The impact of the interaction between Neuroligin3 and CYFIP1 on phenotypes associated with Autism Spectrum Disorders

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Contents

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Abstract

Autism spectrum disorders (ASD) are characterised by alterations in behaviour, brain structure and molecular processes. The underlaying genetic aetiology is complex, with many genes linked to ASD. However, there might be convergence in the function of protein products of these genes, leading to the characteristic phenotypes associated with ASD. The role of two proteins associated with ASD, Neuroligin3 and CYFIP1, in establishing these phenotypes was investigated. The interaction between them was confirmed, in neurons, *in vivo*. Additionally, several other proteins associated with ASD were found to interact with Neuroligin3, indicating that they might contribute to the same biological pathway. Interestingly, the interactors of Neuroligin3 differed between neurons and glia, suggesting that they were cell population specific. Double mutant mice lacking *Nlgn3* and heterozygous for *Cyfip1* were generated to investigate the impact of the Neuroligin3/CYFIP1 interaction on mouse behaviour, dendritic spine density and RNA expression. The double mutant mice phenocopied their littermates with *Nlgn3* deletion in their hyperactivity. However, motor learning, which is impaired in males heterozygous for *Cyfip1*, was restored in the double mutant males, this finding suggesting that Neuroligin3 could inhibit the function of CYFIP1 at the molecular level. Two other parameters modulated the effect of these genetic mutations on behaviour: sex of the mice and the social environment in which they were reared. There might be an increase of dendritic spine density in the motor cortex in the double mutants, but this effect did not extend to the visual cortex, suggesting that increased genetic load led to region-specific alterations in this parameter. On the other hand, the RNA expression in the hippocampus was only affected by the *Nlgn3* deletion and was again modulated by the social environment. In particular, the transcriptome of *WT* and *Nlgn3* knockout males differed between those housed with littermates of the same and of different genotype. In conclusion, the interaction between Neuroligin3 and CYFIP1 affected the behaviour of mice, the dendritic spine density in the motor cortex and the transcriptome in the hippocampus and this effect was further modulated by sex of the mice and their social environment. These findings support the fact that ASD is likely to result from complex interaction between genes and the environment.

Abbreviations

ABI1	Abl interactor 1
ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analysis of Variance
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ARP2/3	Actin-related protein 2/3
ASD	Autism Spectrum Disorders
BDNF	brain-derived neurotrophic factor
Bp	base-pair
°C	Degrees Celsius
Cb	Cerebellum
CREB1	CAMP responsive element binding protein 1
cDNA	complementary DNA
CNS	Central Nervous System
CNV	Copy Number Variants
cm	centimetre
CRISPR	clustered regularly interspaced short palindromic repeats
Cx	Cortex
CYFIP1	Cytoplasmic FMR1-interacting protein 1
DHPG	3,5-Dihydroxyphenylglycine
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
eIF4E	eukaryotic translation initiation factor 4E
ESC	embryonic stem cells
FAST	Flexible Accelerated STOP tetracycline Operator (tetO)-knockin
FMRP	fragile X mental retardation 1
FRET	Förster Resonance Energy Transfer
GABA	gamma-Aminobutyric acid, or γ-aminobutyric acid
GLM	Generalised linear model
GO	Gene Ontology
GWAS	Genome-wide association study
h	hours
HEK293	Human embryonic kidney 293 cells
HSD	Honestly Significant Difference

IgG	Immunoglobulin G
iPSCs	induced pluripotent stem cells
IRES	internal ribosome entry site
LTD	long-term depression
LTP	long-term potentiation
M	molar
M1	Primary motor cortex
mAb	monoclonal antibody
mg	miligram
MGH	mixed genotype housing
mGluR	metabotropic glutamate receptor
min	minutes
ml	mililiter
mM	milimolar
mRNA	messenger RNA
ms	milisecond
NAP1	Nucleosome Assembly Protein
ng	nanogram
NGF	Nerve Growth Factor
NMDAR	N-Methyl-d-aspartic acid receptor
n.s.	not significantly different
OCD	Obsessive Compulsive Disorder
OMP	Olfactory Market Protein
P60	postnatal day 60
pAb	polyclonal antibody
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PDD-NOS	Pervasive Developmental Disorder-Not Otherwise Specified
PI3K	phosphoinositide 3-kinase
PSB	Phosphate-Buffered Saline
Pv+	Parvalbumin-expressing
Q-Q plot	quantile-quantile plot
RNA	ribonucleic acid
rpm	revolutions per minute
s	seconds
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SE	standard error
SGH	single genotype housed
SNP	single-nucleotide polymorphisms
St	Striatum
STED	Stimulated Emission Depletion Microscopy
TBE	Tris-borate-EDTA
TrkB	tropomyosin receptor kinase B
tRNA	transfer RNA
µm	micrometer
µl	microliter
USV	ultrasonic vocalisation
UTR	untranslated region
V1	Primary visual cortex
VCA	verprolin-homology central acidic region
VNO	vomeronasal organ
WAVE1	WASP-family verprolin homologous protein 1
WGCNA	weighted gene correlation network analysis
WIRS	WAVE regulatory complex (WRC) via the WRC interactor receptor sequence
WRC	WAVE regulatory complex
WT	wild type

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Contributions to published work

Work presented in this thesis was published in a series of journal articles which are detailed below.

Chapters 1 and 7 of this thesis contain the discussion of the literature supporting the participation of *Nlgn3*, *Cyfip1* and *Fmr1* in the same biological pathway, which impacts on long-term depression (LTD), dendritic morphology and behaviour. These observations have also been combined to form a review, available here:

Sledziowska M., Galloway J., Baudouin S.J. (2020). Evidence for a Contribution of the *Nlgn3/Cyfip1/Fmr1* Pathway in the Pathophysiology of Autism Spectrum Disorders. *Neuroscience* 445:31-41.

Chapter 3 contains the investigation into the interctome of Neuroligin3 in neurons and glia in the mouse model. These experiments were included as part of the following publication:

Bachmann S.O., **Sledziowska M.**, Cross E., Kalbassi S., Waldron S., Chen F., *et al.* (2019). Behavioral training rescues motor deficits in *Cyfip1* haploinsufficiency mouse model of autism spectrum disorders. *Translational Psychiatry* 9(1):29.

Experiments on the combined impact of *Nlgn3* deletion and *Cyfip1* haploinsufficiency on mouse behaviour, dendritic spine density and RNA expression are contained in chapters 4, 5 and 6. Social environment and the sex of the animals were included as modulating factors. These findings formed the basis for the following publication:

Sledziowska M., Kalbassi S., Baudouin S.J. (2020). Complex interactions between genes and social environment cause phenotypes associated with Autism Spectrum Disorders in mice. *eNeuro* 7(4).

Chapter 1: Introduction

Neurodevelopmental disorders are multifaceted conditions, where the development of the central nervous system is disturbed (Mullins *et al.* 2016). They are characterised by deficits in communication, cognition and motor function resulting from altered brain development. These disorders are diagnosed based on clusters of symptoms that tend to occur together. Autism Spectrum Disorders are defined as a group of neurodevelopmental disorders, where the verbal and non-verbal communication as well as behaviours and interests of a person are affected from an early age.

The aetiology of Autism Spectrum Disorders (ASD) remains elusive. The genetics behind ASD is complex and not entirely understood. The associated symptomology appears to be simple, however many comorbid deficits and conditions are discovered upon a closer look (Geschwind 2009). Autism Spectrum Disorders (ASD) are diagnosed based on two core symptoms: 1) impaired social communication and interaction, and 2) stereotyped behaviour and restricted interests (Figure 1, American Psychiatric Organization 2012). However, in addition to these core symptoms, the clinical picture often includes a range of other deficits, including sensory impairment, developmental delay, motor impairment, sleep disturbance, epilepsy, and some psychiatric conditions (Geschwind 2009). Before 2012, individuals with social and behavioural symptoms accompanied by normal language and cognitive development were diagnosed with Asperger's syndrome (American Psychiatic Association 1980). Patients who met only some of the criteria for diagnosis of autism used to be diagnosed with Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS). The old nomenclature can still be found in some studies. The symptoms of ASD usually appear in the first few years of life, with the child failing to develop speech and social skills according to the expected trajectory, or experiencing a period of regression where already acquired skills are lost (Yirmiya and Charman 2010). These conditions are estimated to affect 1% of the child population (Baird *et al.* 2006; Loomes *et al.* 2017).

A gender difference in the diagnosis of ASD has been repeatedly reported. A 4:1 ratio of males to females is most commonly reported (Fombonne 2005; Baird *et al.* 2006). However, the ratio of 4:1 is only valid across the whole spectrum of autism. When ASD is comorbid with intellectual disability the ratio is closer to 2:1 and to 5:1 in high-functioning individuals (Fombonne 2005; Kim *et al.* 2011). This discrepancy might arise from the fact that ASD presents differently depending on gender, while the diagnostic criteria seem to be biased towards the male presentation. This was demonstrated in a large study showing that girls meeting diagnostic criteria for ASD were more likely to also have low IQ scores and more behavioural problems than boys meeting the same criteria. Moreover, in the absence of these

additional symptoms, girls were less likely to meet the diagnostic criteria for ASD than boys, despite scoring equally high on ASD-like traits (Dworzynski *et al.* 2012). The reason for this discrepancy is not well-documented as the literature on the male presentation of ASD is far more extensive than on the corresponding female profile. One theory is that girls with ASD develop coping strategies more readily which leads to camouflaging of their symptoms (Attwood 2006). Another possibility is that the female profile is simply not adequately captured by the current diagnostic criteria (Kirkovski *et al.* 2013). It is also important to consider the complexity of human gender. A recent large study, involving 641,860 individuals, showed that transgender and gender-diverse people more often have a diagnosis of ASD as well as behavioural traits associated with ASD (Warrier *et al.* 2020).

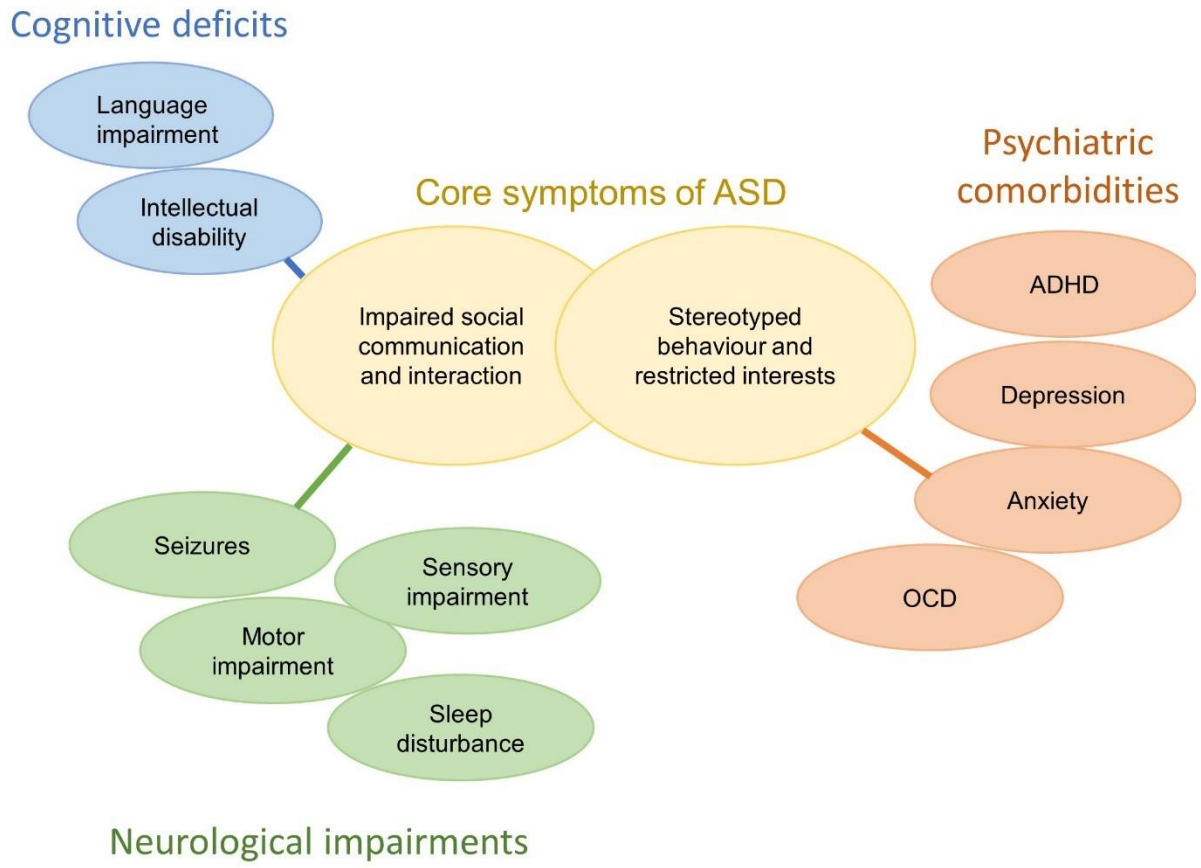


Figure 1 **Core and comorbid symptoms of ASD.** The cognitive deficits are outlined in blue, the consequences of neurological impairments in green and the psychiatric comorbidities in orange. OCD = Obsessive compulsive disorder, ADHD = Attention deficit hyperactivity disorder.

ASD is highly heritable, with 30-99% concordance rate for monozygotic twins (Hallmayer *et al.* 2015; Rosenberg *et al.* 2009; Bailey *et al.* 1995) and 3-30% recurrence risk for siblings (Jorde *et al.* 1991; Bolton *et al.* 1994; Ozonoff *et al.* 2011). The overall heritability for ASD was estimated to be 70-80% (Constantino *et al.* 2012), however a more recent study in a Scandinavian sample showed the heritability rate of 50-60% (Sandin *et al.* 2014). The large range of the heritability estimate is likely the result of the large discrepancy in the estimates of concordance rate for twins and siblings (Ramaswami and Geschwind 2018).

Cases of ASD have traditionally been divided into 'syndromic' and 'non-syndromic'. Syndromic forms result from a single highly penetrant mutation, where the diagnosis of ASD accompanies a range of other symptoms. This group includes tuberous sclerosis complex, caused by mutations in the *TSC1/TSC2* genes; Phelan McDermid syndrome caused by microdeletions within *SHANK3*; fragile X syndrome caused by mutations in *FMR1*; Rett syndrome caused by mutations in *MeCP2*; and Timothy syndrome caused by mutations in *CACNA1C* (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Phelan and McDermid 2012; Blomquist *et al.* 1985). In the non-syndromic forms, ASD is diagnosed based solely on the behavioural symptoms, which accounts for the majority of the cases (Buxbaum *et al.* 2012).

Some genetic mutations associated with non-syndromic ASD have been identified through the study of affected families. For example, the relevance of *NLGN3* was found through a case study of brothers with ASD (Jamain *et al.* 2003). Genome-wide association studies (GWAS) identified several single-nucleotide polymorphisms (SNPs) linked to ASD (Wang *et al.* 2009; Weiss *et al.* 2009; Anney *et al.* 2010). Additionally, 44% of families with at least one child diagnosed with ASD displayed Copy Number Variants (CNVs) that are not present in controls (Sebat *et al.* 2010; Consortium The Autism Genome Project *et al.* 2007; Pinto *et al.* 2010; Krumm *et al.* 2014) and *de novo* CNVs were present in 2-7% of relatives of children diagnosed with ASD (Marshall *et al.* 2008; Sanders *et al.* 2015). Overall, *de novo* mutations were found to account for 15-25% of the estimated heritability of ASD, suggesting that genetic factors account for a large proportion of ASD risk (Ramaswami and Geschwind 2018).

It is important to note, however, that in addition to the genetic factors, ASD has been linked to several environmental risk factors. These include infection during pregnancy, use of antidepressant medication by the mother, and exposure to pollution during pregnancy (Atladóttir *et al.* 2010; Croen *et al.* 2011; Raz *et al.* 2015). The interventions commonly used for individuals diagnosed with ASD are also often interpersonal or environmental in nature. One of the most popular interventions for children with autism is Applied Behavioural Analysis,

based on operant conditioning (DeFilippis and Wagner 2016). Effectiveness of this intervention in improving cognitive and behavioural symptoms was demonstrated in a randomized, controlled trial (Dawson *et al.* 2010). Additionally, a large trial of Sensory Enrichment Therapy for children diagnosed with ASD showed improvement in a range of symptoms including learning, memory, anxiety and attention levels, motor, social and communication skills and well as sensory processing (Aronoff *et al.* 2016). Social environment also impacts on the outcomes for individuals with ASD. Generally, children with ASD benefit in terms of their social competence from having typically developing siblings (McHale *et al.* 2016). However, there are some reports of adjustment problems in the those siblings. Interestingly, the siblings of children diagnosed with ASD were more likely to show impairment in their cognitive, social and communication skills than those with a typically developing sibling (Georgiades *et al.* 2013). Overall, environment is of extreme importance when considering ASD in the human population.

1.1. Convergence in Autism Spectrum Disorders

A vast number of mutations in the genes associated with ASD result in the same diagnosable two core symptoms, even when they are accompanied by comorbid deficits. This raises the possibility that the function of genes linked to ASD converges on a selected set of biological pathways, which when disrupted result in the same set of behavioural changes (reviewed in Quesnel-vallières 2019, Figure 2). Investigating these points of convergence can provide clues as to the underlying mechanisms at play in ASD.

1.1.1. Convergence of functions of genes involved in Autism Spectrum Disorders

Initially, an observation was made that the phenotypes associated with different models of ASD are highly overlapping. Altered synaptic function was noted in Rett syndrome, idiopathic autism arising from mutations in *NLGN3,* and *NLGN4,* and a duplication in region 15q11.13 (Zoghbi 2003; Noh *et al.* 2013). Multiple mutations associated with ASD also resulted in altered glutamatergic (Bear *et al.* 2004; Purcell *et al.* 2001) and inhibitory transmission (Tabuchi *et al.* 2007; Oldham *et al.* 2008). Other processes altered in more than one model of ASD included calcium signalling (Krey and Dolmetsch 2007), serotonin signalling (Chugani 2004), epigenetic regulation (Jiang *et al.* 2004; Hogart *et al.* 2007), cholesterol metabolism (Tierney *et al.* 2006), neuroinflammation and microglia activation (Vargas *et al.* 2004; Estes and McAllister 2016).

Some studies used Gene Ontology (GO) analysis to identify overlaps in the function of genes associated with ASD. The GO database was combined with DAVID (Database for Annotation, Visualization, and Integrated Discovery) and MetaCore by GeneGo (Thompson

Reuters) to identify the function of CNVs associated with ASD (Luo *et al.* 2012). The GO terms resulting from these studies included actin dynamics/cytoskeleton regulation (Gilman *et al.* 2011; Luo *et al.* 2012), neuronal function and development (Gilman *et al.* 2011; Luo *et al.* 2012; Noh *et al.* 2013; Gai *et al.* 2012; Pinto *et al.* 2014; Gandal *et al.* 2018; De Rubeis *et al.* 2014; Voineagu *et al.* 2011), cell adhesion (Gilman *et al.* 2011; Luo *et al.* 2012), transcription regulation (Luo *et al.* 2012; De Rubeis *et al.* 2014), chromatin regulation (De Rubeis *et al.* 2014; Pinto *et al.* 2014) and immune regulation (Voineagu *et al.* 2011; Gandal *et al.* 2018). In line with these observations, RNA sequencing of brain samples from individuals with ASD revealed the upregulation of genes associated with the neuronal function (Gupta *et al.* 2014). Similarly, knocking down several ASD risk genes in primary mouse neurons resulted in alterations in the level of expression of genes associated with neurogenesis and synaptic activity (Lanz *et al.* 2013).

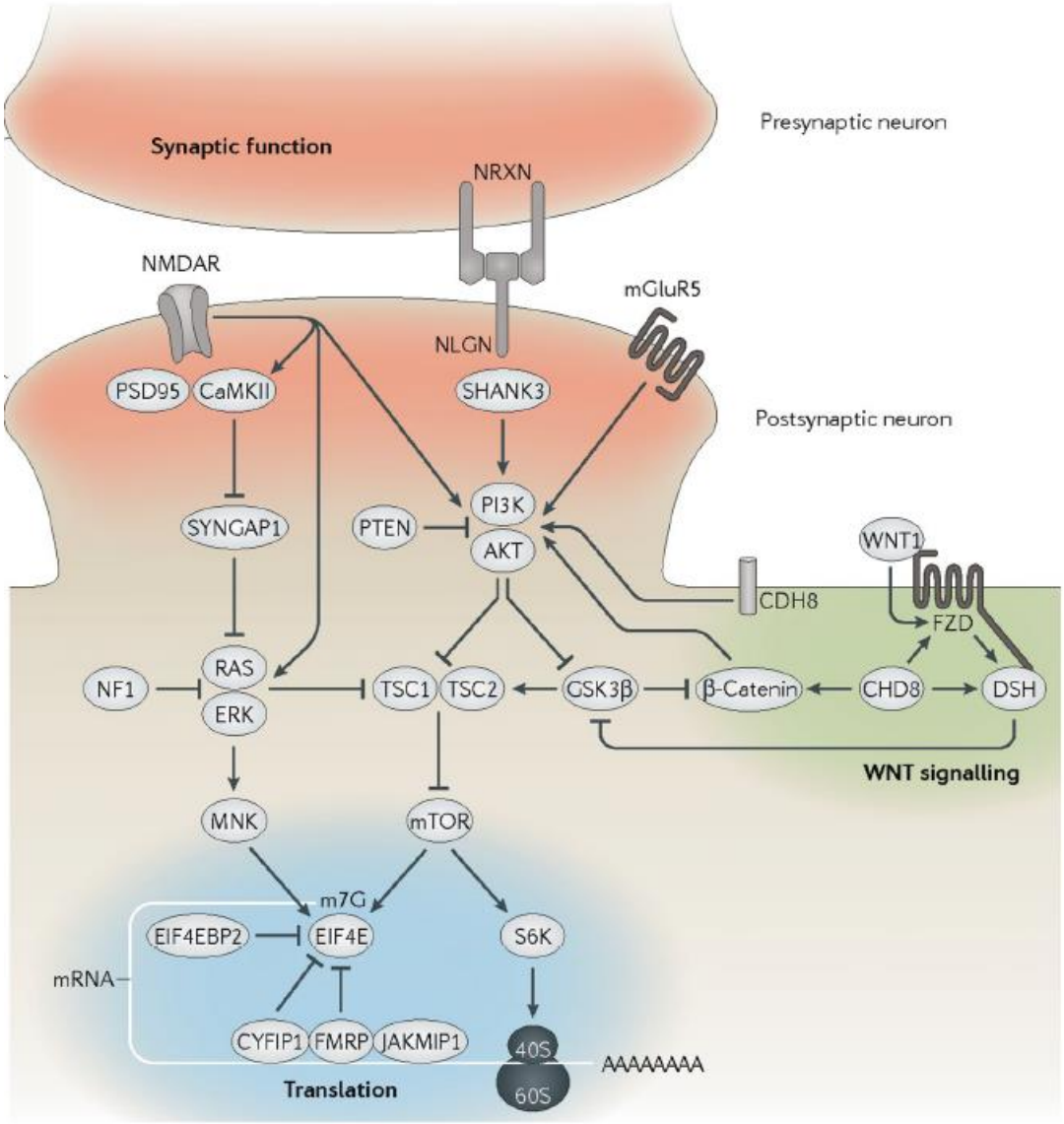


Figure 2 **Three of the proposed converging biological pathways in ASD, based on RNA sequencing.** Adapted from Quesnel-vallières (2019).

1.1.2. Interactions of proteins involved in Autism Spectrum Disorders

Another way to investigate the common molecular pathways in ASD is by directly considering the interactions between proteins involved. These proteins are likely to be part of the same biological pathways and can impact on each other's function readily. In support of this hypothesis, several proteins associated with ASD were predicted to interact with each other (Noh *et al.* 2013). When the genes associated with ASD were overlaid with the database on known human protein-protein interactions, proteins linked to ASD were found to be likely to frequently associated with each other (Correia *et al.* 2014; Neale *et al.* 2012).

Protein complexes associated with chromatin remodelling and intracellular transport contained some of the proteins linked to ASD (Li *et al.* 2015; Roak *et al.* 2012). Some of these proteins and their interactors were further examined using co-immunoprecipitation coupled with mass-spectrometry and were found to play a role in neuron physiology, morphology, and embryogenesis (Li *et al.* 2015). In line with this observation, two-hybrid screening coupled to glutathione-Sepharose affinity copurification assay showed interactions between SHANK3, TSC1*,* and Homer3, all of which are expressed in the postsynaptic compartment and can influence synaptic function (Sakai *et al.* 2011). Furthermore, multiplex co-immunoprecipitation performed on tissue from different models of ASD showed that the presence or absence of ASD-associated genes can alter the nature of protein interactions present in these models (Brown *et al.* 2018; Iossifov *et al.* 2012). Finally, these protein interactions differed depending on the developmental period, with some being expressed at embryonic stage while others were expressed primarily postnatally (Hormozdiari *et al.* 2015).

1.1.3. Limitations of current approaches to the investigation of convergence in Autism Spectrum Disorders

Possible convergence in the biological function of the different genes associated with ASD has been investigated primarily by comparing the phenotypes present in different ASD models and by employing GO analysis. Comparing the phenotypes linked to different genetic models resulted in a purely descriptive and correlational analysis. Similarly, a GO terms analysis resulted in the generation of categories to classify the pre-described functions of the genes of interest. Both of these methods rely on previous research into the function of specific genes, which might not be complete.

Alternatively, the common pathways involved in ASD can be established by investigating the protein complexes involved in ASD. Determining the protein interactions directly allows for the inference of the relationship between them and hypothesising about the possible mechanism leading to phenotypes associated with ASD.

1.1.4. Outstanding questions

There is some research about pathways that might be affected in ASD. However, the precise changes in these processes are often not known. One way to establish the mechanism leading to the behavioural changes seen in ASD is through investigating relevant protein interactions. The potential links between these proteins are not well established and need to be further investigated through biochemical methods. Additionally, it is not clear how a lack of or a reduction in one protein might affect the function of their interaction partners. Investigating these connections could potentially lead to understanding how these proteins collectively lead to the phenotypes observed in ASD.

