taken up into the brain. Therefore the more metabolically active a brain area is, the more glucose it requires, and so the more ^{14}C-2DG accumulates in that region. This technique allowed detection of any differences in regional cerebral metabolism between *Nrxn1α* Hz and Wt mice. Functional changes have been highlighted in a number of brain regions in both Sz and ASD, some of which are common to both disorders (reviewed in Section 1.1 and 1.2). Any changes in cerebral metabolism were assessed for their translational relevance to those seen in Sz and ASD, and relevance to any behavioural changes found in the *Nrxn1α* Hz mice.

6.2 The impact of *Nrxn1α* heterozygosity on cerebral metabolism: Key findings

- ***Nrxn1α* Hz mice show hypofrontality**
- ***Nrxn1α* Hz mice show increased cerebral metabolism in mesolimbic system, retrosplenial cortex and thalamic brain regions**
- ***Nrxn1α* Hz mice show increased cerebral metabolism in the Dorsal Raphé (DR) nucleus at 3 months old but not at any other age**

6.3 Results

6.3.1 *Nrxn1α* Hz mice show hypofrontality

Nrxn1α Hz mice showed decreased cerebral metabolism, as reflected by decreased local cerebral glucose utilisation (LCGU), in the prefrontal cortex ('hypofrontality') as compared to Wt mice (Figure 6.1 A, B and C, See Figure 6.2 A and B for representative autoradiograms). In this way a significant main effect of genotype was found for both the medial Prelimbic (mPrl) [$F_{(1, 62)}$ =16.839, p<0.0010] and anterior Prelimbic (aPrl) [$F_{(1, 62)}$ =4.600, p=0.0359] cortices, supporting significantly decreased cerebral metabolism in these regions. The effects of genotype on the mPrl and aPrl were not significantly modified by age or sex (no significant age x genotype or sex x genotype interactions).

6.3.2 *Nrxn1α* Hz mice show increased cerebral metabolism in mesolimbic system, retrosplenial cingulate cortex and thalamic brain regions

Nrxn1α Hz mice showed significantly increased cerebral metabolism in the Ventral Tegmental Area (VTA), Retrosplenial Cortex (RSC) and Ventrolateral Thalamus (VL) as compared to Wt mice, evidenced by a significant main effect of genotype found for LCGU in these regions (VTA [$F_{(1, 63)}$ =4.690, p=0.0341], RSC [$F_{(1, 63)}$ =5.321, p=0.0244] and VL [$F_{(1, 63)}$ =4.825, p=0.0317]) (Figure 6.1 A, D, E and F, See Figure 6.2 C and D for representative autoradiograms). The impact of genotype on these regions was not significantly modified by age or sex (no significant age x genotype or sex x genotype interactions).

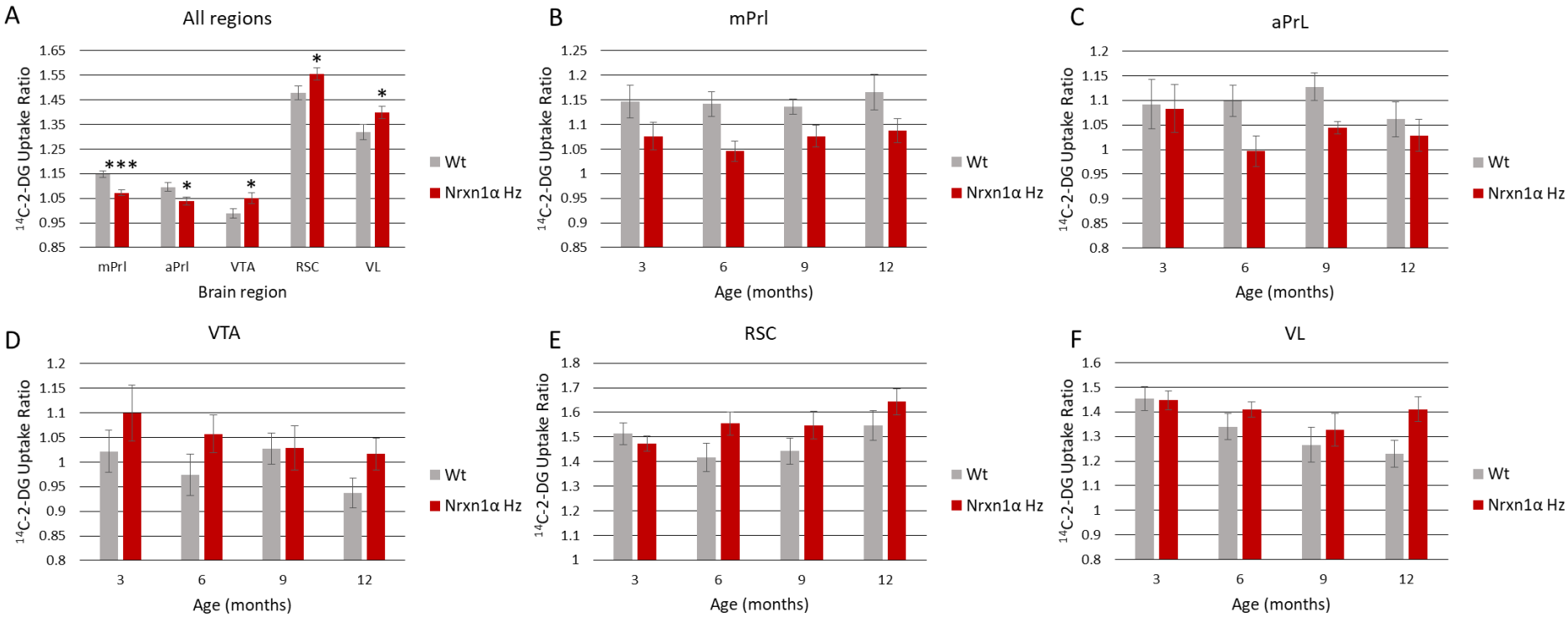

Figure 6.1 Nrxn1α Hz mice show hypofrontality and increased cerebral metabolism in mesolimbic system, retrosplenial cingulate cortex and thalamic brain regions

Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. ^{14}C-2-Deoxyglucose (^{14}C-2-DG) Uptake Ratio for ***(A)*** *all brain regions showing altered cerebral metabolism in Nrxn1α Hz mice and* ***(B-F)*** *each affected brain region plotted by genotype across the age groups for both sexes combined. A significant main effect of genotype was found for the medial Prelimbic cortex (mPrl) [$F_{(1, 62)}$ =16.839, p<0.0010], anterior Prelimbic cortex (aPrl) [$F_{(1, 62)}$ =4.600, p=0.0359], Ventral Tegmental Area (VTA) [$F_{(1, 63)}$ =4.690, p=0.0341], Retrosplenial cortex (RSC) [$F_{(1, 63)}$ =5.321, p=0.0244] and Ventrolateral Thalamus (VL) [$F_{(1, 63)}$ =4.825, p=0.0317]. Sample sizes were* ***(A)*** *Wt n=38-39, Nrxn1α Hz n=40 and* ***(B-F)*** *3 month: Wt n=9, Nrxn1α Hz n=10, 6 month: Wt n=10, Nrxn1α Hz n=10, 9 month: Wt n=10, Nrxn1α Hz n=10, 12 month: Wt n=9-10 Nrxn1α Hz n=10. * denotes p<0.05 and *** denotes p<0.001 significant difference to Wt (main effect, ANOVA).*

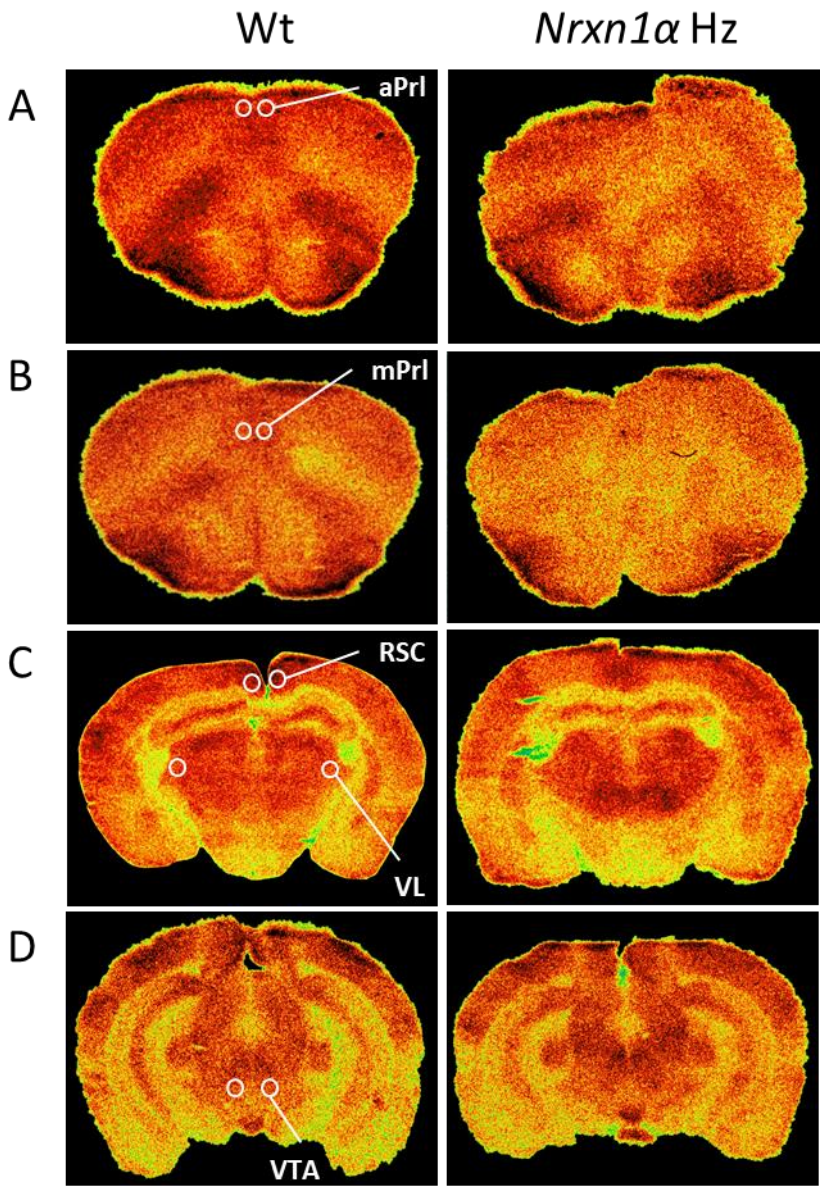

Figure 6.2 Representative autoradiograms for brain regions which show altered cerebral mentalism in Nrxn1α Hz mice compared to Wt

Representative autoradiograms are from one male 3 month old mouse from each genotype at the level of ***(A)*** *anterior Prelimbic cortex (aPrl),* ***(B)*** *medial Prelimbic cortex (mPrl),* ***(C)*** *Retrosplenial cortex (RSC) and Ventrolateral Thalamus (VL) and* ***(D)*** *Ventral Tegmental Area (VTA).*

6.3.3 *Nrxn1α* Hz mice show increased cerebral metabolism in the Dorsal Raphé (DR) nucleus at 3 months old but not at any other age

Nrxn1α Hz mice show significantly increased cerebral metabolism, as reflected by an increase in LCGU, in the DR at 3 months old but not at any other age group analysed (Figure 6.2 A, See B for representative autoradiograms). In this way a significant age x genotype interaction was found for the DR [$F_{(3, 63)}$ =3.031, p=0.0358]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that *Nrxn1α* Hz mice had higher cerebral metabolism in the DR than Wt mice at 3 months old (p<0.0010). Wt mice had significantly increased cerebral metabolism at 6 months old when compared to 3 months old (p=0.0031). By contrast, *Nrxn1α* Hz mice had similar levels of cerebral metabolism in the DR across all age groups analysed. Sex did not significantly impact on the increased cerebral metabolism seen in the DR of *Nrxn1α* Hz mice (no significant sex x age x genotype or sex x genotype interactions).

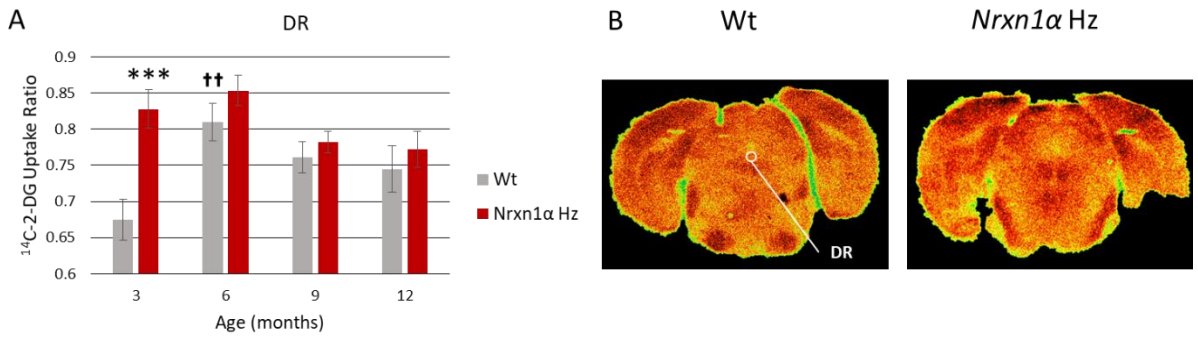

Figure 6.3 Nrxn1α Hz mice show increased cerebral metabolism in the Dorsal Raphé Nucleus (DR) in comparison to Wt mice at 3 months old but not at any other age

Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. ***(A)*** *^{14}C-2-Deoxyglucose (^{14}C-2-DG) Uptake Ratio for Dorsal Raphé (DR) plotted by genotype across the age groups.* ***(B)*** *Representative autoradiograms of Wt and Nrxn1α Hz mouse brains at the level of the DR. A significant age x genotype interaction was found for DR [$F_{(3, 63)}$ =3.031, p=0.0358]. Samples sizes were 3 month: Wt n=9 Nrxn1α Hz n=10, 6 month: Wt n=10 Nrxn1α Hz n=10, 9 month: Wt n=10, Nrxn1α Hz n=10, 12 month: Wt n=10, Nrxn1α Hz n=10. †† denotes p<0.01 and *** denotes p<0.001 significant increase compared to 3 month old Wt (Pairwise t-test with FDR correction). Representative autoradiograms are from one male 3 month old mouse from each genotype.*

Brain region	Impact of *Nrxn1α* heterozygosity on cerebral metabolism	Modified by age?	Modified by sex?
mPrl	↓	×	×
aPrl	↓	×	×
VTA	↑	×	×
RSC	↑	×	×
VL	↑	×	×
DR	↑	✓ 3m only	×

Table 6.1 Summary table of the effects of Nrxn1α heterozygosity on cerebral metabolism
mPrl=medial Prelimbic cortex, aPrl=anterior Prelimbic cortex, VTA=Ventral Tegmental Area, RSC=Retrosplenial Cortex, VL=Ventromedial Thalamus, DR=Dorsal Raphé, ↓=significant decrease in cerebral metabolism, ↑=significant increase in cerebral metabolis, 3m= 3 month old group, ×= effect not significantly modified by age/sex.

6.3.4 Validating the effects of *Nrxn1α* heterozygosity on cerebral metabolism

To check the validity of the ^{14}C-2DG functional brain imaging findings, blood glucose, plasma ^{14}C-2DG concentration and the concentration ratio of plasma ^{14}C-2DG: whole brain average ^{14}C-2DG were tested for significant effects of genotype. It was found that the *Nrxn1α* Hz mice had significantly higher blood glucose levels (Mean=7.4, SD=1.6) as compared to Wt mice (Mean=6.6, SD=1.6). However, *Nrxn1α* Hz had similar plasma ^{14}C-2DG levels and a similar ratio of plasma ^{14}C-2DG: whole brain average ^{14}C-2DG to that seen in Wt mice. In this way there was a significant effect of genotype on blood glucose levels [$F_{(1, 61)}$ =6.830, p=0.0113] (see Appendix, section A2), but no significant effect of genotype or significant genotype interactions for plasma ^{14}C-2DG concentration or the concentration ratio of plasma ^{14}C-2DG: whole brain average ^{14}C-2DG were found. These findings validate the previous genotype differences in LCGU. While blood glucose levels were significantly elevated in *Nrxn1α* Hz mice, this was in the normal physiological range (Bowe *et al.*, 2014) and there was no evidence that the ability of ^{14}C-2DG to enter the brain from the plasma was significantly altered in *Nrxn1α* Hz mice (supported by no significant difference in the ratio of plasma ^{14}C-2DG: whole brain average ^{14}C-2DG).

6.4 The impact of sex and age on cerebral metabolism: Key findings

- Cerebral metabolism shows 3 main patterns of age-dependent variation:
    1. Cerebral metabolism increases with age in a number of cortical regions
    2. Cerebral metabolism decreases with age in a number of thalamic regions
    3. Cerebral metabolism is reduced at 9 months old but normalises by 12 months old in Septal/diagonal band of Broca nuclei
- Sex impacts on the age-dependent alterations in cerebral metabolism seen in infralimbic and entorhinal cortex and the striatum
- Female mice show increased cerebral metabolism in thalamic and Raphé nuclei and decreased cerebral metabolism in the Nucleus Accumbens in comparison to males

6.5 Results

6.5.1 Cerebral metabolism shows 3 main patterns of age-dependent variation

Cerebral metabolism increases with age in a number of cortical regions

As age increased there was an increase in cerebral metabolism in cortical regions, including in the Frontal Association Area (FRA), Dorsolateral Orbital cortex (DLO), Piriform cortex (Piri) and Insular cortex (Ins) (Figure 6.4). In this way, a significant main effect of age was found for FRA [$F_{(3, 62)}$ =7.907, p=0.0002], DLO [$F_{(3, 62)}$ =9.656, p<0.0001], Piri [$F_{(3, 63)}$ =10.791, p<0.0001] and Ins [$F_{(3, 63)}$ =4.915, p=0.0040]. In these regions cerebral metabolism was similar at 3 and 6 month old but was significantly increased in the 9 and 12 month old groups. *Post hoc* testing using Pairwise t-test with FDR correction revealed that in the FRA, 9 and 12 month old mice had significantly higher cerebral metabolism than 3 and 6 month old

mice (3 month vs 9 month p=0.0038, 3 month vs 12 month p=0.0007, 6 month vs 9 month p=0.0025, 6 month vs 12 month p=0.0156) (Figure 6.4 A). In the DLO, cerebral metabolism was significantly increased in the 6 (p=0.0346), 9 (p<0.0001) and 12 (p<0.0010) month old groups compared to the 3 month old group. Cerebral metabolism in the DLO was also significantly increased at 9 months old compared to at 6 months old (p=0.0025) (Figure 6.4 B). In the Piri cerebral metabolism significantly increased in 9 and 12 month old mice as compared to 3 and 6 month old mice (3 month vs 9 month p=0.0012, 3 month vs 12 month p<0.0010, 6 month vs 9 month p=0.0013, 6 month vs 12 month p<0.0010) (Figure 6.4 C). Finally in the Ins, cerebral metabolism was significantly increased at 9 (p=0.0050) and 12 (p=0.0260) months old as compared to mice at 3 months old (Figure 6.4 D). There was no significant impact of genotype or sex on age-dependent increases in cerebral metabolism seen in these cortical regions (no significant genotype x age or sex x age interactions).

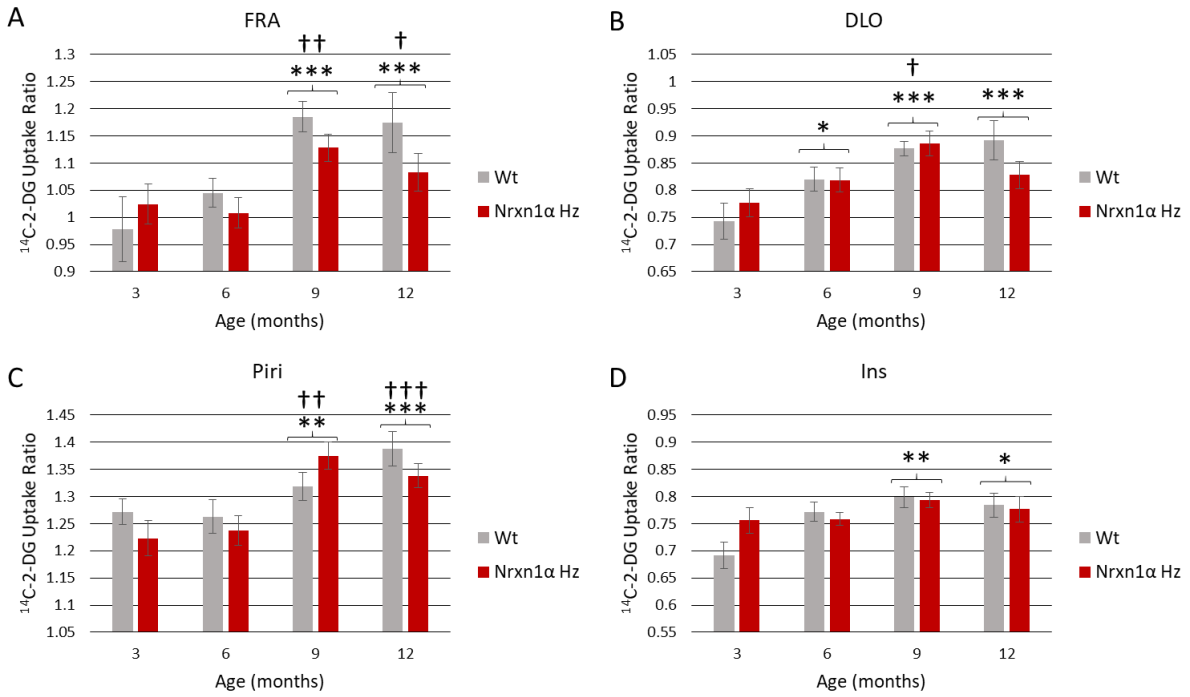

Figure 6.4 Cerebral metabolism increases with age in a number of cortical regions

Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. ^{14}C-2-Deoxyglucose (^{14}C-2-DG) Uptake Ratio for ***(A)*** *Frontal Association Area (FRA),* ***(B)*** *Dorsolateral Orbital cortex (DLO),* ***(C)*** *Piriform cortex (Piri) and* ***(D)*** *Insular cortex (Ins) plotted by genotype per age group. A significant main effect of age was found for FRA [$F_{(3, 62)}$ =7.907, p=0.0002], DLO [$F_{(3, 62)}$ =9.656, p<0.0001], Piri [$F_{(3, 63)}$ =10.791, p<0.0001] and Ins [$F_{(3, 63)}$ =4.915, p=0.0040]. Sample sizes were n=10 per group except for 3 month old Wt which were n=9. *, ** and *** denote, p<0.05, p<0.01 and p<0.001 respectively, significant difference as compared to 3 month group. †, †† and ††† denote, p<0.05, p<0.01 and p<0.001 respectively, significant difference as compared to 6 month group (Pairwise t-test with FDR correction).*

Cerebral metabolism decreases with age in a number of thalamic regions

Cerebral metabolism decreased with age in multiple thalamic regions including the Reticular thalamus (Rt), Medial Geniculate nucleus (MG), the Ventrolateral Thalamus (VL) and the Ventromedial Thalamus (VM) (Figure 6.5). A significant main effect of age was found for Rt [$F_{(3, 63)}$ =3.333, p=0.0250], MG [$F_{(3, 63)}$ =8.081, p=0.0001], VL [$F_{(3, 63)}$ =3.753, p=0.0152] and VM [$F_{(3, 63)}$ =2.831, p=0.0454]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that in the Rt cerebral metabolism was significantly reduced in 9 (p=0.0500) and 12 (p=0.0130) month old mice as compared to 3 month old mice (Figure 6.5 A). Similarly, in the MG the 9 month old group showed significantly decreased cerebral metabolism as compared to the 3 (p=0.0016) and 6 (p<0.0001) month old groups. The 12 month old group also showed significantly decreased cerebral metabolism when compared to the 6 month old group in this region (p=0.0205) (Figure 6.5 B). In the VL the 9 month old group showed significantly decreased cerebral metabolism as compared to the 3 month old group (p=0.0400) (Figure 6.5 C), and in the VM the 12 month old group showed significantly decreased cerebral metabolism as compared to the 3 month group (p=0.0500) (Figure 6.5 D). There was no significant impact of genotype or sex on the age-dependent increase in cerebral metabolism seen in these thalamic regions (no significant genotype x age or sex x age interactions).

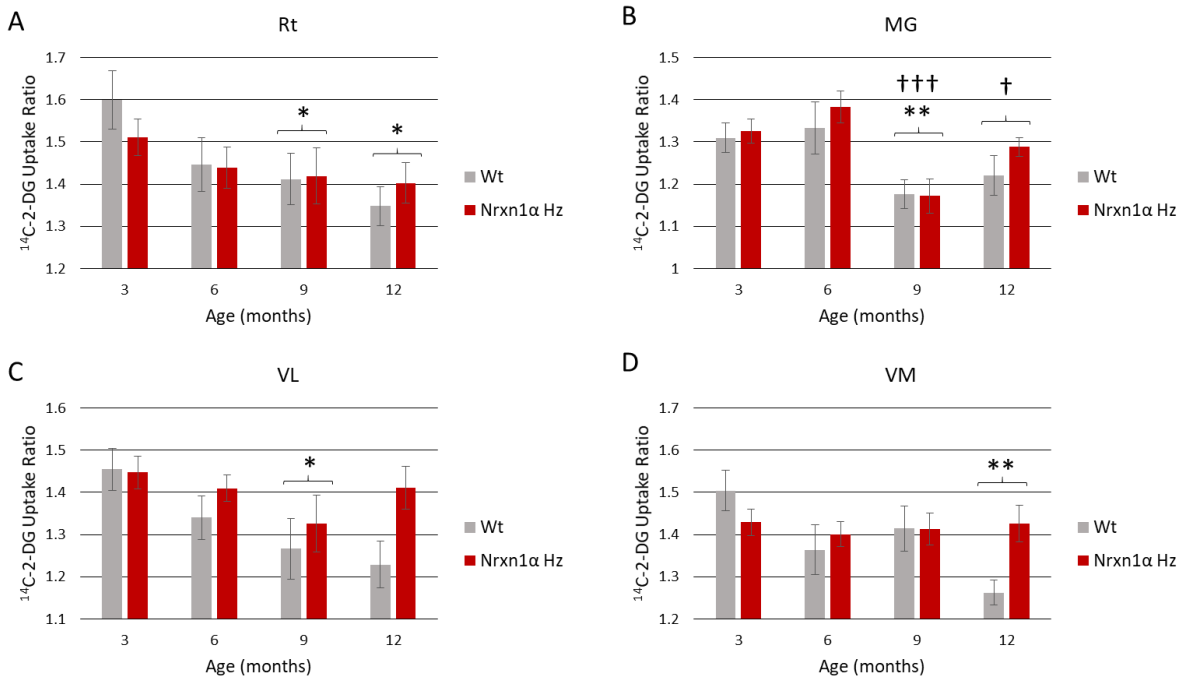

Figure 6.5 Cerebral metabolism decreases with age in a number of thalamic regions

*Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. ^{14}C-2-Deoxyglucose (^{14}C-2-DG) Uptake Ratio for **(A)** Reticular Thalamus (Rt), **(B)** Medial Geniculate Thalamus (MG), **(C)** Ventrolateral Thalamus (VL) and **(D)** Ventromedial Thalamus (VM) plotted by genotype per age group. A significant main effect of age was found for Rt [$F_{(3, 63)}$ =3.333, p=0.0250], MG [$F_{(3, 63)}$ =8.081, p=0.0001], VL [$F_{(3, 63)}$=3.753, p=0.0152] and VM [$F_{(3, 63)}$ =2.831, p=0.0454]. Sample sizes were n=10 per group except for 3 month old Wt which were n=9. * and ** denote, p<0.05 and p<0.01 respectively, significant difference as compared to 3 month group. † and ††† denote, p<0.05 and p<0.001 respectively, significant difference as compared to 6 month group (Pairwise t-test with FDR correction).*

Cerebral metabolism is reduced at 9 months old but normalises by 12 months old in Septal/Diagonal Band of Broca nuclei

In Septal/Diagonal Band of Broca regions cerebral metabolism decreased in 9 month old mice as compared to 6 month old mice, but then increased in 12 month old mice as compared to 9 month old mice, with metabolism in 12 month old mice not being significantly different to that seen at 3 and 6 months old (Figure 6.6). This pattern emerged in the Medial Septal Nucleus (MS), the Lateral Septal Nucleus (LS), the nucleus of the vertical limb of the Diagonal Band of Broca (VDB) and the nucleus of the horizontal limb of the Diagonal Band of Broca (HDB). A significant main effect of age was found for MS [$F_{(3, 63)}$ =9.930, p<0.0001], LS [$F_{(3, 63)}$ =6.0402, p=0.0007], VDB [$F_{(3, 63)}$ =4.453, p=0.0067] and HDB [$F_{(3, 63)}$ =3.994, p=0.0114]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that in the MS cerebral metabolism was reduced at 9 months old as compared to 6 months old (p=0.0004) and increased at 12 months old as compared to 9 months old (p=0.0004). Cerebral metabolism in 12 month old mice in the MS was not significantly different to that seen in 3 and 6 month old mice (Figure 6.6 A). A similar pattern was also seen in the LS (6 month vs 9 month p=0.0067, 9 month vs 12 month p=0.0067). But in addition the LS showed increased cerebral metabolism in 6 (p=0.0067) and 12 (p=0.0067) month old mice as compared to that seen in 3 month old mice. However, cerebral metabolism in the LS was not significantly different between 12 and 6 month old mice (Figure 6.6 B). In both the VDB and HDB, cerebral metabolism was also significantly reduced at 9 months old as compared to 6 months old (6 month vs 9 month: VDB p=0.0250, HDB p=0.0280) (Figure 6.6 C and D). Cerebral metabolism was also significantly increased in the 12 month old group as compared to the 9 month old group in the VDB (p=0.0250), however this failed to reach significance in the HDB. Again cerebral metabolism was not significantly different in these

regions between animals that were 12 months old as compared to animals that were 3 and 6 months old (Figure 6.6 C and D). There was no significant impact of genotype or sex on age-dependent differences in cerebral metabolism in Septal and Diagonal Band of Broca regions (no significant genotype x age or sex x age interactions).