1.2. The role of Neuroligin3 in Autism Spectrum Disorders

The genetic aetiology of ASD is complex, with many genes linked to the disorder. Among them is the X-linked gene *NLGN3,* which codes for the protein Neuroligin3. It is an example of a gene that, when mutated, gives rise to a non-syndromic form of ASD, which has been particularly understudied. Due to its location at the synapse, Neuroligin3 is an interesting candidate that might affect synaptic transmission profoundly. In this section, the literature relating to its function and association with ASD is reviewed.

1.2.1. Molecular function and known interactors of Neuroligin3

NLGN3 is an X-linked gene located on Xq13.1 (Jamain *et al.* 2003). It is one of five neuroligin genes present in the human genome: *NLGN1, NLGN2, NLGN3, NLGN4, NLGN4Y*, all of which have transmembrane and PDZ binding domains. However only *NLGN3* and *4* are located on the X chromosome. These two members of the neuroligin family are likely to be linked to ASD, as discussed in Section 1.2.2.

Neuroligin3 is expressed in the brain, in excitatory and inhibitory neurons (Budreck and Scheiffele 2007). Additionally, Neuroligin3 has been found to be present in cultured astrocytes where it regulates their development and morphology (Stogsdill *et al.* 2017). *Nlgn3* mRNA was also detected in glial cells and the protein Neuroligin3 was present in the secretion of gliomas (Gilbert *et al.* 2001; Li *et al.* 2018; Venkatesh *et al.* 2015). In neurons, Neuroligin3 is expressed in the post-synaptic compartment (Budreck *et al.* 2007). It belongs to a family of single-pass transmembrane proteins and consists of a large extracellular domain, a small transmembrane domain and a short cytoplasmic tail (Figure 3).

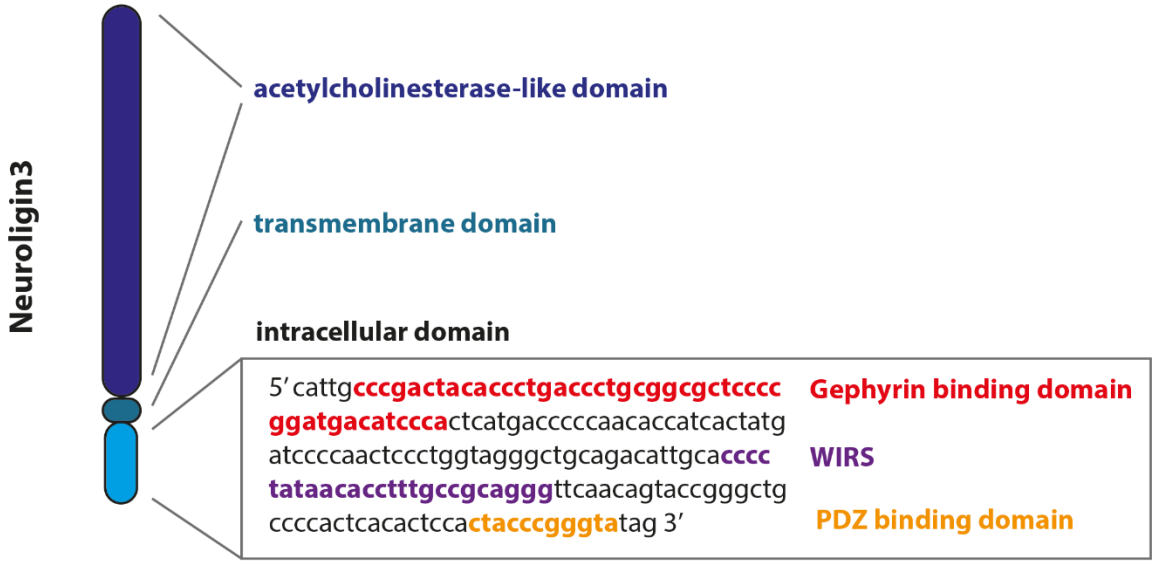
There is evidence for a role of Neuroligin3 in synapse formation and function. Under physiological conditions, Neuroligin3 forms homodimers or heterodimers with other neuroligins (Shipman and Nicoll 2012). Dimerization of Neuroligin3 affected synaptic

morphology, AMPAR-mediated synaptic transmission, and expression of other synaptic proteins (Shipman and Nicoll 2012), and a triple deletion of *Nlgn1, 2, 3* was found to lead to a reduction in the number of synaptic contacts (Varoqueaux *et al.* 2006). While the effect of the absence of *Nlgn3* on the number of synapses is not known, overexpression of Neuroligin3 was found to lead to an increase in the number of synapses in hippocampal neurons, *in vitro* (Chih *et al.* 2004).

The involvement of Neuroligin3 in synaptic function was further demonstrated in a series of studies about the effect of *Nlgn3* deletion on synaptic transmission. Lack of Neuroligin3 resulted in a deficit in tonic endocannabinoid signalling, leading to altered GABAergic signalling in hippocampal interneurons (Foldy *et al.* 2013). A localised deletion of *Nlgn3* in the nucleus accumbens led to a decrease of inhibitory postsynaptic potentials (Rothwell *et al.* 2014), while a deletion in parvalbumin-expressing cells in the hippocampus led to a decrease in N-Methyl-d-aspartic acid receptor (NMDAR)-mediated synaptic transmission and increased glutamate release (Polepalli et al. 2017). Finally, the absence of *Nlgn3* was associated with a deficit in metabotropic glutamate receptor 1(mGluR1)-mediated long-term depression (LTD) in the cerebellum (Baudouin 2014).

The information about the known interactors of Neuroligin3 is limited. The extracellular, acetylcholine esterase-like domain allows it to bind to neurexins, typically Neurexin 1β (Baig *et al.* 2017; Ichtchenko *et al.* 1996). Within the cell, the short cytoplasmic trail contains the PDZ-binding domain, which allows Neuroligin3 to bind to PSD95, and -93 (Irie *et al.* 1997). Neuroligin1 also contains this domain, which was found to aid the internalisation of the protein during LTD, suggesting that a similar process might be valid for Neuroligin3 (Jeong *et al.* 2019). Additionally, the cytoplasmic tail contains a gephyrin-binding domain (Poulopoulos *et al.* 2009). This binding domain, as well as another unnamed short stretch of the cytoplasmic tail, were found to impact on the inhibitory transmission in neurons (Nguyen *et al.* 2016).

A



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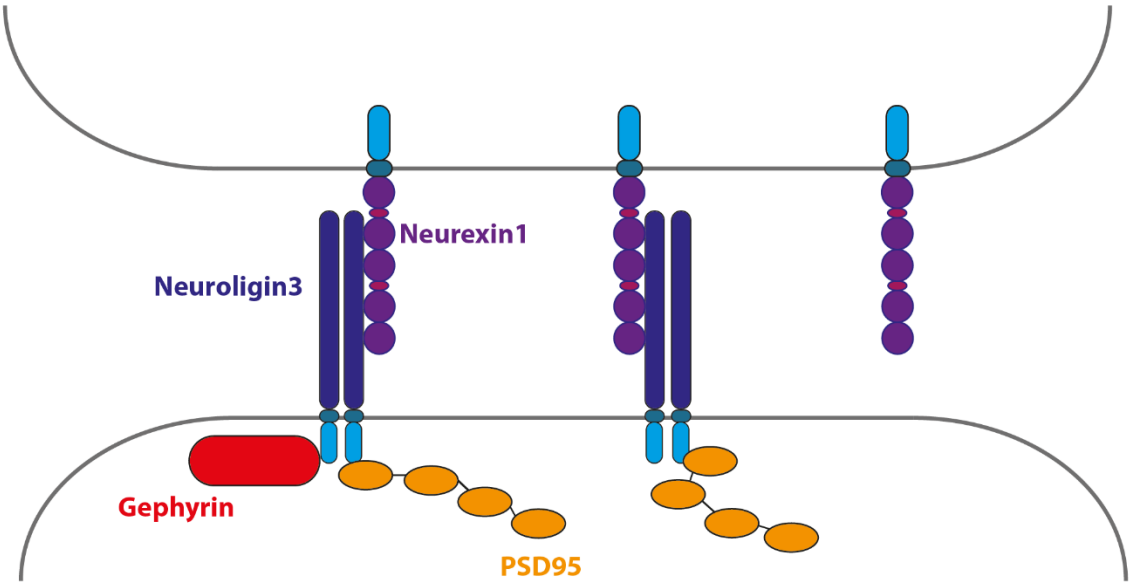


Figure 3 **The structure and known interactors of Neuroligin3. A** The structure of Neuroligin3, with a large external acetylcholinesterase-like domain, small transcellular domain and a short cytoplasmic tail. **B** Known interactors of Neuroligin3 at the synapse.

A potential novel interaction between Neuroligin3 and Cytoplasmic FMR1-interacting protein 1 (CYFIP1) was proposed recently (Chen et al. 2014). Neuroligin4, as well as several other adhesion proteins, were found to bind the WAVE regulatory complex (WRC) via an WRC interactor receptor sequence (WIRS, Chen et al. 2014). The interacting surface in the WRC was found to be composed of CYFIP1 and Abl interactor 1 (ABI1), suggesting that there could be binding between CYFIP1 and proteins containing a WIRS. Among them is Neuroligin3, however, this association has not been confirmed *in vivo.* While the Neuroligin3 could potentially interact with CYFIP1 via WIRS, there is a possibility that other binding domains of Neuroligin3 could be involved. For example, interaction via PDZ-binding domain as well as the gephyrin-binding domain remain to be excluded. Alternatively, the interaction between Neuroligin3 and CYFIP1 could occur indirectly, through another protein. Interestingly, *Nlgn3* mRNA was also found to be bound by fragile X mental retardation 1 (FMRP), which is an interactor of CYFIP1 (Chmielewska *et al.* 2018). Thus, the interaction between Neuroligin3 and CYFIP1 might occur indirectly through FMRP.

1.2.2. Evidence for the association between *NLGN3* and Autism Spectrum Disorders

There is evidence for the association between *NLGN3* and ASD. In the first instance, a point mutation in *NLGN3,* where cysteine 451 was substituted for arginine (R451C), was found in two brothers, one with classical autism and the other diagnosed with Asperger's syndrome (Jamain *et al.* 2003). The R451C mutated protein was thought to be retained in the endoplasmic reticulum, leading to a decrease in the protein available at the membrane (Jaco *et al.* 2010). Additionally, the mutated protein had a lower affinity for Neurexin1, its transsynaptic binding partner, potentially disrupting its cell-adhesion function (Jaco *et al.* 2010). This however was not confirmed in HEK293 cells (Xu *et al.* 2017).

A truncated *NLGN3,* missing exon 7 was found in a lymphoblastoid cell line derived from patients diagnosed with ASD, alongside the full-length version of the protein (Talebizadeh *et al.* 2006). This smaller *NLGN3* product might play a regulatory role, by modulating the function of the full-length isoform and reducing the availability of mature protein. In line with this observation, the mRNA levels of *NLGN3* were found to be reduced in lymphoblastoid cell line derived from individuals with ASD (Yasuda *et al.* 2011).

The results of GWAS studies relating to *NLGN3* are somewhat mixed. Sanders *et al.* (2015) reported *de novo* deletions in *NLGN3*, however deletions were not observed in another cohort (Glessner *et al.* 2009). Mutations in *NLGN3* in affected individuals were found in Greek and Japanese populations, however not in a Chinese sample or in high functioning individuals (Volaki *et al.* 2009; Yanagi *et al.* 2012; Liu *et al.* 2012; Wermter *et al.* 2008). Interestingly, a case of microduplication in the region encompassing *NLGN3* in a boy with autistic features

was reported (Gumus 2019), suggesting that both deletion and duplication of *NLGN3* might result in ASD phenotype. The summary of the studies presented in this section is available in Table 1.

Table 1 Details of studies about the association between mutations in *NLGN3* and ASD.

Type of study	**Mutation**	**Symptoms**	**Reference**
Case study	Arginine residue into cysteine (R451C) substitution within the esterase domain	Classical autism Asperger's syndrome	(Jamain *et al.* 2003)
Sequencing of lymphoblastoids from 10 patients with ASD and 30 controls	*NLGN3* isoform missing exon7	Classical autism	(Talebizadeh *et al.* 2006)
Lymphoblastoids obtained from 35 patients with ASD	mRNA level of *NLGN3* was reduced	Classical autism Asperger's syndrome PDD-NOS	(Yasuda *et al.* 2011).
GWAS	*De novo* deletion	Classical autism	(Sanders *et al.* 2015)
GWAS	No SNPs observed	Classical autism	(Glessner *et al.* 2009)
Exon sequencing of 40 individuals with ASD in Greek population	SNPs	Classical autism	(Volaki *et al.* 2009)
Sequencing of 62 Japanese patients with ASD	Synonymous substitution and three intronic substitutions in NLGN3	Classical autism	(Yanagi *et al.* 2012)
Sequencing of 285 patients with ASD and 384 controls in Chinese population	No SNPs observed	Classical autism	(Liu *et al.* 2012)
Single Strand Confirmation Polymorphisms Analysis in in 107 individuals	No SNPs observed	Asperger's syndrome High-functioning individuals with ASD	(Wermter *et al.* 2008)
Case study	Microduplication	Classical autism	(Gumus 2019)

Most of the mutations in *NLGN3* were found to occur in the region coding for the extracellular cholinesterase-like domain (Yan *et al.* 2005). On the other hand, a mutation in the cytoplasmic tail of Neuroligin4 was observed in some individuals with ASD (Etherton *et al.* 2011). When this mutation was introduced into the gene encoding Neuroligin3, a decrease of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) transmission in the hippocampus was observed, implying that mutations in the cytoplasmic tail of the protein might also affect its function (Etherton *et al.* 2011).

1.3. The role of *CYFIP1* in Autism Spectrum Disorders

The presence of a WIRS domain in Neuroligin3 supports the possibility that it might bind CYFIP1 (Chen *et al.* 2014). The two proteins could modulate each other's functions, having a combined effect on cellular processes. These might be relevant to ASD, as both proteins have been shown to be linked to these conditions. Here, the function of CYFIP1 in the cell is described and the evidence for the link between *CYFIP1* and ASD is reviewed.

1.3.1. Molecular function and known interactors of CYFIP1

CYFIP1 is localised at 15q11.2 and gives raise to the protein CYFIP1. It is expressed in the cerebellum and cortex during development and postnatally, as well as in the tissues outside of the brain (Bonaccorso *et al.* 2015). A more recent study found *Cyfip1* mRNA in neuronal and non-neuronal cells throughout the brain (Zhang *et al.* 2019). CYFIP1 was found to play a role in carcinogenesis (Silva *et al.* 2009), and in neuronal development (Abekhoukh *et al.* 2017; Bonaccorso *et al.* 2015). The function of CYFIP1 in neurons is discussed in this section.

CYFIP1 plays a dual role in the cell, as a regulator of both protein translation and actin polymerisation. Regarding protein translation, it was identified as a direct binding partner of FMRP in yeast two-hybrid screening (Schenck et al. 2001). FMRP itself is a protein translation initiation inhibitor. This process is dependent on the eukaryotic translation initiation factor 4E (eIF4E) binding translational initiation complex. However, this connection can be prevented by CYFIP1-FMRP complex binding eIF4E instead (Figure 4A). In line with the involvement of CYFIP1 in protein translation regulation, it was observed that the protein level of FMRP targets increased upon a reduction of CYFIP1 (Napoli *et al.* 2008). This process was found to depend on neural activity, as CYFIP1-eIF4E complex was found to be dissociated and an increase in protein translation was observed, alongside an increase in brain-derived neurotrophic factor (BDNF) and -3,5-Dihydroxyphenylglycine (DHPG) levels (Napoli *et al.* 2008). Additionally, both FMRP and CYFIP1 might impact on mTOR signalling. In *Drosophila,* deletions of both *dCyfip1* and *dFmr1* were associated with a reduction in the neuromuscular junction length. However, the neuromuscular junction in flies lacking both genes was restored to the usual

length, suggesting that FMRP and CYFIP1 might act on this pathway in opposing manner (Abekhoukh *et al.* 2017). There is also evidence for FMRP regulating the levels of Neuroligin3 (Chmielewska *et al.* 2018), indicating another potential connection between Neuroligin3 and CYFIP1.

With regard to actin polymerisation, CYFIP1 can influence actin dynamics by participating in the WAVE regulatory complex (WRC, Figure 4B). Initially, CYFIP1 was found to bind RAC1 and was found to be co-localised with filamentous actin (Kobayashi *et al.* 1998). The link between CYFIP1 and actin regulation became clear following characterisation of the WRC. This complex consists of WASP-family verprolin homologous protein 1 (WAVE1), CYFIP1/2, nucleosome assembly protein (NAP1), ABI1/2, and HSPC300 (Chen *et al.* 2010). CYFIP1 has the capacity to inhibit the verprolin-homology central acidic region (VCA) motif of WAVE1. Only when this binding site is released, the binding and activation of actin-related protein 2/3 (ARP2/3) can occur, leading to actin polymerisation. To allow the release of VCA, the CYFIP1 has to undergo a conformational change, as a result of the binding of RAC1 (De Rubeis *et al.* 2013).

According to the data presented above, CYFIP1 could be considered an inhibitor of actin polymerisation. However, there is some indication that CYFIP1 may actually be important for WRC stability in the *Drosophila* model (Kunda *et al.* 2003; Zhao *et al.* 2013). Therefore, WRC could be first activated and then degraded once separated from CYFIP1. In line with this prediction, reduction of *CYFIP1* in human neural progenitors impaired cytoskeleton remodelling, while induced pluripotent stem cells (iPSCs) derived from patients with 15q11.2 microdeletions showed alterations in cell junctions and apical polarity (Nebel *et al.* 2016; Yoon *et al.* 2014). Additionally, there appeared to be a co-regulation between CYFIP1 and other members of WRC, supported by the observation that the mRNA levels of WRC components were decreased in neurons alongside *Cyfip1* depletion as well as in the mRNA obtained from individuals with 15q11.2 deletion (Abekhoukh *et al.* 2017).

An impact of Neuroligin3 on the levels of polymerised actin was also demonstrated in *Drosophila*. A reduction in the fly orthologs of neuroligins, dNlg1-3 was associated with a reduction of polymerised actin. This effect was no observed when mutant proteins lacking the WIRS binding domain were introduced, and rescue of the reduction was possible only by proteins with the intact domain. Thus, suggesting that Neuroligin3 might impact on one of the canonical functions of CYFIP1.

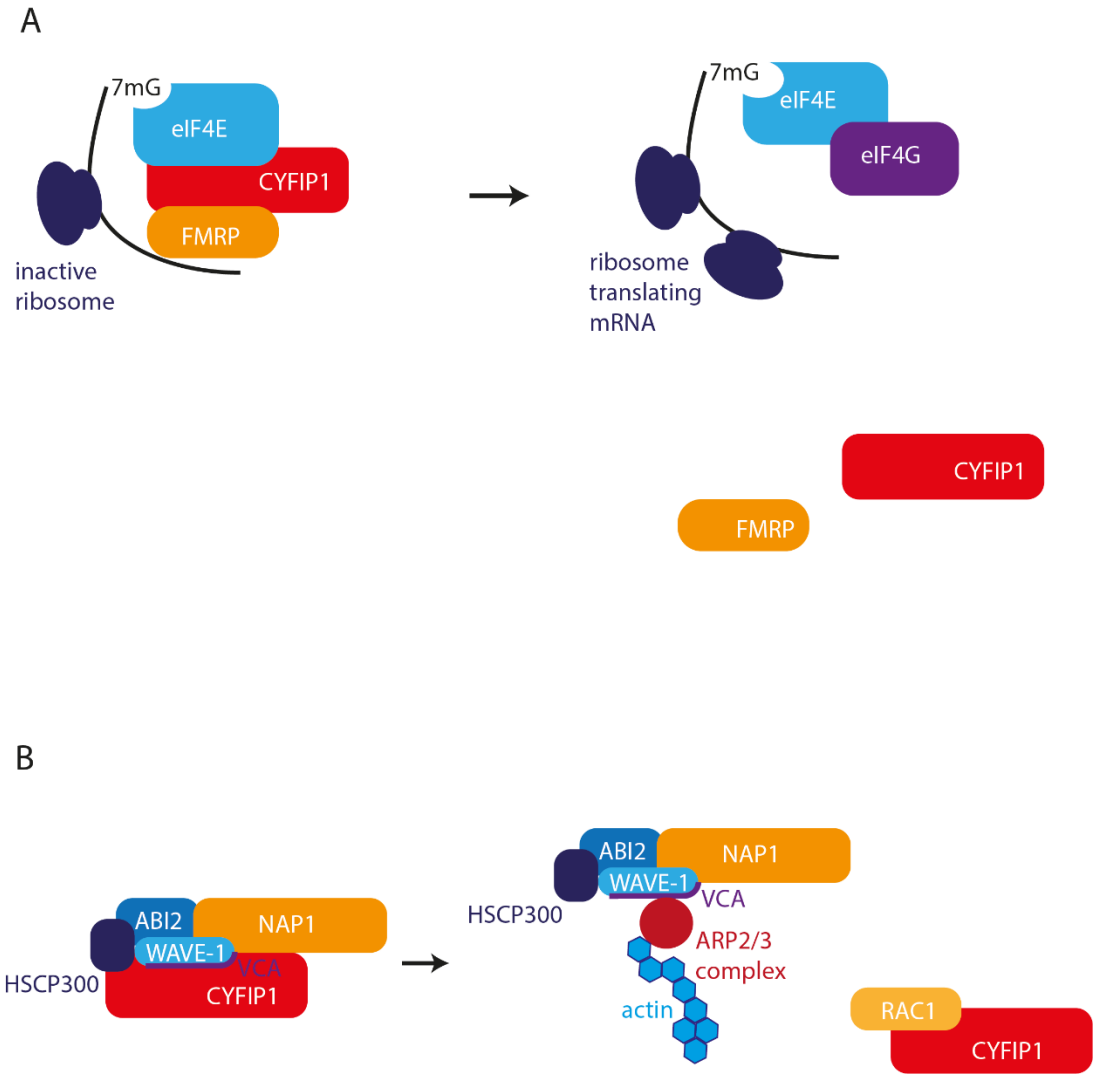


Figure 4 **Molecular function of CYFIP1 A** CYFIP1 inhibits protein translation. CYFIP1 can bind both eIF4E and FMRP preventing translation of mRNAs. When CYFIP1 is released, eIF4E can form a complex with eIF4G, leading to protein translation. **B** CYFIP1 regulates actin polymerisation via its participation in the WAVE regulatory complex. CYFIP1 covers the VCA binding site of WAVE-1 preventing the binding of the ARP2/3 complex. When RAC1 binds CYFIP1, the VCA binding site is exposed allowing for the binding of ARP2/3 and actin polymerisation.

1.3.2. Evidence for the association between *CYFIP1* and Autism Spectrum Disorders

There is accumulating evidence for the association of *CYFIP1* with ASD. *CYFIP1* is one of the four genes located in the 15q11.2 region. Deletions in this region are associated with Prader-Willi syndrome or Angelman syndrome (Doornbos *et al.* 2009), which are imprinted genetic disorders. Prader-Willi syndrome results from the lack of paternally inherited genes, while Angelman syndrome is caused by the absence of maternally inherited genes (Nicholls and Knepper 2001). Microdeletions in the 15q11.2 region were associated with a decrease in *CYFIP1* mRNA levels in lymphoblastoid cell lines derived from patients with Prader-Willi syndrome, highlighting the potential involvement of CYFIP1 in this condition (Bittel *et al.* 2006).

ASD symptoms are often present in individuals with this syndrome (Dykens *et al.* 2011; Peters *et al.* 2004). In line with this observation, both deletion and duplications of *CYFIP1* were associated with behavioural changes characteristic of ASD and other neurodevelopmental disorders (Leblond *et al.* 2012; Picinelli *et al.* 2016; Zwaag *et al.* 2009). Additionally, a GWAS study found that SNPs in CYFIP1 are linked to ASD diagnosis (Wang *et al.* 2015). Finally, decreased *CYFIP1* mRNA levels were found in leukocytes originating from patients diagnosed with ASD (Nowicki *et al.* 2007). In contrast, another study found that *CYFIP1* mRNA levels were increased in the blood of patients diagnosed with ASD, supporting the possibility that both a decrease and an increase in the levels of CYFIP1 might be detrimental (Zwaag *et al.* 2009). The findings of these studies are summarised in Table 2.

Table 2 Details of studies about the association between mutations in *CYFIP1* and ASD.

Type of study	**Mutation**	**Symptoms**	**Reference**
mRNA from lymphoblastoids from patients with Prader-Willi	*CYFIP1* mRNA levels reduced	Prader-Willi	(Bittel *et al.* 2006)
Sequencing of 243 families with neurodevelopmental disorders	15q11.2 duplication and deletions	Classical autism Obsessive Compulsive Disorder Intellectual disability	(Picinelli *et al.* 2016)
SNP microarray	15q11.2 microduplication	Classical autism	(Zwaag *et al.* 2009)
Odds ratios based on existing databases	SNPs in *CYFIP1*	Classical autism	(Wang *et al.* 2015)
Sequencing of 455 patients with ASD and 431 controls	*CYFIP1* deletion	Classical autism	(Leblond *et al.* 2012)
mRNA levels in 13 cases	*CYFIP1* mRNA levels reduced	Classical autism	(Nowicki *et al.* 2007)

1.4. Behavioural phenotypes in mouse models of *Nlgn3* deletion and *Cyfip1* haploinsufficiency

ASD is diagnosed based on behavioural symptoms. Therefore, the face validity of the mouse models used to investigate ASD relies on replicating some of these behavioural symptoms. This section focuses on the behavioural phenotypes present in three mouse models of ASD: *Nlgn3* deletion, Neuroligin3 R451C knock-in model, and *Cyfip1* haploinsufficiency. An ubiquitous deletion of *Cyfip1* is embryonic lethal (Pathania *et al.* 2014) and the behaviour of mice with conditional deletion of *Cyfip1* in a subset of cells has not yet been investigated. A summary of the behavioural phenotypes is available in Table 3.