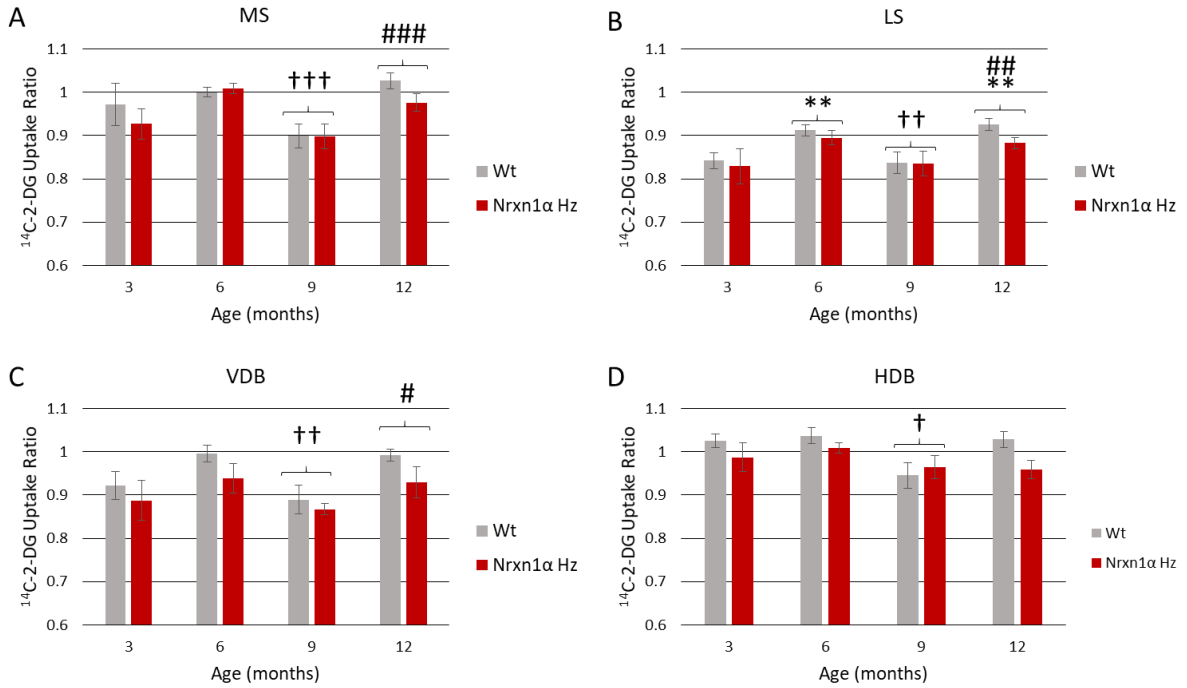

Figure 6.6 Cerebral metabolism is reduced at 9 months old but normalises by 12 months old in the Septal and Diagonal Band of Broca nuclei

*Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous. ^{14}C-2-Deoxyglucose (^{14}C-2-DG) Uptake Ratio plotted for **(A)** Medial Septal Nucleus (MS), **(B)** Lateral Septal Nucleus (LS), **(C)** nucleus of the vertical limb of the Diagonal Band (VDB) and **(D)** nucleus of the horizontal limb of the Diagonal B and (HDB) by genotype per age group. A significant main effect of age was found for MS [$F_{(3, 63)}$ =9.930, p<0.0001], LS [$F_{(3, 63)}$ =6.0402, p=0.0007], VDB [$F_{(3, 63)}$ =4.453, p=0.0067] and HDB [$F_{(3, 63)}$ =3.994, p=0.0114]. Sample sizes were n=10 per group except for 3 month old Wt which were n=9. †, †† and ††† denote, p<0.05, p<0.01 and p<0.001 respectively, significant decrease as compared to 6 month group. #, ##, ### denote, p<0.05, p<0.01 and p<0.001 respectively, significant increase as compared to 9 month group (Pairwise t-test with FDR correction).*

6.5.2 Sex impacts on age-dependent alterations in cerebral metabolism in the Infralimbic and entorhinal cortex and the striatum

Male, but not female, mice show increased cerebral metabolism in the Infralimbic and Entorhinal Cortex with age

Male mice showed significantly increased cerebral metabolism in the Infralimbic Cortex (IL) and Entorhinal Cortex (EntoC) at 12 months old compared to at 3 months old, an effect that was not seen in female mice (Figure 6.7). A significant sex x age interaction was found for the IL [$F_{(3, 62)}$ =3.497, p=0.0206] and the EntoC [$F_{(3, 61)}$ =2.912, p=0.0415]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that male mice showed significantly increased cerebral metabolism at 12 months old as compared to at 3 months old in the IL (p=0.0500) (Figure 6.7 A) and the EntoC (p=0.0320) (Figure 6.7 B). By contrast, there was no significant difference in cerebral metabolism in the IL or EntoC between the different age groups in female mice. Genotype did not significantly impact on cerebral metabolism in the IL or EntoC (no significant main effect of genotype or sex x age x genotype or sex x genotype interactions).

Male, but not female, mice show decreased cerebral metabolism in the Dorsolateral Striatum (DLST) at 6 and 9 months old as compared to 3 months old

Male mice showed significantly reduced cerebral metabolism in the DLST at 6 and 9 months old as compared to at 3 months old, an effect that was not seen in female mice (Figure 6.7 C). A significant sex x age interaction was found for the DLST [$F_{(3, 63)}$ =4.111, p<0.0100]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that 6 and 9 month old males

had significantly, or trending towards significantly, decreased cerebral metabolism in the DLST compared to 3 month old males (3 month vs 6 month p=0.0410, 3 month vs 9 month p=0.0510). However, cerebral metabolism in the DLST was similar at 3 and 12 months old. No significant difference in cerebral metabolism in the DLST were found between the different age groups in female mice.

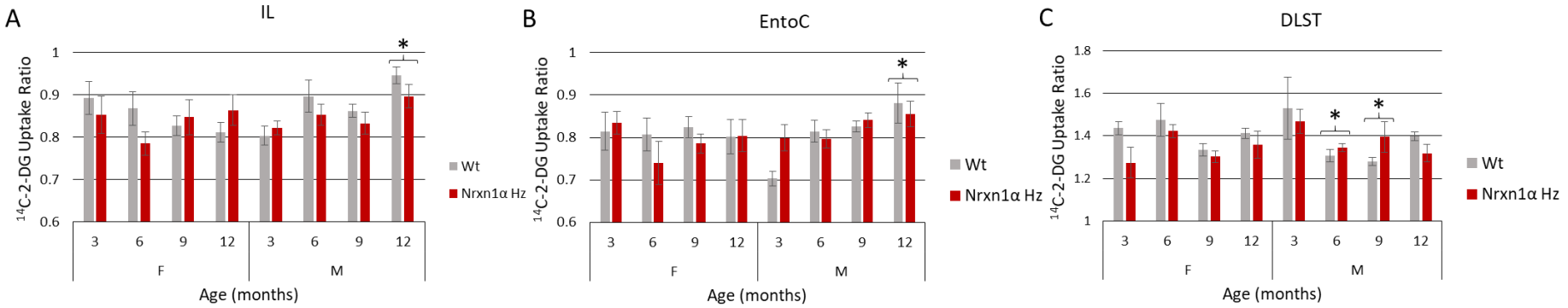

Figure 6.7 Sex impacts on age-dependent alterations in cerebral metabolism in Infralimbic cortex, entorhinal cortex and striatal regions

*Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous, F=female, M=male. ^{14}C-2-Deoxyglucose (^{14}C-2-DG) Uptake Ratio plotted for **(A)** Infralimbic cortex (IL), **(B)** Entorhinal Cortex (EntoC) and **(C)** Dorsolateral Striatum (DLST) by genotype per sex across the age groups. A significant sex x age interaction was found for IL [$F_{(3, 62)}$=3.497, p=0.0206], EntoC [$F_{(3, 61)}$ = 2.912, p=0.0415] and DLST [$F_{(3, 63)}$ =4.111, p<0.0100]. Sample sizes were n=5 per group except for 3 month old Wt males which were n=4 * denotes p<0.05 significant difference as compared to 3 month old M group (Pairwise t-test with FDR correction).*

Female mice show increased cerebral metabolism in thalamic and Raphé nuclei and decreased cerebral metabolism in the Nucleus Accumbens

Female mice showed increased cerebral metabolism in the Medial Raphé (MR) and the Anteromedial Thalamus (AM) and decreased cerebral metabolism in the Nucleus Accumbens Core (NaC) compared to male mice (Figure 6.8), evidenced by a significant main effect of sex found for each region (MR [$F_{(1, 62)} = 6.662$, $p=0.0122$], AM [$F_{(1, 63)} = 4.054$, $p=0.0483$], NaC [$F_{(1, 63)} = 10.617$, $p=0.0018$]). There was no significant evidence that genotype or age influenced the impact of sex on cerebral metabolism in the Nac, AM or MR (no significant sex x age or sex x genotype interactions).

6.5.3 Validating the effects of age and sex on cerebral metabolism

To validate the ^{14}C-2DG functional brain imaging findings, blood glucose, plasma ^{14}C-2DG concentration and the concentration ratio of plasma ^{14}C-2DG: whole brain average ^{14}C-2DG were tested for significant effects of sex and age. It was found that plasma ^{14}C-2DG levels increased significantly with age in mice and that male mice had significantly higher blood glucose levels than female mice. However there were no significant effects of age or sex on the concentration ratio of plasma ^{14}C-2DG: whole brain average ^{14}C-2DG. In this way there was a significant effect of age for plasma ^{14}C-2DG concentration [$F_{(1, 60)}$ =4.695, p=0.0052] and a significant effect of sex for blood glucose levels [$F_{(1, 61)}$ =9.222, p=0.0035] (see Appendix, section A2). *Post hoc* testing using Pairwise t-test with FDR correction revealed that 12 month old mice had significantly higher plasma ^{14}C-2DG levels than 3 and 6 month old mice (p=0.0081 and p=0.0182, respectively). These findings validate the previous age and sex differences in LCGU, since there was no significant effect of age or sex on concentration ratio of plasma ^{14}C-2DG: whole brain average ^{14}C-2DG and blood glucose levels of both sexes were in the normal physiological range.

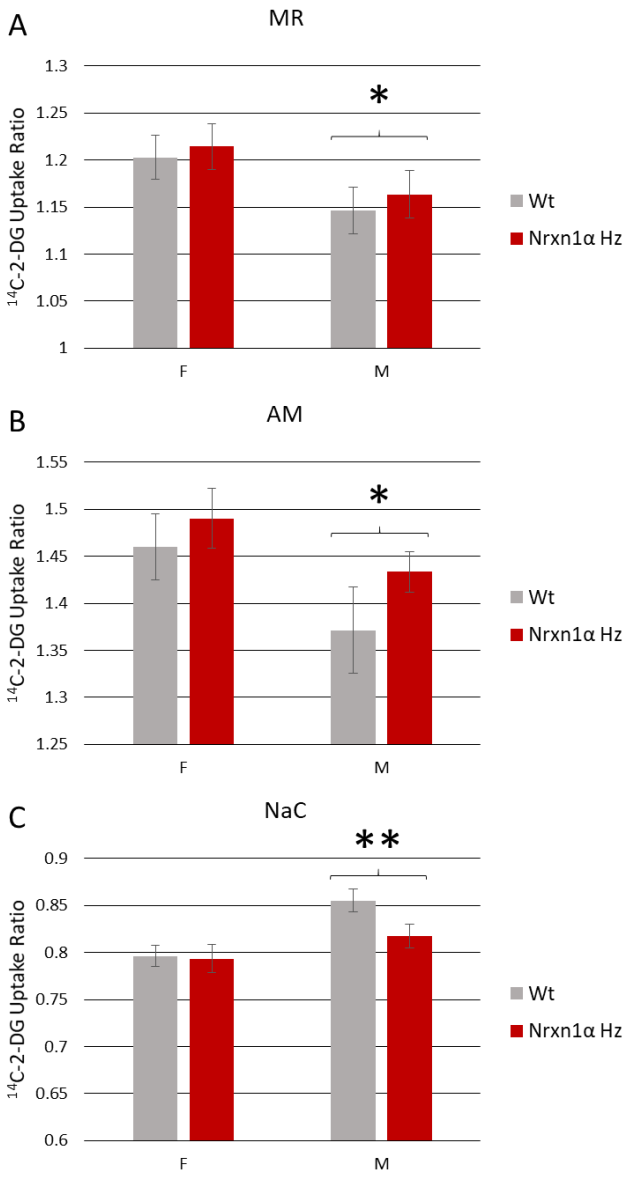

Figure 6.8 Female mice show increased cerebral metabolism in thalamic and Raphé nuclei and decreased cerebral metabolism in the Nucleus Accumbens compared to males

*Data shown are Mean ± SEM. Wt=Wild-type, Nrxn1α Hz=Nrxn1α Heterozygous, F=female, M=male. ^{14}C-2-Deoxyglucose (^{14}C-2-DG) Uptake Ratio plotted for **(A)** Medial Raphe (MR), **(B)** Anteromedial Thalamus (AM) and **(C)** Nucleus Accumbens Core (NaC) by genotype for each sex, with data for all age groups combined. A significant main effect of sex was found for MR [$F_{(1, 62)} = 6.662$, $p=0.0122$], AM [$F_{(1, 63)} = 4.054$, $p=0.0483$] and NaC [$F_{(1, 63)} = 10.617$, $p=0.0018$]. Sample sizes were Wt: F n=20 and M n=19, Nrxn1α Hz: F n=20 and M n=20. * and ** denote, $p<0.05$ and $p<0.01$ respectively, significant difference as compared to female mice (main effect, ANOVA).*

6.6 Discussion

Nrxn1α Hz mice show altered cerebral metabolism

There were 3 main findings of altered cerebral metabolism in the *Nrxn1α* Hz mice: (1) *Nrxn1α* Hz mice show hypofrontality, (2) *Nrxn1α* Hz mice show increased cerebral metabolism in the mesolimbic system, thalamus and retrosplenial cingulate cortex and (3) *Nrxn1α* Hz mice show increased cerebral metabolism in the Dorsal Raphe (DR) nucleus at 3 months old but not at any other age tested (Table 6.1).

Nrxn1α Hz mice show hypofrontality

Decreased cerebral metabolism in the prefrontal cortex (PFC), also known as hypofrontality, was found in both the mPrl and aPrl in the *Nrxn1α* Hz mice (Figure 6.1 A, B and C, See Figure 6.2 A and B for representative autoradiograms). Hypofrontality was not impacted on by sex or age of mice and thus appears to be a prominent effect in both male and female mice across the 4 age groups tested.

Hypofrontality is well established in Sz and has been shown in both resting-state (Ingvar and Franzen, 1974; Buchsbaum *et al.*, 1982; Farkas *et al.*, 1984; Wolkin *et al.*, 1985; Weinberger *et al.*, 1986) and task-based (Andreasen *et al.*, 1992; Steinberg *et al.*, 1996; Hazlett *et al.*, 2000; Hazlett and Buchsbaum, 2001; Mitelman *et al.*, 2018) functional brain imaging studies. Hypofrontality and has also been shown in ASD in both resting-state (Ohnishi *et al.*, 2000) and task-based (Luna *et al.*, 2002; Silk *et al.*, 2006; Kana *et al.*, 2007; Mitelman *et al.*, 2018) functional brain imaging studies. The ^{14}C-2-DG functional brain imaging undertaken in mice in the current study is arguably more closely related to resting functional brain imaging in patients. The ^{14}C-2-DG signal represents regional neuronal activity over a prolonged

period of time (45 minutes), during which mice are not performing a cognitive task. Under rest conditions Sz patients showed decreased cerebral metabolism in the areas of the PFC including the Dorsolateral Prefrontal Cortex (DLPFC) (Weinberger *et al.*, 1986). Since the Prl cortex in rodents is functionally and anatomically homologous to the DLPFC (Brodman's areas 9 and 46) in humans, in terms of its projections from the mediodorsal nucleus of the thalamus and its functional role in cognitive flexibility (Brown and Bowman, 2002), this suggests that the decreased cerebral metabolism found in the Prelimbic (mPrl and aPrl) cortex of *Nrxn1α* Hz mice has translational relevance to the DLPFC dysfunction seen in Sz and ASD. Under task conditions the DLPFC (Brodman's area 46) has also been shown to have decreased cerebral metabolism in both Sz and ASD patients in comparison to healthy controls when analysed using ^{18}F-Fludeoxglucose PET imaging, a technique similar to the ^{14}C-2-DG imaging approach used in our study (Mitelman *et al.*, 2018). Further studies in ASD patients have found the DLPFC to have reduced activation during task related fMRI (Luna *et al.*, 2002; Silk *et al.*, 2006). In addition to task based studies, resting state SPECT imaging revealed that ASD patients showed reduced regional cerebral blood flow in the DLPFC (Brodman's area 9) compared to control subjects (Ohnishi *et al.*, 2000), further supporting reduced activity in this PFC subfield. However, it should be noted that reduced cerebral metabolism in the DLPFC is not always found in Sz (Andreasen *et al.*, 1992; Schneider *et al.*, 2007) and ASD (Zilbovicius *et al.*, 2000) patients.

There have been no previous studies investigating cerebral metabolism in *Nrxn1α* Hz or KO mice, however hypofrontality has also been found in other rodent models of Sz and ASD. For instance the sub-chronic PCP rat model, modelling NMDA hypofunction as an aetiological mechanism in Sz (Olney *et al.*, 1999), showed reduced cerebral metabolism in the aPrl, Dorsolateral Orbital cortex (DLO) and Lateral Orbital Cortex (LO) using ^{14}C-2-DG functional

brain imaging (Dawson *et al.*, 2012), which somewhat paralells the hypofrontality observed in the *Nrxn1α* Hz mice. Interestingly this model also had a reversal learning deficit caused by increased regressive errors during a set-shifting task (Dawson *et al.*, 2012), as seen in the *Nrxn1α* Hz mice. The shared nature of the cognitive deficit and the PFC hypometabolism seen in these models supports their translational relevance to the functional deficits seen in Sz. Another study characterising heterozygous truncated *DISC1* mice, modelling a genetic risk factor for Sz (Song *et al.*, 2008), also showed hypofrontality in the Orbital cortex using ^{14}C-2-DG functional brain imaging (Dawson *et al.*, 2015). The MAM rat model, which models a number behavioural and neurophysiological of aspects of Sz (Moore *et al.*, 2006), has also been shown to have hypofrontality in the orbital cortex using fMRI (Kaneko *et al.*, 2017). In terms of ASD rodent models, the BTBR T^{+}Itpr3^{tf}/J (BTBR) mouse, originally bred for diabetes related research but found to display a number of ASD-like behaviours (Bolivar *et al.*, 2007; Moy *et al.*, 2007), was also shown to have hypofrontality in an fMRI study (Dodero *et al.*, 2013). In addition heterozygous 16p11.2 deletion mice, modelling a genetic risk factor for ASD (Weiss *et al.*, 2008), were found to have reduced functional connectivity in the prefrontal cortex (Bertero *et al.*, 2018). By contrast, the *Fmr1* KO mouse model of Fragile X syndrome, a syndrome that includes ASD (Geschwind and State, 2015), was shown to have increased cerebral metabolism in the PFC using ^{14}C-2-Deoxyglucose functional brain imaging, although increases in cerebral metabolism were found in all brain regions measured in these mice (Qin *et al.*, 2002). Overall, these studies suggest that PFC hypofunction is a key mechanism of brain dysfunction in multiple rodent models relevant to Sz and ASD, and this aligns with hypofrontality identified in *Nrxn1α* Hz mice in the current study.

The cerebral metabolism findings in the prefrontal cortex of the *Nrxn1α* Hz mice partially support findings from the CFT in the current study. Lesions to the Orbital Frontal Cortex (OFC) have been shown to cause a deficit in reversal learning in rats (Mcalonan and Brown, 2003; Chase *et al.*, 2012) and mice (Bissonette *et al.*, 2008) in set shifting tasks similar to the CFT used in the current study. Studies which found that OFC lesions cause reversal learning deficits have found this to be accompanied by increased perseverative errors in a 2-choice odour discrimination digging task (Kim and Ragozzino, 2005), a touch screen based task (Chudasama and Robbins, 2003) and a maze based task (Ghods-Sharifi *et al.*, 2008) in rats. No alterations in cerebral metabolism were found in the OFC of the *Nrxn1α* Hz mice in the current study (data not shown) which contrasts with the reversal learning deficit seen in these animals in the CFT. However, the reversal learning deficit seen in the *Nrxn1α* Hz mice was due to increased regressive rather than perseverative errors (Figure 5.4), which aligns with the unaffected cerebral metabolism in the OFC of *Nrxn1α* Hz mice. Rats with lesions to the OFC are thought to have intact attentional set shifting (Extra-dimensional shift (EDS)) due to having a reversal learning deficit which results in the lack of formation of an attentional set, and thus performance on the EDS phase is found to better than the reversal phase (OD2R) (Chase *et al.*, 2012). This theory could be applied to the *Nrxn1α* Hz mice since they show intact ability on the SS phase and selectively impaired reversal learning.

The Prl in mice has been associated with working memory since lesions to this area cause deficits in working memory tasks in rats (Granon *et al.*, 1994; Kesner *et al.*, 1996; Taylor *et al.*, 2003). Reduced cerebral metabolism in the Prl of *Nrxn1α* Hz mice may induce a working memory deficit in these animals, which in turn could be responsible for increased regressive errors seen in these animals during reversal learning. Lesions in the medial prefrontal cortex (Prl) have also been associated with specific impairment of shifting attentional set (EDS) in

rats (Birrell and Brown, 2000) and mice (Bissonette *et al.*, 2008). In contrast to these findings, while *Nrxn1α* Hz mice showed decreased cerebral metabolism in the Prl they were unimpaired in the SS phase of the CFT, where the mice must switch their attentional set from the stimulus of odour to that of location (right or left side). One argument could be that the SS phase in the current study was less challenging than the EDS phase in previous set-shifting studies, since both Wt and *Nrxn1α* Hz mice performed better on the SS phase than the OD2R phase in terms of PC (Section 5.7.5). However both rat (Chase *et al.*, 2012) and mouse (Bissonette *et al.*, 2008) set-shifting studies have shown that the completion of the reversal and EDS phases can be similar in terms TTC and PC in control animals. Taken together these findings may suggest while lesions to the Prl cause a deficit in attentional set shifting (EDS) in rats and mice, decreased cerebral metabolism in the Prl in the *Nrxn1α* Hz mice is not sufficient to cause this effect. Alternatively, this could be due to methodological differences between the set-shifting behavioural paradigms applied in other studies and the test applied here. For example, the EDS phase in previous studies and the SS phase in the current study are not identical. Previous set-shifting studies have employed multiple associative and reversal stages in testing, that would likely strengthen the formation of an attentional set, while the current study only employed one reversal learning phase. In addition the stimulus dimensions used in previous studies were floor texture or digging medium vs odour, as compared to odour vs location in the current study, which could also modify the outcome of the results. Therefore, it is possible that the altered medial PFC (Prl) function seen in *Nrxn1α* Hz would be sufficient to cause a deficit in the EDS phase during set-shifting using these other testing parameters, however does not cause a deficit in the SS phase of the CFT employed here.

Nrxn1α Hz mice show increased cerebral metabolism in mesolimbic system, Retrosplenial Cingulate Cortex and thalamus

Nrxn1α Hz mice showed increased cerebral metabolism in the VTA, RSC and the VL (Figure 6.1 A, D, E and F, See Figure 6.2 C and D for representative autoradiograms). Hypermetabolism in these areas was not impacted on by the sex or age of mice. The brain regions showing increased cerebral metabolism in the *Nrxn1α* Hz mice have been implicated as being dysfunctional in both Sz and ASD patients. For instance Sz patients show decreased resting state functional connectivity of the VTA, including reduced connectivity to the dorsal Anterior Cingulate Cortex (ACC) and the Thalamus (Hadley *et al.*, 2014). Sz patients also show reduced activation of the VTA during a probabilistic reasoning task (Rausch *et al.*, 2014). In ASD patients, reduced functional connectivity between Nucleus Accumbens and VTA has also been reported during a perception of social vs non-social stimuli task (Supekar *et al.*, 2018). These studies provide support for the potential translational relevance of altered cerebral metabolism found in the VTA of *Nrxn1α* Hz mice.

The RSC is part of the cingulate cortex, which also includes the Anterior and Posterior Cingulate Cortex (PCC). Both the RSC and ACC are brain areas that are functionally altered in Sz and ASD. In Sz patients, reduced activation in the ACC has been reported during emotional picture rating (Nelson *et al.*, 2015) and semantic (Tendolkar *et al.*, 2004) tasks, and resting state functional connectivity of the RSC has been shown to be reduced in pateints with Sz (Bluhm *et al.*, 2009). One study found that while the RSC had reduced connectivity to the prefrontal cortex, connectivity between the RSC and the lingual gyrus (medial occipitotemporal gyrus) was increased in Sz patients (Wang *et al.*, 2015), suggesting that alterations in RSC connectivity are region-dependent in Sz. RSC dysfunction and altered connectivity is also supported in ASD. Mitelman *et al.* found that cerebral metabolism in RSC

(Brodman's area 29 and 20) was increased in ASD patients which parallels the finding in *Nrxn1α* Hz mice, but they found no difference in RSC metabolism in Sz patients compared to healthy controls in task related PET imaging (Mitelman *et al.*, 2018). By contrast, another study found that ASD patients showed reduced activation in Cingulate/RSC (Brodman's area 29) during task related fMRI (Luna *et al.*, 2002). Similarly there are mixed findings regarding the resting state functional connectivity of the RSC in ASD. While one study found this to be reduced RSC connectivity (Starck *et al.*, 2013), another found overconnectivity between anterior insula and RSC, and between DLPFC and RSC (Hogeveen *et al.*, 2018). The studies showing altered function of the RSC and ACC in Sz and ASD patients support the potential translational relevance of altered RSC cerebral metabolism in *Nrxn1α* Hz mice.

The VL thalamic nucleus receives input from basal ganglia and cerebellum (Kuramoto *et al.*, 2011). While there is little literature on the VL specifically in Sz and ASD, the thalamus as a whole has been studied. In Sz patients, reduced activation in the thalamus has been found during working memory task (Andrews *et al.*, 2006) and increased thalamo-cortical resting state connectivity has been found for prefrontal, motor and sensory cortex connections (Klingner *et al.*, 2014). In ASD, cerebral metabolism has been shown to be lower in the thalamus during a verbal learning PET task (Haznedar *et al.*, 2006). Resting state functional connectivity studies have shown mixed findings of both increased (Cerliani *et al.*, 2015) and decreased (Chen, H. *et al.*, 2016) thalamo-cortical connectivity in ASD. Therefore altered cerebral metabolism in the VL thalamus in *Nrxn1α* Hz mice may have translational relevance to thalamic dysfunction seen in both Sz and ASD.