1.4.1. Social behaviour

The sociability of mice is studied in numerous ways: direct interaction, three-chamber test, discrimination and preference for social olfactory cues, as well as courtship behaviour. When males lacking *Nlgn3* were placed in an arena with juvenile male mice, they spent less time interacting with their partners than their *WT* littermates, indicating a deficit in their sociability or social memory (Bariselli *et al.* 2018; Kalbassi *et al.* 2017). The social memory can be tested in mice using a three-chamber test. In this assay, a mouse is habituated to the presence of another mouse, which becomes the familiar subject. Then the mouse being tested is offered a choice to interact with a familiar mouse or a novel mouse. Mice prefer to investigate novel stimuli, social or otherwise, so *WT* mice on average spend more time with the novel mouse. However, both the Neuroligin3 R451C and mice lacking *Nlgn3* showed no preference for the novel mouse, indicating that they might not remember having met the familiar mouse or that they are unable to distinguish between the two (Cao *et al.* 2018; Radyushkin and Hammerschmidt 2009; Tabuchi *et al.* 2007). This deficit appeared to be limited to social memory, as these mice did show a preference for an unfamiliar object.

Olfaction plays an important role in mouse social behaviour and therefore mice tend to generally show a lot of interest in social pheromones. However, mice with *Nlgn3* deletion were shown to have reduced preference for social odours (Bariselli *et al.* 2018; Kalbassi *et al.* 2017). Nevertheless, this deficit was not replicated in these mice in another study (Dere *et al.* 2018), nor in the Neuroligin3 R451C model (Burrows *et al.* 2017). The lack of interest in social odours or perhaps an inability to discriminate between them could be a possible explanation of the previously described deficit in the three-chamber test.

Male mice with *Nlgn3* deletion also showed a deficit in their courtship behaviour. As part of the courtship, male mice tend to emit ultrasonic vocalisations. The number and duration of these vocalisations were shown to be reduced in males lacking *Nlgn3* (Kalbassi *et al.* 2017; Fischer & Hammerschmidt 2011; Radyushkin *et al.* 2009). Additionally, the latency to follow

when presented with a female was increased. These mice also showed less preference for the bedding of a female in oestrus containing the social odorants over fresh bedding (Dere, 2018). In the Neuroligin3 R451C model, the time of interaction with the female was found to be decreased (Burrows *et al.* 2017). Overall, males with mutations in *Nlgn3* showed a reproducible deficit in their courtship behaviour.

The social behaviour of mice heterozygous for *Cyfip1* has not been extensively studied. However, a reduced interest in social odours was reported in this model, similar to that of mice lacking *Nlgn3* (Bachmann *et al.* 2019). This deficit might imply that the social behaviour of these mice could be altered, but more assays need to be included in the analysis.

1.4.2. Motor behaviour and motor learning

Motor activity and exploratory behaviour can be measured in an open field arena. In this assay, mice are allowed to freely explore an arena for a set period of time and their activity is tracked. Both mice lacking *Nlgn3* and Neuroligin3 R451C mice showed hyperactivity in the open field (Cao *et al.* 2018; Ding *et al.* 2014; Kalbassi *et al.* 2017; Radyushkin and Hammerschmidt 2009; Rothwell *et al.* 2014; Qin *et al.* 1997). Increased activity was also observed in the elevated plus maze, where mice were allowed to remain in the arms of a maze with raised walls or to explore the open arms. Males lacking *Nlgn3* also explored more holes in the hole board test, where they were placed on a floor with gaps, into which they had the choice to dip their heads into (Radyushkin and Hammerschmidt 2009). This behaviour could potentially be explained by increased anxiety or by an increased reaction to the novel environment. The measures of anxiety, however, such as reduced time spent in the centre of the open arena or a reduction in the exploration of open arms of the elevated plus maze were not changed in these mice (Radyushkin and Hammerschmidt 2009; Rothwell *et al.* 2014). The exploratory behaviour observed was unlikely to result from increased motivation to obtain rewards, as these mice showed a similar level of digging for food and sucrose preference (Radyushkin and Hammerschmidt 2009). The hyperactivity phenotype was also found to follow a selective deletion of *Nlgn3* in Purkinje cells, but deletion of *Nlgn3* in all parvalbumin-expressing neurons resulted in hypoactivity, suggesting that the activity of these mice is regulated by this neuronal population (Rothwell *et al.* 2014). In contrast, mice heterozygous for *Cyfip1* tended to be hypoactive in the open field (Bozdagi *et al.* 2012), although this deficit has not been replicated (Bachmann *et al.* 2019).

The ability to learn motor routines can be approximated by training on the rotarod. In this assay, mice are placed on a rod, which rotates with increasing speed, and their ability to walk on the rod is measured. Both mice lacking *Nlgn3* and Neuroligin3 R451C mice showed an enhanced rate of learning on rotarod compared to their *WT* littermates (Chadman *et al.*

2009; Rothwell *et al.* 2014). In males lacking *Nlgn3,* this improvement was accompanied by a fast reduction in the variability in the location and length of steps (Rothwell *et al.* 2014). Interestingly, deletion of *Nlgn3* also led to increased time of steps on the Erasmus ladder further suggesting changes in motor behaviour (Baudouin *et al.* 2012). This phenotype was restored by re-expressing Neuroligin3 in Purkinje cells in the cerebellum, suggesting this cellular population might be important for this behaviour. In contrast, mice heterozygous for *Cyfip1* showed an impairment in their ability to learn new motor routines on the rotarod (Bachmann *et al.* 2019).

1.4.3. Cognition

Mice lacking *Nlgn3* showed reduced contextual and cued fear conditioning, indicating a possible deficit in their learning ability (Radyushkin and Hammerschmidt 2009), an impairment that was not present in the Neuroligin3 R451C mice (Chadman *et al.* 2009). However, in both models a minimal change in spatial learning in the Morris Water Maze was present (Chadman *et al.* 2009). Interestingly, novel object recognition was not impaired in either model (Cao *et al.* 2018; Bariselli *et al.* 2018; Tabuchi *et al.* 2007), suggesting that the lack of interest in novel mice reported in the previous section was unlikely to be related to an inability to discriminate between subjects. No cognitive impairment was reported in mice heterozygous for *Cyfip1,* which could be due to limited behavioural analysis performed in this model.

1.4.4. Behaviour of female mice

It is important to note that the behavioural findings described above arise primarily from the investigation of adult males. Only two studies included either *Nlgn3* heterozygous females or those lacking *Nlgn3* entirely. A reduction in preference for bedding originating from a cage of males and therefore containing social pheromones was observed in *Nlgn3* heterozygous females indicating a potential social behaviour deficit (Dere *et al.* 2018). In contrast, a later study showed that *Nlgn3* heterozygous females did not differ from their *WT* littermates in their interaction time with unfamiliar mouse, activity in the open field, and activity in the elevated plus maze (Kalbassi *et al.* 2017). Nevertheless, the females with a complete deletion of *Nlgn3* interacted less with other females, they were hyperactive in the open field, and they tended to spend more time in the centre of the arena (Kalbassi *et al.* 2017). These findings suggest that mice lacking *Nlgn3* show a largely similar behavioural phenotype, regardless of sex. No information about the behavioural phenotype of females heterozygous for *Cyfip1*, however, has been published.

1.5. Dendritic spine density, morphology and turnover in mouse models of *Nlgn3* deletion and *Cyfip1* haploinsufficiency

A commonly reported phenotype in ASD is a change in dendritic spine density, morphology, and plasticity (Martínez-Cerdeño 2016; Forrest *et al.* 2018; Phillips and Pozzo-miller 2015). Dendritic spines are small protrusions where primarily excitatory synapses are located and the input from neurons is received. The formation of a new dendritic spine requires local protein translation and actin polymerisation (Nakahata and Yasuda 2018). As discussed previously in Section 1.3.1., CYFIP1 is a regulator of both processes. Additionally, Neuroligin3 might also play a role in dendritic spine formation via its interaction with CYFIP1 (as discussed in Section 1.2.1.). This section reviews literature on dendritic spine density, morphology, and plasticity in mouse models of *Nlgn3* deletion and *Cyfip1* haploinsufficiency (Table 3).

1.5.1. Dendritic spine density

Neuroligin3 impacts synaptic formation and function as discussed in Section 1.2.1. However, the effect of *Nlgn3* deletion on dendritic spine density has not been extensively documented. A study of a triple knockout of *Nlgn1, 2,* and *3* showed a reduction in spine number *in vitro* (Chih et al. 2004). In contrast, when dendritic spine density was analysed in Neuroligin3 R451C mice, no differences were reported (Isshiki et al., 2014, Tabuchi et al. 2007).

In line with its role in suppressing actin polymerisation, in hippocampal neurons originating from mice heterozygous for *Cyfip1,* an increase in protein synthesis and actin assembly was observed (Hsia *et al.* 2016). Consistent with the importance of these processes in dendritic spine formation, CYFIP1 localised in clusters in dendritic spine heads and dendritic shafts (Hsiao *et al.* 2016; Pathania *et al.* 2014). Mice heterozygous for *Cyfip1* showed a decrease in spine density in the motor cortex and olfactory bulb (Abekhoukh *et al.* 2017; Bachmann *et al.* 2019).

1.5.2. Dendritic spine morphology

The possibility that the morphology of spines might be affected by *Nlgn3* deletion has not yet been verified. However, an increase in synapse remodelling and dendritic branching was observed in the Neuroligin3 R451C knock-in model (Etherton et al. 2011; Isshiki et al. 2014).

The morphology of individual spines was found to be altered in mice heterozygous for *Cyfip1.* An increase in filamentous spines was observed in neurons in these animals (Abekhoukh *et al.* 2017; De Rubeis *et al.* 2013; Pathania *et al.* 2014). Interestingly, the same

phenotype was observed in mice, in which *Cyfip1* was deleted in a population of excitatory neurons in the forebrain (Davenport *et al.* 2019). The filamentous appearance of the spines signifies that they are immature, as the spines adopt a long and thin shape in the initial phase of their formation (Berry and Nedivi 2017). The immature spine phenotype present in the neurons *in vitro* was reversed by transfecting them with a full-length *Cyfip1* construct, reinforcing the idea that *Cyfip1* is important for spine development (De Rubeis *et al.* 2013).

1.5.3. Dendritic spine turnover

Dendritic spines are highly plastic, with new spines being continuously formed and eliminated. An increase in the turnover of dendritic spines was observed in mice with Neuroligin3 R451C mutation, in the PSD95 positive and gephyrin positive cells of the cortex (Isshiki *et al.* 2014). However, the turnover of spines in this model was found to be unaffected by sensory modulation, which is the case in *WT* mice. A similar phenotype was present in mice heterozygous for *Cyfip1,* where the base turnover of spines was increased in relation to their *WT* littermates (Bachmann *et al.* 2019). However, these mice showed, an increase of dendritic spine in response to motor learning, like the control animals.

Table 3 Summary of phenotypes in mouse models of *Nlgn3* deletion, Neuroligin R451C substitution and *Cyfip1* haploinsufficiency

	***Nlgn3* deletion**	**Neuroligin3 R451C**	***Cyfip1* haploinsufficiency**
Behaviour			
Social interaction	Reduced	Not determined	Not determined
Three chamber test	No preference for social stimulus	No preference for social stimulus	Not determined
Interest in social odours	Reduced	Comparable to *WT*	Reduced
Courtship behaviour	Reduced	Reduced	Not determined
Open field activity	Increased	Increased	Comparable to *WT*
Motor learning on rotarod	Increased	Increased	Reduced
Elevated plus maze	Comparable to *WT*	Comparable to *WT*	Not determined
Fear conditioning	Reduced	Comparable to *WT*	Not determined
Spatial learning in Morris Water Maze	Reduced	Reduced	Not determined
Novel object recognition	Comparable to *WT*	Comparable to *WT*	Not determined
Dendritic spines			
Density	Not determined	Comparable to *WT*	Reduced in motor cortex, olfactory bulb
Morphology	Not determined	Not determined	Increase in filamentous spines
Turnover	Not determined	Not determined	Increased

1.5.4. Outstanding questions

While the function of Neuroligin3 is reasonably well characterised, only a few of its interactors are known. The interactome of Neuroligin3 and the combined impact of the interacting proteins on traits associated with ASD remains to be investigated. There is a predicted binding between Neuroligin3 and CYFIP1 (Chen *et al.* 2014), however, it has not yet been confirmed *in vivo.* The potential relationship between these two proteins and their impact on biological processes remains to be determined. As both of these proteins are associated with ASD, this investigation might elucidate the mechanism behind the convergence of biological pathways seen in these conditions. Specifically, there is some indication that Neuroligin3 might impact on actin remodelling and protein translation, the two canonical functions of CYFIP1. The behaviour of mice lacking *Nlgn3* is well characterised, however, the analysis of the behaviour of mice heterozygous for *Cyfip1* is quite limited. For example, the behaviour of females heterozygous for *Cyfip1* has not been previously investigated. Since ASD is diagnosed based on behavioural symptoms, it is crucial to confirm the face validity in these models. There are few similarities in the behavioural phenotypes associated with *Cyfip1* haploinsufficiency and *Nlgn3* deletion, suggesting that combining these two mutation might have a cumulative effect. While the dendritic spine phenotypes associated with *Cyfip1* haploinsufficiency have been previously described, little is known about dendritic spine density, morphology, and turnover in mice lacking *Nlgn3.*

1.6. The effect of mixed genotype housing on behaviour and transcriptome of mouse models of Autism Spectrum Disorders

Using mice as a model is inherently tied to the fact that they are social animals. Mice are typically housed together in a laboratory environment, where they can interact with each other and form social structures. As such, the social environment might be a modulating factor of their behaviour and their physiology. In this section, the literature surrounding the impact of the social environment on the behaviour and physiology in mice is reviewed in the context of ASD.

1.6.1. Evidence for an effect of social environment on the behaviour of mice

The first social contact mice experience is with their mother and their littermates. Pups separated from their mother showed hyperactivity, impairment in their hippocampal-dependent learning, an increase in depressive-like and anxiety-like behaviours, altered stress response and changes in their dominance behaviour in adulthood (Benner *et al.* 2014; Champagne and Meaney 2007; George *et al.* 2010; Ibi *et al.* 2008; Martini and Valverde 2012; Rice *et al.* 2008). It is important to note that these consequences of separation from the mother were often more severe for the female than the male pups (Bondar *et al.* 2018; Romeo *et al.*

2003). In contrast, housing multiple mothers with their litters together led to an increased social investigation, grooming and sniffing of other mice in adulthood and readily establishing social hierarchies (Branchi *et al.* 2006; D'Andrea *et al.* 2007).

The behaviour of mice could also be affected by manipulations of the social environment introduced in adulthood. Social isolation of mice following weaning, where mice are individually housed, was shown to lead to increased interaction with novel mice in the three-chamber test and hyperactivity (Naert *et al.* 2011). However, the hyperactivity phenotype resulting from social isolation was only found in females in another study (Palanza *et al.* 2001). In addition, it was observed that not only social isolation but also adding novel mice to the existing social structure can result in a rise of anxiety-like behaviours (Schmidt *et al.* 2007). Interestingly, merely being housed with a familiar mouse which underwent fear conditioning could reduce conditioned fear response and promote fear extinction (Bredy and Barad 2008). These results suggested that both social isolation as well as social housing might affect behaviour. Furthermore, social housing could mediate the impact of other factors on behaviour. For example, an enriched environment, which consisted of additional stimuli such as tubes and houses, reduced anxiety-like behaviours, but only in socially housed animals and not in the socially isolated animals (Chourbaji *et al.* 2005). This suggested that not only does social environment affect behaviour, it also has the capacity to modulate the effect of other variables.

Another aspect of the social environment that might affect the behavioural phenotypes of mice is the genotype of their littermates. The behaviour of BTBR mice, which showed deficits in social behaviour and memory as a result of inbreeding, was found to be affected by the littermates they were housed with. BTBR mice housed with other BTBR mice had reduced sociability, however, BTBR mice kept with C57BL/6 mice showed normalisation of social behaviour (Yang *et al.* 2011). The memory deficit in BTBR mice was also eradicated through co-learning, the engagement of two familiar mice in the same learning task, with C57BL/6 but no other BTBR mice (Lipina and Roder 2013). In contrast, mice with 16p11.2 deletion were found to only present with a deficit in the ultrasonic vocalisations in the context of courtship when they were housed with *WT* mice. Meanwhile, mice with the 16p11.2 deletion housed together showed a normal level of vocalisation (Yang *et al.* 2015). A similar effect was shown with mice lacking the oestrogen receptor α. Interestingly, in this case, it was the wild type mice that were affected. The wild type males that were housed with mutant mice showed increased aggression compared to those housed with other wild type mice. The behaviour of the mutant females was also altered; the level of social interaction was decreased in the mutant-only housed females compared to mixed housing (Crews *et al.* 2009).

The effect of housing on behaviour might arise from the inability to establish the usual hierarchy in mixed genotype housing conditions. This theory was supported by an experiment in males with an extra copy of the gene *Cdknlc,* which resulted in higher dominance of these mice (McNamara *et al.* 2018). Introducing these mice into a cage of *WT* males led to hierarchy destabilisation, as compared to cages of only *WT* males. A recent study on the impact of the social environment on the behaviour of mice with *Nlgn3* deletion and their *WT* littermates reinforced this hypothesis (Kalbassi *et al.* 2017). Males lacking *Nlgn3* were found to be submissive to the *WT* littermates they were housed with. Additionally, the social hierarchy in cages with mice of mixed genotypes was found not to be correlated with vocalisation indicating hierarchy destabilisation. Interestingly, here the behaviour of *WT* littermates housed with males lacking *Nlgn3* was affected, as they showed reduced interest in social odours and time spent interacting with a female. This effect was observed primarily in male mice. Collectively, these studies demonstrated that housing mice of different genotypes together has the capacity to impact on behaviour of both mutant and *WT* mice.

1.6.2. Evidence for an effect of social environment on the physiology of mice

The effect of the social environment is not limited to mouse behaviour but extends to various aspects of their physiology. The housing of multiple mothers and litters together resulted in higher nerve growth factor (NGF) and BDNF levels in the brain of the pups once they have reached adulthood (Branchi *et al.* 2006). On the other hand, social isolation in adulthood was found to correlate with decreased myelination in the prefrontal cortex and structural connectivity changes (J. Liu *et al.* 2016; C. Liu *et al.* 2016). These findings suggested that social housing can have functional and structural consequences in the brain. Interestingly, the effect of the social environment also extended to recovery following an injury. Socially housed mice were found to recover from traumatic head injury quicker than socially isolated mice (Doulames *et al.* 2015). However, social housing only had a positive impact on the recovery when the other mouse in the cage was healthy and no effect when the other mouse also received an injury (Venna *et al.* 2014). Similarly, the social environment could modulate how other factors impacted on physiology. For example, the effect of stress on wound healing was increased in socially isolated mice compared to socially housed mice (Glasper and DeVries 2005). Wound healing was also differentially influenced by social housing with mice of the same or different genotype (Baud *et al.* 2017).

Social structure was also shown to impact on molecular events such as RNA expression. Altering the existing social structure had the capacity to alter the expression of mineralocorticoid and glucocorticoid receptor mRNAs in the hippocampus (Schmidt *et al.* 2007). This observation was in line with differentially expressed mRNAs depending on the

position in the social hierarchy. In particular, corticotropin-releasing hormone mRNA, glucocorticoid receptor and BDNF mRNA were found to be upregulated in the dominant mice (So *et al.* 2015), as well as serotonin receptors, tropomyosin receptor kinase B (TrkB, Horii et al. 2017), and CAMP responsive element binding protein 1 (CREB1, Horii et al. 2017). Meanwhile, synapsin IIb mRNA and protein were found to be upregulated in the submissive mice (Nesher *et al.* 2015). Even social defeat, where a mouse interacts with a partner displaying a high level of aggressive behaviour, was found to be sufficient to modulate the transcriptome. Specifically, it altered the expression of mRNAs associated with autism in the hippocampus, striatum, and hypothalamus, including *Nlgn3* (Kudryavtseva *et al.* 2018).

1.6.3. Outstanding questions

The impact of several aspects of the social environment on behaviour and physiology of mice has been investigated. However, the directionality of the effect on a given phenotype varies depending on the population of mice selected. The information on the effects of the social environment is only available for a handful of different models. While there is some information about the way social environment might impact on mice lacking *Nlgn3* and their *WT* littermates, there have been no studies of mice with *Cyfip1* haploinsufficiency. Thus, the way the social environment might modulate the effect of *Cyfip1* on phenotypes associated with ASD is unknown.

1.7. Aims and objectives of the thesis

The aim of this thesis is to investigate the impact of the Neuroligin3/CYFIP1 interaction on phenotypes associated with ASD. Initially, the interactome of Neuroligin3 in neurons and glial cells was characterised. In the course of these experiments the interaction between Neuroligin3 and CYFIP1, as well as the known interactors of CYFIP1, FMRP, and WAVE-1 were verified. This was followed by investigating the impact of the Neuroligin3/CYFIP1 interaction on behaviour in mice. The activity, motor learning, and social behaviour of mice lacking *Nlgn3,* heterozygous for *Cyfp1,* or double mutants were compared. Dendritic spine density in the cortex was also contrasted between these different groups of mice in order to determine if the Neuroligin3/CYFIP1 interaction might impact dendritic spine formation. Finally, the transcriptome of mice lacking *Nlgn3*, or heterozygous for *Cyfip1,* or both, were investigated. Additionally, sex and social environment were considered as possible factors that might modulate the effect of *Nlgn3* and *Cyfip1* mutations on these phenotypes associated with ASD.

Chapter 2: Materials and Methods

2.1. Experimental animals

2.1.1. Husbandry and legislation

All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 (amended 2012). The mice were housed in 16 x 48 x 14 cm cages. They were provided with sawdust bedding, nesting material, a wooden chew stick, and a cardboard tube. The cages were cleaned once a week. The mice also had free access to food and water in their cages. The temperature in the room was maintained between 18 and 22 °C. There was a 12 h light/dark cycle in the room with the lights being turned on at 6 am and switched off at 6 pm.

All mice were backcrossed to a C57BL/6 background for at least eight generations. Females and males used for breeding were at least P60 (postnatal day 60). The males used for breeding were removed from the cage once the female was determined to be pregnant. A biopsy of the ear was taken between ages P21 and P30 for genotyping and identification. The pups were weaned from their mother between ages P21 and P30. Mice were then kept with their littermates of the same sex, 2-5 within a cage. If there were more than 5 littermates of the same sex, they were separated randomly into two cages.

2.1.2. Mouse lines

Several mouse lines were used in the experiments throughout this thesis, which are detailed in Table 4 below.

Table 4 The mouse lines used in the thesis.

Strain	Supplier	Relevant sections	Simplified notation
(FAST) Neuroligin 3 conditional knock-in	#RBRC05451 (Tanaka et al., 2010)	Chapters 3,4,5,6	*Nlgn3$^{y/-}$* *Nlgn3$^{+/-}$* *Nlgn3$^{-/-}$* *Nlgn3$^{y/fl}$*
Omptm4(cre)Mom/MomJ	JAX: #006668 (Li et al. 2004)	Chapter 3	*Omp$^{Cre/+}$* *Omp$^{Cre/Cre}$*
Pvalbtm1(cre)Arbr/J	JAX: #017320 (Hippenmeyer et al. 2005)	Chapter 3	*Pvalb$^{Cre/+}$* *Pvalb$^{Cre/Cre}$*
Cyfip1tm2a(EUCOMM)Wtsi	MDCK: #EPD0555_2_B11	Chapter 4,5,6	*Cyfip1$^{+/-}$*
Tg(Thy1-EGFP)MJrs/J	JAX: # 007788 (Feng *et al.* 2000)	Chapter 4,5	*Thy1^{EGFP}*

Flexible Accelerated STOP tetracycline Operator (tetO)-knockin (FAST) *Nlgn3* conditional knock-in mice contained a *loxP*-flanked STOP cassette in the promoter region of *Nlgn3,* resulting in premature termination of transcription (#RBRC05451, Tanaka et al., 2010). Therefore, the mice containing the construct had a functional deletion of *Ngln3.* In the presence of *Cre*, the STOP cassette was excised resulting in re-expression of *Nlgn3.* For simplicity, throughout the thesis the *Nlgn3*$^{y/fl}$ mice are referred to as *Nlgn3*$^{y/-}$ and the *Nlgn3*$^{+/fl}$ females are referred to as *Nlgn3*$^{+/-}$, with the exception of the re-expression experiments.

In Omp$^{tm4(cre)Mom}$/MomJ mice, part of the *Omp* gene was replaced with *Cre* recombinase. In these mice, *Cre* was therefore expressed in the same cells as *Omp*. Similarly, *Pvalb*$^{tm1(cre)Arbr/J}$ mice had a construct that consisted of an internal ribosome entry site (IRES), *Cre*, and a polyadenylation sequence, in the 3' untranslated region (UTR) of exon 5 of the parvalbumin gene. Therefore, *Cre* was expressed in the same cells as parvalbumin.

In *Cyfip1*$^{tm2a(EUCOMM)Wtsi}$ (MDCK: #EPD0555_2_B11) animals, a Flp-recombinase cassette was inserted upstream from one of the exons, trapping the transcript. Finally, in Tg$^{(Thy1\text{-}EGFP)MJrs/J}$ mice (JAX: # 007788,(Feng *et al.* 2000), EGFP was expressed under the *Thy1* promoter resulting in sparse labelling of neurons throughout the brain, including the cortex.

2.1.3. Breeding schemes

For experiments described in Chapter 3, *Nlgn3*$^{y/fl}$*Omp*$^{Cre/+}$ and *Nlgn3*$^{y/fl}$*Pvalb*$^{Cre/+}$ mice were generated, with the aim to re-express *Nlgn3* in neurons containing OMP and parvalbumin respectively. In order to generate *Nlgn3*$^{y/fl}$*Omp*$^{Cre/+}$ mice, *Nlgn3*$^{y/fl}$ males were crossed with *Omp*$^{Cre/+}$ females. Only the *Nlgn3*$^{y/fl}$*Omp*$^{Cre/+}$ mice were included in the analysis. Similarly, to generate *Nlgn3*$^{y/fl}$*Pvalb*$^{Cre/+}$ mice *Nlgn3*$^{+/fl}$ females were crossed with *Nlgn3*$^{y/+}$*Pvalb*$^{Cre/Cre}$ males. Out of the resulting litters we selected *Nlgn3*$^{y/fl}$*Pvalb*$^{Cre/+}$ males for the experiments. The rest of the offspring was excluded.

For the experiments described in Chapters 4, 5 and 6 *Nlgn3*$^{+/-}$ females were crossed with *Cyfip1*$^{+/-}$ males resulting in males with one of the following genotypes: *WT*, *Nlgn3*$^{y/-}$, *Cyfip1*$^{+/-}$, *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ and females with one of the following genotypes: *WT*, *Nlgn3*$^{+/-}$, *Cyfip1*$^{+/-}$, *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$. Additionally, some of the *Cyfip1*$^{+/-}$ sires carried the *Thy1-EGFP* transgene resulting in a proportion of the offspring also carrying the *Thy1-EGFP* transgene but lacking *Nlgn3* or being heterozygous for *Cyfip1.*

For the experiments described in Chapters 4 and 6, additional cohorts of *WT* animals were generated. In Chapters 4 and 6, a single genotype housed (SGH) cohort of *WT* animals was created by crossing males and females with C57BL/6 genetic background purchased

from JAX, which resulted in all *WT* offspring, which were only housed with each other. In Chapter 6, an additional cohort of SGH *WT* animals was generated where the parents were taken from the *Nlgn3* colony. *Nlgn3*$^{+/-}$ females were crossed with *WT* males in order to obtain cages of SGH *WT*, MGH *WT,* SGH *Nlgn3*$^{y/-}$ and MGH *Nlgn3*$^{y/-}$ males.The details of all of the breeding and resulting offspring can be found in Table 5.

Table 5 Summary of mouse breeding schemes, with associated genotyping required.

Parents	**Offspring**		**Genotyping**	**Experiments**
***Omp*$^{Cre/+}$ males *Nlgn3*$^{+/fl}$ females**	*Males:* *WT* *Omp*$^{Cre/+}$ *Nlgn3*$^{y/fl}$ *Omp*$^{Cre/+}$	*Females:* *WT* *Nlgn3*$^{+/fl}$ *Nlgn3*$^{+/fl}$ *Omp*$^{Cre/+}$	STOP-tetO for *Nlgn3* deletion *Nlgn3* Cre	Chapter 3
***Pvalb*$^{cre/cre}$ males *Nlgn3*$^{+/-}$ females**	Males: *Pvalb*$^{Cre/+}$ *Nlgn3*$^{y/fl}$*Pvalb*$^{Cre/+}$	Females: *Pvalb*$^{Cre/+}$ *Nlgn3*$^{+/fl}$*Pvalb*$^{Cre/+}$	STOP-tetO for *Nlgn3* deletion *Nlgn3* Cre	Chapter 3
***Cyfip1*$^{+/}$*Thy1*^{EGFP} males *Nlgn3*$^{+/-}$ females**	Males: *WT* *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *Thy1*^{EGFP} *Nlgn3*$^{y/-}$ *Thy1*^{EGFP} *Cyfip1*$^{+/-}$ *Thy1*^{EGFP} *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *Thy1*^{EGFP}	Females: *WT* *Nlgn3*$^{+/-}$ *Cyfip1*$^{+/-}$ *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *Thy1*^{EGFP} *Nlgn3*$^{+/-}$ *Thy1*^{EGFP} *Cyfip1*$^{+/-}$ *Thy1*^{EGFP} *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *Thy1*^{EGFP}	STOP-tetO for *Nlgn3* deletion *Nlgn3* *Cyfip1* *Cyfip1* construct for deletion *Thy1-EGFP*	Chapter 4, 5, 6
***WT* C57BL/6 males from JAX *WT* C57BL/6 females from JAX**	*WT*		Not required	Chapter 4, 6
***WT* C57BL/6 males from *Nlgn3* colony *WT* C57BL/6 females from *Nlgn3* colony**	*WT*		Not required	Chapter 6
***WT* males *Nlgn3*$^{+/-}$ females**	Males: *WT* *Nlgn3*$^{y/-}$	Females: *WT* *Nlgn3*$^{+/-}$	STOP-tetO for *Nlgn3* deletion *Nlgn3*	Chapter 6

2.1.4. DNA extraction and genotyping

In order to extract the DNA, ear biopsies were heated with 150 µl of 0.5 M NaOH for 1 h at 90°C. The samples were then placed on ice for 5 min. Next, 50 µl of TrisEDTA (Tris 1 M, Ethylenediaminetetraacetic acid (EDTA) 4 mM, pH 7.5) was added. The samples were then vortexed and frozen at -20°C. Polymerase chain reaction (PCR) was used to confirm the presence of the genetic construct. The reagents included in the PCR reaction, primers used and the programme for each genetic construct are detailed in Table 6. The buffer and taq polymerise were obtained from New England Biolabs and dNTPs from Promega. The PCR was performed using a T100 Thermal Cycler (Biorad), and the resulting product was loaded on a 2% agarose gel in 1X Tris-borate-EDTA (TBE) buffer, with 1 µl of SaveView nuclei acid stain (NBS Biologicals) per 100 ml of gel.

Table 6 PCR reagents, primers, and cycle settings used for mouse genotyping. [1]

Construct	PCR reagents	Primers	Cycle	Product
STOP-tetO for *Nlgn3* deletion	2.5 µl buffer 0.5 µl 10 mM dNTP 0.5 µl each primer (10 mM) 0.12 µl taq polymerase 1µl DNA 19.88 µl water	Sense: 5' TCCGTGGGCA CATACACATTCA GA 3' Antisense: 5' AGCAGAGCTC GTTTAGTGAACC GT 3'	1. 2 min at 95°C 2. (30 s at 95°C, 30 s at 61°C, 30 s at 72°C) x 29 3. 5 min at 72°C 4. Hold at 4°C.	700 bp
Nlgn3	2.0 µl buffer 0.5 µl 10 mM dNTP 0.4 µl DMSO 1.0 µl each primer (10 mM) 0.36 µl taq polymerase 1 µl DNA 12.24 µl water	Sense: 5' TCCGTGGGCA CATACACATTCA GA 3' Antisense: 5' GGGCTGGATG TTGCAATTGGAG TT 3'	1. 5 min at 95°C 2. (30 s at 95°C, 30 s at 57°C, 1 min at 68°C) x 35 3. 5 min at 72°C 4. Hold at 4°C.	1000 bp
Cre	2.5 µl buffer 0.5 µl 10 mM dNTP 0.2 µl each primer (10 mM) 0.12 µl taq polymerase 1 µl DNA 20.48 µl water	Sense: 5' GGTTATGCGG CGGATCCGAAAA GAAA 3' Antisense: 5' ACCCGGCAAA ACAGGTAGTTAT TCGGATCA 3'	1. 3 minutes at 94°C 2. (10 seconds at 94°C, 30 seconds at 60°C, 30 seconds at 68°C) x 40 3. 10 minutes at 72°C 4. Hold at 4°C	381 bp
Cyfip1	2.5 µl buffer 0.5 µl 10 mM dNTP 0.5 µl each primer (10 mM) 0.12 µl taq polymerase 19.38 µl water.	Sense: 5' CAGGCTGTCT TTTCCTCCTG 3' Antisense: 5' ACTGCAAACAT CCCCTTCAG 3'	1. 1 min at 95°C 2. (30 s at 95°C, 40 s at 60°C, 1 min at 68°C) x 40 3. 5 min at 68°C 4. Hold at 4°C	273 bp
***Cyfip1* construct for deletion**	2.5 µl buffer 0.5 µl 10 mM dNTP 0.5 µl each primer (10 mM) 0.12 µl taq polymerase 19.38 µl water.	Sense: 5' CAGGCTGTCTT TTCCTCCTG 3' Antisense: 5' GAACTTCGGA ATAGGAACTTCG 3'	1. 1 min at 95°C 2. (30 s at 95°C, 40 s at 60°C, 1 min at 68°C) x 40 3. 5 min at 68°C 4. Hold at 4°C	146 bp

[1] Bp = base-pair, dNTP = deoxyribonucleotide triphosphate, DMSO = dimethyl sulfoxide.

Thy1-EGFP	2.5 µl buffer 0.5 µl 10 mM dNTP 0.5 µl each primer (10 mM) 0.12 µl taq polymerase 19.38 µl water.	Sense: CTAGGCCACAGA ATTGAAAGATCT Antisense: CGGTGGTGCAGA TGAACTT	1. 1 min at 95°C 2. (30 s at 95°C, 40 s at 60°C, 1 min at 68°C) x 34 3. 5 min at 68°C 4. Hold at 4°C	415 bp

2.2. Behavioural assays

All of the behavioural tests took place in the light phase of the light/dark cycle. Only adult mice, P60 or later, were tested. All mice were handled for at least two days prior to the experiments or until they were comfortable being picked up. Mice were allowed to habituate to the experimental room for at least 30 min prior to every test. They were tested on the behavioural assays in the following order: activity in the open field, rotarod, interest in social odours, courtship vocalization. A maximum of one test a day was conducted.

2.2.1. Open field activity

The spontaneous activity of mice was recorded. Mice were tested on two consecutive days. During the test, they were individually placed in an open field arena (40 cm x 20 cm x 48 cm, Figure 5) and allowed to explore for 20 min. Although the test was conducted in the dark, the bottom of the arena was illuminated from the bottom by an infrared lamp in order to allow the tracing of the mice, which was recorded using a video camera placed above the arena. The traces were recorded and quantified in EthoVision XT (Noldus). The same software was used to define the centre of the arena (area 5 cm or more away from the walls) and to quantify the time spent in this area.

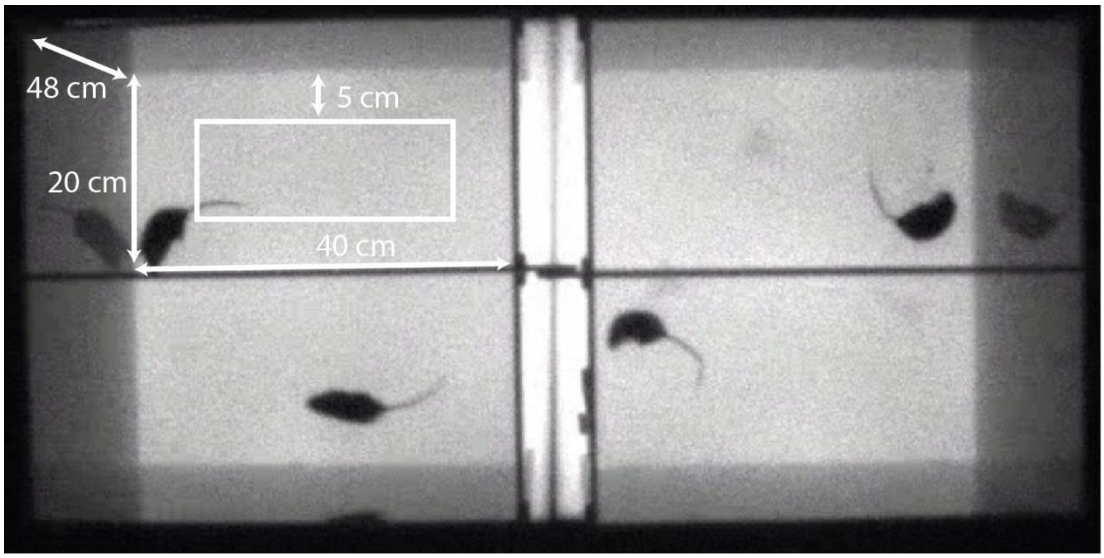


Figure 5 **The open field arena.** The arena was 40 cm x 20 cm x 48 cm. Four arenas were placed in a grid and up to four mice could be tested at simultaneously.

2.2.2. Rotarod

Motor learning of the mice was assessed using rotarod (Ugo Basile 7650, Jones and Roberts 1968). The latency to fall off the rod was assessed across 10 trials lasting 5 min, repeated during three subsequent days.

During the trial, mice were placed on the rod. The rotarod was then switched on and accelerated from 4 to 40 revolutions per minute (rpm) over the course of 5 min. Mice were allowed to walk on the rod until they fell off, gripped to the rod and the rod made a full revolution, or 5 min had passed. Falling off or gripping the rod was interpreted as an inability to cope with the task any longer and signalled the end of the trial. Latency to fall was measured using a stopwatch (Casio©). After each trial, mice were allowed to rest for 5 min at the bottom of the apparatus.

2.2.3. Social odour interest

Social odours originated from a cage of 3-4 *WT* mice, that were maintained with the same home cage bedding to allow for the concentration of odorants present in the urine. For some of the trials, the cage also contained a maximum of one *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ or *Nlgn3*$^{+/-}$ *Cyfip1*$^{+/-}$ mice. Prior to the trial, cotton buds were wiped on the bottom of the home cage in a zig-zag fashion to obtain the social odour cue. Next, mice were placed in the experimental arena and were allowed to habituate for 2 min. Then they were exposed to a clean cotton bud for 2 min, which was then swapped for a new clean cotton bud, and mice were allowed to interact with it for another 2 minutes. Finally, mice were exposed to a cotton bud with the odour cue for 2 minutes, which was then swapped for a new cotton bud with an olfactory cue for another 2 minutes. Male mice were exposed to olfactory cues originating from a cage of male mice, while female mice were exposed to olfactory cues originating from a cage of female mice. The trials were recorded with a video camera placed above the experimental arena, using EthoVision XT (Noldus). Time spent sniffing the cotton bud was scored manually, blinded to the genotype.

2.2.4. Ultrasonic vocalisation during courtship

Female mice in oestrus were identified using vaginal lavage followed by cytological staining (Giemsa solution, Polysciences Inc.). The stains were examined under the microscope and the morphology of the cells was assessed to determine which stage of the oestrus cycle the mice were in. Female mice in oestrus were then used as stimulus for this assay.

Male mice were habituated to the experimental arena (40 cm x 20 cm x 48 cm) for 3 min. Next, an unfamiliar female mouse in oestrus was added to the arena and the mice were allowed to interact freely for 3 min. Ultrasonic vocalisations (USVs) between 40 and 250 Hz produced by the male mice were recorded using a preamplifier UltraSoundGate 416 H, Avisoft Bioacoustics) connected to a microphone (UltraSoundGate CM16, Avisoft Bioacoustics), located at the top of the wall of the arena. It has been previously reported that only males emitted USVs towards females, thus all of the vocalisation was attributed to the males (Whitney *et al.* 1973). The total number of USVs and their duration was analysed using SASLabPro (Avisoft Bioacoustics). A USV needed to fall within the frequency of 30 to 200 Hz and last longer than 5 ms (Holy and Guo 2005) to be included in the analysis.

The trials were recorded with a video camera placed above the experimental arena, using EthoVision XT (Noldus). The interaction time between the mice was manually scored blinded to the genotype of the mice. Duration of interaction between two animals was reported as time mice spent within 2 cm of each other, without counting tail-to-tail interactions.

2.3. Biochemistry and molecular biology

2.3.1. Brain dissection

Mice were culled by cervical dislocation. Death was confirmed by decapitation (ASPA, Home Office 1986). The brain was extracted and transferred into an ice-cold brain matrice (Electron Microscopy). The brain was then sliced with ice-cold blades (Electron Microscopy). The cuts were made coronally from the anterior to the posterior side. After cutting off the olfactory bulb, cuts were performed at 1.5 mm, 1 mm, 1mm, 1.5 mm, 1.5 mm, and 1.5 mm, as illustrated in Figure 6. Next, the slices were transferred onto the ice-cold metal block and micro-dissected in Phosphate-Buffered Saline (PSB, Thermo Fisher Scientific), according to the Mouse Brain Atlas in Stereotaxic coordinates (Paxinos and Franklin 2004). Samples for protein extraction and RNA extraction were snap-frozen in liquid nitrogen and stored at -80°C. The samples for co-immunoprecipitation were immediately transferred to an appropriate buffer as described in Section 2.3.3.

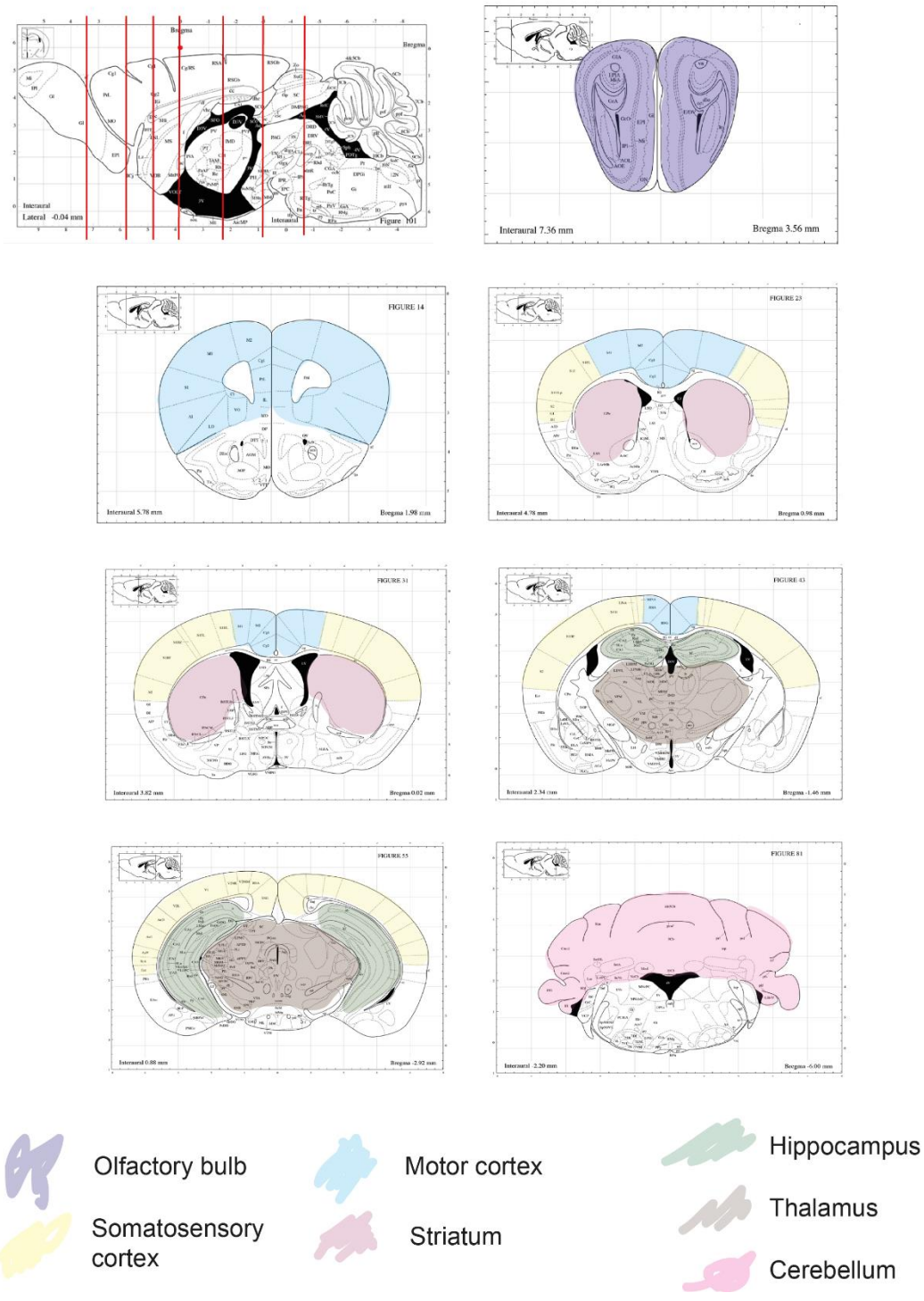


Figure 6 **Consecutive coronal cuts in the mouse brain during dissection.** Illustration adapted from the Mouse Brain Atlas in Stereotaxic coordinates (Paxinos and Franklin 2004).

2.3.2. Protein extraction

Tissue samples were weighted and lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 50 mM NaCl, 1% triton, 10 mM NaF, 1mM $NaVO_4$, 1 mM dithiothreitol, 10 µl per 1 ml complete protease inhibitor cocktail (Merck)) was added, at 100 µl per 10 mg of tissue. The samples were then homogenised in the buffer and placed on a rotator for 30 min at 4°C. Next, samples were centrifuged at 14,000 *g* for 30 min. The supernatant was removed and mixed with sample buffer (106 mM Tris HCl, 141 mM Tris base, 2% Lithium dodecyl sulfate, 10% Glycerol, 0.51 mM EDTA, 0.22 mM Brilliant Blue, 0.175 mM Phenol Red, pH 8.5) and 50 mM dithiothreitol. The samples were then heated for 10 min at 70°C and stored at -20°C prior to Western Blotting.

2.3.3. Co-immunoprecipitation

Tissues dissected as described in Section 2.3.1 were homogenized in 100 µL of lysis buffer for each 10 mg of tissue (20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 10 mM NaF, 1 mM Na_3VO_4, 10 µl per 1 ml complete protease inhibitor cocktail (Merck)). Samples were centrifuged at 14,000 *g* for 10 min at 4°C and the supernatant containing the proteins was removed. Ethanol was removed from Protein A-Sepharose beads (GE Healthcare Life Sciences) by two washes with ice-cold phosphate-buffered saline, at 2000 *g* for 2 min.

The supernatant was pre-cleared by incubating with Protein A-Sepharose beads for 30 min at 4°C. For the input sample, 10% of the protein extract was removed at this stage. Protein A-Sepharose beads for immunoprecipitation were washed with lysis buffer at 2000 *g* for 2 min. Proteins were then incubated for 2 h at 4°C with Protein A-Sepharose beads and 2 µl anti-Neuroligin3 antibody (#129311, Synaptic Systems, 1 mg/ml). Unbound proteins were removed by three washes with lysis buffer. Peptides were then eluted in lithium dodecyl sulfate buffer (106 mM Tris-HCl, 141 mM Tris-base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM Brilliant Blue, 0.175 mM Phenol Red, 10 mM DTT) at 70°C for 10 minutes. Samples were frozen at -20°C for storage.

2.3.4. Western blot

Prior to blotting, the samples were incubated at 70˚C for 10 min and centrifuged at 14000 *g* for 10 min. Proteins were then separated on 4-12% NuPAGE Bis-Tris gradient gel (Invitrogen) and transferred to nitrocellulose membrane (GE Healthcare) using wet transfer. The blots were blocked with 5% milk (BioRad) in TBS-T for 1 h and incubated with primary antibody overnight at 4° C. The primary antibodies listed in Table 7 were diluted in 5% milk (BioRad) in TBS-T at 1:1000 dilution. Following the overnight incubation, membranes were

washed 3 times with TBS-T. To visualize the binding of primary antibodies, anti-rabbit IgG or anti-mouse IgG HRP-conjugated secondary antibody (1:20,000 dilution, Promega) were used. The membranes were incubated with the secondary antibodies diluted in 5% milk in TBS-T for 2 h at room temperature. The membranes were then washed 3 times with TBS-T. Chemiluminescence was developed using the WesternBright ECL substrate (Advansta). Blots were imaged using imaging hardware (Bio-Rad) and Image Lab 5.0 software (Bio-Rad). Band sizes were quantified using the Image Lab 5.0 software (Bio-Rad).

Table 7 Primary antibodies used for Western Blotting.[2]

Antibody	Type	Host	Cat. no	Distributor	Concentration
Anti-Neuroligin3	mAb	mouse	335B8	Synaptic Systems	1 mg/ml
Anti-Neuroligin3	mAb	rabbit	EPR16158	Abcam	1.921 mg/ml
Anti-CYFIP1	pAb	rabbit	07-531	Millipore	1 mg/ml
Anti-FMRP	pAb	rabbit	4317	Cell Signalling	1 mg/ml
Anti-CYFIP1	pAb	rabbit	Ab108220	Abcam	1 mg/ml
Anti-WAVE1	mAb	mouse	MABN503	Millipore	1 mg/ml
Anti-Neuroligin1	mAb	mouse	4C12	Synaptic Systems	1 mg/ml
Anti-Neuroligin2	mAb	mouse	5E6	Synaptic Systems	1 mg/ml

[2] mAb = monoclonal antibody pAb = polyclonal antibody Cat. no = Catalogue number.

2.3.5. Mass-spectrometry analysis

Four samples from the striatum of *Nlgn3*$^{y/-}$*Pvalb*$^{Cre/+}$ mice and four samples from the cerebellum of the same animals were co-immunoprecipitated for Neuroligin3 as described in Section 2.3.4. The samples were then analysed by mass-spectrometry at the University of Bristol Proteomics Facility in collaboration with Dr. Kate Heesom.

Samples were separated on a sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) gel, until the samples entered 3 cm into the gel. Each lane of the gel was then cut into three pieces and each of them was digested using DigestPro automated digestion unit (Intavis Ltd.). Peptides were then fractioned with the Ultimate 300 nano-LC system with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Data were processed with Proteome Discoverer software v1.4 (Thermo Scientific) and compared against the UniPort Mouse database with the SEQUEST algorithm. Peptide precursor mass tolerance was 10 ppm, and MS/MS tolerance was 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of one missed cleavage was permitted. The data were then filtered by a false discovery rate of 1%. The complete dataset is available as part of Bachmann *et al.* (2019) publication.

One sample originating from the cerebellum was excluded because Neuroligin3 was not detected. A striatum sample was excluded because Neuroligin1 and 2 were not present despite being known interactors of Neuroligin3 (Shipman and Nicoll 2012). The samples were analysed in combination with an existing dataset of samples co-immunoprecipitated for Neuroligin-3 from the vomeronasal organ (VNO) of *WT* and *Nlgn3*$^{y/-}$ mice. The VNO dataset was established by Dr. Ellen Cross.

Cluster analysis was performed on the data using RStudio and SPSS Statistics software. Any proteins precipitated in the *Nlgn3*$^{y/-}$ samples were excluded, as they were likely to be false positives. The dataset was binarized because the mass-spectroscopy analysis did not provide quantifiable results concerning the amount of protein detected. The set of proteins present in all samples was considered. Proteins detected in a particular sample were assigned a score of 1, while proteins not detected was assigned a score of 0. A hierarchical cluster analysis was performed using R Studio. Next, a two-step cluster analysis was conducted with the obtained number of clusters in SPSS Statistics.