Other rodent models relevant to Sz and ASD have also shown altered cerebral metabolism in some of the same areas identified as being dysfunctional in *Nrxn1α* Hz mice in the current

study. These include the *Fmr1* KO ASD mouse model, modelling Fragile X syndrome which is a known cause of ASD, which was found to have increased cerebral metabolism in cingulate cortex and ventral thalamus (Qin *et al.*, 2002). Also the heterozygous truncated *DISC1* mouse model, relevant to both Sz and ASD, was found to have increased cerebral metabolism in the VTA (Dawson *et al.*, 2015), while the sub-chronic PCP rat model of Sz shows reduced cerebral metabolism in the RSC (Dawson *et al.*, 2012). This suggests that altered RSC, VL and VTA function are key alterations induced by aetiologically relevant risk factors for both Sz and ASD.

The VTA is part of the mesolimbic dopaminergic pathway in the brain. While the nigrostriatal dopaminergic pathway is most strongly associated with movement, dysfunction of the dopaminergic mesolimbic system can also cause alterations in movement. Therefore increased cerebral metabolism in the VTA in *Nrxn1α* Hz mice could be linked to the hyperlocomotor phenotype observed in the OF test (Figure 3.1). In support of this notion, selective activation of the excitatory neurons expressing CMLKIIα in the VTA of rats results in increased locomotor activity (Guo *et al.*, 2014). The VL receives input from basal ganglia and cerebellum (Kuramoto *et al.*, 2011) and projects to motor cortex in mice (Hooks *et al.*, 2013; Yamawaki and Shepherd, 2015), and so increased cerebral metabolism in this thalamic nucleus may also contribute to the increased locomotor activity in the *Nrxn1α* Hz mice. Although the VTA mainly contains dopaminergic neurons there is evidence for glutamatergic co-transmission in neurons which project from the VTA to the ACC (Mingote *et al.*, 2015), which may explain the increased cerebral metabolism seen in both the VTA and RSC, since the latter is part of the cingulate cortex.

The RSC, which is part of the cingulate cortex, was found to have increased cerebral metabolism in the *Nrxn1α* Hz mice. Lesions in to the ACC in a 4-choice odour based digging task caused impaired reversal learning that was not due to perseverative errors, and intact initial odour discrimination in mice (Ragozzino and Rozman, 2007). This has some parallels to observations in *Nrxn1α* Hz mice in the current study. *Nrxn1α* Hz mice showed altered (increased) cerebral metabolism in the RSC and showed intact associative learning, but impaired reversal learning with no change in perseverative errors. Thus, altered cerebral metabolism in the RSC may contribute to impaired reversal learning in the *Nrxn1α* Hz mice.

Nrxn1α Hz mice show increased cerebral metabolism in the Dorsal Raphé (DR) nucleus at 3 months old but not at any other age

Cerebral metabolism was significantly increased in the DR in *Nrxn1α* Hz mice at 3 months old but not at any other age tested. Wt mice showed developmental changes in cerebral metabolism in the DR since cerebral metabolism significantly increased at 6 months old as compared to 3 months old, then showed non-significant alterations at 9 and 12 months old. *Nrxn1α* Hz mice do not show this trajectory, as cerebral metabolism is at a similar level across all age groups (Figure 6.3). This suggests that the normal developmental alterations in DR function, seen in Wt mice, are disrupted in *Nrxn1α* Hz mice. Whether DR function shows an earlier developmental trajectory than that seen in Wt animals, or whether the DR lacks any developmental change at 3 months in comparison to earlier time points remains to be determined.

The DR contains a large group of serotonergic neurons that have widespread connections throughout the brain that play a key neuromodulatory function in the regulation of CNS activity (Andrade and Haj-Dahmane, 2013; Olivier, 2015). There is little evidence for altered

cerebral metabolism in the DR in Sz and ASD patients, although the DR is difficult to image using human imaging methods as it is a small structure. There is however evidence for altered serotonergic transmission in both Sz (Selvaraj *et al.*, 2014) and ASD (Muller *et al.*, 2016). In support of the findings in the current study, male and female *Fmr1* KO mice showed increased cerebral metabolism in the DR using ^{14}C-2-DG functional brain imaging (Qin *et al.*, 2002; 2005). Interestingly the *Fmr1* KO mice used in the study were 4-5 months old. The authors did not measure DR metabolism at later ages (beyond 6 months) and so whether this difference resolves at later ages in *Fmr1* KO mice, as seen in *Nrxn1α* Hz mice, has not been determined. Overall, these data suggest that the function of the serotonin system, and DR, is impacted by risk genes for ASD and that these effects may be developmentally regulated.

The DR is structurally and functionally connected to the VTA (Gervais and Rouillard, 2000; Taylor, S. R. *et al.*, 2014) which supports increased cerebral metabolism in both these brain areas in the *Nrxn1α* Hz mice. Compensatory mechanisms working against *Nrxn1α* heterozygosity may prevent increased cerebral metabolism in the DR from 6 months old onwards explaining why increased cerebral metabolism is only seen in 3 month old mice.

Summary 1

The *Nrxn1α* Hz mice show decreased cerebral metabolism in the prefrontal cortex (mPrl and aPrl) and increased cerebral metabolism in mesolimbic (VTA), Cingulate (RSC) and thalamic (VL) brain regions. *Nrxn1α* Hz mice also showed increased cerebral metabolism in the DR at 3 months old but not at any other age. In support of the translational relevance of the findings in *Nrxn1α* Hz mice, there is evidence for hypofrontality in both Sz and ASD patients and some animal models relevant to these disorders. The functional brain imaging findings

in the *Nrxn1α* Hz mice show some alignment with altered cognition found in the CFT. For instance, lesions in the OFC have been associated with reversal learning deficits due to perseveration. *Nrxn1α* Hz mice do not show altered cerebral metabolism in the OFC or perseveration which supports previous findings, however they do show impaired reversal learning. Furthermore, lesions in the medial PFC in rodents (Prl) have been shown to cause deficits in set shifting (EDS), by contrast *Nrxn1α* Hz mice do not show impaired ability to shift their attention from the stimulus dimension of odour to location during the SS phase of the CFT. This could be due to the severity of altered Prl function (lesion vs hypometabolism) or differences in the EDS phase in previous studies and the SS phase in the current study. The Prl has also been implicated in working memory in rodents and so reduced cerebral metabolism in this area may contribute to the reversal learning deficit in *Nrxn1α* Hz mice, due to poor working memory which may induce the increased regressive errors seen in these animals.

The VTA and RSC were shown to have increased cerebral metabolism in *Nrxn1α* Hz mice, and have been found to have altered function in Sz and ASD and in animal models relevant to these disorders. Lesions to the cingulate cortex in rodents have also been shown to cause reversal learning deficits without perseveration, which supports the potential role of cingulate cortex (RSC) dysfunction in the reversal learning deficit identified in *Nrxn1α* Hz mice in this study. Furthermore, increased cerebral metabolism in the VTA may be linked with hyperlocomotor activity in *Nrxn1α* Hz mice since activity in the mesolimbic dopaminergic system has been associated with movement. The VL and DR also showed increased cerebral metabolism in *Nrxn1α* Hz mice. While functional activity of these brain areas has not been directly implicated in Sz and ASD patients, there is evidence for both altered thalamic connectivity and serotonin system function in these disorders.

Furthermore, other animal models relevant to Sz and ASD also show altered cerebral metabolism in the VL and DR. In conclusion, the *Nrxn1α* Hz mice show altered cerebral metabolism in brain areas that are: (1) linked to Sz and ASD, (2) known to be involved in cognitive functions found to be disrupted in these mice and (3) dysfunctional in other rodent models relevant to Sz and ASD.

Regional cerebral metabolism shows three main patterns of age-dependent variation

There were three main patterns of age-dependent variation in cerebral metabolism (Figure 6.4-6.6). These were: (1) increased cerebral metabolism with age in a number of cortical regions, (2) decreased cerebral metabolism with age in a number of thalamic regions and (3) reduced cerebral metabolism selectively at 9 months old in Septal/Diagonal Band of Broca regions.

Cerebral metabolism increased with age in a number of cortical regions (Figure 6.4). In the frontal (FRA and DLO), Piri and Ins cortex cerebral metabolism was similar in mice at 3 and 6 month old and was increased significantly in 9 and 12 month old mice, when compared to the 3 month old mice. In humans, evidence suggests that there is increased activation in the PFC and Ins with age, using cross-sectional task-based fMRI (Kennedy *et al.*, 2015; Li, H. J. *et al.*, 2015), mirroring our findings in these cortical regions (FRA, DLO and Ins) in mice. By contrast resting state ^{18}F-DG PET imaging studies in humans, which are more methodologically related to ^{14}C-2-DG imaging in mice in the current study than task based fMRI, found the PFC and Ins showed decreased cerebral metabolism with age (Berti *et al.*, 2014). The exact reason for the differences between these studies is not known. However, both the human functional brain imaging studies previously mentioned include subjects that

are elderly, whereas the oldest group of mice in the current study were middle-aged and therefore direct comparisons should be made with caution. However, overall the available data from human brain imaging studies and the study in mice conducted here suggests that neuronal function in cortical regions is affected by age, and that we can detect translationally relevant age-dependent alterations in cerebral metabolism in mice using ^{14}C-2-DG functional brain imaging.

The OFC, of which the DLO is part, is involved in reversal learning during set-shifting tasks in rodents (Mcalonan and Brown, 2003; Bissonette *et al.*, 2008; Chase *et al.*, 2012). The DLO showed increased cerebral metabolism with age and reached its highest level at 9 months old (Figure 6.4 B). Interestingly, during reversal learning in the CFT (OD2R phase) Wt mice show the poorest performance at 9 months old in terms of TTC (Figure 5.3 B), which reaches significance when compared to 12 month old Wt mice, but not when compared to 3 and 6 month old Wt mice. It is possible that increased cerebral metabolism in the DLO at 9 months old is associated with poor performance during reversal, however further study would be required to confirm this.

Cerebral metabolism was found to be decreased with age in a number of thalamic regions (Figure 6.5). This was evident in the Rt, MG, VL and VM nuclei of the thalamus, with cerebral metabolism being significantly decreased in 9 and 12 month old mice as compared to 3 month old mice. The thalamic nuclei act as functional and anatomical hubs that allow bidirectional relay of information between cortical and subcortical areas. While there is little evidenced for altered cerebral metabolism in the thalamus as age increases in humans using resting state ^{18}F-FDG PET imaging (Berti *et al.*, 2014), there is evidence for an age-related decrease in thalamus volume using structural MRI scanning (Hughes *et al.*, 2012) and for

altered thalamic low frequency oscillations using resting state fMRI (Mather and Nga, 2013). Thus there is evidence for age-dependent alterations in thalamus structure and function in humans that may relate to the age-dependent alterations in thalamus metabolism identified here in mice. The decrease in cerebral metabolism observed in the mice at 9 and 12 months old compared to younger ages, an effect that isn't seen with increased age in humans, may be due to mice being middle-aged in the current study. Another limitation in comparing these mouse and human studies is the poor anatomical resolution of human brain imaging studies, that are not currently able to selectively measure the discrete thalamic nuclei (Rt, VL, VM, MG) identified as showing age-dependent alterations in function in mice.

The current study also found that cerebral metabolism was selectively decreased in mice at 9 months old as compared to 6 months old in Septal (MS and LS) and Diagonal Band (VDB and HDB) of Broca nuclei (Figure 6.6), from here on collectively referred to as the septum/DB. The septum/DB nuclei lie in close anatomical proximity and project to a number of brain regions including the hippocampus, entorhinal cortex, RSC, hypothalamus, thalamic nuclei and the midbrain nuclei (including the DR and VTA) (Swanson and Cowan, 1979; Unal *et al.*, 2015). The septum/DB has been associated with pleasure (Olds and Milner, 1954; Ikemoto, 2010) and regulation of the generation of theta oscillations in the hippocampus and entorhinal cortex (Rawlins *et al.*, 1979; Jeffery *et al.*, 1995). While the mechanistic basis and relevance of the age-dependent alteration in septum/DB function identified in this study is currently unknown, age-dependent alterations in septum/DB function are supported by some data from human studies. A human post-mortem study found evidence for increased metabolic activity in the VDB in patients over 70 years old as compared those under 70, as reflected by neuronal Golgi apparatus size (Ishunina and Swaab, 2003). This provides some support for functional alterations in the septum/DB with age, however there

are few human or animal studies which report on function of the Septal/DB regions in middle-age or old age and so cannot directly support the pattern of age-dependent alterations in cerebral metabolism seen in this study. Further studies are required to elucidate the basis and relevance of the age-dependent alterations in septum/DB function identified here.

Sex influences the impact of age on cerebral metabolism in a number of brain regions

Male, but not female, mice showed increased cerebral metabolism in the IL, a subfield of the PFC, and EntoC at 12 months old as compared to 3 months old, and decreased cerebral metabolism in the Striatum (DLST) at 6 and 9 months old as compared to 3 months old (Figure 6.7).

A study in humans found that the intrinsic functional connectivity of the striatum altered between childhood and middle-age. It was found that while connectivity between the PCC and the dorsal Striatum decreased with age, connectivity between the anterior Ins and dorsal ACC areas and the ventral striatum increased with age (Porter *et al.*, 2015). While this study supports functional age-related changes in the cortical and striatal networks, as seen in the mice in the current study, there was no evidence in the study that sex modified age-related effects on function in humans. An fMRI study in humans found that cognitively normal elderly adults (average age 76 years old) were less able to deactivate their EntoC than young adults (average age 21 years old) during an episodic memory task (Huijbers *et al.*, 2014). This provides some support for altered EntoC function as a result of age, and may relate to the increased resting state cerebral metabolism in the EntoC found in mice in the current study. However in the current study this effect was only found in middle-aged male,

and not female, mice using 14C-2-DG imaging which measures brain activity under resting conditions. The study in humans reported increased activation in the EntoC during task-activated conditions, and while the sex of subjects was controlled for in the analysis it was not reported that sex influenced the impact of age on EntoC function (Huijbers *et al.*, 2014). Overall, there is a lack of data relating to the potential influence of sex on age-dependent changes in brain activity in humans.

Female mice showed increased cerebral metabolism as compared to male mice in the AM, MR and NaC (Figure 6.8). In a human resting-state ^{18}F-FDG PET imaging study, females had higher cerebral metabolism than males in the thalamus (Murphy *et al.*, 1996) which supports the sex difference in cerebral metabolism in the AM thalamic nucleus in the current study. A study in rats also found that the spine synapse density and percentage of spines with a large heads in the NaC was higher in females than males (Wissman *et al.*, 2012), which may relate to the higher glucose utilisation seen in the NaC of females in comparison to males, as the ^{14}C-2-DG signal is thought to largely reflect the activity of synapses in the defined brain regions.

Summary 2

There were three patterns of age-dependent variation in cerebral metabolism seen in this study. Cerebral metabolism increased in cortical areas and decreased in thalamic regions with age. Also cerebral metabolism was reduced at 9 months old in the septum/DB and normalised by 12 months old to levels seen in 3 and 6 month old animals. There were also sex-dependent effects of age on PFC, entorhinal and striatal brain regions, and sex effects in raphé, thalamus and nucleus accumbens. Human functional brain imaging studies provide some support for altered cerebral metabolism due to age and sex observed in the current

study, however comparisons between human studies and the current study should be made with caution due to differences in age of subjects, the imaging techniques utilised and imaging conditions employed (resting vs task).

Future studies

The findings from the ^{14}C-2-DG functional imaging in the *Nrxn1α* Hz mice highlighted Sz and ASD relevant brain endophenotypes at the brain region level. Further analysis at the network-level in these mice may reveal relevant brain endophenotypes in terms of resting state functional connectivity, this could be carried out on the current regional data by applying graph theory, as previously outlined (Dawson *et al.*, 2012; Dawson *et al.*, 2015). To investigate how resting state functional brain imaging relates to behaviour in *Nrxn1α* Hz mice correlational analysis could be carried out between performance levels in reversal learning in the CFT and regional cerebral metabolism. To understand the mechanism underlying hypofrontality in *Nrxn1α* Hz mice, ligand binding studies could be carried out in the PFC to test whether levels of different neurotransmitter receptors, for instance NMDA receptors to investigate glutamate function, differ compared to Wt mice. Furthermore, analysis of different neurotransmitter levels in the brain could be carried out using HPLC, to test if they differ in *Nrxn1α* Hz mice. An initial candidate would be serotonin, since there was altered cerebral metabolism in the DR which contains a large number of serotonergic neurons.

Network analysis could be carried out to investigate age-dependent effects on functional connectivity using regional data from the current study. Also, to further investigate developmental and ageing effects on brain function ^{14}C-2-DG functional imaging could be completed in both younger and older mice than used in the current study. Assessing mice at

older ages would be of particular interest given our finding that *Nrx-1* modulates ageing in the fruit fly (Chapter 8). This would add information to current findings in middle-aged mice, and allow better comparison with humans studies of functional brain changes during ageing, since few functional brain imaging studies exist which investigate changes in young and middle-aged human subjects.

Chapter 7 Characterising altered *Nrx-1* expression in the P{XP}Nrx^{d08766} fly

7.1 Introduction

P{XP}Nrx^{d08766} flies were obtained from Bloomington *Drosophila* stock centre (Flybase ID FBti0042917) since they had previously been shown to have reduced levels of *Nrx-1* protein by western blot (Zeng *et al.*, 2007; Tong *et al.*, 2016). This made them a suitable candidate to model *Nrx-1* hypofunction in relation to heterozygous deletions in *NRXN1α* which increase the risk of developing Sz and ASD. However, when *Nrx-1* expression and protein level in the P{XP}Nrx^{d08766} flies was validated in-house by qPCR and western blot following backcrossing onto a w^{1118} control fly strain, these flies were found to be overexpressing *Nrx-1*. Although the findings in the current study contrasted with those in the literature, and meant the P{XP}Nrx^{d08766} flies no longer provided a suitable model for heterozygous deletions in *NRXN1α* found in humans, the flies were used to characterise the effects of *Nrx-1* hyperfunction on life span, behavioural senescence (Chapter 8) and sleep (Chapter 9).

7.2 Results

P{XP}Nrx^{d08766} flies overexpress *Nrx-1*

P{XP}Nrx^{d08766} flies were found to have significantly increased expression of *Nrx-1* using qPCR (Figure 7.1). A significant effect of genotype was found for fold change of *Nrx-1* expression [$F_{(1, 14)}$ =7.140, p=0.0182], revealing significantly increased expression of *Nrx-1* in both male and female P{XP}Nrx^{d08766} flies as compared to control w^{1118} flies. A significant effect of sex was also found for fold change of *Nrx-1* expression [$F_{(1, 14)}$ =28.844, p<0.0001], revealing significantly higher expression of *Nrx-1* in male compared to female flies of both

genotypes. Sex did not significantly modify the effect of genotype on *Nrx-1* expression (no significant sex x genotype interaction).

P{XP}Nrx^{d08766} flies were also found to have significantly increased *Nrx-1* protein level by western blot (Figure 7.2). A significant effect of genotype was found for percentage change in *Nrx-1* protein level [$F_{(1, 15)}$ =7.678, p=0.0143], revealing a significantly increased level of *Nrx-1* protein in both male and female P{XP}Nrx^{d08766} flies as compared to control w^{1118} flies. A significant effect of sex was also found for *Nrx-1* protein level [$F_{(1, 15)}$ =28.551, p<0.0001], revealing a significantly higher level of *Nrx-1* protein in female compared to male flies of both genotypes. Sex did not significantly modify the effect of genotype on *Nrx-1* protein level (no significant sex x genotype interaction).

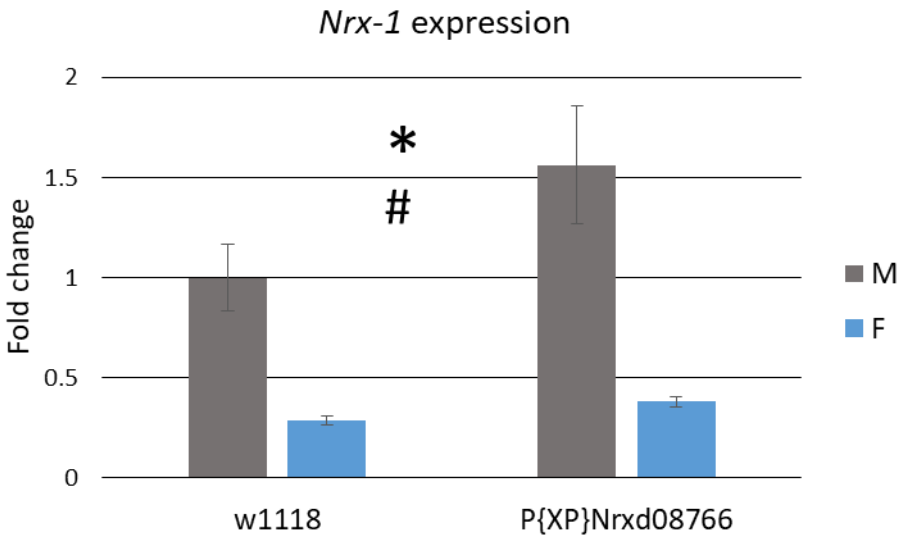

Figure 7.1 P{XP}Nrx^{d08766} flies show increased Nrx-1 expression in both sexes
*Data are mean ± SEM except the male w^{1118} group which are plotted at 1x fold change with group SEM. W1118=w^{1118} control fly, P{XP}Nrx d08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly, M=male, F=female. Nrx-1 expression was measured in fly heads by qPCR and data from each sample was normalised to a single male w^{1118} sample, thus the male w^{1118} group are plotted at 1x fold change in the figure. Sample sizes were M w^{1118} n=6, M P{XP}Nrx^{d08766} n=5, F w^{1118} n=5 and F P{XP}Nrx^{d08766} n=6. A significant effect of genotype [$F_{(1, 14)}$=7.140, p=0.0182] and sex [$F_{(1, 14)}$=28.844, p<0.0001] was found for Nrx-1 expression fold change. * denotes p<0.05 significant increase in P{XP}Nrx^{d08766} flies compared to w^{1118} in both males and females (main effect genotype, ANOVA). # denotes p<0.0001 significant increase in male and compared to female flies of both genotypes (main effect sex, ANOVA).*

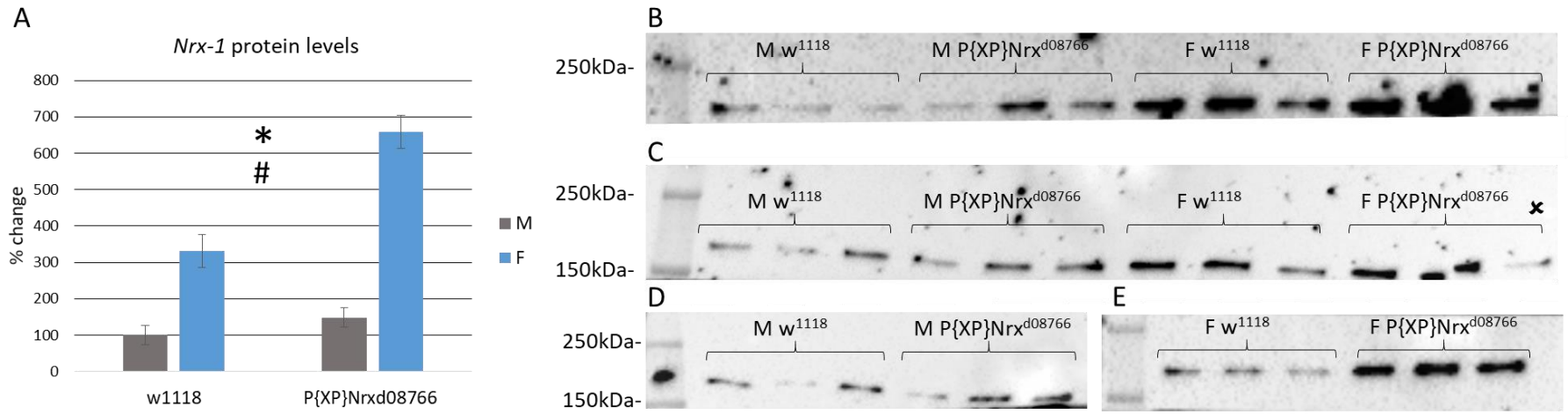

Figure 7.2 P{XP}Nrx^{d08766} flies show increased Nrx-1 protein levels in both sexes

W1118=w^{1118} control fly, P{XP}Nrx d08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly, M=male, F=female. Nrx-1 protein level was measured by western blot in fly heads and data from each sample was normalised to a single male w^{1118} sample. Samples sizes were n=3 per genotype per sex. ***(A)*** *Nrx-1 protein level % change per group, data are mean ± SEM except for the male w^{1118} group plotted at 100% fold change with group SEM.* ***(B)*** *western blot image for all samples.* ***(C)*** *repeat of* ***(B)****.* ***(D)*** *western blot image for repeat of male samples only.* ***(E)*** *western blot image repeat for repeat of female samples only.* ***(B)*** *and* ***(C)*** *images were used for statistical analysis. All bands in* ***(A)-(E)*** *appear at ~200kDa. A significant effect of genotype [$F_{(1, 15)}$=7.678, p=0.0143] and sex [$F_{(1, 15)}$=28.551, p<0.0001] was found for % change of Nrx-1 protein level. * denotes p<0.05 significant increase in P{XP}Nrx^{d08766} flies compared to w^{1118} in male and female flies (main effect genotype, ANOVA). # denotes p<0.0001 significant decrease in male compared to female flies of both genotypes (main effect sex, ANOVA). × denotes sample excluded from analysis.*

7.3 Discussion

In contrast to previous studies (Zeng *et al.*, 2007; Tong *et al.*, 2016), the P{XP}Nrx^{d08766} flies were found to overexpress *Nrx-1* at both the RNA level (Figure 7.1) and the protein level (Figure 7.2) following backcrossing onto the in-house w^{1118} genetic background. This suggests that genetic background of the flies modified the effect of the p-element inserted into the *Nrx-1* gene, bringing about overexpression rather than underexpression. Although the P{XP}Nrx^{d08766} flies received from the stock centre already had a w^{1118} genetic background, the genetics of w^{1118} fly strains are highly likely to differ between institutions (Colomb and Brembs, 2014), due to being separated for many generations. For instance they may differ in the ability of their transposable DNA elements (transposons) to move around within the genome (transposon landscapes) (Rahman *et al.*, 2015). Although the P{XP}Nrx^{d08766} flies were found not to be *Nrx-1* hypomorphs, and so not modelling heterozygous deletions in *NRXN1α* which increase the risk of developing Sz and ASD, they were used to test the effects of *Nrx-1* overexpression on life span, behavioural senescence and sleep.

There was a significant effect of sex for both *Nrx-1* expression (Figure 7.1) and protein level (Figure 7.22), although these were in opposite directions. Male flies expressed significantly more *Nrx-1* than female flies at the RNA level (Figure 7.1) and by contrast female flies had significantly higher levels of *Nrx-1* protein than males (Figure 7.2). This suggests that post-transcriptional modifications (Maier *et al.*, 2009) for *Nrx-1* may differ between male and female flies, resulting in significantly decreased *Nrx-1* protein following significantly increased *Nrx-1* RNA, in male compared to female flies.

Chapter 8 *Nrx-1* hyperfunction affects life span, behavioural senescence and locomotor activity

8.1 Introduction

Both life span analysis and Exploratory Walking (EW) senescence were used to measure ageing in P{XP}Nrx^{d08766} flies, providing information about survival and function during ageing in comparison to w^{1118} control flies. EW parameters gradually and robustly change with age (Ismail *et al.*, 2015). Walking distance, walking velocity, walking duration, rotation frequency and frequency of visits to the central zone decrease with age, while duration of time spent in the central zone of the arena increases with age. The fly brain and CNS controls locomotor behaviour (Strauss *et al.*, 1992; Strauss and Heisenberg, 1993; Martin *et al.*, 1998; Strauss, 2002; Besson and Martin, 2005) and thus EW parameters indirectly measures CNS function and its decline with age. However most EW parameters are confounded by peripheral effects such as neuromuscular functioning, which also decline with age. Duration and frequency of time spent in the central zone, and rotation frequency parameters are thought to be indicative of 'decision making' behaviours controlled by the fly brain (Strauss, 2002; Besson and Martin, 2005; Serway *et al.*, 2009; Ismail *et al.*, 2015) and are less confounded by peripheral effects. Therefore investigating multiple EW parameters allows determination of whether effects are due to alterations in CNS ageing or neuromuscular ageing. Furthermore, a previous study indicated that female flies show a steeper (earlier) age-related decline in spontaneous locomotor activity compared to male flies (Le Bourg and Minois, 1999). Sex differences in locomotor activity have also been found at young age (non-age-related sex differences), whereby males show hyperlocomotor

activity compared to female flies (Martin, 2004; Besson and Martin, 2005; Woods *et al.*, 2014).