2.3.6. RNA Extraction

All procedures were conducted in ribonuclease free conditions. Tissues were dissected and stored as described in Section 2.3.1. TRIzol Reagent (Invitrogen) was added to the samples, at 1 ml per 50 - 100 mg of tissue. Tissue was homogenised and incubated at room temperature for 5 min. The samples were transferred to Phrasemaker Tubes (Invitrogen) and 0.2 ml of chloroform was added. Then, samples were shaken for 15 s, incubated at room temperature for 15 min and centrifuged for 5 min at 14,000 *g*, at 4°C. The RNA-containing upper phase was mixed with 0.5 ml of isopropanol and the samples were incubated for 1 h at - 80 °C. Following incubation, samples were centrifuged for 10 min at 10,000 *g*. The supernatant was removed, and the pellet was washed using 75% EtOH. The pellet was dissolved in RNA free water and treated with deoxyribonuclease 1 (QIAGEN) as per manufacturer instructions. The resulting samples were stored at - 80 °C until further processing as described in Section 2.3.7.

2.3.7. RNA Sequencing

RNA sequencing was performed at the School of Bioscience's Genome Hub of Cardiff University in collaboration with Angela Marchbank and Dr Daniel Pass. Quality control of the RNA samples was confirmed by Tape Station and Qubit. The minimum RIN score was 7.3. The library was prepared according to manufacturer instructions (Illumina TruSeq), briefly described below.

Total RNA was purified with magnetic beads to remove ribosomal and non-messenger RNA. mRNA was then transferred for first-strand cDNA synthesis with superscript II. Next, the second strand of the cDNA was synthesised, and the template eradicated. Adapters were ligated to the cDNA. The cDNA was then amplified to enrich the libraries, tested on a DNA chip for quality control and the library size was normalised. The RNA sequencing was performed according to manufacturer instructions using the Illumina NextSeq500 system in 1x75 bp cartridge. A strand of cDNA was bound to a docking site and fluorescent dNTPs were added one at a time.

After the sequencing, the sequences were trimmed with Trimmomatic (Bolger *et al.* 2014) and assessed for quality with FastQC. All of the samples have passed the quality assessment, including the percentage of duplicate reads, sequence quality score, sequence length distribution, and possible adaptor contamination. STAR was used to map the reads onto the Mouse Genome Assembly GRCm38 (Dobin *et al.* 2012). Transcripts were assigned using Feature Counts (Liao *et al.* 2014). Downstream analysis was done in R 2.6.2 (R Core

Team 2019). Version 1.28.1 of DESeq2 package was used for differential gene expression (Love *et al.* 2014). The log-fold shrinkage was turned off and the adjusted p-value was set to < 0.1. The relative expression of genes was assessed in pair-wise fashion to include all housing and genotype conditions. The values were normalised using the implementation of variance-stabilizing transformation from the DESeq2 package. Principal component analysis (PCA) of the top 100 genes with the greatest fold expression differences was conducted using the R function procomp. Weighted Gene Correlation Network Analysis (Langfelder and Horvath 2008) was performed on normalised data, with a power of 5 and a minimum module size of 200.

2.4. Dendritic spine density quantification

Dendritic spine quantification was conducted in the motor and visual cortices of mice which also expressed enhanced green fluorescent protein (EGFP) under the *Thy-1* promoter. The straining was present primarily in the layer V of the cortex. Mice were anesthetised with Euthatal and perfused with 4% paraformaldehyde in 0.1 M PBS. The entire brain was dissected and post-fixed overnight in 4% paraformaldehyde in 0.1 M PBS, then kept in 30% sucrose solution until saturated and stored at -80 °C. The brains were cut coronally into 50 µm sections on a cryostat (Leica Biosystems, Germany) and immediately mounted on glass slides. Four animals per condition were included and six slides per animal were imaged. Two dendrites per slides were selected in order to sample the whole area of the brain region of interest. The regions of interest were identified using a mouse brain atlas (Paxinos and Franklin 2004) andZ-stack images spaced 0.5 µm apart were acquired on a Zeiss LSM700 upright confocal microscope (Carl Zeiss, Welwyn Garden City, UK), using a ×40 water immersion lens. The images were reconstructed into two dimensions using Z-stack maximum intensity projection in ImageJ (NIH, USA, public domain). The images were analysed blinded to the genotype of the animals. Spines were identified manually and counted on a 20 – 250 µm long stretch of a dendrite, with a minimum of 24 dendrites from four mice, per condition. Spine density was calculated as number of spines per 10 µm of a dendrite.

2.5. Statistical analysis

Data analysis was conducted using R software, version 3.6.2. (R Core Team 2019) or when an appropriate package was not available using Graph Pad Prism version 8.3.1. (La Jolla California USA). Histograms and boxplots of the data were scanned for potential outliers, which were confirmed using Rosner's test. Two outliers were removed out of the activity in the open field data and five out of the social olfaction dataset.

For the behavioural data, when an outcome variable was compared between two groups only, a t-test or a non-parametric equivalent (Mann Whitney U test or Wilcoxon Signed Rank test) was used. To determine if a t-test could be used, the assumptions of normal distribution and homogeneity of variance of raw data were tested. The raw data was assessed using the Shapiro-Wilk test and by visual inspection of the Q-Q plot. When Shapiro-Wilk test was significant, a non-parametric test was used. The homogeneity of variance was assessed using Levene's test. If the resulting p-value was less than 0.05 the assumption of homogeneity of variance was determined to be violated and therefore a non-parametric test was used.

In cases where an outcome variable was compared between more than two groups of one variable, one-way Analysis of Variance (ANOVA) was used. The assumption of the normal distribution of data was tested by visual inspection of a histogram of residuals and Shapiro-Wilk test conducted on residuals rather than raw data, while the homogeneity of variance assumption was evaluated by visual inspection of a plot of residuals vs fitted values. If the Shapiro-Wilk test resulted in a p-value smaller than 0.05 or heteroscedasticy was observed in the data, non-parametric test was used instead. If the resulting p-value was smaller than 0.05, a post hoc test was used to determine which groups differed from each other. To correct for multiple comparisons, Tukey's Honestly Significant Difference (HSD) was used following a classical ANOVA or Dunn's test following the Kruskal-Wallis test, and Games-Howell correction was used following Welch's ANOVA.

When the effect of more than one factor on the outcome variable was investigated, two-way or mixed ANOVA was used. Mixed ANOVA was utilised in cases where an independent factor as well as a repeated measures factors were included in the analysis. The assumptions of normality and homogeneity of variance were checked on the residuals. If they were violated, a non-parametric test was used. The non-parametric mixed ANOVA was conducted according to Noguchi et al. (2012). When a two-way ANOVA was followed by simple effects analysis, Holm-Sidak correction for multiple comparisons was used.

The dendritic spine data were analysed using generalised linear model (GLM). The data was separated by sex and by brain region. Then a linear mixed-effect model was used, where a fixed covariate was the genotype of the animal. In order to account for the dependency among the observations we included the animal identifier as a random effect.

The details of significant results are reported in the text. The details of all the analyses, as well as the Shapiro-Wilk and Levene's tests, can be found in Appendix 1. The mean value and the standard error (SE) of the mean can be found in tables throughout the text.

Chapter 3: Characterisation of the interactome of Neuroligin3 in a mouse model

3.1. Introduction

There is increasing evidence for the convergence of biological pathways in ASD as outlined in Chapter 1. However, the mechanism by which mutations in the relevant genes lead to the same set of phenotypical outcomes remains elusive. Investigating the proteins they encode and their interactions can be utilised as a means to identify the members of a given biological pathway and to disentangle the potential combined effects these proteins have on phenotypes associated with ASD.

Neuroligin3 plays a role in synapse formation and function (Etherton *et al.* 2011; Foldy *et al.* 2013; Hutsler and Zhang 2010; Pizzarelli and Cherubini 2013; Tabuchi *et al.* 2007) and mutations in *Nlgn3* have been liked to ASD (Glessner *et al.* 2009; Jamain *et al.* 2003; Sanders *et al.* 2015). Neurolign3 is composed of a large cholinesterase-like domain outside of the cell, a small transmembrane domain, and a short cytoplasmic tail (Südhof 2008). To date, known interactors of Neuroligin3 include Neuroligin1, -2, and -4, which can form cis-heterodimers embedded in the plasma membrane of neurons and glia (Südhof 2008). Neuroligin3 interacts trans-synaptically with neurexins, allowing for cell-adhesion and synaptic transmission (Jaco *et al.* 2010). Within the cytoplasmic tail of the protein, there is a PDZ-binding domain allowing for the binding of PSD95 and -93, as well as indirect interaction with SHANK1 (Irie *et al.* 1997). The cytoplasmic portion of the protein also contains a gephyrin-binding domain and a newly described WAVE regulatory complex interacting receptor sequence (WIRS) (Chen *et al.* 2014). The WIRS sequence was shown to mediate the interaction between Neuroligin4 and the WAVE regulatory complex, possibly via a surface formed by CYFIP1 and ABI2. Thus, the interaction between Neuroligin3, which contains the WIRS domain, and the WAVE regulatory complex is expected, although it has not yet been confirmed.

Protein interactions can vary depending on the cellular population in which they are expressed (Brown *et al.* 2018; Iossifov *et al.* 2012). Neuroligin3 is present in the brain, where it is produced by both excitatory and inhibitory neurons (Budreck and Scheiffele 2007; Ichtchenko *et al.* 1996). This protein has also been observed in astrocytes, in cell culture, as well as in the secretion of gliomas; and its mRNA has been found in glial cells (Gilbert *et al.* 2001; Li *et al.* 2018; Stogsdill *et al.* 2017; Venkatesh *et al.* 2015). Interestingly, Neuroligin3 expression is present in the olfactory ensheathing cells populating the mouse vomeronasal organ (VNO) (Gilbert *et al.* 2001). As the VNO is devoid of synapses, this raises the possibility that the function of Neuroligin3 in these cells might be different from that described in neurons.

In this chapter, we used the VNO as an example of tissue where Neuroligin3 is non-synaptic and likely expressed in glia.

Neuroligin3 is also present in parvalbumin-expressing (Pv+) interneurons. These cells are GABAergic interneurons, including chandelier and basket cells in the cortex, basket cells in the hippocampus, and Purkinje cells, basket and stellate interneurons in the cerebellum (Gabbott and Bacon 1996; Klausberger *et al.* 2005; Kosaka *et al.* 1993; Rudy *et al.* 2010; Schwaller *et al.* 2002). Some Pv+ cells are also found in the thalamus, striatum, brainstem, and olfactory bulb (Arai *et al.* 1994; Bennett-clarke *et al.* 1992; Kita *et al.* 1990; Miyamichi *et al.* 2013; Teramoto *et al.* 2003). The level of parvalbumin was shown to be decreased in mouse models of ASD including mice lacking *Nlgn3* (Gogolla *et al.* 2009; Dong *et al.* 2016; Martins *et al.* 2011; Penagarikano *et al.* 2011; Selby *et al.* 2007). Expression of a number of genes associated with ASD such as *MECP2, COX10,* and *CNTNAP2* specifically in the Pv+ cells is linked to changes in motor behaviour, learning and social behaviour (Inan *et al.* 2016; Ito-ishida *et al.* 2015; Selimbeyoglu *et al.* 2017; Wöhr *et al.* 2015). Interestingly, optogenetic activation of Pv+ cells was found to restore social behaviour in Neuroligin3 R451C mutant mice (Cao *et al.* 2018). Similarly, selective re-expression of *Nlgn3* in Pv+ cells in the brainstem, striatum, thalamus, cortex, and cerebellum resulted in the restoration of social behaviour in male mice (Kalbassi *et al.* 2017). The association between *Nlgn3* in Pv+ cells and behaviour could be mediated by its effect on synaptic transmission. Deletion of *Nlgn3* specifically in Pv+ cells led to a reduction in NMDAR-mediated synaptic transmission, increased glutamate release related to mGluR3 signalling and changes in the theta and gamma rhythms (Dickinson *et al.* 2015; Larrain-Valenzuela *et al.* 2017; Maxwell *et al.* 2015; Polepalli *et al.* 2017). Thus, function of Neuroligin3 in Pv+ neurons might play a role in establishing the phenotypes associated with ASD.

In this chapter, we explored the interactome of Neuroligin3 present at synapses in Pv+ neurons. We also investigated the interacting proteins of non-synaptic Neuroligin3 present in the VNO, presumably in glial cells. We compared the resulting interactomes and discussed interesting targets.

3.2. Aims and objectives

1. To confirm that Neuroligin3 is expressed in glial cells in the VNO.
2. To compare the interactome of Neuroligin3 is Pv+ neurons in the brain and ensheathing cells in the VNO.
3. To validate known interactors of Neuroligin3 and identify new targets, in neurons.

3.3. Results

3.3.1. Neuroligin3 is not expressed in neurons in mouse vomeronasal organ

To confirm that *Nlgn3* is not present in neurons in the VNO, we used an $Omp^{Cre/Cre}$ mouse line, where *Cre* is produced in cells containing Olfactory Marker Protein (OMP). This protein is expressed selectively in olfactory neurons in mice (Chang and Parrilla 2016). We crossed the $Omp^{Cre/Cre}$ females with $Nlgn3^{y/fl}$ males to obtain $Omp^{Cre/+}$ $Nlgn3^{y/fl}$ mice (Figure 7 A). The $Nlgn3^{y/fl}$ mice contained a STOP codon preventing transcription of *Nlgn3*, flanked by *loxP* sites. Thus, *Cre* was able to excise the STOP condom, resulting in a re-expression of *Nlgn3* in neurons containing OMP. However, no re-expression of *Nlgn3* in the VNO of these mice was observed (Figure 7 B), suggesting that neurons in this tissue do not normally express *Nlgn3*. Neuroligin3 in the VNO (Figure 7 B) might be present in non-neuronal cells such as ensheathing glia instead, which do not express OMP (Gilbert et al. 2001).

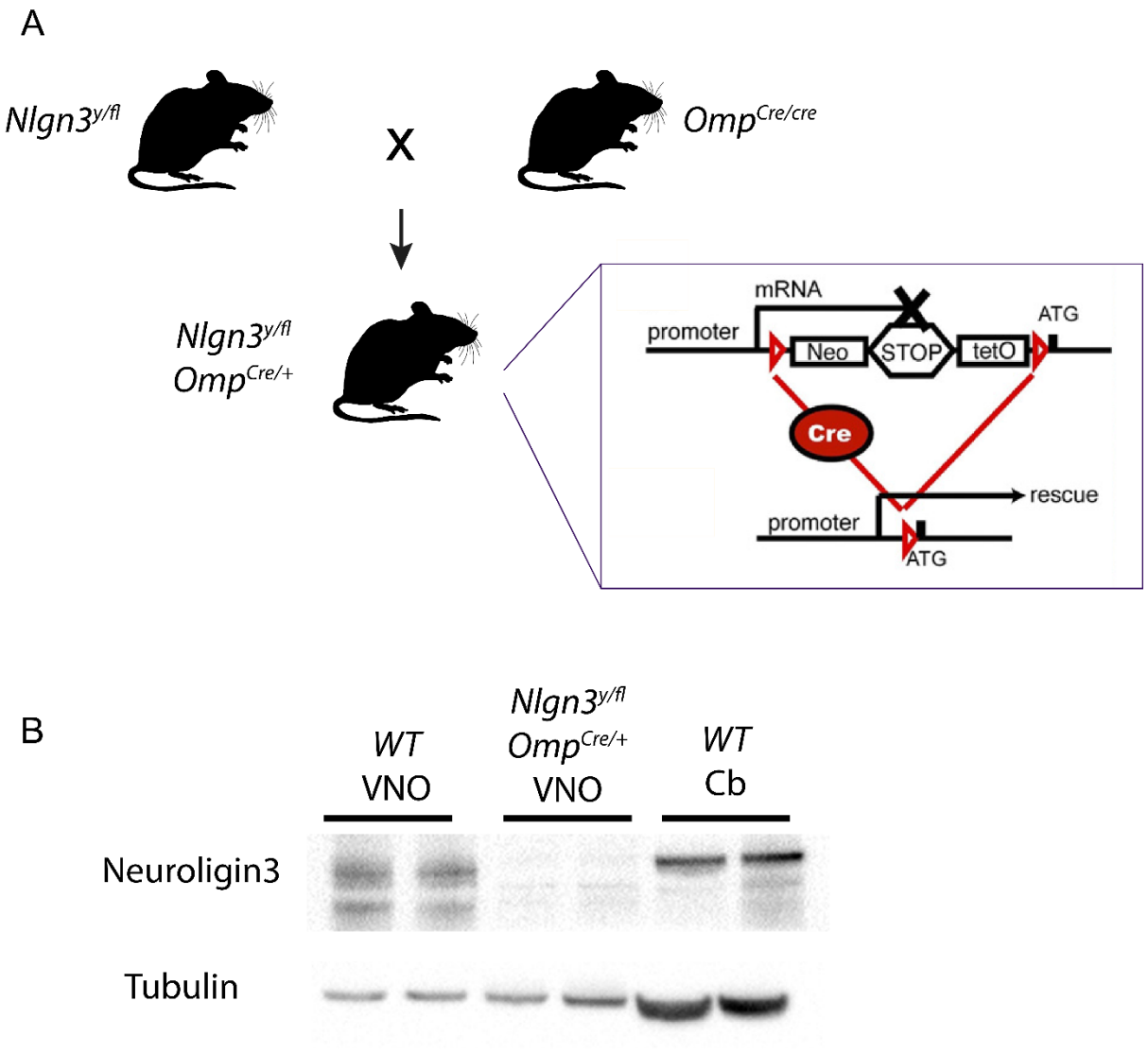


Figure 7 **Neuroligin3 was not expressed in neurons in the VNO. A** Breeding scheme to obtain the *Omp*$^{Cre/+}$*Nlgn3*$^{y/fl}$ mice. *Omp*$^{Cre/Cre}$ females, where *Cre* was activated in the neurons containing Olfactory Marker Protein were crossed with *Nlgn3*$^{y/fl}$ males. In the *Omp*$^{Cre/+}$*Nlgn3*$^{y/fl}$ offspring the STOP cassette preventing expression of *Nlgn3* was excised by *Cre,* allowing for the re-expression of *Nlgn3* in cells containing OMP, adapted from Tanaka *et al.* (2010) **B** Western Blot showing Neuroligin3 expression in the *WT* VNO and cerebellum, as well as the VNO of *Omp*$^{Cre/+}$*Nlgn3*$^{y/fl}$ mice. While Neuroligin3 is present in both the cerebellum and the VNO of WT mice, there is no signal for the *Omp*$^{Cre/+}$*Nlgn3*$^{y/fl}$ VNO. VNO = vomeronasal organ, Cb = cerebellum.

3.3.2. Neuroligin3 interacts with other ASD-associated proteins

To investigate the interactome of Neuroligin3, we combined co-immunoprecipitation to isolate the interactors of Neuroligin3 with mass-spectrometry to identify them (Figure 8 A). Neuroligin3 is expressed both in neurons and in glia in the brain. In order to detect interactors of Neuroligin3 that are specific to the neuronal population, we used striatum and cerebellum of *Nlgn3*$^{y/fl}$*PvalbCre*$^{/+}$ mice (*n* = 3). In these mice, *Nlgn3* is re-expressed only in Pv+ neurons. Those brain samples were compared to the VNO, where Neuroligin3 is likely present in non-neuronal cells (*n* = 3). Additionally, samples of VNO of *Nlgn3*$^{y/-}$ mice were included, where no Neuroligin3 was present to account for unspecific binding during co-immunoprecipitation (*n* = 2).

Overall, there were fewer interactors of Neuroligin3 in the VNO than in the brain. There was little overlap in terms of protein identity between the brain and the VNO. VNO and striatum shared only 1% of the found proteins, VNO and cerebellum shared 6% of the proteins, while all three tissues shared 12% of them (Figure 8 B). The overlap between the two brain regions was much greater, with 28% of interacting proteins in common. Therefore, we conducted a cluster analysis to determine if the division of the proteins by anatomical region of origin described the dataset well. Following the exclusion of the proteins co-immunoprecipitated in the *Nlgn3*$^{y/-}$ samples, hierarchical cluster analysis determined that three clusters described the dataset best. The subsequent two-step cluster analysis was therefore performed to divide the proteins into three clusters. The first, second, and third clusters contained 19.3%, 46.0%, and 34.7% of the proteins respectively (Figure 9 A). In order to determine if these clusters corresponded to the brain regions analysed, the number of proteins originating in each of the brain regions was analysed per cluster. The first cluster contained proteins from striatum and cerebellum but not VNO, the second cluster contained a mix of proteins originating from all three tissues, and the third cluster contained almost exclusively proteins from the striatum (Figure 9 B). This data suggested that interactors of Neuroligin3 in neuronal cells in the brain and non-neuronal cells in the VNO were likely to vary.

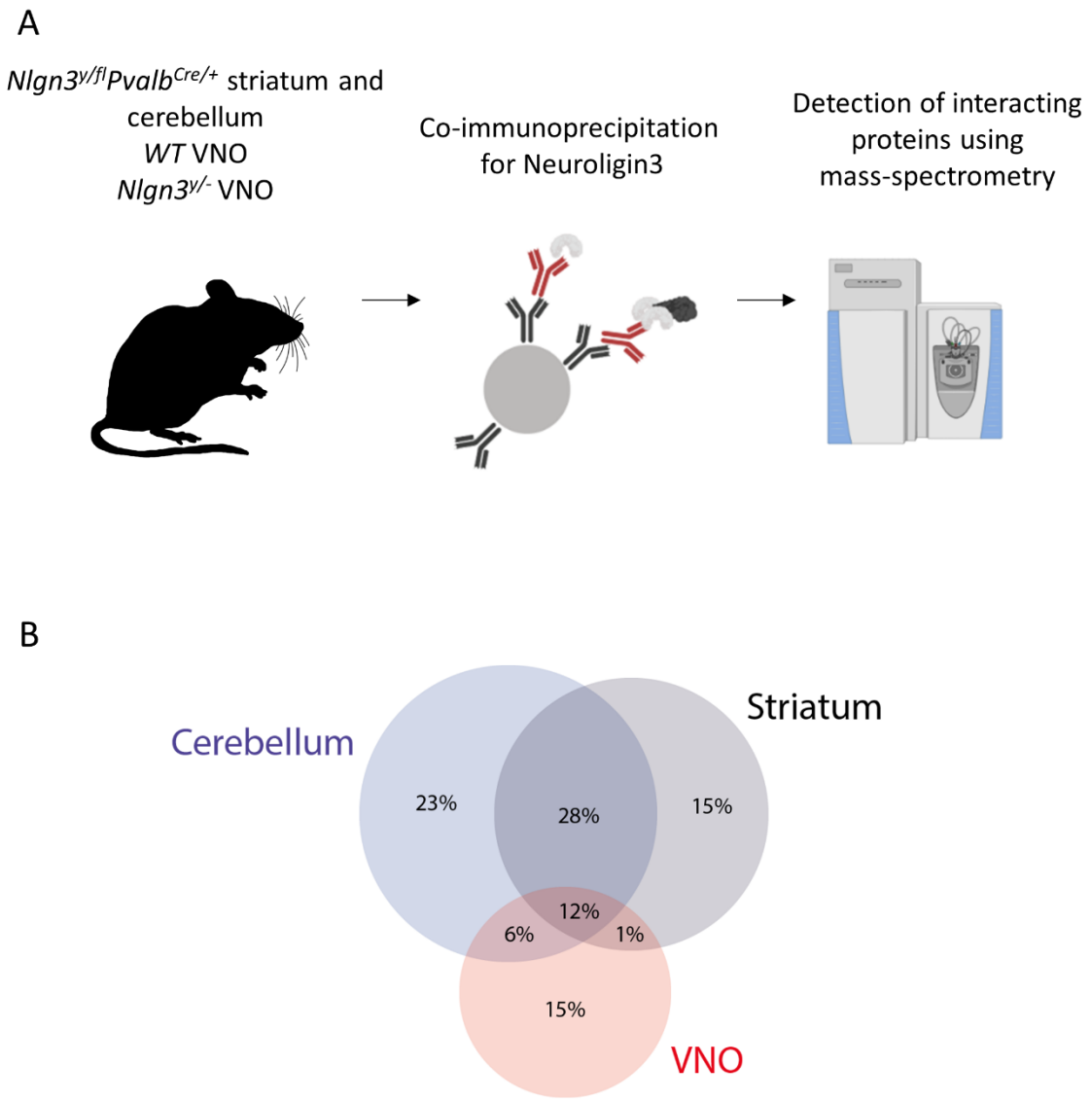


Figure 8 **Neuroligin3 interactors differed between VNO and brain. A** Schematic illustrating the experimental flow. Striatum and cerebellum from *Nlgn3*$^{y/fl}$*Pvalb*$^{Cre/+}$ mice as well as VNO from *WT* and *Nlgn3*$^{y/fl}$ were obtained and proteins were extracted. Co-immunoprecipitation was conducted on the samples, and the interacting proteins were identified using mass-spectrometry. **B** Venn diagram showing the percentage of shared interacting proteins between the three tissues (the cerebellum and striatum of *Nlgn3*$^{y/fl}$*Pvalb*$^{Cre/+}$ mice and the VNO of *WT* mice). Striatum and cerebellum shared many more Neuroligin3 interactors than either of the brain tissues with the VNO.