8.2 Key findings

- **P{XP}Nrx^{d08766} flies show a small life span extension**
- **P{XP}Nrx^{d08766} flies have delayed age-related decline in walking distance and rotation frequency**
- **P{XP}Nrx^{d08766} flies have ameliorated age-related increase in duration of time spent in the central zone of the arena**
- **Male, but not female, P{XP}Nrx^{d08766} flies have increased walking distance, walking velocity and rotation frequency and decreased time spent in the central zone**
- **Sex impacts on EW parameters and their alterations with age**

8.3 Results

8.3.1 P{XP}Nrx^{d08766} flies show a small life span extension

When fly cohorts and sexes were analysed independently using log rank tests, it was found that life span was variably extended by a small amount in P{XP}Nrx^{d08766} flies and the effect was mostly seen in males. Life span was extended in male P{XP}Nrx^{d08766} flies compared to male w^{1118} in all 4 cohorts (Figure 8.1 A-D). Log rank tests on survival data revealed that male P{XP}Nrx^{d08766} life span extension was close to significance in cohort 1 (p=0.0532, log rank chi squared), reached significance in cohort 2 (p=0.0457, log rank chi-squared) but did not reach significance in cohorts 3 and 4 (p=0.1450, p=0.0901, respectively, log rank chi-squared) compared to w^{1118} males. Log rank tests on survival data also revealed that female P{XP}Nrx^{d08766} flies showed no significant difference in life span in cohorts 1 and 3, but

significant life span extension in cohort 4 (p=0.0427, log rank chi squared) (Figure 8.2 A-C). Females were excluded from cohort 2 since they became trapped in moisture in the stock bottles prior to entering life span analysis, and appeared sickly and died earlier than expected.

In addition to the statistical analysis of independent cohorts, survival analysis of the whole data set gained from all lifespan experimental cohorts was performed using a Cox proportional-hazards model (Cox, 1972). This approach allows for the regression of life span in relation to multiple predictor variables, in the case of this experiment fly genotype, sex and the experimental cohort. This analysis allows us to determine the impact of these different factors on the hazard rate (rate of death) and the relative hazard ratio, the likelihood of death at any particular point in time, between the different factors. Using this analysis it was found that being a w^{1118} control fly was associated with a significant increase in the hazard ratio for death in comparison to being a P{XP}Nrx^{d08766} *Nrx-1* hypermorph fly (hazard ratio=1.283, 28% greater likelihood of death in w^{1118} in comparison to P{XP}Nrx^{d08766} genotype, 95% confidence interval=1.149 - 1.433, p<0.0001). Given the previous observation, from the various cohorts, that sex may influence the impact of *Nrx-1* genotype on life span we undertook exploratory analysis in male and female flies separately. Cox proportional-hazards regression model confirmed that in males being w^{1118} was significantly associated with an increased risk of death (*males*; w^{1118}: P{XP}Nrx^{d08766} hazard ratio=1.366 , 37% greater likelihood of dying if w^{1118}, 95% confidence interval= 1.180-1.581, p<0.0001). However, this effect failed to reach significance in female flies (*females*; w^{1118}: P{XP}Nrx^{d08766} hazard ratio=1.158, 16% greater likelihood of dying if w^{1118}, 95% confidence interval=0.980-1.369, p=0.0850).

When the effect of genotype was analysed, being w^{1118} was found to significantly increase the likelihood of death in cohort 1 (hazard ratio=1.288, 29% increased likelihood of death, 95% confidence interval=1.043-1.591, p=0.0190), cohort three (hazard ratio=1.237, 24% increased likelihood of death, 95% confidence interval=1.005-1.523, p=0.0451) and cohort 4 (hazard ratio=1.294, 29% increased likelihood of death, 95% confidence interval=1.057-1.583, p=0.0124) and trended towards significance in cohort 2 (hazard ratio=1.317, 32% increased likelihood of death, 95% confidence interval=0.988-1.757, p=0.0609) in comparison to being a P{XP}Nrx^{d08766} fly.

Female flies show significant life span extension compared to male flies

When cohorts and sexes were analysed independently it was found that sex had a variable impact on life span. Log rank tests on individual fly cohorts revealed that w^{1118} females showed significant life span extension compared to males in cohort 1 and 4 ($p<0.0001$ and $p<0.0001$ respectively, log rank chi-squared), but showed no difference in life span in cohort 3. In P{XP}Nrx^{d08766} flies, females showed significant life span extension compared to males in cohort 1, 3 and 4 ($p<0.001$, $p=0.0381$ and $p<0.0001$ respectively, log rank chi-squared).

Cox proportional-hazard analysis showed that being male was associated with a significantly increased risk of death (male: female hazard ratio=1.481, 48% greater likelihood of death in males in comparison to females, 95% confidence interval=1.312-1.673; $p<0.0001$). In addition, there was also significant evidence that the risk of death varied significantly between the cohorts of flies characterised (cohort 1: cohorts 4, hazard ratio=0.853, 15% decreased likelihood of death in cohort 1, 95% confidence interval=0.739-0.986, p=0.0312; cohort 2: cohort 4, hazard ratio=1.581, 58% increased likelihood of death in cohort 2, 95%

confidence interval=1.311-1.908, p<0.0001, cohort 3: cohort 4, hazard ratio=1.515, 52% increased likelihood of death in cohort 3, 95% confidence interval 1.308-1.754, p<0.0001).

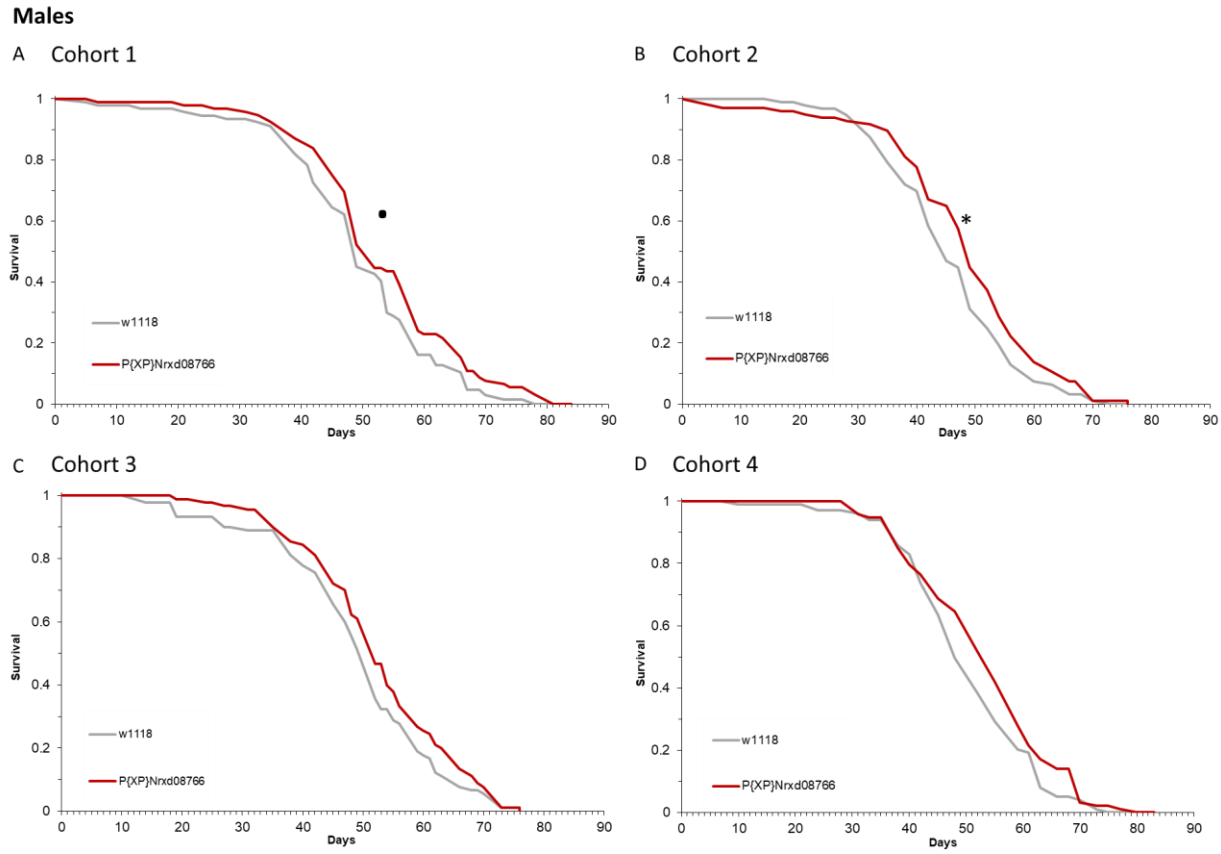

Figure 8.1 Male P{XP}Nrx^{d08766} flies show a small life span extension

*Survival curves comparing male P{XP}Nrx^{d08766} and control flies in **(A)** Cohort 1, **(B)** Cohort 2, **(C)** Cohort 3 and **(D)** Cohort 4. w1118=white^{1118} control fly, P{XP}Nrxd08766= P{XP}Nrx^{d08766}Nrx-1 hypermorph fly. Sample sizes were w^{1118} n=100, P{XP}Nrx^{d08766} n=100. * denotes p<0.05 significant life span extension compared to control, ● denotes p<0.06 trend towards significant life span extension compared to white^{1118} (log rank chi squared).*

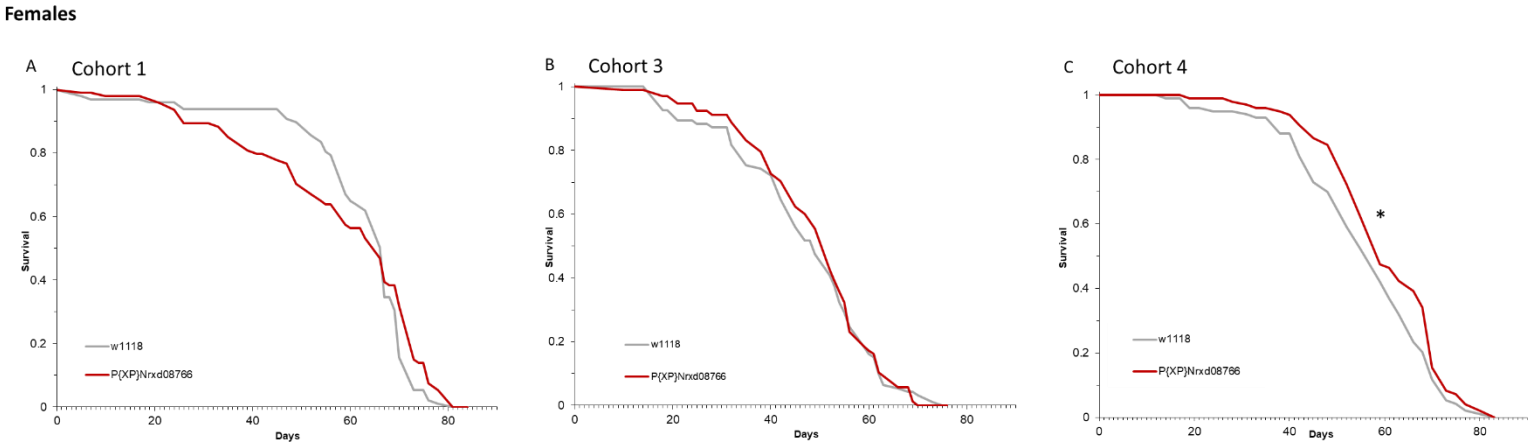

Figure 8.2 Female P{XP}Nrx^{d08766} flies variable life span extension

Survival curves comparing female P{XP}Nrx^{d08766} and control flies in ***(A)*** *Cohort 1,* ***(B)*** *Cohort 3 and* ***(C)*** *Cohort 4. w1118=white^{1118} control fly, P{XP}Nrxd08766= P{XP}Nrx^{d08766} Nrx-1 hypermorph fly. Sample sizes were w^{1118} n=100, P{XP}Nrx^{d08766} n=100. * denotes significant life span extension compared to w^{1118} (log rank chi squared).*

8.3.2 *Nrx-1* hyperfunction reduces behavioural senescence

P{XP}Nrx^{d08766} flies have delayed age-related decline in locomotor activity

P{XP}Nrx^{d08766} flies showed a delay in age-related decline in distance walked during the EW test (Figure 8.3 A). In this way a significant age x genotype interaction was found for distance walked [$F_{(6, 406)}$ =2.293, p=0.0345]. This effect was not modified by sex (no significant sex x age x genotype interaction) and so the analysis of the age x genotype interaction on walking distance was based on flies from both sexes. No age x genotype interactions were found for walking velocity, duration or frequency. Although age does have an effect on these parameters (see section 8.3.3), these effects are not modified by genotype. *Post hoc* testing using Pairwise t-test with FDR correction revealed that P{XP}Nrx^{d08766} flies walked a significantly greater distance than w^{1118} flies at 32 (p= 0.0013) and 46 (p=0.0231) days old. However there was no difference in walking distance between P{XP}Nrx^{d08766} and w^{1118} flies at the oldest time point (53 days old).

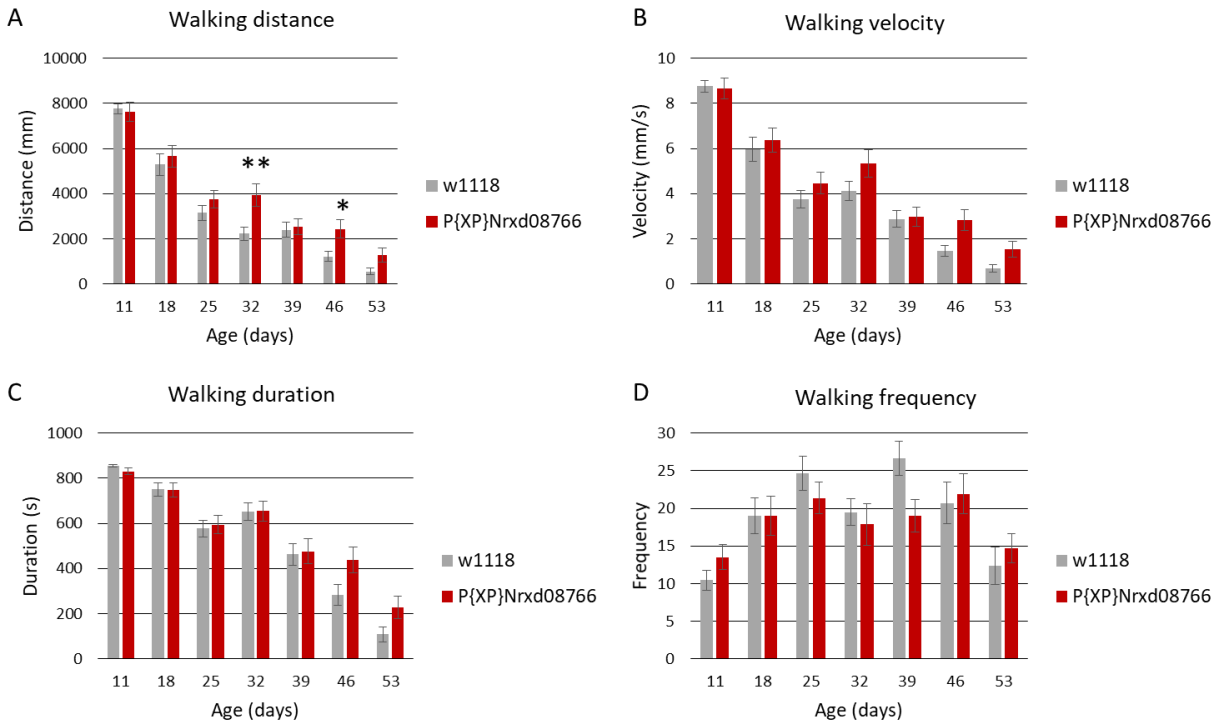

Figure 8.3 P{XP}Nrx^{d08766} flies have delayed age-related decline in walking distance

Data shown are mean ± SEM. w1118=white^{1118} control fly, P{XP}Nrxd08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly. Measures of locomotor activity include ***(A)*** *Walking distance,* ***(B)*** *Walking velocity,* ***(C)*** *Walking duration and* ***(D)*** *Walking frequency. A significant age x genotype interaction was found for Walking distance only [$F_{(6, 406)}$ =2.293, p=0.0345]. Sample sizes were w^{1118} n=23-32, P{XP}Nrx^{d08766} n=29-32 per time point. * and ** denote, p<0.05 and p<0.01 respectively, significant increase compared to w^{1118} (Pairwise t-test with FDR correction).*

P{XP}Nrx^{d08766} flies have reduced rotation frequency at young age and delayed age-related decline in rotation frequency

P{XP}Nrx^{d08766} flies had reduced rotation frequency at 11 days old and showed a delay in age-related decline in rotation frequency (Figure 8.4 A). A significant age x genotype interaction was found for rotation frequency [$F_{(6, 408)}$ =4.930, p<0.0001]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that P{XP}Nrx^{d08766} flies rotated significantly less times than w^{1118} flies at 11 days old (p=0.0115). They also showed significantly increased rotation frequency compared to w^{1118} flies at 32 days (p=0.0116) and a trend towards significantly increased rotation frequency at 46 days (p=0.0517) old, before reaching similar levels to w^{1118} at 53 days old. Sex did not modify the effects of *Nrx-1* hyperfunction on age-related decline in rotation frequency (no significant sex x age x genotype interaction), however sex did impact on the effect of genotype on rotation frequency (significant sex x genotype interaction) which is described later in section 8.3.4. Sex also impacted on age-related changes in rotation frequency described in section 8.3.3.

P{XP}Nrx^{d08766} flies show ameliorated age-related increase in time spent in the central zone of the arena

P{XP}Nrx^{d08766} flies showed an amelioration of the age-related increase in time spent in the central zone of the arena (Figure 8.4 B). A significant age x genotype interaction was found for duration of time spent in the central zone [$F_{(6, 402)}$ =3.090, p=0.0057]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that P{XP}Nrx^{d08766} flies spent significantly less time in the central zone of the arena at 46 (p=0.0033) and 53 (p=0.0009) days old as compared to w^{1118} flies, but not at younger ages. Sex did not impact on the effect of genotype on changes in age-related increase in time spent in the central zone (no significant

sex x age x genotype interaction), however sex did impact on the effect of genotype on duration of time spent in the central zone (significant sex x genotype interaction) which is described in section 8.3.4.

Young female, but not male, P{XP}Nrx^{d08766} flies have increased number of visits to the central zone of the arena

Female P{XP}Nrx^{d08766} flies visited the central zone of the arena more times than female w^{1118} flies at young age, by contrast male P{XP}Nrx^{d08766} flies visit the central zone a similar number of times to male w^{1118}. Both male and female flies show a reduction in the number of visits to the central zone of a novel arena as they age (Figure 8.4 C and D). A significant sex x age x genotype interaction was found for frequency in the central zone [$F_{(6, 408)}$ =2.735, p=0.0129]. Therefore the data were split by sex and ANOVAs were run separately for males and females. A significant age x genotype interaction was found in female [$F_{(6, 202)}$ =3.186, p=0.0052], but not male, flies. A significant effect of age was found in male flies [$F_{(6, 202)}$ =13.231, p<0.0001]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that P{XP}Nrx^{d08766} females visit the central zone significantly more times than w^{1118} females at 11 days old (p=0.0003), an effect that was not seen at any other age (Figure 8.4 C).

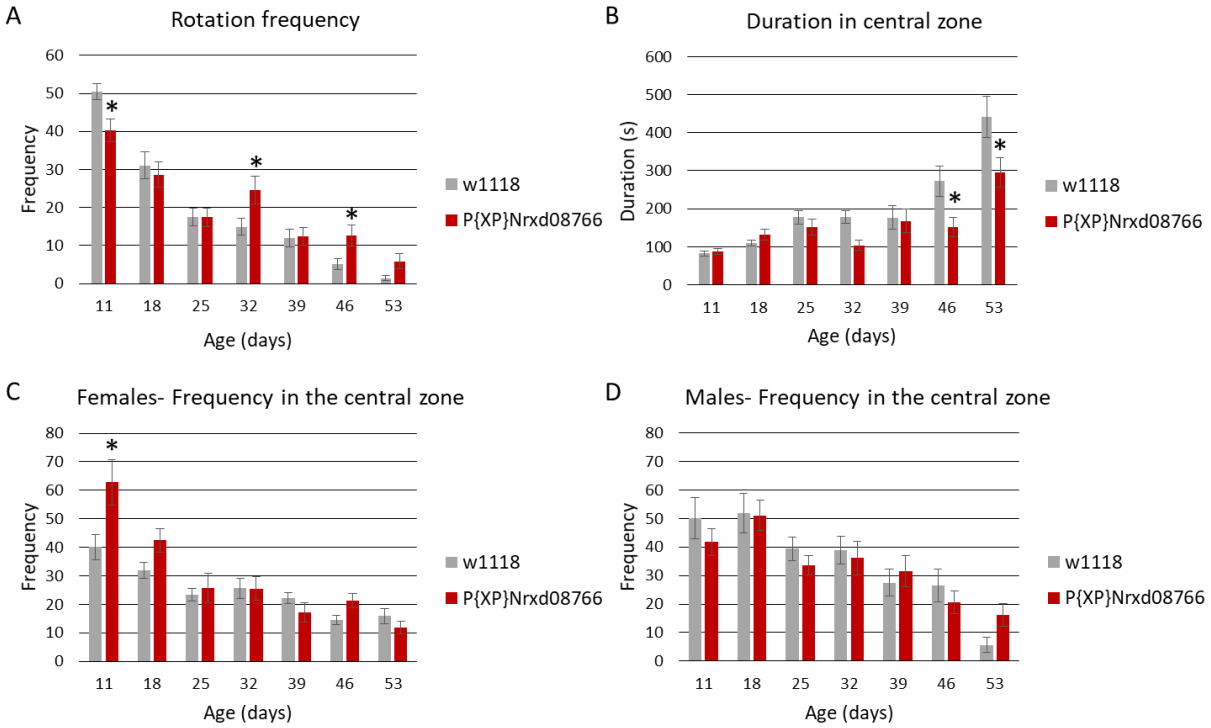

Figure 8.4 Nrx-1 hyperfunction delays age-related decline in rotation frequency, ameliorates age-related increase in time spent in the central zone, and alters rotation frequency and frequency in the central zone at young age

Data shown are mean ± SEM. w1118=white^{1118} control fly, P{XP}Nrxd08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly. Exploratory Walking (EW) parameters include ***(A)*** *Rotation frequency,* ***(B)*** *Duration in the central zone,* ***(C)*** *Frequency in the central zone for females and* ***(D)*** *Frequency in the central zone for males. A significant sex x age x genotype interaction was found for Frequency in the central zone [$F_{(6, 408)}$ =2.735, p=0.0129]. When data were split by sex a significant age x genotype interaction was found in females [$F_{(6, 412)}$ =3.186, p=0.0052] and a significant effect of age was found in males [$F_{(6, 202)}$ =13.231, p<0.0001]. A significant age x genotype interaction was found for Rotation frequency [$F_{(6, 408)}$ =4.930, p<0.0001] and Duration in the central zone [$F_{(6, 402)}$ =3.090, p=0.0057]. Sample sizes were w^{1118} n=23-32, P{XP}Nrx^{d08766} n=29-32 per time point for* ***(A)*** *and* ***(B)****, w^{1118} F n=14-16, P{XP}Nrx^{d08766} F n=14-16 per time point for* ***(C)*** *and w^{1118} M n=12-16, P{XP}Nrx^{d08766} M n=15-16 per time point for* ***(D)****. * denotes p<0.05 significant difference compared to w^{1118} of the same age group (Pairwise t-test with FDR correction).*

Ageing parameter	Impact of *Nrx-1* hyperfunction	Modified by Sex?
Life span	↑	×
Walking distance (decrease)	Delay	×
Walking velocity (decrease)	-	-
Walking duration (decrease)	-	-
Rotation frequency (decrease)	Delay	×
Duration in central zone (increase)	↓	×
Frequency in central zone (decrease)	-	-

Table 8.1 The effects of Nrx-1 hyperfunction on life span and behavioural senescence summary
↑ =significant increase, ↓ =significant decrease, Delay =significant delay in age-related change in parameters, - =no significant effect, × =genotype effect not significantly modified by sex.

8.3.3 Sex impacts on age-related changes in EW behaviour

Sex impacts on age-related changes in walking distance, velocity, duration and frequency

It was confirmed that walking distance, velocity, duration and frequency changed significantly with age (Ismail *et al.*, 2015), however the current study also found that sex impacted on these age-related changes (Figure 8.5 A-D). All flies showed decreased walking distance, velocity and duration as they aged. However male flies walked further, faster, for longer durations and in less bouts than female flies, during ageing. In this way we found significant sex x age interactions for walking distance [$F_{(6, 406)}$ =7.213, p<0.0001], velocity [$F_{(6, 406)}$ =9.891, p<0.0001], duration [$F_{(6, 399)}$ =3.845, p=0.0010] and frequency [$F_{(6, 408)}$ =4.813, p<0.0001]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that male and female flies were similar at 11 days old, for all four parameters. However at each time point from 18-53 days old males flies had significantly increased walking distance (M v F: 18 days p<0.0001, 25 days p<0.0001, 32 days p<0.0001, 39 days p<0.0001, 46 days p=0.0011, 53 days p=0.0663), velocity (M v F: 18 days p<0.0001, 25 days p<0.0001, 32 days p<0.0001, 39 days p<0.0001, 46 days p=0.0005, 53 days p=0.0448) and duration (M v F: 18 days p=0.0014, 25 days p<0.0001, 32 days p<0.0001, 39 days p<0.0001, 46 days p<0.0001, 53 days p= 0.0005) and significantly decreased walking frequency (M v F: 18 days p<0.0001, 25 days p<0.0001, 32 days p<0.0001, 39 days p=0.0010, 46 days p=0.01655, 53 days p= 0.0112) compared to female flies. This demonstrates that age-related changes in locomotor activity are ameliorated in male compared to female flies. Both male and female flies showed changes in all locomotor parameters at 53 days old as compared to those at 11 days old, except for walking frequency were males showed no change. Interestingly, it was found that

females showed age-related changes earlier than males for walking distance, velocity and duration. *Post hoc* testing revealed that females show significant changes for all locomotor parameters at 18 days old when compared to 11 days old (F 11 days v 18 days: distance $p<0.0001$, velocity $p<0.0001$, duration $p=0.0008$, frequency $p<0.0001$), whereas males only show significant changes at 25 days old when compared to 11 days old (M 11 days v 25 days: distance $p<0.0001$, velocity $p<0.0001$, duration $p<0.0001$).