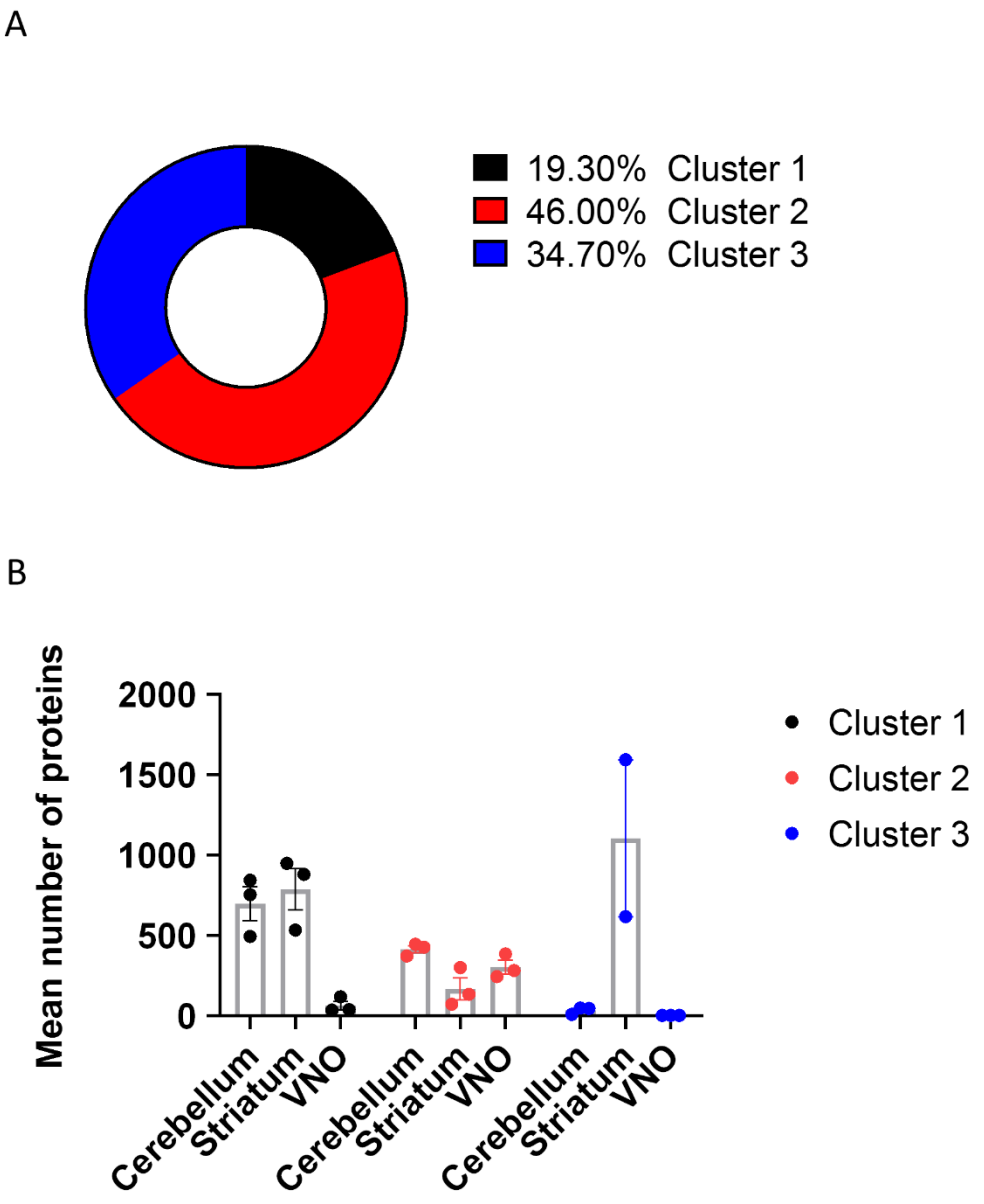


Figure 9 **Clusters of Neuroligin3 interactors corresponded to the tissue of origin. A** Sizes of individual clusters following two-step clustering. **B** Number of proteins in each cluster originating in the cerebellum or striatum of the *Nlgn3*$^{y/-}$*Pvalb*$^{Cre/+}$ mice or the VNO of *WT* mice. Cluster 1 contained primarily interactors from cerebellum and striatum and Cluster 3 contained interactors from the striatum only.

Among the interactors of Neuroligin3 found in the striatum and cerebellum were proteins characteristic of excitatory and inhibitory neurons. Proteins likely originating from excitatory neurons included PSD93, PSD95, SAP97, SAP102. Inhibitory synaptic proteins comprised Neuroligin2, Neuroligin3, and Gephyrin. Additionally, Neuroligin3 interacted with FMRP, WAVE1, and ABI1. While FMRP was detected in the Pv+ cells in both cerebellum and striatum, WAVE1 and AB1 were only identified in the striatum. CYFIP1, the known interactor of both FMRP, WAVE1, and ABI, was also found among the interactors of Neurolign3 in the striatum and cerebellum. Interestingly, several other proteins linked to ASD were also present in this analysis of Neuroligin3 interactors in the Pv+ cells in the brain (Table 8).

Table 8 Neuroligin3 interactors in the parvalbumin-expressing neurons of striatum and cerebellum were associated with ASD.[3]

Protein	SFARI score	Striatum/Cerebellum	Description
FMRP	S	Both	FMRP has a role in synaptic protein synthesis, synaptic plasticity and mRNA regulation. Deletion of *FMR1* is associated with Fragile X Syndrome.
ALDH5A1	S	Both	An enzyme which catalyses the degradation of GABA.
SLC1A2	S	Both	A protein involved in clearing of glutamate from extracellular spaces at the synapse, associated with a number of neurodevelopmental disorders.
SYNGAP1	1S	Striatum	SYNGAP1 is involved in NMDA and AMPA receptor mediated plasticity, axon formation and dendritic spine regulation.
MECP2	2S	Both	MECP2 has the capacity to bind methylated DNA and repress transcription. Mutations in *MECP2* are associated with Rett Syndrome.
GABRB3	2	Striatum	GABRB3 is a ligand-gated ion channel, that serves as a receptor for GABA.
ILF2	2	Both	ILF2 modulates the transcription of IL2 during T-cell activation.
USP7	2	Cerebellum	USP7 is a hydrolyse, which plays a role in deubiquitination of a number of other proteins.

[3] SFARI score was obtained from a database of genes associated with ASD, where the lower score indicates stronger association and S marks the syndromic conditions. In this table, only proteins with a score of 2 or less were included, which had a strong association with ASD.

3.3.3. Neuroligin3 interacts with other Neuroligins, CYFIP1 and FMRP in parvalbumin-expressing neurons

The interaction between Neuroligin3 and CYFIP1, as well as with the known interactors of CYFIP1: FMRP, ABI1, and WAVE1, was detected using mass-spectrometry. To confirm these associations, we used co-immunoprecipitation combined with Western Blot (Figure 10). We have included tissue from *WT* animals, where Neuroligin3 is expressed in both neurons and glia to check if the same interactions can be detected in this heterogenous sample. In addition to striatum and cerebellum, protein interactions in the cortex were investigated. Neuroligin3 was found to interact with Neuroligin2 in both brain regions, both in Pv+ cells and in the heterogeneous sample originating from *WT* mice. However, no interaction between Neuroligin3 and Neuroligin1 was detected in the Pv+ neurons, despite it being present in the *WT* samples. The interaction between Neuroligin3 and CYFIP1 was confirmed both in the Pv+ cells and in the *WT* sample. However, the one between Neuroligin3 and FMRP was only detectable in the Pv+ cells and not in the heterogeneous sample originating from the *WT*, which was likely to contain both neurons and glia. These results suggested that there was a degree of cellular specificity in the interactions between these proteins. The confirmation of interactions between Neuroligin3 and WAVE1 as well as Neuroligin3 and ABI1 was attempted, however was unsuccessful due to the lack of effective antibodies.

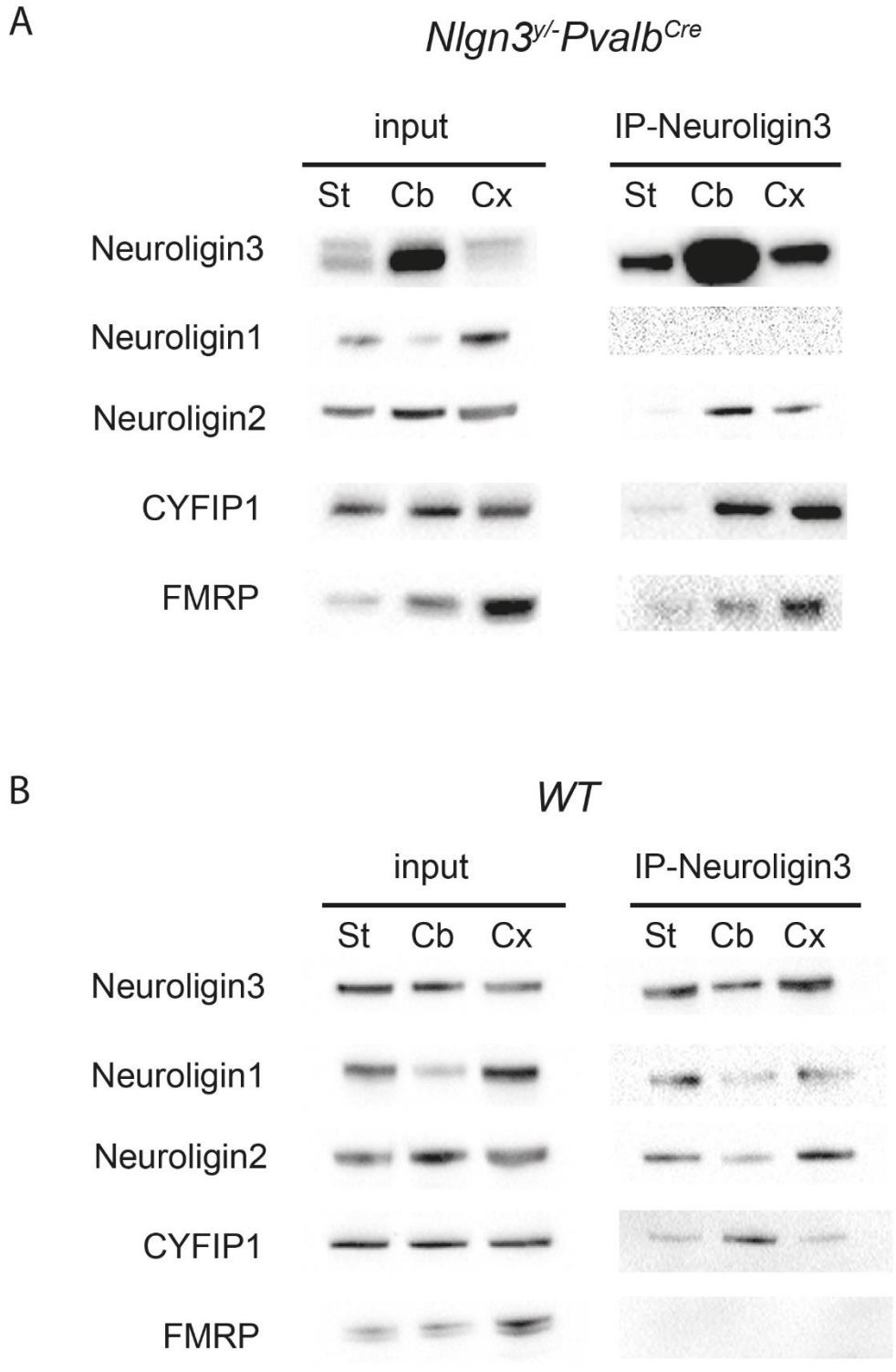


Figure 10 **Western Blot analysis confirmed Neuroligin3 interactors. A** Western Blots following co-immunoprecipitation for Neuroligin3 in *Nlgn3$^{y/-}$PvalbCre* striatum, cerebellum, and cortex. **B** Western Blots following co-immunoprecipitation for Neuroligin3 in *WT* mice striatum, cerebellum, and cortex. St = striatum, Cb = cerebellum, Cx = cortex. Please note the lack of *Nlgn3$^{y/-}$* and loading control.

3.4. Discussion

In this chapter, the interactomes of Neuroligin3 in Pv+ cells of striatum and cerebellum and in ensheathing glia of the VNO were compared. Initially, we confirmed that Neuroligin3 is unlikely to be expressed in neurons in the VNO, suggesting that Neuroligin3 is exclusively expressed in glia in this tissue. Overall, there were fewer interactors of Neuroligin3 in this cellular population than in the Pv+ neurons. There was also little overlap in the identity of the interacting proteins between the VNO and the brain suggesting that these interactions occur in a cell-specific manner. We found a number of other proteins associated with ASD among the interactors of Neuroligin3, supporting the notion of convergence in ASD. Additionally, Neuroligin3 was found to interact with FMRP and CYFIP1, which regulate protein translation and actin polymerisation.

The interactors of Neuroligin3 varied between the glial cells of the VNO and the neurons in the striatum and cerebellum. There were also considerably fewer interactors detected in the VNO than in the brain. This could potentially be an artefact of an uneven amount of starting tissue originating from the VNO and the brain or of differences in the co-immunoprecipitation efficacy between samples. On the other hand, the differences in the number and identity of the interactors between the two cellular populations could potentially be explained by different isoforms of Neurolign3 being present in these tissues. There are two splice insertion sites (A1 and A2) in *Nlgn3*, which result in four possible Neuroligin3 splice isoforms. The expression of these isoforms was examined in the hippocampus and was found to vary within that structure, having a differential impact on synaptic transmission (Uchigashima *et al.* 2020). To determine if the different isoforms of Neuroligin3 have different binding affinity for their interactors, the analysis presented here should be repeated in cells transfected with individual isoforms of Neuroligin3. Additionally, RNA sequencing could be used to investigate the expression of the different isoforms in the striatum, cerebellum and the VNO.

A number of known interactors of Neuroligin3 were detected. Neuroligin3 was found to interact with Neuroligin2 and, in some cellular populations, with Neuroligin1, in line with the observation that Neuroligin3 can form heterodimers with other Neuroligins (Südhof 2008). Neuroligin3 is found both at excitatory and inhibitory synapses, Neuroligin2 is only specific to the inhibitory synapses, while Neuroligin1 is present primarily at excitatory ones (Song *et al.* 1999). This difference in the expression pattern of these two proteins might be responsible for the fact that Neuroligin2 was found to interact with Neuroligin3 in both Pv+ cells and in the heterogeneous sample arising from *WT* tissue, while the interaction between Neuroligin1 and Neuroligin3 was only detectable in the *WT* brain. The formation of heterodimers between

different neuroligins was shown to be important for a range of processes at the synapse. Specifically, mutated Neuroligin3 unable to form dimers was associated with changes in synapse morphology, leading to altered dendritic spine turnover and changes in AMPAR-mediated synaptic transmission (Shipman and Nicoll 2012). Interestingly, the dimerization of neuroligins was also linked to variation in the expression of other synaptic proteins, suggesting that there might be profound downstream consequences of a disruption in the subtle balance of protein interactions (Shipman *et al.* 2011).

The known interactions between Neuroligin3 and PSD95 and PSD93 were also confirmed in the Pv+ cells, using mass-spectrometry. PSD95 and PSD93 are thought to be found exclusively in excitatory neurons. The presence of these interaction may arise from that fact that the re-expression of Neuroligin3 might not have been entirely specific to the Pv+ neurons. Alternatively, binding of these proteins might have occurred in solution, following the lysis of the cells. All of these proteins are part of the postsynaptic compartment and are known to interact with SHANK3, TSC1, and Homer3 (Sakai *et al.* 2011). This network of interactions in the postsynaptic compartment beginning with Neuroligin3 could potentially explain the effect Neuroligin3 had been reported to have on synaptic function. This possibility is further discussed in Section 7.5.

We also uncovered a novel interaction between Neuroligin3 and CYFIP1. CYFIP1 is a member of the WAVE regulatory complex (WRC) and it is generally assumed that CYFIP1 prevents activation of the WRC, that only occurs upon Rac1 binding (Chen *et al.* 2010; Derivery *et al.* 2009; Kobayashi *et al.* 1998). However, there was some indication that it might be important for WRC stability in *Drosophila* (Kunda *et al.* 2003; Zhao *et al.* 2013). CYFIP1 also forms a binding surface along ABI1, which allows for interaction with proteins containing the WIRS domain (Chen *et al.* 2014). Presence of this domain in the cytoplasmic tail of certain adhesion proteins, in combination with the presence of Rac1, was found to enhance the activity of WRC (Chen *et al.* 2014), suggesting that these two ligands can act cooperatively to induce actin polymerisation. Among the adhesion proteins containing the WIRS domain are Neuroligin1, -3, and -4. While the interaction between WRC and Neuroligin4 was previously confirmed, this is the first instance that the association between WRC and Neuroligin3 has been reported, in neurons, *in vivo.* Neuroligin3 was found to interact with CYFIP1, ABI, and WAVE1, all members of the WRC, suggesting that as predicted the interaction with CYFIP1 is likely to occur via the WIRS domain. In line with the data reported in Chen *et al.* 2014, decreasing levels of Neuroligins in *Drosophila*, or deleting the WIRC domain, led to a decrease in the level of polymerised actin (Xing *et al.* 2018). This finding suggests that like other

adhesion proteins, Neuroligins might be able to inhibit the action of CYFIP1, promoting the activation of WRC and subsequent actin assembly.

Interestingly, an interaction between Neuroligin3 and FMRP was noted in the Pv+ neurons. FMRP is a known interactor of CYFIP1 (Abekhoukh, H. Bahar Sahin, *et al.* 2017; Schenck *et al.* 2001). This raises the possibility that the interaction between Neuroligin3 and FMRP occurs via CYFIP1, as the co-immunoprecipitation did not allow for differentiation between direct and secondary interactions. However, a recent report that has shown that FMRP can bind *Nlgn3* mRNA and regulate the levels of Neuroligin3 (Chmielewska et al 2018). In order to determine if the interaction between FMRP and Neuroligin3 is direct, Förster Resonance Energy Transfer (FRET) assay could be used, provided availability of suitable antibodies. Alternatively, the interaction between FRMP and Neuroligin3 could be investigated using co-immunoprecipitation in a population of cells which do not express CYFIP1. The interaction between Neuroligin3 and FMRP, direct or occurring via CYFIP1, implies that Neuroligin3 could potentially have an impact on protein translation.

The discovery of novel interactions between Neuroligin3 and other proteins linked to ASD validates the existence of convergence in biological pathways in these conditions. Previous studies identified several pathways that are likely to be affected in ASD, including cytoskeleton regulation, cell adhesion (Gilman *et al.* 2011; Luo *et al.* 2012), transcription regulation (Luo *et al.* 2012; De Rubeis *et al.* 2014), chromatin regulation (De Rubeis *et al.* 2014; Pinto *et al.* 2014) and immune regulation (Voineagu *et al.* 2011; Gandal *et al.* 2018). In addition to those pathways, neuronal development and maintenance, including synaptic function are the most often reported processes altered in ASD (Bear *et al.* 2004; De Rubeis *et al.* 2014; Gai *et al.* 2012; Gandal *et al.* 2018; Gilman *et al.* 2011; Luo *et al.* 2012; Noh *et al.* 2013; Pinto *et al.* 2014; Purcell *et al.* 2001; Tabuchi *et al.* 2007; Voineagu *et al.* 2011; Zoghbi 2003). Still, while these studies focused on the previously described functions of proteins involved in the ASD, here we propose how these biological pathways might be regulated through interaction between proteins. Neuroligin3 mutation is likely to result in downstream changes in synaptic processes, cell adhesion regulation, cytoskeleton, and transcription regulation via its interactions with other proteins involved in ASD.

3.5. Conclusions

We compared the interactome of Neuroligin3 in neuronal and glial cells. The interactors were different between these two cellular populations, possibly due to different isoforms of Neuroligin3 being present. Among these interactors was a number of other proteins associated with ASD, suggesting that there might be a convergence in the biological function

of these proteins, leading to the same behavioural phenotype. These biological processes might include actin polymerisation and protein translation, as Neuroligin3 was also found to interact with CYFIP1 and FMRP. However, the role of Neuroligin3 in these two processes needs to be investigated further.

Chapter 4: The effect of *Nlgn3* deletion and *Cyfip1* haploinsufficiency on mouse behaviour

4.1. Introduction

In the previous chapter, a molecular interaction between Neuroligin3 and CYFIP1 was confirmed by co-immunoprecipitation, mass-spectrometry and Western Blot. This finding indicates that both proteins are part of the same pathway. Next, we investigated if this pathway impacted on phenotypes associated with ASD.

As described in Chapter 1, ASD is diagnosed based on behavioural symptoms, which include deficits in social communication and interaction as well as stereotyped behaviour and restricted interests (American Psychiatric Organization 2012). As such, the validity of mouse models of ASD is often evaluated in the light of their behavioural reproducibility. The behaviour of mice lacking *Nlgn3* and of those heterozygous for *Cyfip1* has been previously investigated. In both cases, deficits in social behaviour were observed. The social behaviour of mice is strongly informed by olfactory cues (Haga *et al.* 2010; Hoffman *et al.* 2015; Pérez-Gómez *et al.* 2014; Roberts *et al.* 2010). A decrease in the ability to discriminate between odours or in the interest in social olfactory cues were demonstrated in both the mouse models of *Nlgn3* deletion and *Cyfip1* haploinsufficiency (Bachmann *et al.* 2019; Dere *et al.* 2018). However, the behaviour of the two mouse models diverged when other assays were used.

In the context of courtship, males lacking *Nlgn3* tended to vocalise less in response to a female in oestrus (Radyushkin *et al.* 2009; Fischer and Hammerschmidt 2011), while the response of males heterozygous for *Cyfip1* was within the *WT* range. Also, while mice lacking *Nlgn3* tended to be hyperactive in the open field (Radyushkin and Hammerschmidt 2009; Rothwell *et al.* 2014), the mice heterozygous for *Cyfip1* were shown to either be hypoactive (Bozdagi *et al.* 2012) or no different from their *WT* littermates (Bachmann *et al.* 2019). The reverse pattern of impairment was present when the ability to acquire new motor routines was considered. While males with *Cyfip1* haploinsufficiency showed impairment in motor learning (Bachmann *et al.* 2019), males lacking *Nlgn3* outperformed their *WT* littermates (Rothwell *et al.* 2014). The complete comparison of the behavioural phenotypes present in the two models is available in Table 9.

Table 9 Behavioural phenotypes of *Nlgn3*$^{y/-}$ and *Cyfip1*$^{+/-}$ male mice.

Behavioural test	***Nlgn3*$^{y/-}$**	***Cyfip1*$^{+/-}$ male**
Open field activity	Increased distance travelled and no difference in the time spent in the centre from *WT*	Decreased distance travelled / no change from *WT*
Rotarod	No difference / faster improvement across trials than *WT*	No improvement across trials
Three chamber test	No preference for a stranger mouse over an object	Not determined
Interest in social olfactory cues	Less interest than *WT*	Less interest than *WT*
Vocalization during courtship	Fewer calls when exposed to a female than *WT*	No difference from *WT*
Marble burying	Not determined	More marbled buried / no difference from *WT*
Contextual fear conditioning	Reduced compared to *WT*	No difference compared to *WT*
Cued fear conditioning	Reduced compared to *WT*	Reduced compared to *WT*

Investigating how these mutations affect mouse behaviour provides information about the individual roles of *Nlgn3* and *Cyfip1*. However, in order to understand the additive effect of the two genes in shaping behaviour, we generated double mutant mice, both lacking *Nlgn3* and heterozygous for *Cyfip1*. Double mutant mice have been used previously to explore the consequences of interactions between proteins associated with ASD, including FMRP and eIF4E (Huynh *et al.* 2015), as well as, FMRP and CYFIP2 (Han *et al.* 2014).

Deletions of *Fmr1* and *eIF4E* were associated with increased anxiety, enhanced repetitive behaviour and deficits in sociability, in mice. However, mice with a double deletion also presented with cognitive impairment, indicating that the accumulation of genetic mutations resulted in increased severity of the phenotype. This finding could also be interpreted as the two genes regulating the behaviour via two parallel, independent pathways. Similarly, mice lacking *Fmr1* and mice heterozygous for *Cyfip2* showed alteration in dendritic spine morphology and misregulation of mGluR signalling. Both phenotypes were aggravated in the double mutant animals. Therefore, investigating the phenotypes present in mice carrying multiple mutations allowed for an inference of a possible relationship between two proteins.

Mouse behaviour is further affected by sex and social environment. The impact of these factors has not been extensively characterised in the context of the mouse models of ASD. However, the behaviour of female mice lacking *Nlgn3* or heterozygous for *Nlgn3* was previously reported (Kalbassi *et al.* 2017). The females heterozygous for *Nlgn3* did not show a behavioural phenotype, however females with a complete deletion of *Nlgn3* presented with deficits in social behaviour, and an increase in the time spent in the middle of the open field. Therefore, only females lacking *Nlgn3* phenocopied some aspects of the behaviour of the males with *Nlgn3* deletion. In the same study, a deficit in the sociability of *WT* male mice raised with their *Nlgn3* littermates (mixed genotype housing, MGH) in comparison to *WT* males kept only with *WT* siblings (single genotype housing, SGH) was detected. This suggests that the social environment in which the *WT* animals were raised impacted on their social behaviour. Interestingly, while the males lacking *Nlgn3* had the capacity to shape the behaviour of their *WT* littermates, the females without *Nlgn3* had no role in regulating the behaviour of their littermates. These findings indicate that both sex and social environment could have the capacity to module behaviour in mouse models of ASD. However, how these factors impact on the behaviour of mice with *Cyfip1* haploinsufficiency remains unknown.

In this chapter, we describe the behavioural characterisation of double mutant mice lacking *Nlgn3* and carrying only one allele of *Cyfip1.* These mice were compared to their *Nlgn3*$^{y/-}$, *Cyfip1*$^{+/-}$ and *WT* littermates. Behavioural tests where either of the single mutants were previously shown to display deficits were chosen. These included activity in the open

field, motor learning on the rotarod, interest in social olfactory cues, as well as vocalisation and interaction during courtship. To account for the effect of sex on the behaviour we also included female mice heterozygous both for *Nlgn3* and *Cyfip1* along with their single mutant and *WT* littermates. While it was not possible to obtain female mice lacking both alleles of *Nlgn3* as they would require a different breeding scheme than the males, the double mutants lacking one allele of each of the genes of interest could still provide insight into the possible effect of sex on behaviour. Finally, we included a comparison between MGH *WT* mice and a cohort of SGH *WT* mice. This was done only for the males as the females were previously shown not to mediate the behaviour of their *WT* littermates (Kalbassi *et al.* 2017).

4.2. Aims and objectives

1. To investigate if *Nlgn3* deletion, *Cyfip1* haploinsufficiency, or the combination of both affects the behaviour of male mice.
2. To investigate if there is a sex difference in the behaviour of mice heterozygous for *Cyfip1.*
3. To determine if being housed with males lacking *Nlgn3* or heterozygous for *Cyfip1* affects the behaviour of their *WT* littermates.

4.3. Results

To determine if mutations in *Nlgn3* and *Cyfip*1 had a combined effect on the behaviour of mice, *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ male mice and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ female mice were generated. Their behavioural phenotypes were compared against their littermates carrying the single mutation (*Nlgn3*$^{y/-}$ or *Cyfip1*$^{+/-}$ males, *Nlgn3*$^{+/-}$*or Cyfip1*$^{+/-}$ females) as well as their *WT* littermates. The exploratory behaviour of these mice was tested in the open field and their ability to learn motor routines was evaluated using rotarod. The interest of mice in social odours was also assessed, as was their tendency to engage in ultrasonic vocalisations and social interaction during courtship. The descriptive statistics for the measures of behaviour in male and female mice are available in Table 10 and in Table 11 respectively.

4.3.1. *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ male mice were hyperactive in the open field

An open field protocol was used to examine if mutations in *Nlgn3* and *Cyfip1* impacted on the exploratory behaviour of mice. Some of these mice had a *Thy1-EGFP* transgene in addition to mutations in *Nlgn3* and *Cyfip1,* that was utilised in the experiments described in Chapter 5. However, the presence of the transgene did not affect the distance travelled in the open field or the time spent in the centre of the arena (Appendix 1).

Overall, all of the mice tended to cover more distance on the first day of testing rather than the second day (Mixed ANOVA, main effect of day: $F(1, 62) = 35.54$, $P < 0.001$).

Regardless of the day of testing, there was a difference in the distance travelled depending on the genotype (Mixed ANOVA, main effect of genotype: $F(3, 62) = 6.45$, $P < 0.001$). The effect of genotype on this parameter did not vary between the two days of testing. Both *Nlgn3*$^{y/-}$ ($n = 24$) and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ ($n = 12$) mice covered more distance than their *WT* littermates (($n = 18$, Tukey HSD: $t(1, 62) = 2.70$, $P = 0.04$, $t(1, 62) = 3.53$, $P < 0.01$ respectively) as well as their *Cyfip1*$^{+/-}$ littermates ($n = 12$, Tukey HSD: $t(1, 62) = 2.82$, $P = 0.03$, $t(1, 62) = 3.61$, $P <$ 0.01, respectively). This finding indicated that both *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice tended to be hyperactive in the open field. The hyperactivity in these mice might arise from a more pronounced response to the novel environment. To investigate this possibility, we compared the distance travelled on the first and second day of testing. Interestingly, only *WT* mice showed a significant decrease in the distance travelled (Tukey's HSD: $t(1, 62) = -5.30$, $P <$ 0.001), indicative of habituation (Figure 11 A). This suggests that all the mutants lacked habituation to the known environment.

Next, only the general level of activity was considered, regardless of the habituation to the novel environment. While the behaviour on the first day of testing was likely to reflect a response to the novel environment, the second day of testing was a more accurate measure of the general level of activity. The genotype had an effect on the activity on the second day of testing (One-way ANOVA, main effect of genotype: $F(3, 62) = 6.55$, $P = < 0.001$, Figure 12 A), where both *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice covered more distance than *WT* mice (Tukey's HSD*:* $t(1, 62) = 3.88$, $P < 0.001$ and $t(1, 62) = 3.86$, $P < 0.01$, respectively). While there was no significant difference between *WT* and *Cyfip1*$^{+/-}$ mice or between *Nlgn3*$^{y/-}$ and *Cyfip1*$^{+/-}$, *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice also covered more distance than *Cyfip1*$^{+/-}$ mice (Tukey's HSD*:* $t(1, 62) = 2.75$, $P = 0.043$). These findings indicate that *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice phenocopied *Nlgn3*$^{y/-}$ mice in their hyperactivity. There were no significant differences in the time spent in the centre of the arena, suggesting that this phenotype was likely independent of anxiety (Figure 11 B and Figure 12 B).

To confirm these findings, the data was re-analysed such that the absence of at least one of *Nlgn3* or *Cyfip1* alleles was considered as an independent factor. As previously, the distance travelled on the second day was smaller across the different groups (Mixed ANOVA, main effect of day: $F(1, 62) = 35.54$, $P < 0.0001$) and the *Nlgn3* deletion was associated with hyperactivity (Mixed ANOVA, main effect of *Nlgn3* absence: $F(1, 62) = 16.99$, $P < 0.0001$). There were no differences in the time spent in the centre of the arena.

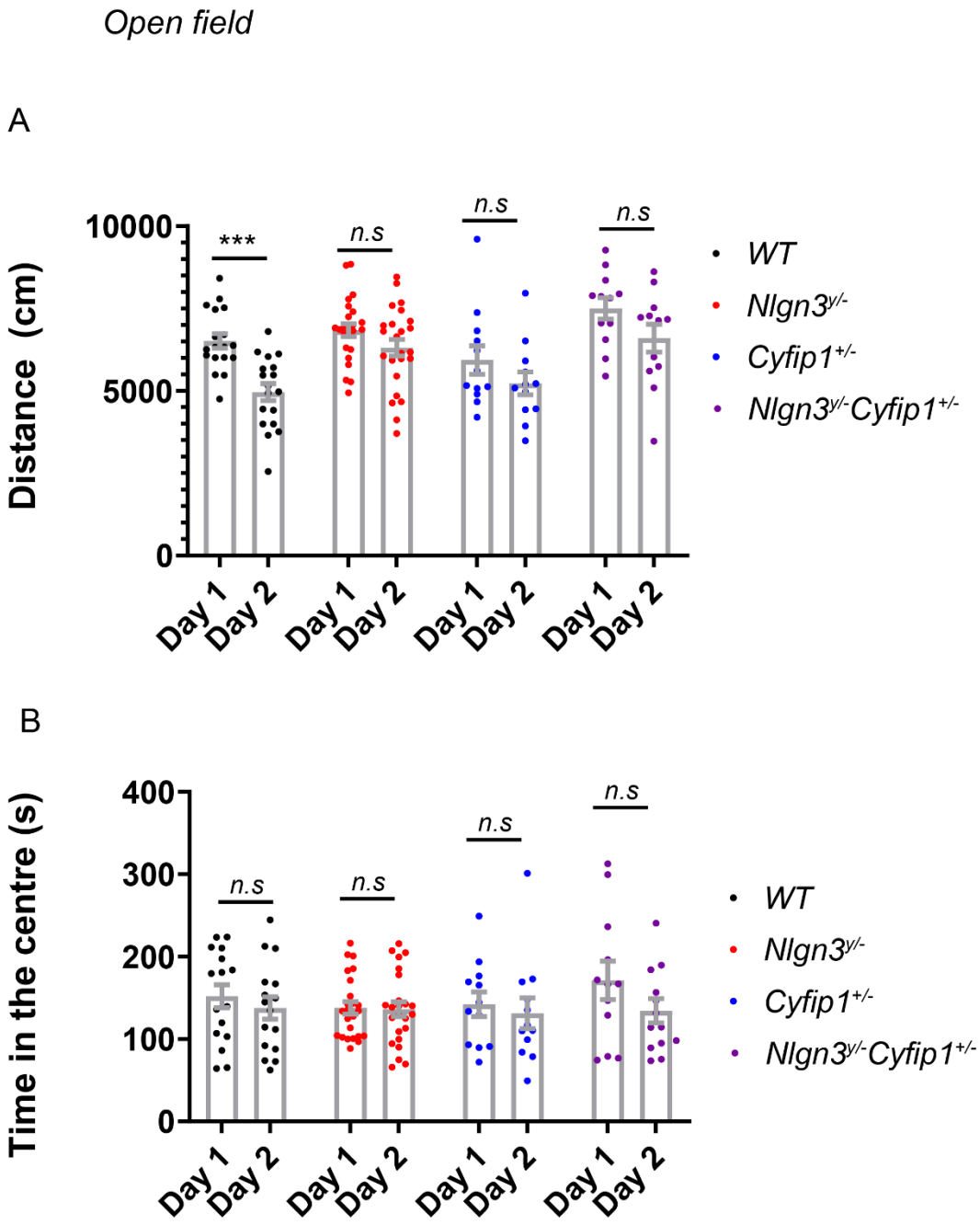


Figure 11 **Both deletion of *Nlgn3* and *Cyfip1* haploinsufficiency caused a deficit in habituation in the open field. A** Distance travelled in the open field by *WT, Nlgn3*$^{y/-}$*, Cyfip1*$^{+/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ males. *WT* males showed a decrease in the distance travelled across two days of testing characteristic of habituation. **B** Time spent in the centre of the open field by *WT, Nlgn3*$^{y/-}$*, Cyfip1*$^{+/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ males. The day of testing had no effect on the time spent in the centre. *** = $P < 0.001$, *n.s* = not significantly different.

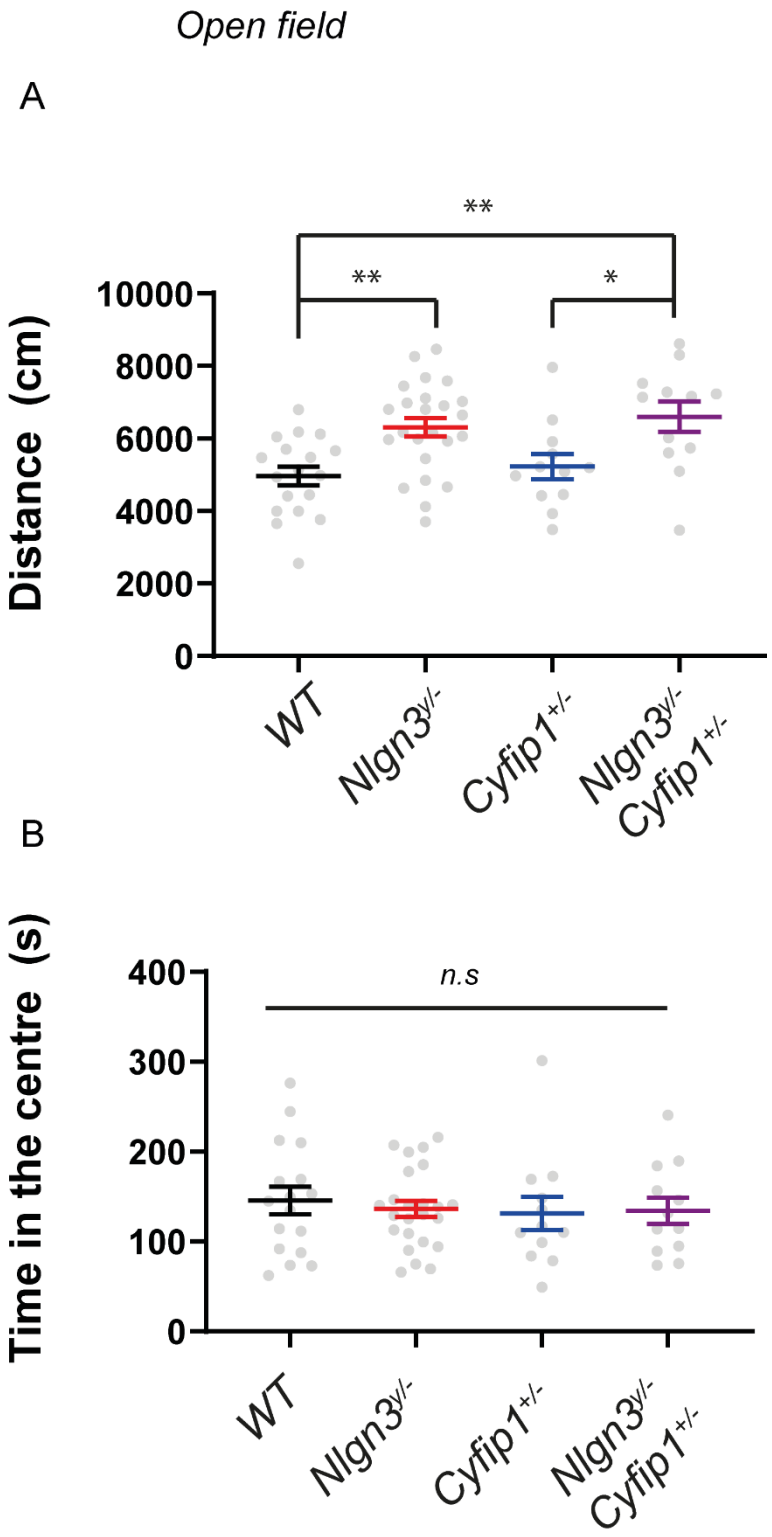


Figure 12 ***Nlgn3$^{y/-}$* and *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* males were hyperactive in the open field. A** The distance travelled in the open field during Day 2 of testing, for *WT*, *Cyfip1$^{+/-}$*, *Nlgn3$^{y/-}$*, and *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* mice. Both *Nlgn3$^{y/-}$* and *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* were hyperactive in the open field. **B** Time spent in the centre of the open field during Day 2 of testing, for *WT*, *Cyfip1$^{+/-}$*, *Nlgn3$^{y/-}$*, and *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* mice. There were no differences in the time spent in the centre depending on genotype. * = $P < 0.05$, ** = $P < 0.01$, *n.s* = not significantly different.

4.3.2. There were some changes in the exploratory behaviour of *Nlgn3$^{+/-}$Cyfip1$^{+/-}$* female mice

In addition to *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* male mice and their littermates, *Nlgn3$^{+/-}$Cyfip1$^{+/-}$* female mice and their littermates were also tested in the open field, to investigate the possible sex differences and the differences arising from the gene dosage of *Nlgn3*. A proportion of females also possessed the *Thy1-EGFP* transgene. However, the presence of the transgene did not affect the distance travelled in the open field or the time spent in the centre of the arena (Appendix 1).

Similar to what we observed in the males, the females tended to cover more distance on the first day of testing than on the second day, regardless of the genotype (Mixed ANOVA, main effect of day: $F(1, 59) = 92.13$, $P < 0.01$). There was also a significant difference in the distance travelled in the open field depending on the genotype, averaged across the two days of testing (Mixed ANOVA, main effect of genotype: $F(3, 59) = 4.51$, $P < 0.001$). However, the difference in the distance travelled was limited to *Nlgn3$^{+/-}$* ($n = 20$) mice which covered more distance than *Cyfip1$^{+/-}$* mice ($n = 10$, Tukey's HSD: $t(1, 59) = 3.42$, $P < 0.01$). The *Nlgn3$^{+/-}$ Cyfip1$^{+/-}$* females ($n = 17$) did not differ from their *WT* ($n = 16$) littermates. This finding was confirmed by re-analysis which showed that absence of an allele *Nlgn3* was significantly associated with hyperactivity (Mix ANOVA, main effect of *Nlgn3* absence $F(1, 59) = 5.78$, $P = 0.0193$), while absence of an allele of *Cyfip1* was associated with hypoactivity ($F(1, 59) = 7.58$, $P = 0.0078$).

To confirm that lack of hyperactivity in relation to *WT* was accompanied by habituation, the distance travelled on the first and second day was compared within each genotype group. Each of the groups of mice covered more distance on the first day of testing than on the second (Tukey's HSD: *WT*: $t(1, 59) = 5.61$, $P < 0.001$, *Nlgn3$^{+/-}$*: $t(1, 59) = 4.42$, $P < 0.01$, *Cyfip1$^{+/-}$*: $t(1, 59) = 3.83$, $P < 0.01$, *Nlgn3$^{+/-}$Cyfip1$^{+/-}$*: $t(1, 59) = 5.30$, $P < 0.001$, Figure 13 A). Like the male mice, there were no significant differences in the time spent in the centre of the open field (Figure 13 A).

Next, only the second day of testing was considered to obtain a measure of a general level of activity independent from the response to the novel environment. As previously reported across two days of testing, *Nlgn3$^{+/-}$* mice covered more distance in the open field than *Cyfip1$^{+/-}$* mice (One-way ANOVA, main effect of genotype: $F(3, 59) = 4.64$, $P = < 0.001$, Tukey's HSD: $t(1, 59) = 3.34$, $P < 0.01$) and *Nlgn3$^{+/-}$Cyfip1$^{+/-}$* mice (Tukey's HSD: $t(1, 59) = 2.67$, $P < 0.047$, Figure 14 A). However, there were no significant differences between the *WT* and any of the mice of the other genotypes. Therefore, there was some indication that *Nlgn3$^{+/-}$* animals are hyperactive similar to their *Nlgn3$^{y/-}$* male littermates. There were no significant

differences in time spent in the centre in the arena depending on the genotype (Figure 13 B and Figure 14 B).

To investigate if sex had an impact on the behaviour in the open field the distance travelled, and the time spent in the centre of the open field, were directly compared between the male and females. While the heterozygous females could not be directly contrasted with the males entirely lacking *Nlgn3*, due to gene dosage discrepancy, it was possible to compare the behaviour of *WT* and *Cyfip1*$^{+/-}$ males and females. However, no significant differences in the distance travelled or the time spent in the centre of the open filed were found. Thus, there was no sex difference in the exploratory behaviour of these mice (Figure 15 A and B).

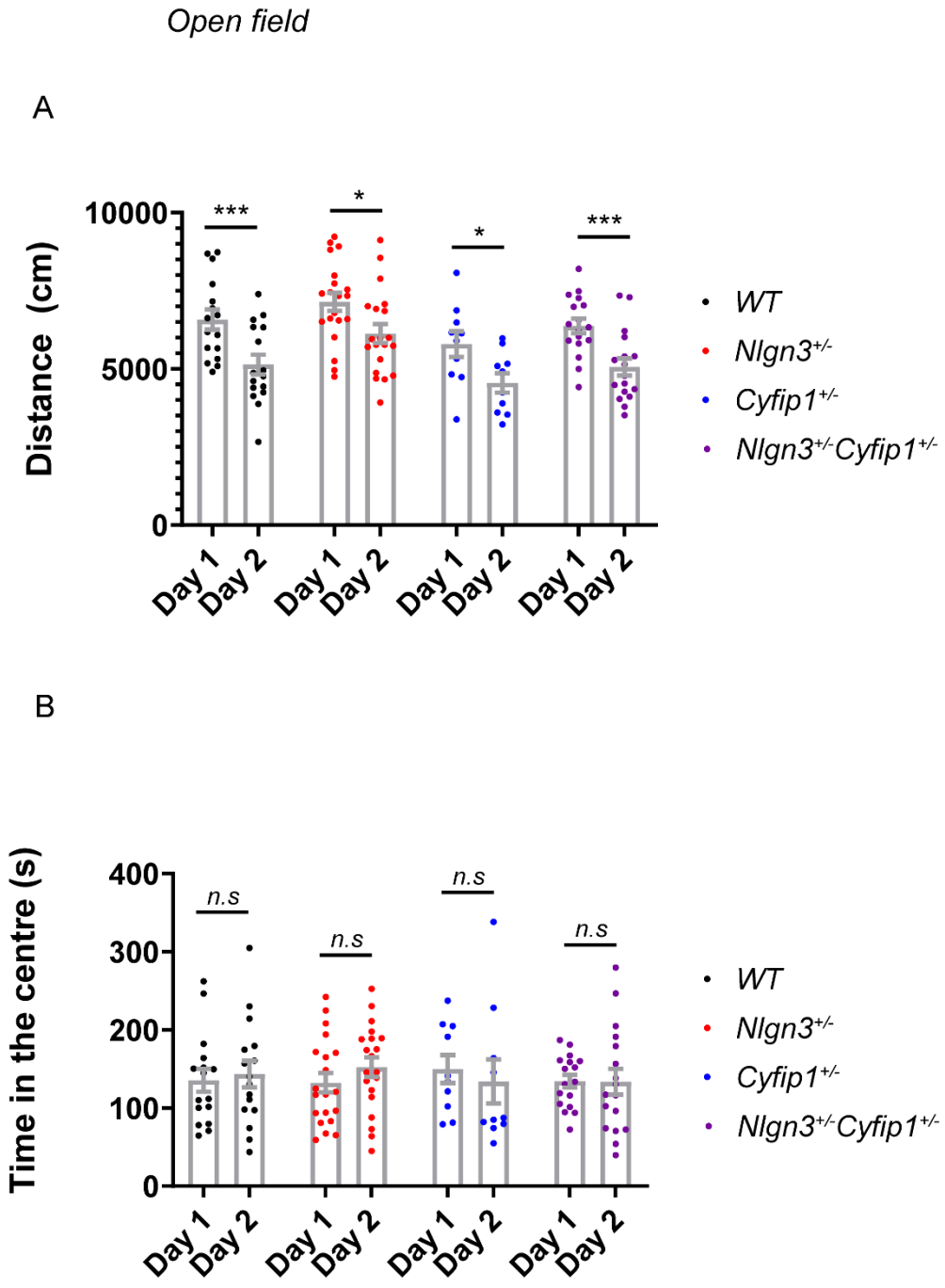


Figure 13 **All female mice showed habituation in the open field. A** The distance travelled in the open field by *WT, Nlgn3*$^{+/-}$*, Cyfip1*$^{+/-}$*, Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ females. All of the females covered more distance on the first day of testing than one the second one, indicating they habituate to the arena. **B** Time spent in the centre of the open field arena by *WT, Nlgn3*$^{+/-}$*, Cyfip1*$^{+/-}$*, Nlgn3*$^{+/-}$ *Cyfip1*$^{+/-}$ females. There were no differences in the time spent in the centre of the arena between the two days of testing. * = $P < 0.05$, *** = $P < 0.001$, *n.s* = not significantly different.

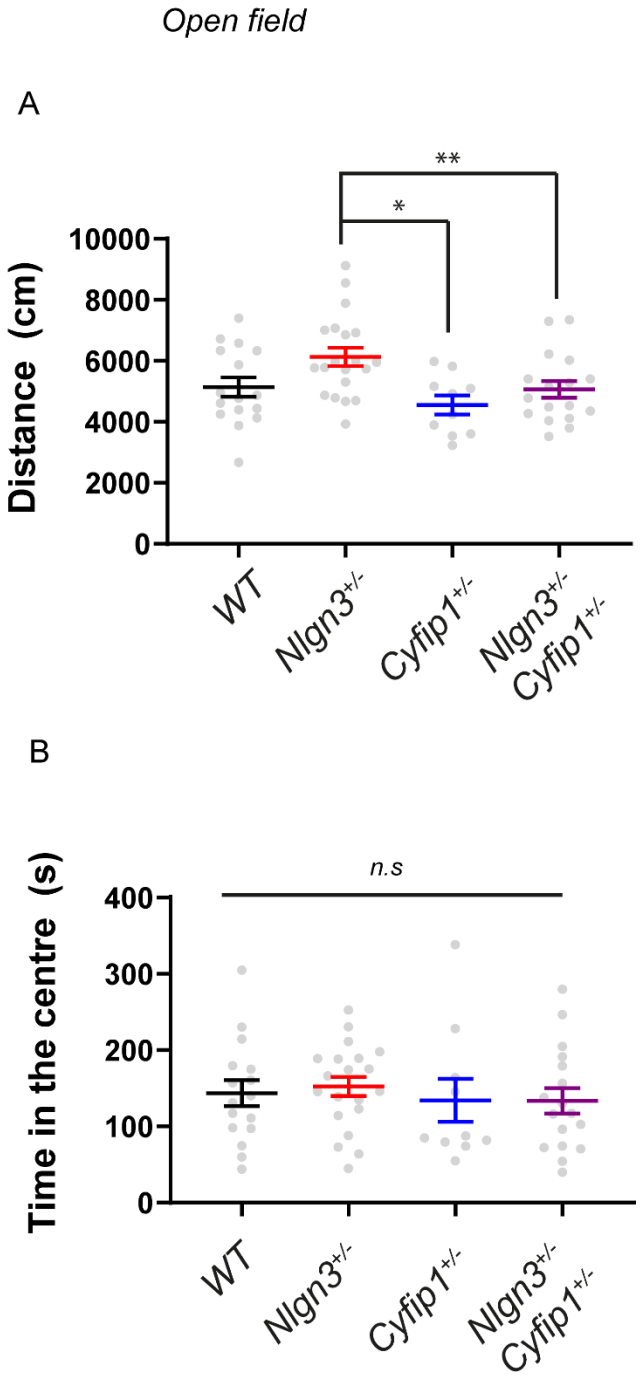


Figure 14 **Females heterozygous for *Nlgn3* and *Cyfip1* showed the same level of activity in the open field as their *WT* littermates. A** The distance travelled in the open field on the second day of testing by the *WT, Nlgn3*$^{+/-}$*, Cyfip1*$^{+/-}$, and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ females. None of the mutant females were different from their *WT* littermates. However, the *Nlgn3*$^{+/-}$ were hyperactive in relation to their *Cyfip1*$^{+/-}$ and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ littermates. **B** Time spent in the centre of the open field arena on the second day of testing by the *WT, Nlgn3*$^{+/-}$*, Cyfip1*$^{+/-}$, and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ females. There were no differences between the females. * = $P < 0.05$, ** = $P < 0.01$, *n.s* = not significantly different.