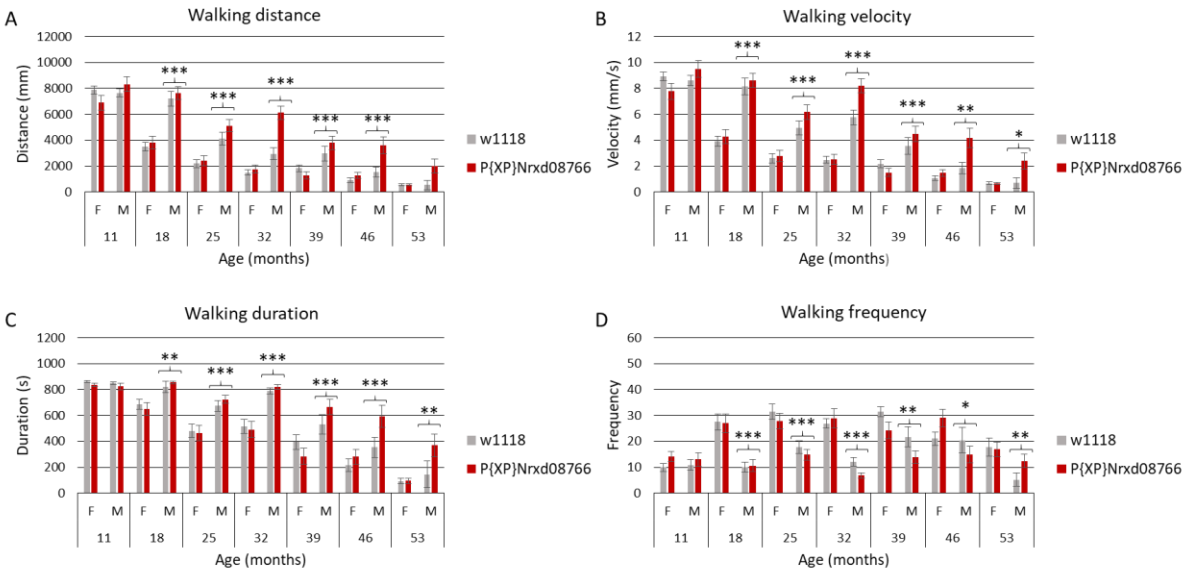

Figure 8.5 Sex impacts on age-related change in walking distance, velocity, duration and frequency *Data shown are mean ± SEM. w1118=white^{1118} control fly, P{XP}Nrxd08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly, F=female, M=male. Measures of locomotor activity include* ***(A)*** *Walking distance,* ***(B)*** *Walking velocity,* ***(C)*** *Walking duration and* ***(D)*** *Walking frequency plotted by genotype per sex for each age group. A significant sex x age interaction was found for Walking distance [$F_{(6, 406)}$ =7.213, p<0.0001], Walking velocity [$F_{(6, 406)}$ =9.891, p<0.0001], Walking duration [$F_{(6, 399)}$ =3.845, p=0.0010] and Walking frequency [$F_{(6, 408)}$ =4.813, p<0.0001]. Sample sizes were w1118; 11 days: F n=14, M n=15, 18 days: F n=16, M n=15, 25 days: F n=16, M n=16, 32 days: F n=16, M n=16, 39 days: F n=16, M n=16, 46 days: F n=15-16, M n=14-16, 53 days: F n=16, M n=7-10, P{XP}Nrxd08766; 11 days: F n=14, M n=15, 18 days: F n=16, M n=15, 25 days: F n=16, M n=16, 32 days: F n=16, M n=16, 39 days: F n=16, M n=16, 46 days: F n=16, M n=16, 53 days: F n=16, M n=15-16. *, **, *** denote, p<0.05, p<0.01, p<0.001 respectively, significant difference compared to female flies of the same age group (Pairwise t-test with FDR correction).*

Sex impacts on age-related changes in rotation frequency

Age-related changes were significantly impacted on by sex for rotation frequency (Figure 8.6 A). All flies showed decreased rotation frequency and frequency in the central zone, and increased duration of time spent in the central zone, as they aged. Although both sexes showed similar levels of rotation frequency at young age (11 days old) and old age (53 days old), male flies had a higher rotation frequency compared to female flies between 18 and 46 days old. There was no difference in age-related changes in duration of time spent in the central zone between male and female flies (Figure 8.6 B). A significant sex x age interaction was found for rotation frequency [$F_{(6, 408)}$ =7.353, p<0.0001 and a significant effect of age [$F_{(6, 402)}$ =21.715, p<0.0001] and sex [$F_{(1, 402)}$ =4.119, p=0.0431] was found for duration in the central zone. *Post hoc* testing using Pairwise t-test with FDR correction revealed that males showed a delay in age-related decline of rotation frequency compared to females. Male flies had significantly higher rotation frequency compared to females for all time points between 18 and 46 days old (18 p<0.0001, 25 p<0.0001, 32 p<0.0001, 39 p=0.0007, 46 p=0.0018) but not at 11 and 53 days old. Frequency of visits to the central zone was significantly impacted on by sex and genotype at young age, described in section 8.3.2 (Figure 8.4 C and D).

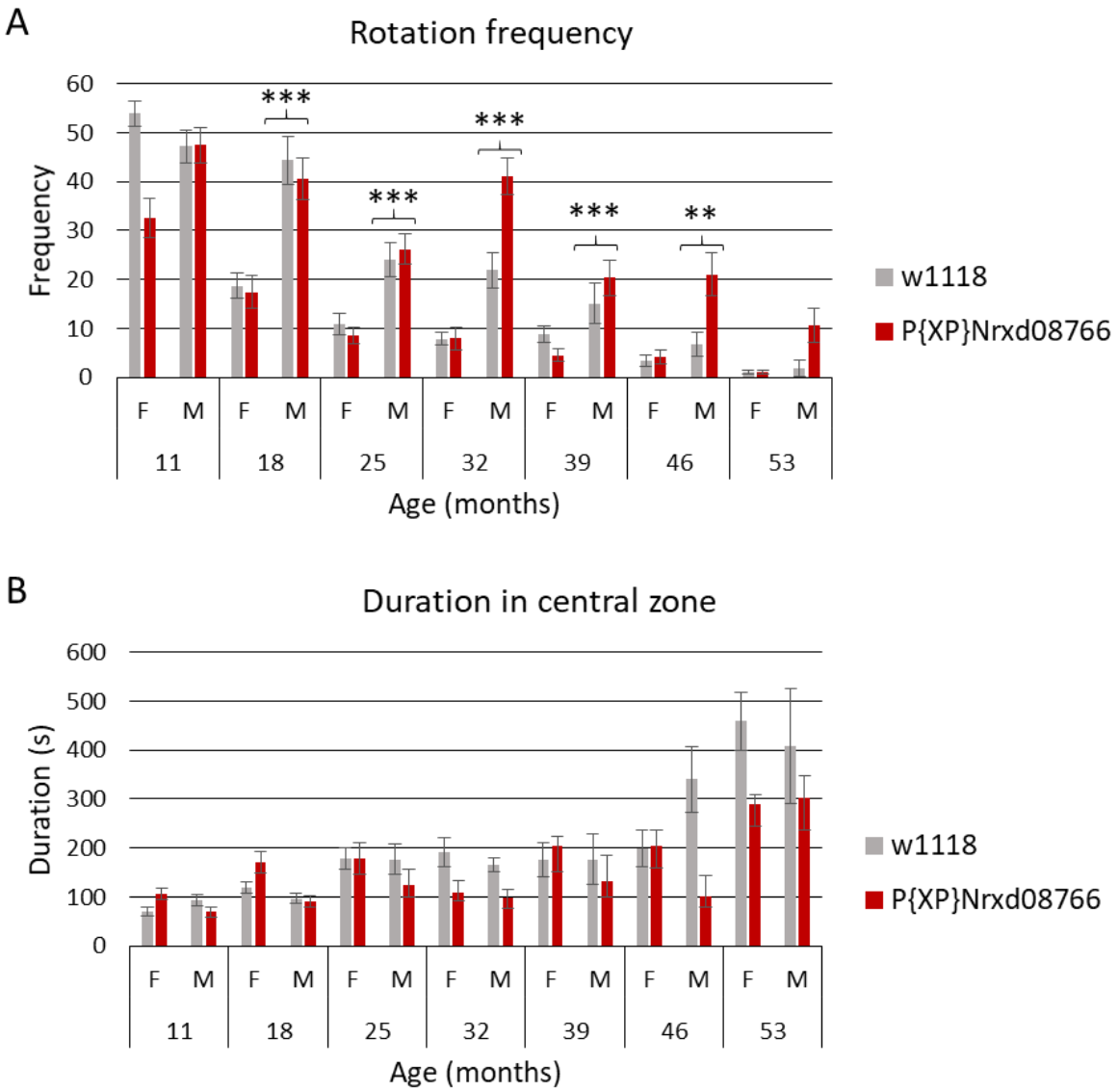

Figure 8.6 Sex impacts on age-related decline in rotation frequency but not duration in the central zone

Data shown are mean ± SEM. w1118=white^{1118} control fly, P{XP}Nrxd08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly, F=female, M=male. Exploratory Walking (EW) parameters include ***(A)*** *Rotation frequency and* ***(B)*** *Duration in the central zone. A significant sex x age interaction was found for Rotation frequency [F (6, 408) =7.353, p<0.0001]. Sample sizes were w1118; 11 days: F n=14, M n=15, 18 days: F n=16, M n=15, 25 days: F n=16, M n=16, 32 days: F n=16, M n=16, 39 days: F n=16, M n=16, 46 days: F n=15-16, M n=14-16, 53 days: F n=16, M n=8-10, P{XP}Nrxd08766; 11 days: F n=14, M n=15, 18 days: F n=16, M n=15, 25 days: F n=16, M n=16, 32 days: F n=16, M n=16, 39 days: F n=16, M n=16, 46 days: F n=16, M n=16, 53 days: F n=16, M n=15-16. **and *** denote, p<0.01 and p<0.001 respectively, significant difference compared to female flies of the same age group (Pairwise t-test with FDR correction).*

8.3.4 *Nrx-1* hyperfunction has non-age-related effects on locomotor activity

Male, but not female, P{XP}Nrx^{d08766} flies show increased walking distance and velocity

Male, but not female, P{XP}Nrx^{d08766} flies showed significantly increased walking distance and velocity as compared to w^{1118} (Figure 8.7 A and B). A significant sex x genotype interaction was found for walking distance [$F_{(1, 406)}$ =19.014, p<.0.0001], velocity [$F_{(1, 406)}$ =17.153, p<0.0001] and duration [$F_{(1, 399)}$ =8.390, p=0.0040]. By contrast no significant sex x genotype interaction or significant effect of genotype was found for walking frequency. *Post hoc* testing, using Pairwise t-test with FDR correction, showed that male P{XP}Nrx^{d08766} flies had significantly greater walking distance (p=0.0015) and velocity (p=0.0048) than male w^{1118} flies. No differences in locomotor activity were found between P{XP}Nrx^{d08766} females and w^{1118} females.

Sex differences in locomotor activity have also been found in previous studies (Martin, 2004; Besson and Martin, 2005; Woods *et al.*, 2014) (Figure 8.7 A-D). In the current study male flies walked faster and more continuously covering a greater distance than females, whereas female flies walked more slowly with more stop-starting and so covered less distance than males. *Post hoc* testing using Pairwise t-test with FDR correction revealed that males had significantly greater walking distance (w^{1118} M v F p=0.0002, P{XP}Nrx^{d08766} M v F p<0.0001), velocity (w^{1118} M v F p<0.0001, P{XP}Nrx^{d08766} M v F p<0.0001) and duration (w^{1118} M v F p<0.0001, P{XP}Nrx^{d08766} M v F p<0.0001) than females. A significant effect of sex on walking frequency was also found [$F_{(1, 408)}$ =100.288, p<0.0001], revealing that female flies have a significantly greater walking frequency (more walking bouts) than male flies.

Male, but not female, P{XP}Nrx^{d08766} flies show alterations in rotation frequency and duration of time spent in the central zone

Male P{XP}Nrx^{d08766} flies showed increased rotation frequency and decreased duration in the central zone as compared to w^{1118} males (Figure 8.8 A and B). Significant sex x genotype interactions were found for rotation frequency [$F_{(1, 408)}$ =20.746, p<0.0001] and duration in the central zone [$F_{(1, 402)}$ =4.278, p=0.0392]. *Post hoc* testing using Pairwise t-test with FDR correction revealed that male P{XP}Nrx^{d08766} flies had increased rotation frequency (p<0.0001) and decreased duration in the central zone (p=0.0180) compared to male w^{1118} flies. By contrast, the only difference between P{XP}Nrx^{d08766} females and w^{1118} females was in frequency of visits to the central zone of the arena selectively at young age (11 days old only), as previously described in in section 8.3.2 (Figure 8.4 C).

There were also sex differences for rotation frequency, but not for duration in the central zone (Figure 8.8 A and B). *Post hoc* testing using Pairwise t-test with FDR correction revealed that w^{1118} males had higher rotation frequency than w^{1118} females (p=0.0003), and P{XP}Nrx^{d08766} males also had higher rotation frequency than P{XP}Nrx^{d08766} females (p<0.0001). No sex differences were found in w^{1118} flies for duration of time spent in the central zone, however a close to significant decrease in duration of time spent in the central zone was found for male P{XP}Nrx^{d08766} flies compared to female P{XP}Nrx^{d08766} flies (p=0.0530).

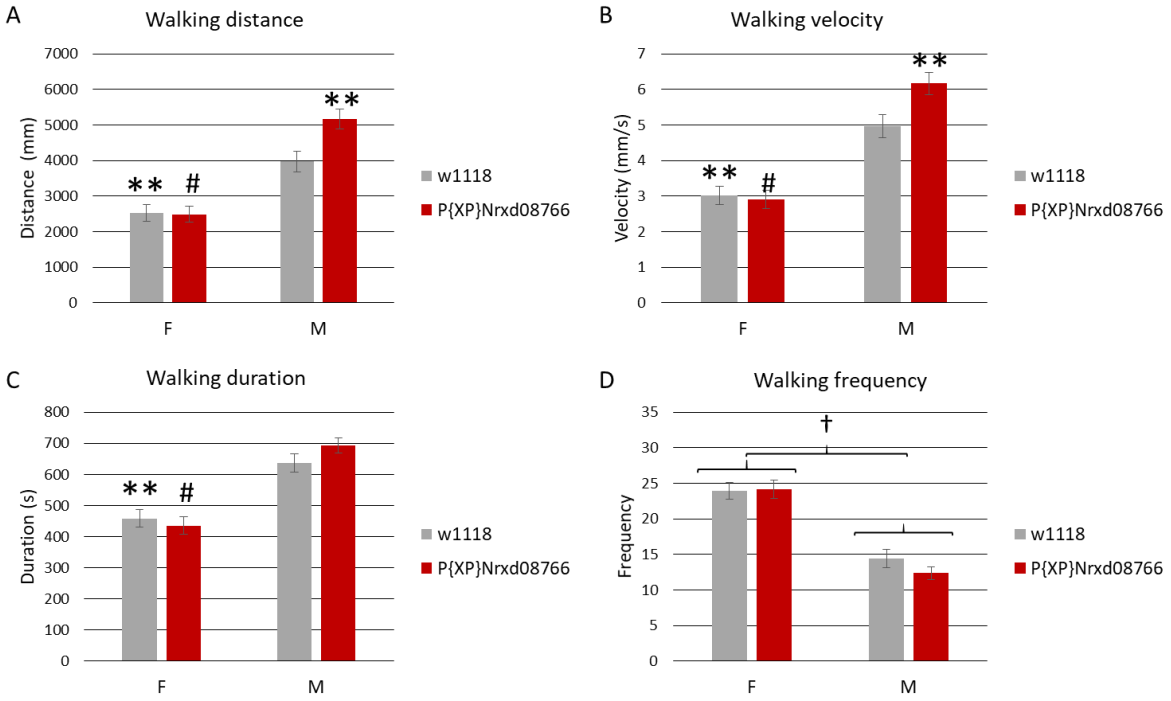

Figure 8.7 Male, but not female, P{XP}Nrx^{d08766} flies show increased walking distance and velocity
Data shown are mean ± SEM. F=female, M=male, w1118=white^{1118} control fly, P{XP}Nrxd08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly. Measures of locomotor activity include ***(A)*** *Walking distance,* ***(B)*** *Walking velocity,* ***(C)*** *Walking duration and* ***(D)*** *Walking frequency (all time points combined). A significant sex x genotype interaction was found for walking distance [$F_{(1, 406)}$ =19.014, p<.0.0001], velocity [$F_{(1, 406)}$ =17.153, p<0.0001] and duration [$F_{(1, 399)}$ =8.390, p=0.0040]. A significant effect of sex was found for Walking frequency [$F_{(1, 408)}$ =100.288, p<0.0001]. Sample sizes were w^{1118} M n=99-106, P{XP}Nrx^{d08766} M n=109-110, w^{1118} F n=109-110, P{XP}Nrx^{d08766} F n=110. ** denotes p<0.01 significant difference as compared to w^{1118} M, # denotes p<0.0001 significant decrease as compared to P{XP}Nrx^{d08766} M (Pairwise t-test with FDR correction). † denotes p<0.0001 significant increase in females compared to males (Main effect of sex, ANOVA).*

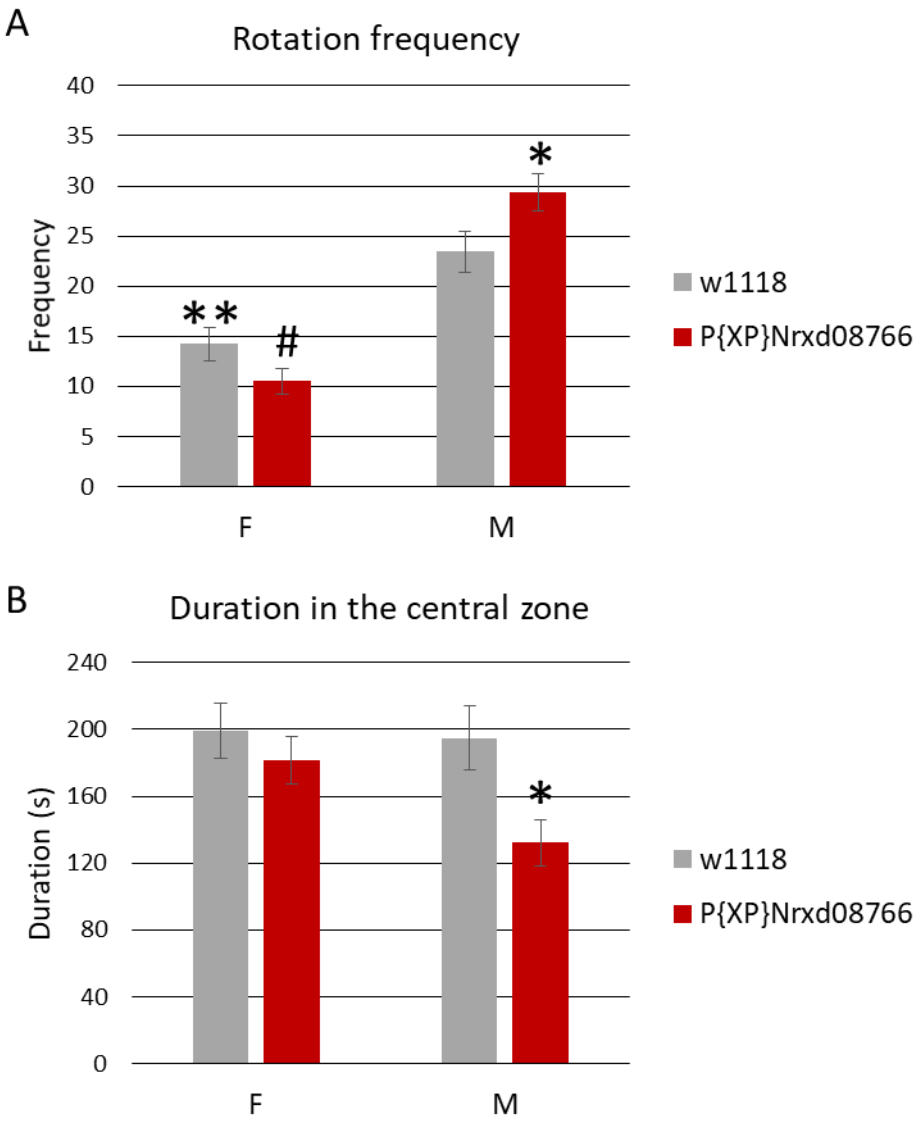

Figure 8.8 Male, but not female, P{XP}Nrx^{d08766} flies show increased rotation frequency and decreased duration of time spent in the central zone.

Data shown are mean ± SEM. F=female, M=male, w1118=white^{1118} control fly, P{XP}Nrxd08766=P{XP}Nrx^{d08766} Nrx-1 hypermorph fly. Exploratory walking parameters shown include ***(A)*** *Rotation frequency and* ***(B)*** *Duration in the central zone (all time points combined). Significant sex x genotype interactions were found for Rotation frequency [$F_{(1, 408)}$ =20.746, p<0.0001] and Duration in the central zone [$F_{(1, 402)}$ =4.278, p=0.0392]. Sample sizes were w^{1118} M n=99-106, P{XP}Nrx^{d08766} M n=109-110, w^{1118} F n=109-110, P{XP}Nrx^{d08766} F n=110. * and **denote, p<0.05 and p<0.01 respectively, significant difference as compared to w^{1118} M, # denotes p<0.0001 significant decrease as compared to P{XP}Nrx^{d08766} M (Pairwise t-test with FDR correction).*

Exploratory Walking parameter	Impact of *Nrx-1* hyperfunction	Modified by Sex?
Walking distance	↑	✓ (M only)
Walking velocity	↑	✓ (M only)
Walking duration	-	-
Walking frequency	-	-
Rotation frequency	↑	✓ (M only)
Duration in central zone	↓	✓ (M only)
Frequency in central zone	-	-

Table 8.2 Non-age-related effects of Nrx-1 hyperfunction on Exploratory Walking (EW) parameters
↑=significant increase, ↓=significant decrease, - =no significant change, M=male.

8.4 Discussion

There were 3 main themes in the life span and Exploratory Walking (EW) test findings: (1) *Nrx-1* hyperfunction causes a small but significant life span extension, mainly in males, and reductions in behavioural senescence; (2) Male, but not female, P{XP}Nrx^{d08766} flies show hyperlocomotor activity and increased rotation frequency; and (3) Confirmation that sex impacts on life span, locomotor activity and behavioural senescence.

8.4.1 *Nrx-1* hyperfunction causes life span extension and reductions in behavioural senescence

Nrx-1 hyperfunction reduced measures of ageing (Table 8.1). P{XP}Nrx^{d08766} flies showed a small but significant life span extension, mainly in males (section 8.3.1, Figure 8.1 and 8.2), a delay in age-related decline in walking distance (Figure 8.3 A) and rotation frequency (Figure 8.4 A), and ameliorated age-related increase in duration in the central zone (Figure 8.4 B). This suggests that not only does *Nrx-1* hyperfunction extend life span it also improves function of flies as they age.

P{XP}Nrx^{d08766} flies show a small life span extension

A small life span extension in P{XP}Nrx^{d08766} flies was found when data analysis included all cohorts and both sexes (section 8.3.1). This is supported by shortened life span observed in male *Nrx-1* KO flies (Zeng *et al.*, 2007), strongly suggesting that *Nrx-1* protein levels affect life span in both directions, since increased *Nrx-1* protein increased life span and KO of *Nrx-1* protein decreased life span in male flies. Increased *Nrx-1* protein levels were found in both male and female P{XP}Nrx^{d08766} flies by western blot in the current study (Figure 7.2), however small increases in life span were mainly found in male P{XP}Nrx^{d08766} flies (section

8.3.1). *Nrx-1* protein levels were found to be increased by more than 2 fold in P{XP}Nrx^{d08766} flies however there was only a small extension in life span in these flies, which suggests only large changes in levels of *Nrx-1* protein affect life span. It cannot be determined whether the reduced life span in *Nrx-1* KO flies (Zeng *et al.*, 2007) was due to accelerated ageing or due to a sickly phenotype which caused early death. Increased life span in P{XP}Nrx^{d08766} *Nrx-1* hypermorph flies in the current study may however provide some support for *Nrx-1* KO affecting the ageing process, rather than causing a sickly phenotype, since increased and decreased (KO) *Nrx-1* protein levels have opposite effects on life span.

Sz and (to a lesser extent) ASD are disorders associated with reduced life span (Shavelle and Strauss, 1998; Shavelle *et al.*, 2001; Pickett *et al.*, 2006; Laursen *et al.*, 2012; Laursen *et al.*, 2014), and risk of developing both disorders is increased by *NRXN1α* heterozygosity (Marshall *et al.*, 2008; Morrow *et al.*, 2008; Glessner *et al.*, 2009; Kirov, Rujescu*, et al.*, 2009). Taken together, findings of altered life span in relation to altered *Nrx-1* protein levels in the current study and a previous study (Zeng *et al.*, 2007) in flies suggest that *NRXN1α* heterozygosity may play a role in reduced life span in Sz and ASD, however further investigation is required to confirm whether this is due to an effect on ageing. Also, not all Sz and ASD patients have mutations in *NRXN1α,* and unhealthy lifestyle choices known to affect life span are also prevalent in this group such as obesity and smoking (Broder-Fingert *et al.*, 2014; Laursen *et al.*, 2014; Hill, A. P. *et al.*, 2015). Therefore factors other than *NRXN1α* heterozygosity*,* and its downstream effects, are likely to contribute to reduced life span in Sz and ASD.

P{XP}Nrx^{d08766} flies have reduced behavioural senescence

Nrx-1 hyperfunction caused delayed age-related decline in walking distance (Figure 8.3 A) and rotation frequency (Figure 8.4 A), and ameliorated age-related increase in time spent in the central zone (Figure 8.4 B). Since rotation frequency is likely to indicate 'decision making' behaviour controlled by the fly brain (Ismail *et al.*, 2015), the current findings suggest that *Nrx-1* hyperfunction has protective effects on the ageing fly brain, resulting in the preservation of these 'decision making' functions at older ages compared to w^{1118}. The delay, rather than the amelioration, of age-related decline in rotation frequency in flies may be due these protective effects of *Nrx-1* hyperfunction not continuing into old age (53 days old) or may be due to factors driving age-related decline in this function overriding the protective effects caused by *Nrx-1* hyperfunction. Although walking distance and duration of time spent in the central zone are EW parameters that are controlled by the fly brain (Strauss *et al.*, 1992; Strauss and Heisenberg, 1993; Martin *et al.*, 1998; Strauss, 2002; Besson and Martin, 2005), decline with age in these parameters could be due to decline in brain function and/or neuromuscular function. Therefore although *Nrx-1* hyperfunction appears to have protective effects on brain function during ageing (evidenced by delayed age-related decline in rotation frequency) it may also have protective effects on neuromuscular function causing delayed age-related decline in walking distance and ameliorated age-related increase in duration of time spent in the central zone. *Nrx-1* is expressed in both central and peripheral nerves in adult flies (Sun *et al.*, 2016) which supports the notion that *Nrx-1* hyperfunction causes reduced behavioural senescence via protective effects on both the central and peripheral nerves.

Nrx-1 hyperfunction also has effects at the young time point only (11 days old) including lowering rotation frequency in both sexes, and increasing the number of visits to the central zone in female flies only (Figure 8.4 A and C). This suggests that *Nrx-1* hyperfunction has developmental, as well as age-related, effects on 'decision making' processes (rotation frequency and frequency of visits to the central zone) in the EW test. These 'decision making' processes are altered at 11 days old which likely reflects a developmental abnormality in brain function that is normalised to the same levels as control flies by 18 days old. This suggests that developmental abnormalities may be restricted to brain function since parameters that are confounded by peripheral effects, such as walking velocity and distance, are unaffected at young age.

8.4.2 Male, but not female, P{XP}Nrx^{d08766} flies show hyperlocomotor activity and increased rotation frequency

Nrx-1 hyperfunction also had non-age-related, but sex specific, effects on EW parameters. *Nrx-1* hyperfunction caused hyperlocomotor activity (increased walking distance and velocity, and decreased time spent in the central zone) and increased rotation frequency in male but not female flies (summarised in Table 8.2).

Male, but not female, P{XP}Nrx^{d08766} flies showed significantly increased walking distance, walking velocity (Figure 8.7 A and B) and rotation frequency (Figure 8.8 A), and significantly decreased duration in the central zone of the arena (Figure 8.8 B). In male P{XP}Nrx^{d08766} flies, increased walking distance and decreased duration of time spent in the central zone was likely driven by increased walking velocity, since there was no significant changes in walking duration, walking frequency (number of bouts) (Figure 8.7 C and D) or frequency of visits the central zone (Figure 8.8 B). Therefore male P{XP}Nrx^{d08766} flies walked faster which

resulted in a larger walking distance and less time spent in the central zone, as increased walking velocity would result in faster entry and exit of the central zone as compared to male w^{1118} flies. Walking speed (velocity) is under the control of the fly brain (Strauss *et al.*, 1992; Strauss and Heisenberg, 1993) and so an increase in this parameter is likely due to altered CNS function.

Rotation frequency and central zone behaviour (duration and frequency of visits) are thought of as 'decision making' behaviours that are related to CNS function (Strauss, 2002; Besson and Martin, 2005; Serway *et al.*, 2009; Ismail *et al.*, 2015). Rotation frequency was found to be increased in male P{XP}Nrx^{d08766} flies (Figure 8.8) and since flies rarely rotate when in motion (Strauss and Heisenberg, 1990) it is unlikely that increased rotation frequency is due to increased walking velocity. This suggests increased rotation frequency in male P{XP}Nrx^{d08766} flies is an effect of altered CNS function.

The dopaminergic system in *Drosophila*, like in rodents and humans, is associated with locomotor activity since decreased dopamine in the brain of *Drosophila* is associated with reduced locomotor activity (Riemensperger *et al.*, 2011). Hyperlocomotor activity in rodents has long be used as a model of positive symptoms in Sz (Van Den Buuse, 2010), since sub cortical hyperdopaminergia is linked to positive symptoms in Sz patients (Uchida *et al.*, 2011; Harro, 2015) and hyperlocomotor activity in rodents (Creese and Iversen, 1975; Kelly *et al.*, 1975; Zhou and Palmiter, 1995; Giros *et al.*, 1996a; Antoniou *et al.*, 1998). Hyperactivity has also been implicated in ASD, since ADHD is a common comorbidity of ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012; Antshel *et al.*, 2016). Patients with ADHD, another neurodevelopmental disorder, have been shown to have increased locomotor activity as

part of the hyperactivity symptom domain of the disorder (Garcia Murillo *et al.*, 2015), and have also been found to have dopaminergic system dysfunction (Spencer *et al.*, 2005). Therefore it is possible that *Nrx-1* hyperfunction causes alterations in dopaminergic neurotransmission in male P{XP}Nrx^{d08766} flies which in turn increased their locomotor activity.

Nrx-1 KO *Drosophila* larvae have previously been found to have reduced locomotor activity (Li *et al.*, 2007), supporting findings of hyperlocomotor activity in P{XP}Nrx^{d08766} *Nrx-1* hypermorph flies. Hyperlocomotor activity was also found in the *Nrxn1α* Hz mice in the current study (Figure 3.1). Since the *Nrxn1α* Hz mice have reduced expression of *Nrxn1α* protein (Missler *et al.*, 2003) and showed hyperlocomotor activity, one might expect the P{XP}Nrx^{d08766} flies with increased *Nrx-1* protein levels to have decreased locomotor activity. However mice have 3 *Nrxn* genes with both α and β isoforms, that undergo alternative splicing to produce many splice variants, whereas flies have a single *Nrx-1* gene (Tabuchi and Sudhof, 2002). The complexity of *Nrxn* expression in the mouse compared to the fly may explain why hyperlocomotor activity is seen in both *Nrxn1α* Hz mice and *Nrx-1* overexpression flies, as changes in expression of *Neurexin* may have differential effects on the brain of flies and mice, bringing about opposite effects on locomotor activity across species. The current study provides evidence that *Neurexin1* may play a role in the same types of behaviours in flies and mice, since disruption of *Neurexin1* altered locomotor activity in both species.

8.4.3 Sex impacts on life span, locomotor activity and behavioural senescence

Sex differences were found in life span (section 8.3.1), EW parameters (Figure 8.7 and 8.8) and their alterations with age (Figure 8.5 and 8.6). The current study confirmed previous findings that female flies show a significant life extension compared to male flies (Tower, 2006), although this is not always the case and can be dependent on a number of variables including genotype, genetic background and experimental conditions (Lints *et al.*, 1983; Austad and Fischer, 2016). For all EW parameters, except for duration and frequency in the central zone, male and female flies showed similar levels at 11 days old but significant sex differences at all subsequent ages tested (Figure 8.5 and 8.6). Female flies showed age-related changes in these parameters earlier than males, suggesting that female flies' age quicker than males in terms of these measures (Figure 8.5 and 8.6). These findings are supported by a previous study that found spontaneous locomotor activity showed a steeper decline with age in female flies compared to male flies (Le Bourg and Minois, 1999).

There were also non-age-related sex differences in EW parameters including significantly increased walking distance, walking velocity, walking duration and rotation frequency in male compared to female w^{1118} control flies (Figure 8.7 A-C and 8.8 A). Male flies also showed significantly reduced walking frequency compared to female flies of both genotypes (Figure 8.7 D). Increased locomotor activity (Figure 8.7) and visits to the central zone (Figure 8.4) in male compared to females flies, and the lack of a sex difference in duration of time spent in the central zone, has been shown in previous studies (Martin, 2004; Besson and Martin, 2005; Woods *et al.*, 2014).

8.4.4 Future experiments

Improved age-related EW parameters in the P{XP}Nrx^{d08766} flies suggest that *Nrx-1* hyperfunction increases health span as well as life span, however this has only been evidenced by behavioural measures in the current study. Further studies could be carried out with 4 main aims (1) to test if *Nrx-1* hyperfunction can reduce measures of ageing other than life span and behaviour, (2) to test if reduced measures of ageing by *Nrx-1* hyperfunction is due to factors during development or adulthood, (3) to investigate dopaminergic neurotransmission as a mechanism for hyperlocomotor activity in male P{XP}Nrx^{d08766} flies, and (4) to investigate the mechanism underlying reduced age-related measures in the P{XP}Nrx^{d08766} flies.

Further experiments could be carried out to investigate if *Nrx-1* hyperfunction causes generalised improvement in functioning during ageing or is specific to locomotor behaviour. In this way fecundity could be investigated in P{XP}Nrx^{d08766} female flies. Manipulations that extend life span are often, but not always, coupled with decreased fecundity (Flatt, 2011). Testing fecundity across the life span in female P{XP}Nrx^{d08766} flies would provide more information about whether function (health span) of these flies is improved as well as life span. If fecundity was not altered in these flies this would further support the evidence for increased health span found in the current study. Cellular ageing is associated with the build-up of damaged macromolecules including carbonylated proteins resulting from oxidation by reactive oxygen species (Berlett and Stadtman, 1997). Therefore probes such as 2,4-dinitrophenylhydrazine (DNPH) could be used to detect and quantify carbonylated proteins in P{XP}Nrx^{d08766} fly tissue (Levine *et al.*, 1990). Testing for markers of oxidative stress in P{XP}Nrx^{d08766} flies across the life span could also provide indications of whether

Nrx-1 hyperfunction increases health span as well as life span, as reduced markers in the P{XP}Nrx^{d08766} flies may further evidence improved function during ageing.

Nrx-1 hyperfunction was found to reduce measures of ageing in flies in the current study, however whether this was due to events during development or during adulthood is unknown. To address this question flies with inducible overexpression of *Nrx-1*, using the UAS/GAL80 system (Barwell *et al.,* 2017) could be tested for effects of life span and behavioural senescence (using EW). Flies containing a gene mutation which causes overexpression of *Nrx-1* using the GAL4/UAS system would be crossed with GAL80 flies, GAL80 binds to GAL4 and inhibits it and so the transgene would not be expressed during development. Once adult flies had eclosed an inducer could be administered, e.g. tetracycline, which would prevent expression of GAL80, uninhibiting GAL4, and allowing expression of the *Nrx-1* overexpressing transgene. Therefore flies could develop normally and would only start overexpressing *Nrx-1* as adults. If measures of ageing (life span and EW) were reduced in these flies, as in the P{XP}Nrx^{d08766} flies in the current study, this would suggest that factors during adulthood rather than during development cause this effect.

Alterations in dopaminergic neurotransmission could be investigated as a possible mechanism for hyperlocomotor activity observed in male, but not female, P{XP}Nrx^{d08766} flies. Levels of dopamine or dopamine receptors could be investigated in both males and females of both genotypes. High Performance Liquid Chromatography (HPLC) could be used to measure dopamine and its precursors, and qPCR and western blot could be used to investigate gene expression and protein levels of dopamine receptors.

Investigating insulin/insulin-like growth factor signalling (IIS) may be a good candidate mechanism underlying reduced measures of ageing seen in P{XP}Nrx^{d08766} flies in the current

study. This is due to the pre-existing link between IIS and Sz, and also IIS and life span. Links between IIS and Sz include an increased incidence of diabetes in Sz patients (Bai *et al.*, 2013), alterations in the IIS pathway in Sz post-mortem brain tissue (Zhao *et al.*, 2006) and *Neurexin1α*, which is a risk gene for Sz, being expressed in pancreatic β cells (Maffei *et al.*, 2004; Suckow *et al.*, 2008; Mosedale *et al.*, 2012) and playing a role in insulin release from vesicles (Mosedale *et al.*, 2012). In terms of IIS and life span, there is a large body of evidence that demonstrates that reduced IIS extends life span in model organisms (Broughton and Partridge, 2009; Fontana *et al.*, 2010; Partridge *et al.*, 2011). One study investigated the effects of reduced IIS on EW (Ismail *et al.*, 2015). They found that when IIS signalling was reduced systemically in two fly mutants, life span was extended in males and females in both mutants, and was accompanied by delays in age-related decline in walking distance and velocity and ameliorated age-related decline in walking duration in female flies only, dependant on fly mutant (Ismail *et al.*, 2015). This could suggest that IIS signalling is altered systemically in P{XP}Nrx^{d08766} flies since they too showed increased life span and delayed age-related decline in walking distance. However, reduced systemic IIS signalling did not alter age-related changes in rotation frequency and duration of time spent in the central zone, and delayed age-related decline in walking distance and velocity was observed in female flies only (Ismail *et al.*, 2015). Age-related effects on EW parameters in P{XP}Nrx^{d08766} flies were not sex dependent and included parameters indicative of 'decision making' processes in the fly (rotation frequency and duration in the central zone). Since *Nrx-1* is almost exclusively expressed in neurons in flies, it may be reasonable to think that if *Nrx-1* hyperfunction alters IIS signalling this may only occur in neurons. A study investigating pan-neuronally reduced IIS in flies found that life span was only extended in female flies and had detrimental effects on age-related changes in EW parameters including walking distance,

velocity and duration, and rotation frequency in male and female flies (Ismail *et al.*, 2015). These findings contrast with those of the current study since *Nrx-1* hyperfunction increased life span mainly in male flies, and reduced ageing in EW parameters in both sexes. Taken together the findings from the current study and from Ismail *et al.* suggest that while it may be plausible to investigate if IIS is altered systemically in P{XP}Nrx^{d08766} flies, it seems unlikely that there is specific IIS reduction in neurons. Systemic changes in IIS could be investigated in P{XP}Nrx^{d08766} flies using molecular biology techniques to examine whether different parts of the IIS pathway are altered across the life span, for example testing for differences in *Drosophila* insulin-like peptide (DILP) expression using qPCR or alterations in phosphorylated vs total AKT using western blot.

8.4.5 Summary

Nrx-1 hyperfunction in P{XP}Nrx^{d08766} flies caused a small life span extension and reduced behavioural senescence evidence by delayed age-related decline in walking distance and rotation frequency and ameliorated age-related increase in duration of time spent in the central zone. Taken together, these finding indicate that *Nrx-1* hyperfunction extends life span and health span. Previous work has shown that *Nrx-1* KO flies have reduced life span supporting the current findings and suggesting that *Nrx-1* may play a role in ageing, however further investigation is required to confirm this. *NRXN1* expression, and its downstream effects, may have a role in reduced life span observed in Sz patients. However this is complicated by not all patients having mutations in *NRXN1* and the effect of many other life style factors in Sz patients contributing to reduced life span. Since locomotor behaviour is under the control of the fly brain, *Nrx-1* hyperfunction appears to reduce ageing in the CNS (evidenced by delayed age-related decline in rotation frequency),

however may also affect ageing in the periphery as most locomotor measures of brain function are confounded by neuromuscular effects (walking distance and duration of time spent in the central zone). Effects of *Nrx-1* hyperfunction at young age in flies was restricted to EW parameters that are more indicative of the CNS function (reduced rotation frequency in both sexes and increased frequency in the central zone in females) rather than those confounded by peripheral effects (walking velocity and distance).

Nrx-1 hyperfunction also had non-age-related effects on EW parameters in male flies only, including increased walking distance, walking velocity and rotation frequency, and decreased duration of time spent in the central zone. While increased walking velocity is likely the driver of increased walking distance and decreased duration of time spent in the central zone, increased rotation frequency is less influenced by walking velocity, and reflects 'decision making' processes controlled by the fly brain. Hyperlocomotor activity found in male P{XP}Nrx^{d08766} flies is supported by findings of reduced locomotor activity in *Nrx-1* KO fly larvae. A candidate mechanism for this could be altered dopaminergic neurotransmission. Furthermore there is strong evidence that *Neurexin1* plays a role in locomotor activity since this has shown to be altered in both flies and mice in the current study.

The current study confirmed previous findings of sex differences in life span and locomotor activity, and extended previous findings on sex differences in behavioural senescence in terms of the different EW parameters. Further studies could be carried out with 4 main aims (1) to test if *Nrx-1* hyperfunction can reduce measures of ageing other than life span and behaviour, (2) to test if reduced measures of ageing by *Nrx-1* hyperfunction is due to factors during development or adulthood, (3) to investigate dopaminergic neurotransmission as a

mechanism for hyperlocomotor activity in male P{XP}Nrx^{d08766} flies, and (4) to investigate the mechanism underlying reduced age-related measures in the P{XP}Nrx^{d08766} flies.

Chapter 9 *Nrx-1* hyperfunction alters sleep in flies

9.1 Introduction

The fly (*Drosophila melanogaster*) is a useful model to investigate human sleep. The neurobiology and regulation (homeostatic and circadian) of human sleep is conserved in flies and mammals (Hendricks *et al.*, 2000; Shaw *et al.*, 2000; Huber *et al.*, 2004; Sehgal and Mignot, 2011; Chakravarti *et al.*, 2017; Tomita *et al.*, 2017). In contrast to mammalian sleep, flies sleep in two time periods per day (following dusk and dawn) (Dubowy and Sehgal, 2017) and sleep in multiple short bouts (usually <90 minutes). In flies, sleep is counted as any period of inactivity exceeding 5 minutes (Shaw *et al.*, 2000) and is analysed in the light and dark phases separately. Sleep structure and amount can be investigated in flies using a number of parameters including total sleep, number of sleep bouts and sleep bout length (Andretic and Shaw, 2005). Previous studies have shown that male flies have increased total sleep and sleep in less bouts which are longer in duration than female flies (Koh *et al.*, 2006; Zimmerman *et al.*, 2012). Furthermore, as flies age their sleep has been shown to decrease and become more fragmented (bout number increases and bout length decreases) (Koh *et al.*, 2006; Zimmerman *et al.*, 2012; Metaxakis *et al.*, 2014), however this can vary dependent on light phase and fly genetic background.

9.2 Key Findings

- **P{XP}Nrx^{d08766} flies have decreased total sleep in the light phase across all age groups in male flies and at 11 days old only in female flies**
- **P{XP}Nrx^{d08766} flies have increased total sleep in the dark phase**

- **Female, but not male, P{XP}Nrx^{d08766} flies have increased sleep bout length and decreased sleep bout number**
- **Sex and age modify sleep in flies**

9.3 Results

9.3.1 *Nrx-1* hyperfunction alters sleep amount and structure

P{XP}Nrx^{d08766} flies have decreased total sleep in the light phase but increased total sleep in the dark phase

P{XP}Nrx^{d08766} flies showed reduced total sleep in the light phase, an effect that was dependent on sex and age. In females, P{XP}Nrx^{d08766} flies showed reduced total light phase sleep at 11 days old but not at any other age, compared to w^{1118} (Figure 9.1, 1A). However male P{XP}Nrx^{d08766} flies had reduced total sleep in the light phase across all ages (Figure 9.1, 1C). By contrast, P{XP}Nrx^{d08766} flies showed increased total sleep in the dark phase in both sexes and across all ages (Figure 9.1, 2C). For total sleep in the light phase, a significant sex x age x genotype interaction was found [$F_{(6, 390)}$ =2.832, p=0.0104]. When data were split by sex, a significant age x genotype interaction was found in female flies [$F_{(6, 202)}$ =2.680, p=0.0159] but not in male flies. *Post hoc* testing using Pairwise t-tests with FDR correction revealed that female P{XP}Nrx^{d08766} flies showed significantly reduced sleep compared to w^{1118} females at 11 days old (p=0.0065), but not at any other age (Figure 9.1, 1A). In male flies a significant main effect of genotype [$F_{(1, 188)}$ =4.287, p=0.0398] and age [$F_{(6, 188)}$ = 4.273, p<0.0010] was found. The significant main effect of genotype in male flies supported decreased total sleep in the light phase in P{XP}Nrx^{d08766} flies compared to w^{1118} (Figure 9.1, 1C). A significant main effect of genotype was found for total sleep in the dark phase [$F_{(1, 390)}$ =19.433, p<0.0001], supporting significantly increased total sleep in the dark phase in

P{XP}Nrx^{d08766} flies of both sexes compared to w^{1118} flies (Figure 9.1, 2C). No significant sex x age x genotype or age x genotype interactions were found for this parameter, however significant effects of age [$F_{(6, 390)}$ =24.498, p<0.0001] and sex [$F_{(1, 390)}$ =83.611, p<0.0001] were found separately.

Female, but not male, P{XP}Nrx^{d08766} flies have increased sleep bout length and decreased sleep bout number

Female P{XP}Nrx^{d08766} flies showed altered sleep bouts compared to w^{1118}, an effect not seen in male P{XP}Nrx^{d08766} flies. Female P{XP}Nrx^{d08766} flies slept in less bouts that were of a longer duration than w^{1118} females, in both the light and dark phases (Figure 9.2 Column 1 and 2). In this way significant, or trending towards significant, sex x genotype interactions were found for number of bouts in both light [$F_{(1, 390)}$ =3.344, p=0.0682] and dark [$F_{(1, 390)}$ =8.274, p=0.0004] phases, and bout length in both light [$F_{(1, 390)}$ =3.625, p=0.0577] and dark [$F_{(1, 390)}$ =3.171, p=0.0758] phases. *Post hoc* testing using Pairwise t-tests with FDR correction revealed that female P{XP}Nrx^{d08766} flies had a significantly decreased number of sleep bouts in both light (p<0.0001) and dark (p<0.0001) phases, and significantly, or close to significantly, increased bout length in both the light (p=0.0517) and the dark (p=0.0065) phases.

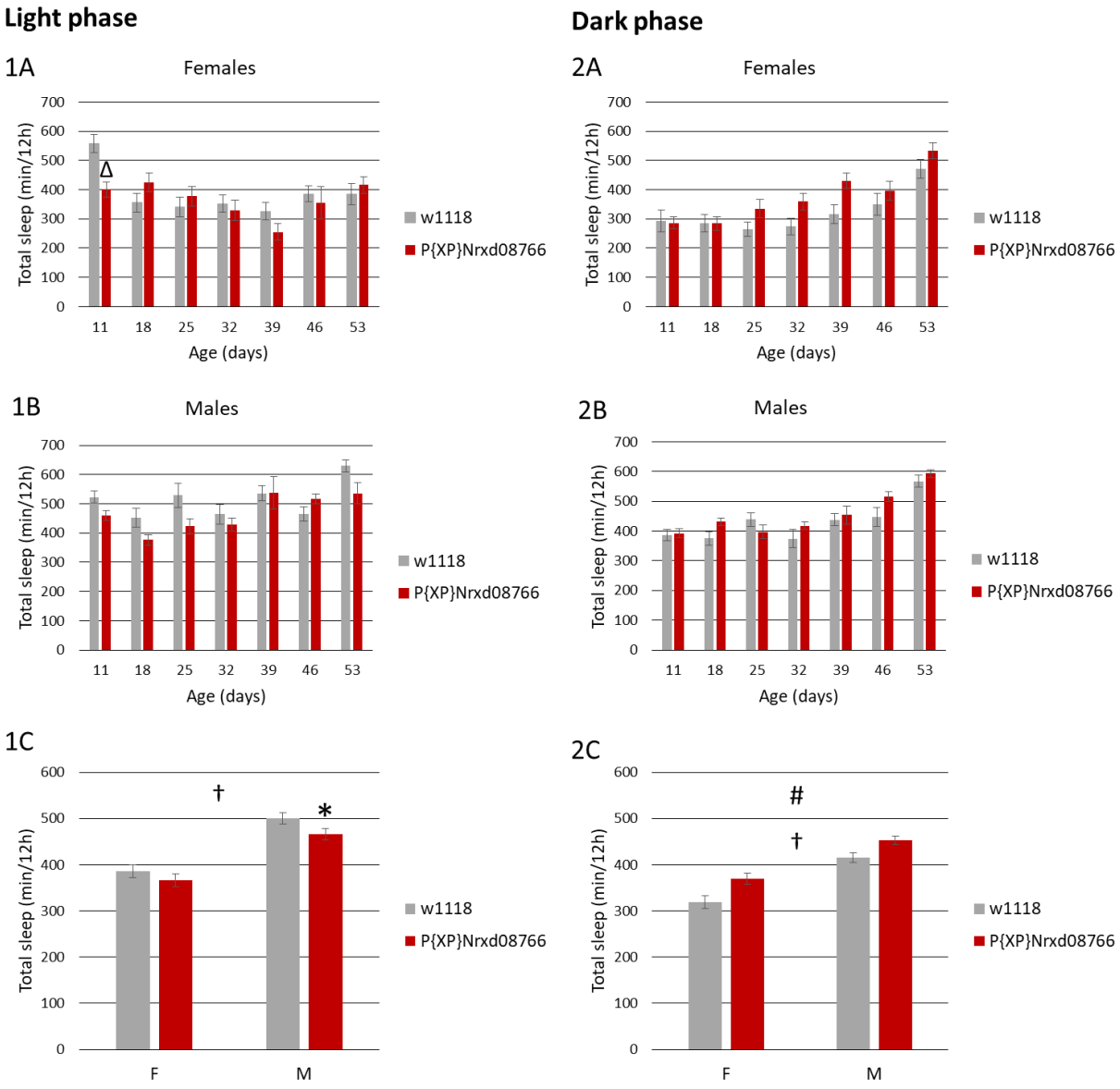

Figure 9.1 P{XP}Nrx^{d08766} flies show decreased total sleep in the light phase and increased total sleep in the dark phase dependent on sex and age

*Data shown are mean ± SEM. w^{1118}=white^{1118} control fly, P{XP}Nrx^{d08766}=Nrx-1 hypermorph fly, F=female, M=male. Total sleep in the light phase **(column 1)** and dark phase **(column 2)** for females only plotted for each time point (w^{1118} F n=14-16, P{XP}Nrx^{d08766} F n=13-16) **(row A)**, males only plotted for each time point (w^{1118} M n=4-16, P{XP}Nrx^{d08766} M n=13-16) **(row B)** and males and females with all time points combined per group (w^{1118} F n=110, P{XP}Nrx^{d08766} F n=106, w^{1118} M n=94, P{XP}Nrx^{d08766} M n=108) **(row C)**. For total sleep in the light phase a significant sex x age x genotype interaction was found [F $_{(6, 390)}$ =2.832, p=0.0104]. When data were split by sex, a significant age x genotype interaction was found in females [F $_{(6, 202)}$ =2.680, p=0.0159] and a significant main effect of genotype [F $_{(1, 188)}$ =4.287, p=0.0398] and age [F $_{(6, 188)}$ =4.273, p<0.0010] were found in males. A significant effect of genotype [F $_{(1, 390)}$ =19.433, p<0.0001] was found for total sleep in the dark phase and a significant effect of sex was found for total sleep in the light [F $_{(1, 390)}$ =74.602, p<0.0001] and dark [F $_{(1, 390)}$ =83.611, p<0.0001] phase. Δ denotes p<0.01 significant decrease in total sleep in the light phase compared to w^{1118} females at 11 days old (Pairwise t-test with FDR correction). * denotes p<0.05 significant decrease in total sleep in the light phase compared to w^{1118} males (main effect of genotype in males, ANOVA). # denotes p<0.0001 significant increase in P{XP}Nrx^{d08766} compared to w^{1118} (main effect of genotype in both sexes combined, ANOVA). † denotes p<0.0001 significant increase in males compared to females (main effect of sex in both genotypes, ANOVA).*

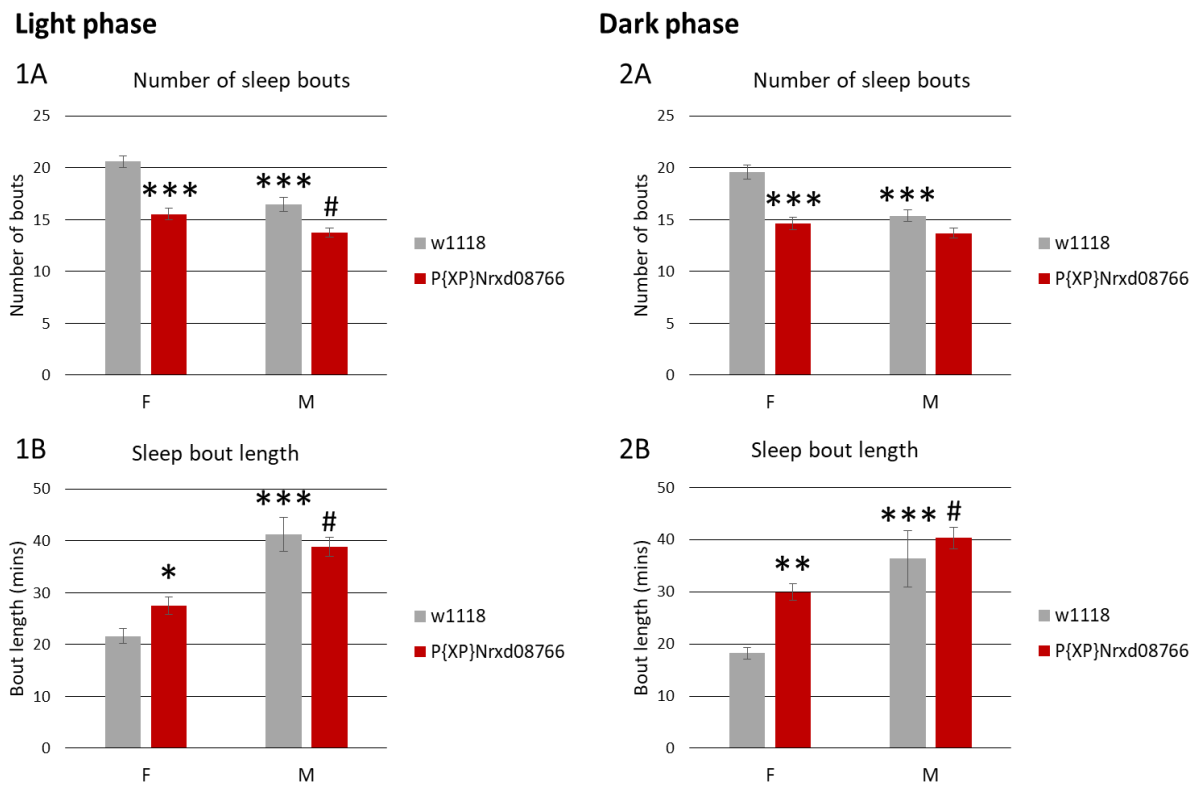

Figure 9.2 Female, but not male, P{XP}Nrx^{d08766} flies show increased sleep bout length and decreased sleep bout number

*Data shown are mean ± SEM. w^{1118}=white^{1118} control fly, P{XP}Nrx^{d08766}=Nrx-1 hypermorph fly, F=female, M=male. Sleep bout parameters measured in the light phase **(column 1)** and dark phase **(column 2)** include number of sleep bouts **(row A)** and sleep bout length **(row B)**. All time points are combined for each group (w^{1118} F n=110, P{XP}Nrx^{d08766} F n=106, w^{1118} M n=94, P{XP}Nrx^{d08766} M n=108). A significant, or close to significant, sex x genotype interaction was found for number of bouts in the light [$F_{(1, 390)}$ =3.344, p=0.0682] and dark [$F_{(1, 390)}$ =8.274, p=0.0004] phase and bout length in the light [$F_{(1, 390)}$ =3.625, p=0.0577] and dark [$F_{(1, 390)}$ =3.171, p=0.0758] phase. *, **, *** denote p=0.05, p<0.01, p<0.001 significant difference compared to w^{1118} females, # denote p<0.05 significant difference compared to P{XP}Nrx^{d08766} females (Pairwise t-test with FDR correction).*

Sleep parameter	Light phase	Impact of *Nrx-1* hypofunction	Modified by Sex?	Modified by Age?
Total sleep	Light	↓	×	✓ (F 11 days old only)
	Dark	↑	×	×
Number of bouts	Light	↓	✓ (F only)	×
	Dark	↓	✓ (F only)	×
Bout length	Light	↑	✓ (F only)	×
	Dark	↑	✓ (F only)	×

Table 9.1 Effects of Nrx-1 hyperfunction on sleep summary table
↑=significant increase, ↓=significant decrease, ×=no significant effect, F=female.

9.3.2 Confirmation that sleep structure is modified by age and sex in flies

Confirmation that total sleep and sleep structure differ between male and female flies

Male w^{1118} flies had significantly higher total sleep and sleep bout length, and a significantly lower number of sleep bouts in both light and dark phases compared to female w^{1118} flies (Figure 9.1 and 9.2). Decreased number of bouts and increased bout duration in males compared to females was found in P{XP}Nrx^{d08766} flies with the exception of bout number in the dark phase, where no difference between male and females was found (Figure 9.2 2A). In this way a significant main effect of sex was found for both total sleep in the light phase [$F_{(1, 390)}$ =74.602, p<0.0001] and total sleep in the dark phase [$F_{(1, 390)}$ =83.611, p<0.0001], revealing that males of both genotypes had significantly increased total sleep compared to females (Figure 9.1 Row C). Also significant or trending towards significant sex x genotype interactions were found for bout number and length in both light and dark phases as previously mentioned in section 9.3.1. *Post hoc* testing using Pairwise t-tests with FDR correction showed that both w^{1118} and P{XP}Nrx^{d08766} males slept in a higher number of bouts and in longer bouts than females of the same genotype, in both light and dark phases (Bout number; light phase: w^{1118} male vs w^{1118} female p<0.0001, P{XP}Nrx^{d08766} male vs P{XP}Nrx^{d08766} female p=0.0309, dark phase: w^{1118} male vs w^{1118} female p<0.0001, Bout length; light phase: w^{1118} male vs w^{1118} female p<0.0001, P{XP}Nrx^{d08766} male vs P{XP}Nrx^{d08766} female p=0.0001, dark phase: w^{1118} male vs w^{1118} female p<0.0001, P{XP}Nrx^{d08766} male vs P P{XP}Nrx^{d08766} female p=0.0141), except for bout number in the dark phase were P{XP}Nrx^{d08766} males and females slept in a similar number of bouts.

Confirmation that age affects total sleep and sleep structure

Total sleep and sleep structure were affected by age. The effects of age were not modified by sex or genotype (no significant sex x age or age x genotype interactions), except for total sleep in the dark phase (Figure 9.1 2C and 9.3 2C). In this way a significant effect of age was found for bout number in light [$F_{(6, 390)} = 4.850$, $p<0.0001$] and dark [$F_{(6, 390)} = 7.463$, $p<0.0001$] phase, bout length in the light [$F_{(6, 390)} = 3.133$, $p=0.0052$] and dark [$F_{(6, 390)} = 5.425$, $p<0.0001$] phase and total sleep in the light [$F_{(6, 390)} = 3.4.698$, $p<0.0001$] and dark phase [$F_{(6, 390)} = 24.498$, $p<0.0001$]. A significant sex x age x genotype interaction was also found for total sleep in the light phase described in section 9.3.1 (Figure 9.1 1A), however has also been presented in terms of the significant main effect of age for visual comparison of all sleep parameters measured (Figure 9.3 1C). Sleep bout parameters showed age dependent alterations that differed in pattern dependent on light phase (Figure 9.3). In the light phase bout number increased with age, bout length decreased with age and total sleep in the light was similar in young and old flies but decreased at middle age (U shape). However in the dark phase, bout number was similar at young and old age but increased in between (inverted U shape), bout length increased only at old age and total sleep increased with age.

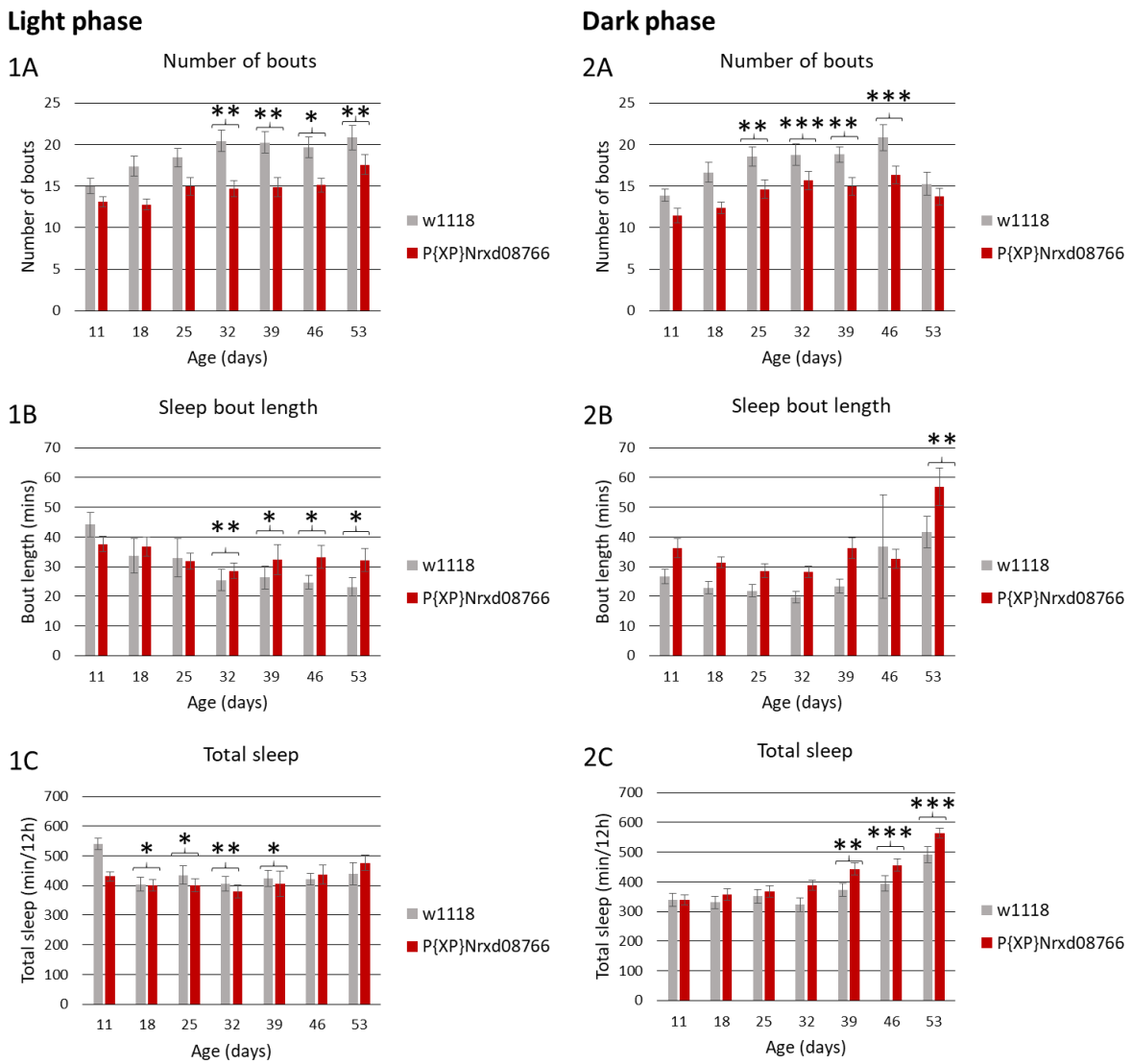

Figure 9.3 Age affects sleep amount and structure

*Data shown are mean ± SEM. w^{1118}=white^{1118} control fly, P{XP}Nrx^{d08766}=Nrx-1 hypermorph fly. Sleep bout parameters measured in the light phase **(column 1)** and dark phase **(column 2)** include number of sleep bouts **(row A)**, sleep bout length **(row B)** and total sleep **(row C)** plotted by genotype per age group. Sample sizes at each time point were w^{1118}: 11 days n=32, 18 days n=32, 25 days n=32, 32 days n=31, 39 days n=30, 46 days n=29, 53 days n=18, P{XP}Nrx^{d0876}: 11 days n=32, 18 days n=32, 25 days n=32, 32 days n=32, 39 days n=30, 46 days n=30, 53 days n=26-29. A significant main effect of age was found for bout number in the light [$F_{(6, 390)} = 4.850$, $p<0.0001$] and dark [$F_{(6, 390)} = 7.463$, $p<0.0001$] phase, bout length in the light [$F_{(6, 390)} = 3.133$, $p=0.0052$] and dark [$F_{(6, 390)} = 5.425$, $p<0.0001$] phase and total sleep in the light [$F_{(6, 390)} = 3.4.698$, $p<0.0001$] and dark phase [$F_{(6, 390)} = 24.498$, $p<0.0001$]. *, **, *** denote p<0.05, p<0.01, p<0.001 significant difference compared to 11 day old flies (Pairwise t-test with FDR correction).*

9.4 Discussion

Total sleep and sleep structure were altered in the P{XP}Nrx^{d08766} flies. P{XP}Nrx^{d08766} flies had decreased total sleep in the light phase and increased total sleep in the dark phase. The number of sleep bouts was decreased and the duration of sleep bouts was increased in female, but not male, P{XP}Nrx^{d08766} flies. In addition to genotype effects, the current study confirmed that sex and age also modify sleep parameters.

Total sleep and sleep structure is altered in the P{XP}Nrx^{d08766} flies

P{XP}Nrx^{d08766} flies showed decreased total sleep in the light phase compared to w^{1118}. This decrease was independent of age in male P{XP}Nrx^{d08766} flies, since there was a decrease across all ages, but was age dependent in female P{XP}Nrx^{d08766} flies whereby significantly decreased total sleep was only seen at 11 days old. By contrast total sleep in the dark phase was increased in P{XP}Nrx^{d08766} flies in both sexes (Figure 9.1). The lack of total sleep in the light phase in P{XP}Nrx^{d08766} flies appeared to be compensated by increased total sleep in the dark phase. The reduction in total sleep in the light phase in female P{XP}Nrx^{d08766} flies at 11 days old only, suggests *Nrx-1* hyperfunction caused a developmental effect on sleep at young age in females that normalises by 18 days old. Female P{XP}Nrx^{d08766} flies also showed an increase in the number of sleep bouts and a decrease in sleep bout duration in both the light and the dark phases as compared to female w^{1118} flies, an effect that was not seen in male flies (Figure 9.2). This effect of *Nrx-1* hyperfunction on sleep bout parameters may have only been observed in female P{XP}Nrx^{d08766} flies due to males having maximal bout length and minimal bout number already, which is supported by sex differences found in both the current study and previous studies. In the current study male w^{1118} control flies had

more consolidated sleep (deceased bout number and increased bout length) compared to females w^{1118} control flies (Figure 9.2), and therefore *Nrx-1* hyperfunction perhaps could not affect these already low levels of sleep bout number and high levels of bout length in male flies. In further support, previous studies have shown increased bout length and decreased bout number in male compared to female flies (Koh *et al.*, 2006; Zimmerman *et al.*, 2012).

***Nrx-1* dysfunction alters sleep structure in flies**

Other studies have found that deletion or hypofunction of *Nrx-1* in flies causes alterations in total sleep and sleep structure. Larkin *et al.* studied sleep in a number of *Nrx-1* KO lines with varying deletions in *Nrx-1.* Overall they found that male *Nrx-1* KO flies showed decreased total sleep in the light phase only, and increased number of sleep bouts and decreased sleep bout duration in both light and dark phases (Larkin *et al.*, 2015). In female *Nrx-1* KO flies they found increased total sleep and increased bout number in both the light and dark phases, and decreased bout duration in the dark phase only (Larkin *et al.*, 2015). Increased number of sleep bouts and decreased bout duration is known as sleep fragmentation. Tong *et al.* also investigated the effects of *Nrx-1* KO and *Nrx-1* hypofunction on sleep in male flies and found that both KO and hypomorph flies had decreased total sleep and bout duration, and increased bout number and in the dark phase (Tong *et al.*, 2016). Although there are some mixed findings regarding how *Nrx-1* deletions or *Nrx-1* hypofunction effects sleep, previous studies agree that increased sleep fragmentation occurs in *Nrx-1* KO and hypomorph flies (Larkin *et al.*, 2015; Tong *et al.*, 2016).

For certain sleep parameters the P{XP}Nrx^{d08766} (*Nrx-1* hypermorph) flies in the current study show significant changes, as compared to control flies, that are in the opposite direction to *Nrx-1* KO and hypomorph flies in previous studies. In this way, the current study

found that *Nrx-1* hyperfunction in the P{XP}Nrx^{d08766} flies caused a reduction in sleep bout number and an increase in sleep bout length in female P{XP}Nrx^{d08766} flies only in both light and dark phases (less sleep fragmentation). By contrast previous studies have shown male and female *Nrx-1* KO flies have increased sleep fragmentation in both light and dark phases (Larkin *et al.*, 2015; Tong *et al.*, 2016), and male *Nrx-1* hypomorph flies have increased sleep fragmentation in the dark phase (Tong *et al.*, 2016). Opposing results for *Nrx-1* KO/hypomorph flies in previous studies and *Nrx-1* hypermorph (P{XP}Nrx^{d08766}) flies in the current study have also been found for total sleep in the dark phase. The current study found increased total sleep in dark phase in both male and female P{XP}Nrx^{d08766} flies, and previous studies have shown that male *Nrx-1* KO and *Nrx-1* hypomorph flies have reduced total sleep in dark phase (Tong *et al.*, 2016). However another study found no difference in total sleep in the dark phase in male *Nrx-1* KO flies, and found female *Nrx-1* KO flies to have increased total sleep in the dark (Larkin *et al.*, 2015), which does not support the findings of the current study.

Findings for total sleep in the light phase in the current study were partially supported by findings from previous studies. The current study found that *Nrx-1* hyperfunction in the P{XP}Nrx^{d08766} flies caused decreased total sleep in the light phase in females at 11 days old only, and Larkin *et al.* found that 3-8 day old female *Nrx-1* KO flies had increased total light phase sleep, supporting this finding. However previous findings in male flies did not support the current study since decreased total sleep in the light phase was found in both male P{XP}Nrx^{d08766} flies, in the current study, and male *Nrx-1* KO flies in a previous study (Larkin *et al.*, 2015).

Findings from *Nrx-1* overexpression studies also support the current findings in the P{XP}Nrx^{d08766} flies. Larkin *et al.* overexpressed *Nrx-1* in female flies and found that both acute overexpression in adult flies and overexpression throughout development increased total sleep and reduced fragmentation (mainly in the dark phase) (Larkin *et al.*, 2015). This supports the current findings of increased total sleep, increased bout length and decreased bout number in the dark phase in female the P{XP}Nrx^{d08766} flies. The reduced sleep fragmentation caused by *Nrx-1* overexpression studies in Larkin *et al.* may have only been reported in female flies as there was no effect in males, which would further support the sex specific effect of *Nrx-1* hyperfunction in the current study. In further support of the current study, Tong *et al.* found that reductions in *Nrx-1* protein in flies dose-dependently reduced total sleep and bout duration and increased bout number in the dark phase when comparing male heterozygous *Nrx-1* KO, *Nrx-1* hypomorph and homozygous *Nrx-1* KO fly strains (Tong *et al.*, 2016). Since female P{XP}Nrx^{d08766} flies in the current study have increased *Nrx-1* protein and show increased total sleep and decreased sleep fragmentation in the dark phase, this supports the notion that *Nrx-1* protein dose-dependently affects total sleep and sleep fragmentation in the dark phase. Increased *Nrx-1* protein and decreased total sleep in the dark phase was also found in male P{XP}Nrx^{d08766} flies in the current study, further supporting the role of level of *Nrx-1* protein in structure of sleep.

As previously described not all the sleep parameters tested in P{XP}Nrx^{d08766} flies in the current study were shown to have changes in the opposite direction to that seen in *Nrx-1* KO or hypomorph flies in previous studies. This could be due to a number of reasons, firstly the current study included 7 age groups of flies with large age range from 11- 53 days old, whereas the previous studies used flies aged 3-8 days old (Larkin *et al.*, 2015; Tong *et al.*, 2016). Also genetic background could play a role in sleep behaviour measured since in the

Larkin *et al.* study the authors do not report that the *Nrx-1* KO flies were backcrossed to the w^{1118} control flies (Larkin *et al.*, 2015), which means that genetic background could confound their comparisons between mutant and control flies (Zimmerman *et al.*, 2012). Furthermore although the flies in Tong *et al.* were backcrossed to w^{1118} flies (Tong *et al.*, 2016), the same genetic background used in the current study, the two w^{1118} fly strains would vary genetically due to being separated for many generations in independent fly labs. Furthermore, Tong *et al.* did not report findings from sleep parameters in the light phase (Tong *et al.*, 2016) so comparisons between the P{XP}Nrx^{d08766} flies in the current study and *Nrx-1* KO and hypomorph flies in their study cannot be made for sleep parameters in the light phase.

Taken together, the findings from the current study in *Nrx-1* hypermorph P{XP}Nrx^{d08766} flies and previous studies in *Nrx-1* KO and *Nrx-1* hypomorph flies suggest that the amount of *Nrx-1* protein affects total sleep and sleep bout parameters, with decreased *Nrx-1* causing decreased total sleep and increased sleep fragmentation in the dark phase and increased *Nrx-1* causing increased dark phase sleep and decreased sleep fragmentation (Larkin *et al.*, 2015; Tong *et al.*, 2016).

Tong *et al.* found that *Nrx-1* in the $\alpha\beta_c$ and $\alpha\beta_s$ neuronal subsets in the mushroom bodies of the fly brain were required for normal total sleep in the dark phase; since experiments rescuing *Nrx-1* in the mushrooms bodies in *Nrx-1* KO flies mostly recovered the lost sleep in the dark phase, and specific KO of *Nrx-1* in the $\alpha\beta_c$ and $\alpha\beta_s$ reduced sleep in the dark phase (Tong *et al.*, 2016).

In addition to alterations in the amount and structure of sleep in *Nrx-1* mutant flies, effects on circadian rhythm and homeostatic sleep regulation have also been found. While one *Nrx-*

1 KO fly strain, 313/241, was found to have an extended free-running period compared to w^{1118} in dark-dark conditions in both male and female flies (Larkin *et al.*, 2015), other *Nrx-1* KO fly strains have been found to show no difference to control (Larkin *et al.*, 2015; Tong *et al.*, 2016). *Nrx-1* is expressed in the clock neurons in the fly brain which also suggests it may play a role in circadian rhythms (Sun *et al.*, 2016). It has also been shown that one *Nrx-1* KO fly strain, $nrx^{\Delta 83/273}$, recovered significantly less sleep after sleep deprivation compared to control, suggesting defective homeostatic regulation of sleep in these flies (Tong *et al.*, 2016).

Sleep disturbance in Sz and ASD

Although sleep disorder has so far not been widely reported in patients with *NRXN1* (2p16.3) mutations per se (Bena *et al.*, 2013), deletions in *NRXN1* increase the risk for developing conditions, Sz and ASD, in which sleep disorder has widely been reported (Tandon *et al.*, 1992; Souders *et al.*, 2009; Anderson and Bradley, 2013; Baker and Richdale, 2015; Klingaman *et al.*, 2015). These include problems with sleep maintenance (reduced total sleep), frequent waking (sleep fragmentation) and problems falling asleep (sleep onset latency) in Sz (Monti and Monti, 2004; 2005; Monti *et al.*, 2013; Chan *et al.*, 2016; Monti *et al.*, 2016) and insomnia and problems with sleep maintenance in ASD (Souders *et al.*, 2009; Baker and Richdale, 2015). The findings from both the current study and previous studies showed that *Nrx-1* is involved in sleep in the fly, particularly sleep structure (fragmentation) and amount of sleep in the dark phase. This suggests that *NRXN1α* deletions may play a role in sleep disturbance in patients, since *Nrx-1* KO caused increased fragmentation and *Nrx-1* hyperfunction caused decreased fragmentation in flies. However, since not all Sz and ASD patients have deletions in *NRXN1α* it may be that *NRXN1α* dysfunction in humans causes

dysfunction of a biological pathway which leads to sleep disturbance, in patients with normal *NRXN1α* another factor (genetic or environmental) could cause dysfunction of this biological pathway thus leading to the same sleep disturbance phenotype. Moreover, since there has been few reports of sleep disturbance specifically in patients with *NRXN1* (2p16.3) deletions (Bena *et al.*, 2013) this may suggest that another contributing factor involved in sleep disturbance in Sz and ASD.

Summary

The current study found *Nrx-1* hyperfunction caused increased sleep bout length and decreased number of sleep bouts in female flies only. *Nrx-1* hyperfunction also caused increased total sleep in the dark in both sexes across all age groups, and decreased total sleep in the light in males across all age groups and in females at 11 days old only. Previous studies in *Nrx-1* KO and hypomorph flies mostly support the findings of the current study as they found opposite effects for some parameters including decreased total sleep and increased sleep fragmentation in the dark phase. However some previous findings do not support the current study which may be due to differences between studies including age of flies used and genetic background. Heterozygous deletions in *NRXN1α* increase the risk of developing Sz and ASD. Both disorders are associated with sleep disturbance including reduced total sleep and increased sleep fragmentation. The findings of the current study support the notion that biological pathways affected by reduced *NRXN1α* may impact on sleep in Sz and ASD, since female *Nrx-1* hypermorph flies showed an opposite phenotype (decreased sleep fragmentation).

Confirmation that sex and age modifies sleep in flies

The sleep parameters measured were modified by sex and age of flies in the current study. Male w^{1118} flies had significantly higher total sleep and sleep bout length and a significantly lower number of sleep bouts in both light and dark phases compared to female w^{1118} flies (Figure 9.1 and 9.2). As flies aged they showed altered sleep amount and structure (Figure 9.3). For instance, there was increased sleep fragmentation with age evidenced by increased sleep bout number and decreased sleep bout length (Figure 9.3), which was not altered by sex or by genotype. Previous studies support the current findings of increased total sleep and bout length and decreased bout number in male compared to female flies (Koh *et al.*, 2006; Zimmerman *et al.*, 2012) and increased sleep fragmentation with age (Koh *et al.*, 2006; Metaxakis *et al.*, 2014), however some previous studies have found these effects to be dependent on light phase, genetic background or age of the flies used. Since sleep bout length and number of sleep bouts showed age-related changes in the current study, sleep analysis could be used as a measure of functional ageing in flies, and would allow detection of factors that could modify ageing. Therefore this would conclude that *Nrx-1* hyperfunction did not affect ageing as measured by sleep parameters. However, although sleep analysis can be used as a measure of ageing in flies, the age-related changes are not as robust as measuring Exploratory Walking (EW) parameters were linear changes are observed with age per week (see chapter 8). Furthermore age-related changes in sleep parameters can be light phase dependent (Koh *et al.*, 2006; Metaxakis *et al.*, 2014).

Future experiments

Since *Nrx-1* hyperfunction caused changes in sleep structure and amount in the P{XP}Nrx^{d08766} flies, it would be interesting to test whether circadian and/or homeostatic regulation of sleep is altered in these flies. This could be done by measuring the free running circadian sleep/wake cycle in dark-dark conditions (circadian regulation) in the P{XP}Nrx^{d08766} flies or by measuring how well the P{XP}Nrx^{d08766} flies recover sleep following deprivation (homeostatic regulation).

A previous study has suggested that reduced total sleep in the dark phase in *Nrx-1* KO flies is due to dysfunctional calcium signaling in αβ Kenyon cells in the mushroom bodies of the fly brain (Tong *et al.*, 2016), therefore it is likely that overexpression of *Nrx-1* in these neurons may cause increase total sleep in the dark phase in the P{XP}Nrx^{d08766} flies in the current study. Experiments involving specific *Nrx-1* overexpression or Knock-down in the αβ Kenyon cells in the mushroom bodies could be performed to confirm this. Experiments could also be performed to investigate which brain areas in the fly require normal functioning of *Nrx-1* for normal sleep structure (sleep bout number and length). This could be done by *Nrx-1* KO or overexpression in specific brain regions in the fly and measuring their effects of sleep bout number and length (sleep fragmentation).

Chapter 10 Discussion

The aim of this project was to bring together two areas of Sz research; genetic risk factors for Sz, in particular *Neurexin1*, and the accelerated ageing hypothesis. The two main research questions were: (1) does *Neurexin1α* heterozygosity, modelling *Neurexin1α* dysfunction that increases the risk of developing both Sz and ASD, bring about Sz or ASD relevant phenotypes (behaviour and cognition) and endophenotypes (brain function), and are these changes temporally regulated across adult development? (2) Does *Neurexin1* dysfunction affect life span and behavioural senescence?

10.1 *Neurexin1α* heterozygosity causes Sz and ASD relevant phenotypes and endophenotypes

Neurexin1α heterozygosity in mice caused a number of behavioural, cognitive and brain function alterations in the current study, that may have translational relevance to alterations seen in individuals with ASD and Sz. Behavioural phenotypes identified in the *Nrxn1α* Hz mice included an increased freezing response on initial exposure to a novel environment, followed by hyperlocomotor activity, and an indication of increased anxiety-like behaviour, as measured by the Open Field (OF) test (Figure 3.1 and 3.4). Cognitive phenotypes identified in the *Nrxn1α* Hz mice included deficits in recognition learning and memory, as measured by Novel Object Recognition Task (NORT), that were observed in both males and females dependant on the analysis parameters used (Figure 4.1 and 4.4). Furthermore, *Nrxn1α* Hz mice showed age-dependant improvement in associative learning, when the association being discriminated was novel, but a deficit in reversal learning due to increased regressive errors, as measured by the Cognitive Flexibility Task (CFT) (Figure 5.1,

5.3 and 5.4). There was also a potential indication of reduced cognitive processing speed in these mice, due to significantly increased trial latency in the CFT (Figure 5.1, 5.2 and 5.3). However, further testing in other paradigms would be required to confirm whether this difference reflects altered processing speed or a difference in motivation. Brain function endophenotypes in the *Nrxn1α* Hz mice included hypofrontality, and increased metabolism in areas that are part of the serotonergic (DR) and dopaminergic (VTA) neurotransmitter systems (Figure 6.1 and 6.2).

The phenotypes and endophenotypes observed in the *Nrxn1α* Hz mice have potential translational relevance to behaviour and brain function alterations seen in individuals with Sz and ASD. For instance, the increased locomotor activity observed in the *Nrxn1α* Hz mice in the OF test may; (1) relate to hyperactivity seen in Sz patients (Perry *et al.*, 2009), (2) be indicative of dopaminergic dysfunction (Creese and Iversen, 1975; Kelly *et al.*, 1975; Antoniou *et al.*, 1998) which is a prominent alteration in Sz, and (3) have translational relevance to positive symptoms of the disorder (Uchida *et al.*, 2011; Harro, 2015). It has also been suggested that the dopaminergic system may be altered in ASD (Hellings *et al.*, 2017; Paval, 2017). Furthermore, it was found that the *Nrxn1α* Hz mice have increased cerebral metabolism in the VTA (Figure 6.1), which further supports a dopaminergic disturbance in these mice. However, how this relates to the hyperlocomotor activity seen in these mice remains to be established experimentally. Intriguingly, paralleling the hyperactivity seen in *Nrxn1α* Hz mice, there is also evidence for hyperactivity in children with deletions in *NRXN1α* (Ching *et al.*, 2010; Schaaf *et al.*, 2012), as well as Sz patients (Perry *et al*., 2009) and individuals with ASD, since ADHD is a common comorbidity of ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012; Antshel *et al.*, 2016). Increased anxiety-like behaviour was also observed in the

Nrxn1α Hz mice, in the form of increased initial freezing upon entry to a novel environment, and decreased time spent in the centre of the arena, during the OF test (Figure 3.1 and 3.4). Again, this has potential translational relevance to both disorders since anxiety is a prominent symptom in Sz (Siris, 2000; Tibbo *et al.*, 2003; Braga *et al.*, 2004; Braga *et al.*, 2013; Young *et al.*, 2013; Temmingh and Stein, 2015; An Der Heiden *et al.*, 2016; Upthegrove *et al.*, 2017) and ASD (Simonoff *et al.*, 2008; Hofvander *et al.*, 2009; Mattila *et al.*, 2010; Lugnegard *et al.*, 2011; Mahajan *et al.*, 2012).

Translationally relevant cognitive phenotypes were also found in *Nrxn1α* Hz mice. Deficits in object recognition memory were found in *Nrxn1α* Hz mice (Figure 4.1 and 4.4), and have also been found in both Sz (Danion *et al.*, 1999) and ASD (Blair *et al.*, 2002). This suggests that *Nrxn1α* plays a role in the brain circuitry underlying recognition learning and memory in mice. However, brain areas involved in short-term recognition learning and memory in rodents, such as the perirhinal cortex (Winters and Bussey, 2005), hippocampus (Hammond *et al.*, 2004), and nucleus accumbens (Sargolini *et al.*, 2003), were not found to be significantly altered in *Nrxn1α* Hz mice, in terms of cerebral metabolism as measured by ^{14}C-2-DG functional brain imaging. However, *Nrxn1α* Hz mice do show altered cerebral metabolism in the prefrontal cortex and VTA, two brain regions that have a prominent role in stimulus novelty detection (Dias and Honey, 2002; Bunzeck and Duzel, 2006; Matsumoto *et al.*, 2007). Thus, the functional brain differences detected in *Nrxn1α* Hz mice suggest that the deficit in NORT performance seen in these animals may be more directly related to their inability to detect novelty rather than a deficit in short-term recognition memory *per se.* Characterising these potential mechanisms certainly warrants further systematic investigation. An immediate problem with this explanation is that *Nrxn1α* Hz mice show age-dependant improvement in associative learning when the discrimination is novel, as

evidenced in the CFT (Figure 5.1 A and B). This suggests that these animals have intact novelty detection. However, novelty detection in *Nrxn1α* Hz mice could be context or stimuli type dependant. An alternative explanation is that the deficits in the NORT detected in *Nrxn1α* Hz mice may be due to deficits in recognition memory, with deficits occurring on the cellular level, such as alterations in synaptic strength, which may not be detected by measuring regional cerebral metabolism. Moreover, while the functional metabolism of individual brain regions implicated in the memory component of NORT may not be affected, the interactions between brain regions may be altered, which could contribute to a recognition memory deficit in these mice.

Deficits in cognitive flexibility have been widely reported in both Sz and ASD in both set-shifting tasks, such as Wisconsin Card Sorting Task (WCST) (Bellini *et al.*; Zanello *et al.*; Bellini *et al.*, 1991; Scarone *et al.*, 1993; Sullivan *et al.*, 1993; Battaglia *et al.*, 1994; Abbruzzese *et al.*, 1995; Haut *et al.*, 1996; Dieci *et al.*, 1997; Stratta *et al.*, 1997; Glahn *et al.*, 2000; Egan *et al.*, 2001; Gooding and Tallent, 2002; Cavallaro *et al.*, 2003; Altshuler *et al.*, 2004; Tsuchiya *et al.*, 2005; Josman and Katz, 2006; Zanello *et al.*, 2006; South *et al.*, 2007; Sumiyoshi *et al.*, 2011; Yasuda *et al.*, 2014; Yeung *et al.*, 2016), and also reversal learning tasks (Coldren and Halloran, 2003; Waltz and Gold, 2007; Mckirdy *et al.*, 2009; D'cruz *et al.*, 2013; Schlagenhauf *et al.*, 2014; Culbreth *et al.*, 2016). Deficits in reversal learning were found in *Nrxn1α* Hz mice in the current study using the CFT. In contrast to findings in Sz and ASD patients in the WCST, where perseverative errors (PEs) underlie the observed deficit, the deficit in reversal learning in *Nrxn1α* Hz mice was caused by increased regressive errors (REs) (Figure 5.4 A). However, PEs are more widely measured in human studies of cognitive flexibility compared to REs, and so more studies may be required to confirm changes in REs in Sz and ASD. In addition, improved associative learning was found in the *Nrxn1α* Hz mice, dependant on

age, when the association was novel (Figure 5.1 A and B). A similar effect has also been shown in high functioning ASD patients (Plaisted *et al.*, 1998a), suggesting that *Nrxn1α* heterozygosity may in fact cause improvements in some cognitive domains, while causing deficits in others. The CFT findings have some alignment with alterations in cerebral metabolism identified in *Nrxn1α* Hz mice (discussed in detail in section 6.6). For instance, altered cerebral metabolism in the Prelimbic cortex (aPrl and mPrl) (Figure 6.1 A-C) may have caused reduced working memory capacity in *Nrxn1α* Hz mice (Granon *et al.*, 1994; Kesner *et al.*, 1996; Taylor *et al.*, 2003) and contributed to an increase in REs during reversal learning. Furthermore, studies have shown that Orbital Frontal Cortex lesions cause increased perseveration during reversal learning in rodents (Chudasama and Robbins, 2003; Kim and Ragozzino, 2005; Ghods-Sharifi *et al.*, 2008), and while a reversal learning deficit was seen in the *Nrxn1α* Hz mice, this was not accompanied by increased Perseverative Errors (PEs), and no change in cerebral metabolism in the OFC was found.

In terms of the translational relevance of the functional brain endophenotypes seen in *Nrxn1α* Hz mice, hypofrontality was found in these animals using ^{14}C-2-Deoxyglucose functional brain imaging (Figure 6.1 A-C). Hypofrontality has been found in resting-state brain imaging studies in both Sz (Ingvar and Franzen, 1974; Buchsbaum *et al.*, 1982; Farkas *et al.*, 1984; Wolkin *et al.*, 1985; Weinberger *et al.*, 1986) and ASD (Ohnishi *et al.*, 2000), and has also been found under task conditions in both disorders (Andreasen *et al.*, 1992; Steinberg *et al.*, 1996; Hazlett *et al.*, 2000; Hazlett and Buchsbaum, 2001; Luna *et al.*, 2002; Silk *et al.*, 2006; Kana *et al.*, 2007; Mitelman *et al.*, 2018). There was also evidence for both dopaminergic (VTA) and serotonergic (DR) neurotransmitter system functional alterations in the *Nrxn1α* Hz mice (Figure 6.1 A ans D and 6.2). These neurotransmitter systems have been

implicated in the aetiology of both Sz and ASD (Uchida *et al.*, 2011; Selvaraj *et al.*, 2014; Harro, 2015; Muller *et al.*, 2016; Hellings *et al.*, 2017; Paval, 2017).

10.2 Some cognitive phenotypes and functional brain endophenotypes found in *Nrxn1α* Hz mice are temporally regulated across adult development

There was evidence for age-dependent changes in cerebral metabolism and cognitive flexibility that were altered in *Nrxn1α* Hz mice. In Wt mice cerebral metabolism in the DR significantly increased between 3 and 6 months of age. By contrast, cerebral metabolism in the DR does not change between these timepoints in *Nrxn1α* Hz mice, and they show increased metabolism in this region in comparison to Wt mice selectively at 3 months old (Figure 6.2). Therefore, the *Nrxn1α* Hz mice do not undergo the developmental increase in cerebral metabolism in the DR seen in Wt mice, between 3 and 6 months of age, and instead cerebral metabolism in the DR remains constant across all age groups measured in *Nrxn1α* Hz mice (Figure 6.2). This suggests that the development of DR function during adult maturation is altered in *Nrxn1α* Hz mice. It may be the case that the developmental events mediating this effect do not occur in *Nrxn1α* Hz mice. Alternatively, *Nrxn1α* heterozygosity may cause this developmental change in DR function to occur earlier in *Nrxn1α* Hz mice (prior to 3 months old), which could be tested by characterising cerebral metabolism in younger mice.

In the CFT, aspects of associative learning and reversal learning were also found to be differentially regulated across adult development in *Nrxn1α* Hz mice, evidenced by changes in specific parameters in OD1 and OD2R phases of the task. These included enhanced associative learning (OD1) in 3 month old *Nrxn1α* Hz mice in terms of significantly increased

Percentage Correct (PC), and in 9 month old *Nrxn1α* Hz mice in terms of significantly increased PC and significantly reduced Trials to Criterion (TTC). No differences were found in either parameter in 6 and 12 month old *Nrxn1α* Hz mice. Furthermore, a reversal learning deficit (OD2R) was found in 12 month old *Nrxn1α* Hz mice, in terms of significantly increased TTC, which was not found in any other age group. PC was significantly reduced in OD2R across all ages of *Nrxn1α* Hz mice. This suggests that some aspects of altered associative learning and reversal learning in *Nrxn1α* Hz mice may be differentially regulated during adult development.

In support of the age-dependant changes in some of the phenotypes and brain imaging endophenotypes found in *Nrxn1α* Hz mice, there is evidence for temporal regulation of *Nrxn1α* expression throughout the life span from both human (Jenkins *et al.*, 2016) and mouse (Kumar and Thakur, 2015) studies. In humans *NRXN1α* and *NRXN1β* expression in the PFC increases as gestational age increases, but then levels off with age postnatally, with the highest peak of expression being between birth and 3 years old (Jenkins *et al.*, 2016). This finding of increased expression of *NRXN1* as gestational age increases is supported by another study, which looked at human cortical tissue at postconceptional weeks 8-12 (Harkin *et al.*, 2017). The findings from human studies suggest that most of the effects of altered *NRXN1* expression may take place early in life, both before birth and up to 3 years old.

While findings from human studies suggest that the biggest changes in *NRXN1* expression occur early in life, findings in the mouse suggest *Nrxn1* expression in the brain does alter during adult development. One study investigated *Nrxn1* expression in the cerebral cortex and hippocampus of male Swiss albino mice at 10 (~2.5 months), 30 (~7.5 months), 50

(~12.5 months) and 80 (20 months) weeks old (Kumar and Thakur, 2015). They found that *Nrxn1* expression was significantly increased at 30 weeks old (~7.5 months) and significantly decreased at 50 weeks old (~12.5 months) when compared to 10 weeks old (~2.5 months), at both the mRNA and protein level. This suggests that cortical and hippocampal *Nrxn1* is temporally regulated across adult development in mice. Therefore, the age-dependent alterations seen in *Nrxn1α* Hz mice DR cerebral metabolism, associative learning and reversal learning may be sensitive to changes in *Nrxn1* expression during the time points measured in the current study. However, the time points which show altered expression of *Nrxn1* in mice do not match exactly with age-dependent cognitive and cerebral metabolism changes found in the *Nrxn1α* Hz mice in the current study. Interestingly, age-dependent alterations in cerebral metabolism in cortical regions were found in the currect study. However, a linear increase in cerebral metabolism in cortical regions as mice age was identified (Figure 6.3), which does not match the pattern of altered *Nrxn1* expression reported by Kumar and Thakur. Moreover, age-dependent changes in cortical cerebral metabolism was not found to be impacted on by *Nrxn1α* heterozygosity. Overall, these data suggest that age-dependent changes in cortical cerebral metabolism are not strongly influenced by alterations in *Nrxn1* expression. Increased cerebral metabolism in the DR was found at 3 months only in *Nrxn1α* Hz mice which does not match the pattern of *Nrxn1* expression in mice during adult development in Kumar and Thakur's study. However, *Nrxn1* expression in this study was only characterised in cortex and hippocampus. This could suggest that this effect in *Nrxn1α* Hz mice is not related to normal *Nrxn1* expression during these time points, or is only indirectly related with other factors that influence cerebral metabolism in the DR. While altered *Nrxn1* expression during mouse adult development may influence associative learning (observed at 3 and 9 months old) and reversal learning

(observed at 12 months) in *Nrxn1α* Hz mice, dependent on parameter measured, this is complicated and would require further study. Furthermore the genetic background of the mice used in Kumar and Thakur's study (Swiss albino) and the current study (C57BL/6NCrl) differed, and so *Nrxn1* expression during adult development may differ between backgrounds.

10.3 Deletions in *NRXN1α* may increase the risk of developing Sz and ASD by altering brain function and cognition

A large amount of genetic association studies have found deletions in *NRXN1α* to increase the risk of developing Sz and ASD, however how this occurs is unknown. The findings of behavioural, cognitive and brain function changes in the *Nrxn1α* Hz mice in the current study, which are similar to changes found in Sz and ASD, provide information about how deletions in *NRXN1α* may increase the risk of developing these disorders in humans.

Nrxn1α has been found to play a role in presynaptic calcium release in neurons, thus influencing neurotransmission (Zhang *et al.*, 2005; Etherton *et al.*, 2009). This altered neurotransmission may differ depending on brain region, possibly due to differing splice variants (Ullrich *et al.*, 1995; Schreiner *et al.*, 2014; Treutlein *et al.*, 2014), which may bring about the different phenotypes and endophenotypes found in *Nrxn1α* Hz mice in the current study. These changes in neurotransmission are likely to bring about changes in behaviour, cognition and brain function in *Nrxn1α* Hz mice (Etherton *et al.*, 2009). This suggests that altered neurotransmission could contribute to alterations in brain function and cognition in Sz and ASD patients. Deletions in *NRXN1α* are estimated to be found in 0.16% of Sz (Kirov, Rujescu*, et al.*, 2009) and 0.5% ASD cases (Etherton *et al.*, 2009), therefore characteristics observed in these disorders cannot be completely explained by

alterations in *NRXN1α.* This means it is likely that the effects of different genetic or other risk factors converge on the same biological pathway, to bring about the same alterations in brain function and cognition in Sz or ASD. Support for this argument comes from similar phenotypes or endophenotypes being reported in different mouse models of genetic or environment risk factors for Sz or ASD. For example, hyperlocomotor activity was observed in the *Nrxn1α* Hz mice in the current study (Figure 3.1) and has been observed in other mouse models of genetic risk factors for Sz and/or ASD. These include mice with mutations in *DISC1* (Hikida *et al.*, 2007; Dachtler *et al.*, 2016) and *Fmr1 KO* (Qin *et al.*, 2002; 2005; Coffee *et al.*, 2009). Hyperlocomotor activity has also been observed in mouse models of environmental risk factors of Sz including maternal stress (Matrisciano *et al.,* 2013; Bronson and Bale, 2014). Similarly, deficits in short-term novel object recognition memory were found in *Nrxn1α* Hz mice in the current study (Figure 4.1 and 4.4), and this phenotype has also been found in male (Duffy *et al.*, 2010), but not female (Chesworth *et al.*, 2012), *Neuregulin1* (*NRG1*) heterozygous mice, which model genetic risk for Sz. Deficits in novel object recognition memory have also been found in mouse models of ASD including 16p11.2 heterozygous deletion mice (Pucilowska *et al.*, 2015; Yang *et al.*, 2015) and *Shank3* homozygous KO mice (Yang *et al.*, 2012; Jaramillo *et al.*, 2016). Environmental risk factors for ASD, such as prenatal exposure to valproic acid, also cause a deficit in short term object recognition learning and memory in mice (Takuma *et al.*, 2014). In the current study *Nrxn1α* Hz mice show enhanced associative learning and a deficit in reversal learning in the CFT (Figure 5.1, 5.3 and 5.4). Other mouse models of genetic and environmental risk factors for ASD have shown similar phenotypes, including *Fmr1* KO mice (Dickson *et al.*, 2013; Nolan and Lugo, 2018) and C57BL/6 mice prenatally exposed to valproic acid (Puscian *et al.*, 2014).

Both models showed intact associative learning and impaired reversal learning, in different cognitive testing paradigms.

Further evidence to support various genetic and environmental risk factors of Sz and ASD converging on common biological pathways, to bring about similar phenotypes in animal models, comes from functional brain imaging studies. Mouse models of genetic risk factors for Sz and ASD that have shown hypofrontality include *Nrxn1α* Hz mice in the current study (Figure 6.1), mice with truncated *DISC1* (Dawson *et al.*, 2015) and *Fmr1* KO mice (Qin *et al.*, 2002). Furthermore mice modelling the 16p11.2 deletion, associated with ASD, also show PFC dysfunction (Bertero *et al.*, 2018).

For some of these models there is also evidence for direct translational relevance to humans. Deletions in 16p11.2, a CNV which like *NRXN1α* increases the risk of developing ASD, have been shown to reduce PFC connectivity in both humans and mice (Bertero *et al.*, 2018). In further support, increased polygene risk score for *MIR137*, a gene that has been strongly associated with Sz (Psychiatric and Consortium, 2014), was found to be associated with poor memory in Sz patients and healthy control subjects (Cosgrove, Harold*, et al.*, 2017). Parelleling this, *MIR137* Hz deletion mice also show memory deficits (Cheng *et al.*, 2018). These include deficits in spatial memory and short-term social recognition memory, the latter being similar to the deficit in short-term object recognition memory seen in the *Nrxn1α* Hz mice in the current study (Chapter 4). These data suggest that multiple genetic risk factors for Sz and ASD, including *Nrxn1α*, increase the risk of developing these disorders by altering brain function in circuits that contribute to short-term memory. However, there are currently no human studies investigating the effects of deletions in *NRXN1α* on

cognition and brain function, which could more directly support translational relevance of these phenotypes found in mice.

10.4 Future investigation: Multiple risk factors may converge on common biological pathways which cause Sz or ASD

The current study highlighted a number of areas that require further investigation in the *Nrxn1α* Hz mice. For instance, further study is required to understand the mechanism underlying the reversal learning deficit and increased trial latency observed in *Nrxn1α* Hz mice in the CFT. The reversal learning deficit and increased regressive errors may be due to dysfunctional working memory, which could be tested using tasks such as touch screen Trial-Unique delayed Nonmatching-to-Location (TUNL) task (Oomen *et al.*, 2013), or the odour-span task (Young *et al.*, 2007). Increased trial latency seen in the *Nrxn1α* Hz mice may be due to slowed cognitive processing speed or reduced motivation to collect food rewards. This could be tested using the 5-choice serial reaction time test (Remmelink *et al.*, 2017) which would enable the separation of task response time and time to collect the food reward, thus indicating a problem with processing speed or motivation for food rewards. Brain function in the the *Nrxn1α* Hz mice could also be investigated further by analysing changes in cerebral glucose metabolism at the network level, for instance using graph theory (Dawson *et al.*, 2012; Dawson *et al.*, 2015). This may reveal brain endophenotypes that are relevant to Sz or ASD or behavioural alterations observed in *Nrxn1α* Hz mice.

To further investigate whether multiple genetic and environmental risk factors cause alterations in common pathways, thus bringing about alterations in brain function and cognition in Sz and ASD, further work must be done comparing different mouse models of these disorders. Different mouse models of risk factors for Sz and ASD could be directly

compared in studies to uncover differences and similarities in phenotypes and endophenotypes. These common phenotypes and endophenotypes could then be investigated in detail to uncover common underlying mechanism or affected biological pathways. For example, common alterations in neurotransmitter system dysfunction, regional brain function or functional brain network connectivity could be characterised. In addition, further studies comparing animal models and human studies of risk factors for Sz and ASD could be performed, in order to test how translatable phenotypes in animal models are to humans. This type of work has already begun (Bertero *et al.*, 2018).

10.5 *Neurexin1* hyperfunction reduces measures of ageing

The current study found that the overexpression of *Nrx-1* in the P{XP}Nrx^{d08766} flies caused a small but significant life span extension (section 8.3.1) and reduced behavioural senescence, evidenced by reduced age-related changes in some Exploratory Walking (EW) parameters (section 8.3.2). *Nrx-1* hyperfunction appears to reduce brain and neuromuscular ageing in flies evidenced by an amelioration of the age-related increase in time spent in the central zone, and a delay in the age-related decline in walking distance and rotation frequency (Figure 8.3 A and 8.4 A). Taken together, the EW findings and life span analysis in the current study, and previous work supporting life span reduction in *Nrx-1* KO flies (Zeng *et al.*, 2007), suggest that *Nrx-1* signaling may play a role in modulating mechanisms of cellular ageing in flies. Further experiments are required to determine if other markers of ageing, such as measures of oxidative stress and fecundity in female flies (discussed in section 8.4.4), are reduced in the P{XP}Nrx^{d08766} flies to confirm the effects of *Nrx-1* on ageing.

The P{XP}Nrx^{d08766} flies used in the current study do not model deletions in *NRXN1α* that increase the risk of developing Sz and ASD in humans, which likely reduces the amount of

NRXN1α protein in the brain. Since overexpression of *Nrx-1* reduced measures of ageing in the current study and KO of *Nrx-1* increased them (shortened life span) (Zeng *et al.*, 2007), one may predict that *NRXN1* hypofunction could increase measures of ageing. This would be in line with the accelerated ageing hypothesis of Sz, since deletions in *NRXN1α* increase the risk of developing Sz. However, this would need to be tested in flies which have lowered, but not completely ablated, *Nrx-1* expression. In line with the current evidence and with further investigation, it may be hypothesised that deletions in *NRXN1α* may contribute to shortened life span (Laursen *et al.*, 2012; Laursen *et al.*, 2014; Lee *et al.*, 2018) and markers of accelerated ageing (Borgwardt *et al.*, 2009; Kirkpatrick and Kennedy, 2018) found in Sz patients. However, a recent study found that increased genetic risk for Sz itself was not associated with early mortality. Laursen *et al.* found that increased polygenic risk score for Sz, a cumulative effect of common loci which increase the risk of developing the disorder, did not increase mortality in a case control study (Laursen *et al.*, 2017). Similarly another study found that polygenic risk score was not associated with shortened telomeres, a possible marker of ageing, in Major Depressive Disorder, BD and Sz patients (Palmos *et al.*, 2018). These studies suggest that genetic predisposition to Sz does not affect measures of ageing such as early mortality or shortened telomeres. However, these studies only looked at common low penetrance genetic variations (SNPs) rather than rare high penetrance variations (e.g. CNVs), and so the effect of these rare mutations, such as deletions in *NRXN1α,* on mortality are unknown. Since studies so far show no association between increased genetic risk for Sz and early mortality or signs of accelerated ageing (Laursen *et al.*, 2017; Palmos *et al.*, 2018), it is likely that early mortality in Sz is due to other factors such as cardiovascular disease (Brink *et al.*, 2017; Correll *et al.*, 2017; Westman *et al.*, 2018).

Since deletions in *NRXN1α* are not present in all individuals with Sz, any potential effects of these deletions on accelerated ageing could not be the only cause or contributing factor.

10.6 Future investigation: Could deletions in *NRXN1α* contribute to accelerated ageing in Sz?

Firstly, experiments are required to confirm the effects of *Nrx-1* on ageing in flies, by testing whether other markers of ageing are reduced in *Nrx-1* hypermorph flies, and also if measures of ageing are increased in *Nrx-1* hypomorph flies (discussed in detail in section 8.4.4). The impact of increased and decreased expression of *Nrx-1* on age-related cognitive decline in the fly could also be tested, using paradigms such as classical conditioning (Burger *et al.*, 2010), to provide further indications about *Nrx-1* function and brain ageing. If the results of these studies confirm the working hypothesis that reduced *Nrx-1* increases measures of ageing in the fly, this could then be investigated in mammals e.g. in the *Nrxn1α* Hz mouse model. Age-related changes in cognition and brain function could be tested using young (3 month) and aged (18-20 months old) *Nrxn1α* Hz and Wt mice, to investigate effects *Nrxn1α* heterozygosity on ageing, in line with the indications from the fly experiments.

10.7 Conclusion

Overall the data presented in this thesis have shown that *Nrxn1α* heterozygosity, which increases the risk of developing Sz and ASD, causes phenotypes and endophenotypes in mice that are relevant to both disorders, including deficits in cognitive flexibility and hypofrontality. Therefore, *Nrxn1α* heterozygosity may increase the risk of developing these disorders by altering brain function and cognition. Furthermore a selection of phenotypes and endophenotypes seen in *Nrxn1α* Hz mice are differentially regulated across adult development, including enhanced associative learning and increased DR cerebral

metabolism. These data have also shown that increased expression of *Nrx-1* causes a small but significant life span extension and reduces some measures of behavioural senescence in the fly. Further work is required to confirm whether *Neurexin1* expression affects the ageing process, and whether this may relate to the accelerated ageing hypothesis of Sz. In conclusion, these data suggest that *Nrxn1α* Hz mice provide a useful translational model for drug discovery. In addition, some effects of *Neurexin* may be mediatiated through the modification of ageing processes, however further research is required to confirm this.

Appendix

A1 CFT testing sheets

Below is an example of the CFT testi sheet for one animal.

Animal ID:
Odour Discrimination 1 (*rewarded): (1) (2)

Trial	Rewarded Side	Correct/Incorrect	Latency (secs)
1	R		
2	L		
3	R		
4	L		
5	R		
6	R		
7	L		
8	R		
9	R		
10	L		
11	L		
12	R		
13	R		
14	L		
15	L		
16	R		
17	R		
18	L		
19	L		
20	L		
21	L		
22	L		
23	R		
24	L		
25	R		
26	L		
27	L		
28	L		
29	L		
30	R		
31	L		
32	R		
33	L		
34	R		
35	L		

36	R		
37	R		
38	L		
39	L		
40	L		

Animal ID:

Odour Discrimination 2 (*rewarded): (1) (2)

Trial	Rewarded Side	Correct/Incorrect	Latency (secs)
1	L		
2	R		
3	R		
4	L		
5	L		
6	R		
7	R		
8	L		
9	L		
10	R		
11	L		
12	L		
13	R		
14	L		
15	R		
16	R		
17	R		
18	L		
19	R		
20	R		
21	L		
22	L		
23	L		
24	R		
25	R		
26	L		
27	R		
28	L		
29	L		
30	R		
31	R		
32	L		
33	L		
34	R		
35	L		

36	R		
37	R		
38	L		
39	R		
40	L		
41	R		
42	R		
43	L		
44	L		
45	L		
46	L		
47	L		
48	R		
49	R		
50	R		
51	R		
52	L		
53	R		
54	R		
55	R		
56	R		
57	R		
58	L		
59	R		
60	R		

Animal ID:

Odour Discrimination 2 Reversal (*rewarded): (1) (2)

Trial	Rewarded Side	Correct/Incorrect	Latency (secs)
1	L		
2	R		
3	L		
4	L		
5	R		
6	R		
7	L		
8	R		
9	R		
10	R		
11	R		
12	L		
13	R		
14	R		

15	L		
16	L		
17	R		
18	L		
19	L		
20	R		
21	L		
22	L		
23	R		
24	R		
25	L		
26	R		
27	R		
28	R		
29	L		
30	L		
31	L		
32	L		
33	R		
34	R		
35	R		
36	L		
37	R		
38	L		
39	R		
40	L		
41	R		
42	R		
43	L		
44	R		
45	L		
46	R		
47	L		
48	R		
49	R		
50	L		
51	L		
52	R		
53	L		
54	L		
55	L		
56	R		
57	R		
58	R		
59	R		

60	R		

Animal ID:
Stimuli Shift (Rewarded side, Left or Right):
Odour pair used: (1) (2)

Trial	Odour (1) Side	Correct/Incorrect	Latency (secs)
1	R		
2	R		
3	L		
4	R		
5	L		
6	R		
7	L		
8	L		
9	R		
10	L		
11	R		
12	R		
13	L		
14	R		
15	L		
16	L		
17	L		
18	R		
19	R		
20	L		
21	L		
22	L		
23	R		
24	L		
25	R		
26	R		
27	L		
28	R		
29	R		
30	R		
31	R		
32	R		
33	R		
34	L		
35	R		
36	R		
37	L		

38	L		
39	L		
40	L		
41	R		
42	L		
43	L		
44	L		
45	L		
46	R		
47	R		
48	R		
49	R		
50	L		
51	L		
52	R		
53	R		
54	L		
55	L		
56	R		
57	L		
58	R		
59	L		
60	R		

A2 The impact of *Nrxn1α* heterozygosity, sex and age on blood glucose and plasma ^{14}C-2DG levels

Nrxn1α heterozygosity and sex impact on blood glucose levels

Genotype	Wt	*Nrxn1α* Hz
Blood glucose average (mmol/L)	6.6	7.4
Standard Deviation	1.6	1.6
p value (ANOVA)	0.0113	

Table 1. *Nrxn1α* Hz mice show higher blood glucose levels than Wt mice. Table shows averages and standard deviations of blood glucose levels (mmol/L) per genotype and p value of significant effect of genotype from ANOVA.

Sex	Female	Male
Blood glucose average (mmol/L)	6.4	7.5
Standard Deviation	1.4	1.7
p value (ANOVA)	0.0035	

Table 2. Male mice show higher blood glucose levels than female mice. Table shows averages and standard deviations of blood glucose levels (mmol/L) per sex and p value of significant effect of sex from ANOVA.

Age impacts on plasma ^{14}C-2DG levels

Age	3	6	9	12
Plasma ^{14}C-2DG average (mmol/L)	5.3E-07	6.1E-07	8.9E-07	1.1E-06
Standard Deviation	3.3E-07	3.2E-07	6.3E-07	6.0E-07
p value (Post hoc)			0.0591†	0.0081†, 0.0182●

Table 3. Plasma ^{14}C-2DG levels increase with age in the age groups tested. Table shows averages and standard deviations of plasma ^{14}C-2DG levels (mmol/L) per age group and p values for groups which have significantly different plasma ^{14}C-2DG levels from post hoc testing with Pairwise t-testing with FDR correction. † denotes significant increase compared to 3 month old group, ● denotes significant increase compared to 6 old month group

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