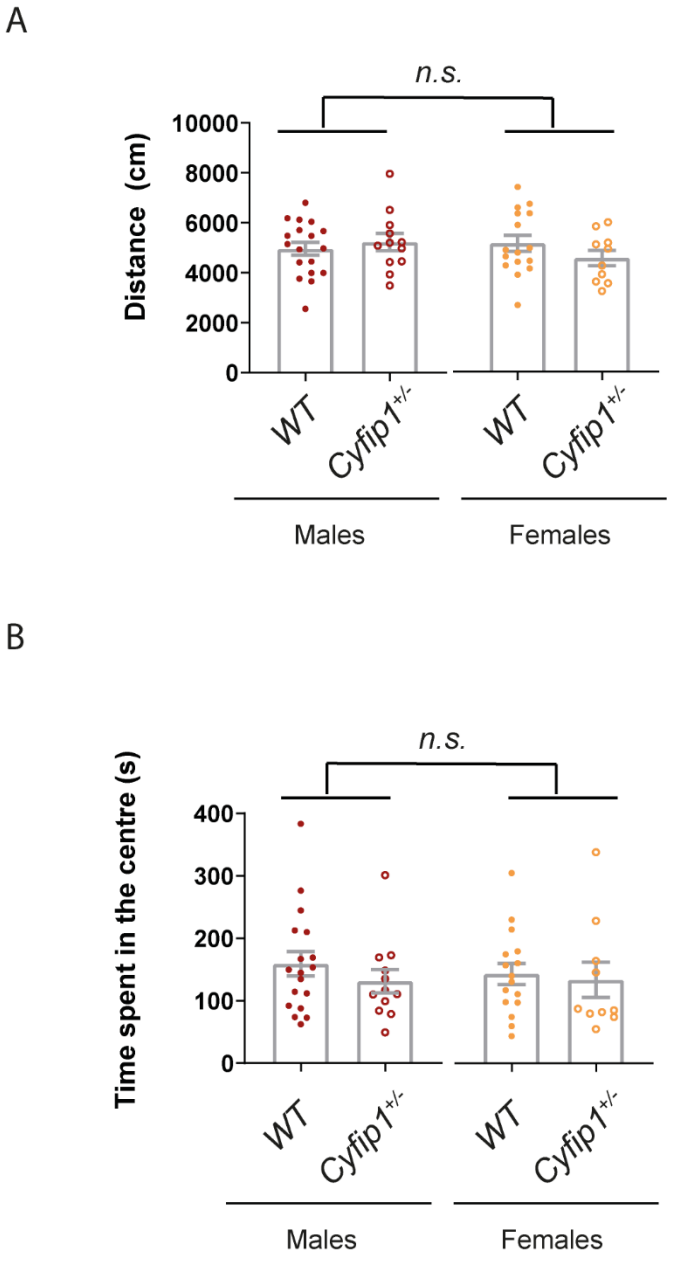


Figure 15 **There was no sex difference in the exploratory behaviour of *WT* and *Cyfip1*$^{+/-}$ mice in the open field. A** The distance travelled in the open field on Day 1 and Day 2 of testing by male and female *WT* and *Cyfip1*$^{+/-}$ mice. **B** The time spent in the centre of the open field on Day 1 and Day 2 of testing by male and female *WT* and *Cyfip1*$^{+/-}$ mice. *n.s* = not significantly different.

4.3.3. Motor routine learning was impaired in *Cyfip1*$^{+/-}$ male mice

The ability to learn new motor routines was evaluated using a rotarod protocol in *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ male mice as well as their littermates. The effects of the genotype of the mouse, day of testing, and trial within each day were considered on the latency to fall off the rotating rod. The presence of *Thy-EGFP* transgene in some of the mice was discerned to have no impact on their performance in this task (Appendix 1).

Regardless of the genotype of the animal, the latency to fall was longer in the latter days of testing (Non-parametric Mixed ANOVA, main effect of day: $F(1, 1577) = 57.36$, $P < 0.001$), and in the trials later in the day (Non-parametric Mixed ANOVA, main effect of trial: $F(9, 1577) = 2.68$, $P < 0.01$). There was no effect of the genotype on the latency to fall when averaged across the day of testing and trial (Figure 16 A). However, the effect of day of testing was different depending on the genotype (Non-parametric Mix model ANOVA, Genotype x Day interaction: $F(3, 1577) = 4.61$, $P < 0.01$;). This suggests that the learning curves across the different days could vary depending on the genotype. This observation was confirmed during a re-analysis of the data where absence of at least one allele of *Nlgn3* or *Cyfip1* were considered as independent factors. Neither the deletion of *Nlgn3* nor *Cyfip1* haploinsufficiency has a significant effect on the latency to fall off rotarod, when averaged over trials and days.

To further investigate the interplay between genotype and day of testing, a simple effects analysis was conducted. For this purpose, the latency to fall was averaged across trials within each day of testing. An increase in the time spent on the rod was observed in the *WT* mice ($n = 17$, Day 1 vs. Day 2: $t(1, 16) = 2.12$, $P = 0.049$, Day 1 vs Day 3: $t(1, 16) = 6.28$, $P < 0.001$, Day 2 vs Day 3: $t(1, 16) = 2.86$, $P < 0.023$), in the *Nlgn3*$^{y/-}$ mice ($n = 16$, Day 1 vs. Day 2: $t(1, 15) = 6.16$, $P < 0.001$, Day 1 vs Day 3: $t(1, 15) = 9.14$, $P < 0.001$, Day 2 vs Day 3: $t(1, 15) = 3.02$, $P < 0.01$), and in *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ ($n = 12$, Day 1 vs. Day 2: $t(1, 12) = 5.05$, $P < 0.01$, Day 1 vs Day 3: $t(1, 12) = 4.70$, $P < 0.01$, Figure 16 B). However, no significant differences in the time spent on the rod were noted in the *Cyfip1*$^{+/-}$ mice ($n = 12$), indicating that they are unable to improve across the days of training. These results indicate the deficit in motor learning seen in *Cyfip1*$^{+/-}$ mice was restored by deleting *Nlgn3* in *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ double mutant mice.

Similarly, a simple effects analysis was conducted to compare the performance of the mice of different genotypes within each day of training. There were no differences in the time spent on the rod during the first two days. However, *Nlgn3*$^{y/-}$ males were found to outperform both *Cyfip1*$^{+/-}$ males ($t(1, 21) = 6.422$, $P = 0.0010$) and *WT* males ($t(1, 31) = 5.024$, $P = 0.0076$) on day three.

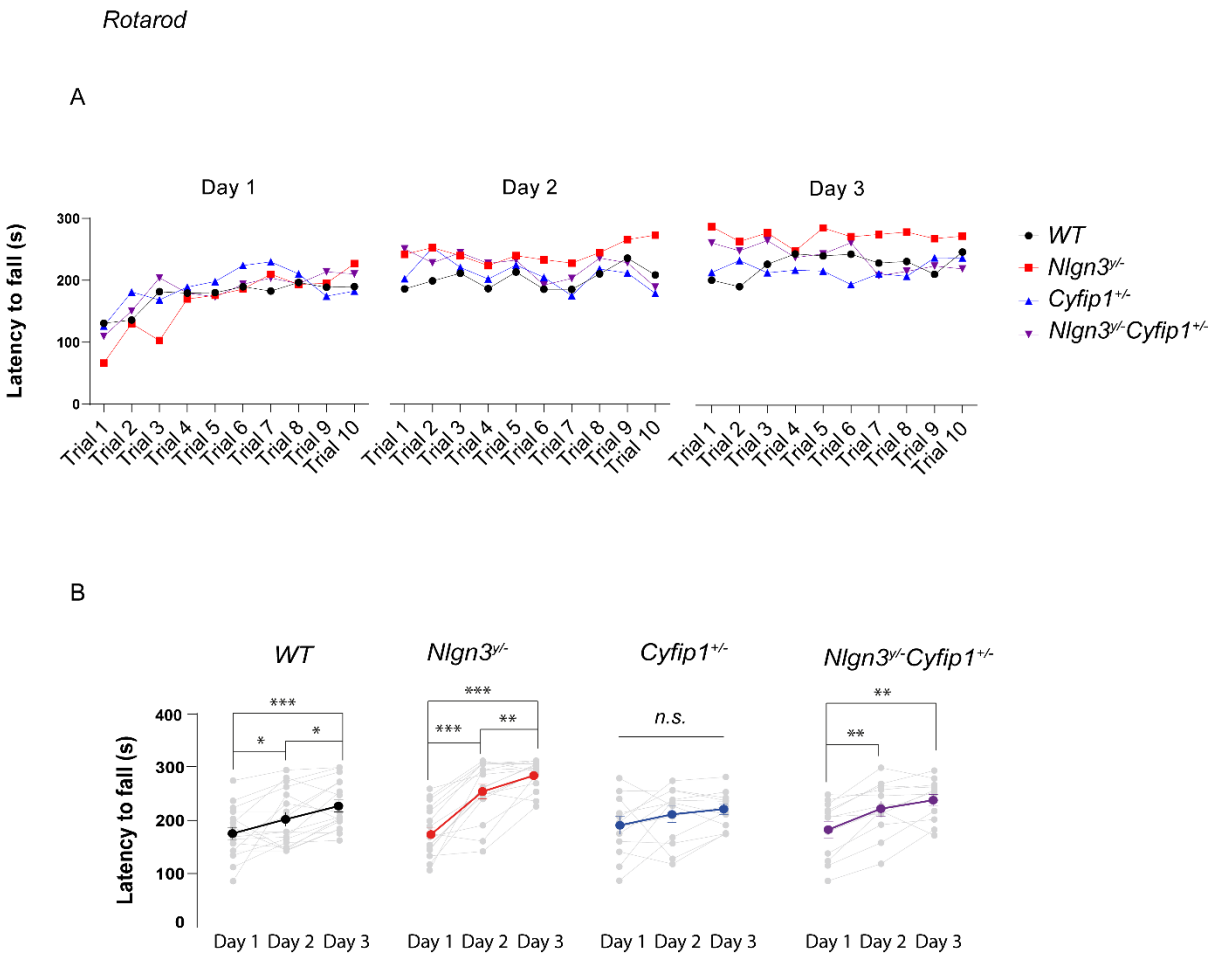


Figure 16 ***Cyfip1*$^{+/-}$ males did not learn new motor routines on rotarod. A** Latency to fall off the rotarod for individual trials for *WT, Nlgn3*$^{y/-}$, *Cyfip1*$^{+/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice. **B** Latency to fall off the rotarod for averaged for every day of testing *WT, Nlgn3*$^{y/-}$, *Cyfip1*$^{+/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, *n.s.* = not significantly different.

4.3.4. Motor routine learning was no different between *WT* and mutant female mice

The ability to learn new motor routines was also investigated in female *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ double mutant mice and their littermates. The latency to fall off the rotarod was compared depending on the trial, day of testing, and the genotype of the animal. The presence of Thy-EGFP transgene did not affect the latency to fall.

Similar to their male littermates, the female mice tended to stay on the rod longer in the latter days of testing (Non-parametric Mixed ANOVA, the main effect of the day: $F(1, 1490) = 66.39$, $P < 0.001$), and in the later trials in the session (Non-parametric Mixed ANOVA, the main effect of trial: $F(1, 1490) = 18.84$, $P < 0.001$). As previously, there was no effect of genotype on the latency to fall when the influence of the day of testing, and the number of the trial was disregarded. Unlike in the male mice, however, there was also no interaction between the effects of genotype and the day of testing (Figure 17 A). This indicates that the learning curves for mice belonging to each genotype group were comparable. This finding was confirmed by re-analysis showing no effect of *Nlgn3* or *Cyfip1* absence on time spent of rotarod, when considered in isolation to the effect of trial and day.

In order to confirm that all the mice learned the new routine in a similar manner a simple effects analysis followed. The latency to fall was averaged across trials and the scores for each genotype groups were compared between days of testing. An increase in time spent on the rod was observed in the *WT* mice ($n = 13$, Day 1 vs. Day 2: $t(1, 13) = 5.09$, $P < 0.01$, Day 1 vs Day 3: $t(1, 13) = 5.85$, $P < 0.01$), in the *Nlgn3*$^{+/-}$ mice ($n = 13$, Day 1 vs. Day 2: $t(1, 13) = 6.15$, $P < 0.01$, Day 1 vs Day 3: $t(1, 13) = 8.06$, $P < 0.001$, Day 2 vs Day 3: $t(1, 13) = 4.23$, $P = 0.028$), in the *Cyfip1*$^{+/-}$ mice ($n = 14$, Day 1 vs Day 3: $t(1, 13) = 5.95$, $P < 0.01$), and in the *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ mice ($n =14$, Day 1 vs. Day 2: $t(1, 14) = 7.34$, $P < 0.001$, Day 1 vs Day 3: $t(1, 14) = 7.41$, $P < 0.001$, Figure 17 B).

To confirm there was a sex difference in the motor learning we tested the latency to fall between the sexes directly for *WT* and *Cyfip1*$^{+/-}$ animals. On average, females performed better than the males (Mix model ANOVA, main effect of sex: $F(1, 52) = 4.88$, $P = 0.035$, Figure 18). Therefore, unlike males, all female mice showed evidence of learning across days.

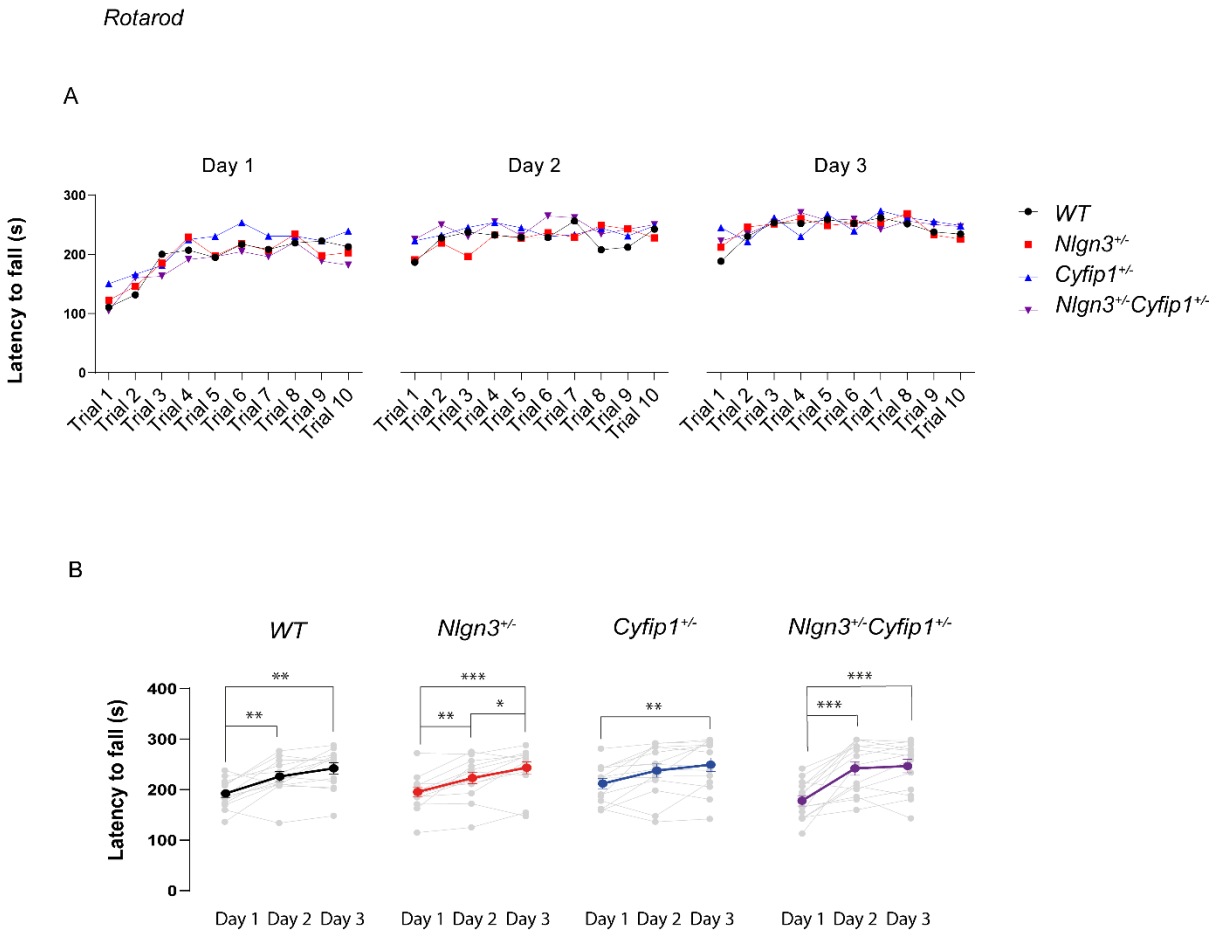


Figure 17 **Female mice heterozygous for *Nlgn3* and *Cyfip1* or both showed *WT*-level motor learning. A** Latency to fall off rotarod across for individual trials for *WT, Nlgn3*$^{+/-}$, *Cyfip1*$^{+/-}$ and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ mice. **B** Latency to fall off rotarod averaged for each day of testing for *WT, Nlgn3*$^{+/-}$, *Cyfip1*$^{+/-}$ and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ mice. All of the female mice showed improvement across the days of testing suggesting they are able to learn new motor routines. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

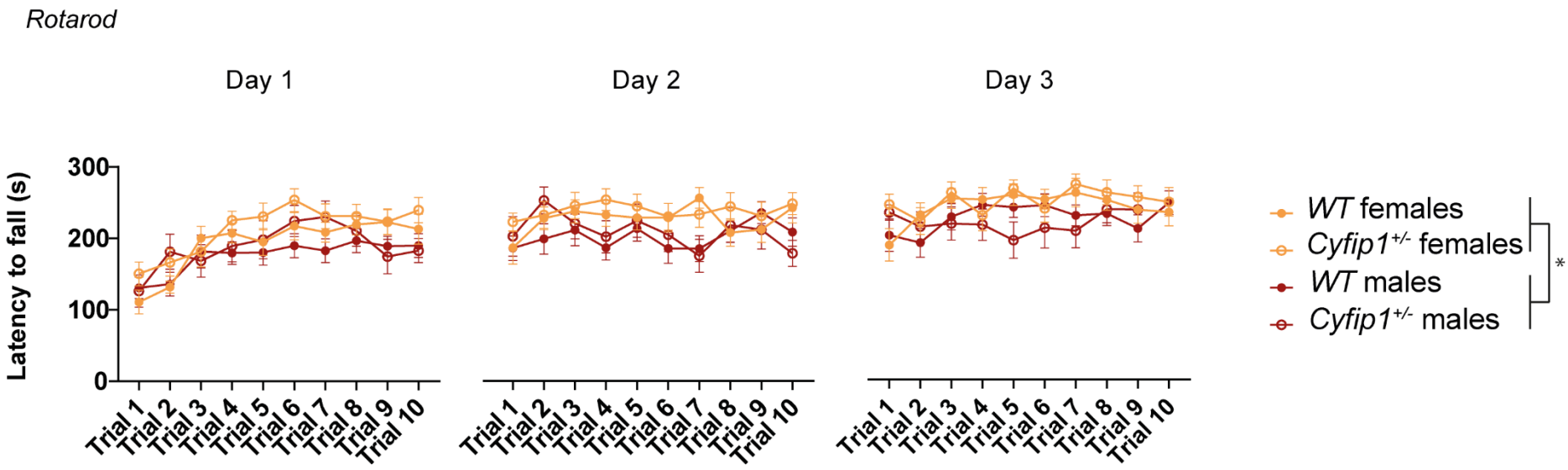


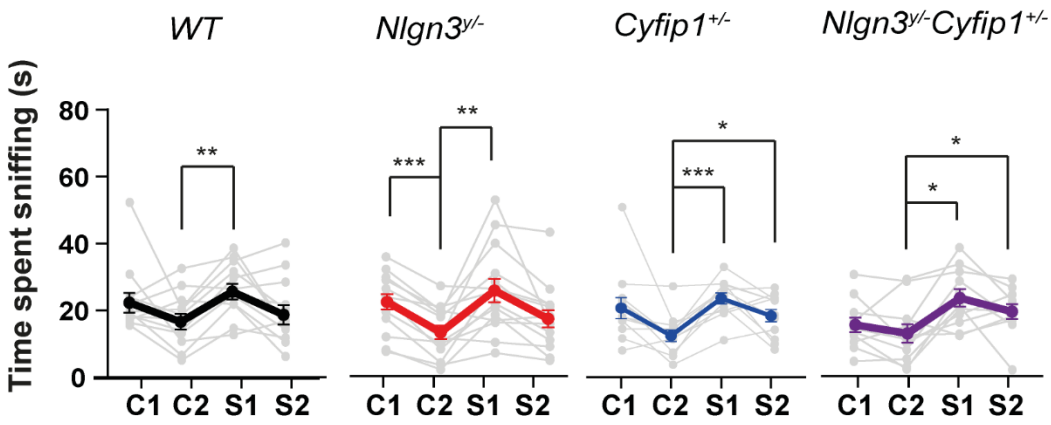
Figure 18 **The motor learning ability varied depending on sex.** The latency to fall off the rotarod across the three days of testing for the *WT and Cyfip1*$^{+/-}$ male and female mice. Regardless of the genotype, the female mice outperformed the males. * = $P < 0.05$

4.3.5. There were no differences in the interest in social olfactory cues between *WT* and mutant male mice

Interest in social olfactory cues was investigated in the *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ male mice as well as in their littermates. The time spent in contact with a clean cotton bud as well as with another containing the olfactory cue was evaluated. The presence of *Thy1-EGFP* transgene in some of the mice did not affect the time spent sniffing the cue.

The genotype did not have an impact on the time spent with the olfactory cue, averaged over the different types of cues. However, there was an overall effect of the cue on the time spent sniffing (Non-parametric Mixed ANOVA, the main effect of cue: $F(2, 90) = 36.37$, $P < 0.001$). This parameter did not vary as a function of genotype, suggesting that mice of different genotypes tended to interact with the olfactory cues in a similar pattern. This was confirmed by analysis showing that neither absence of *Nlgn3* nor absence of *Cyfip1* has an overall effect on the time spent sniffing when considered as independent factors. In line with this observation, an increased interest in the social olfactory cue compared to the control was present in *WT* mice ($n = 12$, Simple effect: $t(1, 11) = 6.48$, $P = 0.0037$), in the *Nlgn3*$^{y/-}$ mice ($n = 14$, Simple effect: $t(1, 13) = 5.41$, $P < 0.01$), in the *Cyfip1*$^{+/-}$ ($n = 12$, Simple effect: $t(1, 12) = 8.74$, $P < 0.001$), and in the *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice ($n = 11$, Simple effect: $t(1, 10) = 5.06$, $P = 0.02$, Figure 19 A). This suggests that all the mice were able to discriminate between the control and social olfactory cue. An increase in interest between the control cue and the second presentation of the social odour was observed only in the *Cyfip1*$^{+/-}$ mice (Simple effect: $t(1, 12) = 4.70$, $P = 0.029$), and in the *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice (Simple effect: $t(1, 10) = 4.40$, $P < 0.046$). The level of interest in the social olfactory cue did not differ depending on the genotype of the mice (Figure 19 B).

A



B

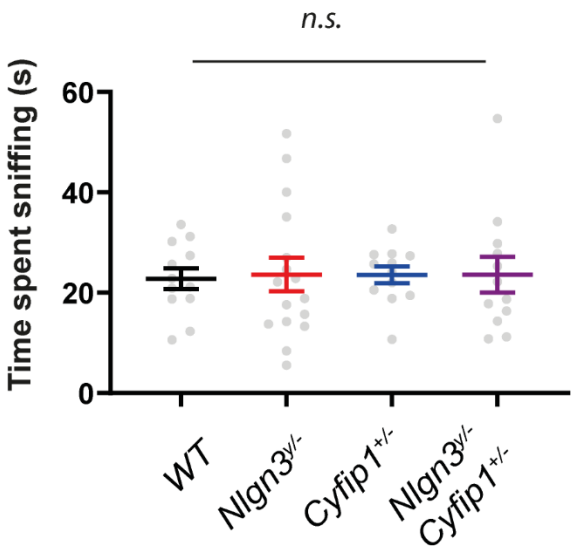


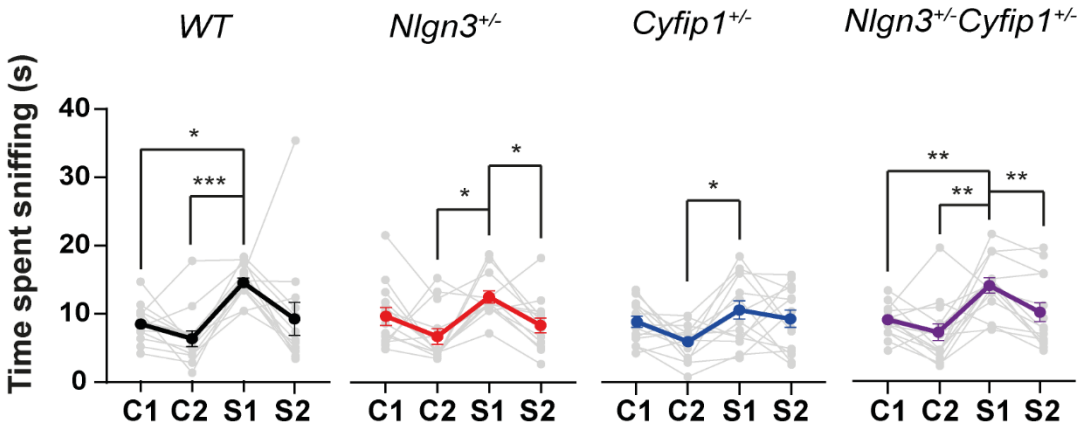
Figure 19 ***Nlgn3* deletion and *Cyfip1* haploinsuficiency had no effect on interest in social odours in males. A** Time spent sniffing the olfactory cue, for *WT, Nlgn3*$^{y/-}$, *Cyfip1*$^{+/-}$, and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice. All the mice spent more time sniffing the social cue compared to the control cue. **B** Time spent sniffing during the first presentation of social olfactory cue depending on the genotype by male mice. There were no differences in the interest in social cue between mice of different genotype. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, *n.s.* = not significantly different.

4.3.6. Interest in the social olfactory cues was no different between *WT* and mutant female mice

Interest in social odours was also evaluated in the *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ female mice and their littermates. The *Thy1-EGFP* transgenes present in some of the mice did not impact on the time spent with the olfactory cue.

Similar to observations in males, the genotype of the female mice did not impact on the time spent with the olfactory cues, when the type of cue was not taken into account. However, there was a difference in the time spent sniffing depending on the type of cue, regardless of the genotype (Non-parametric Mixed ANOVA, the main effect of the cue: $F(2, 100) = 41.07$, $P < 0.001$). The genotype also did not impact on the effect of the type of cue on the time spent sniffing. The absence of *Nlgn3* or the absence of *Cyfip1* also had no effect on the interaction with the olfactory cue, when considered independently. These findings suggest that female mice of different genotypes tended to interact with the olfactory cues in a comparable manner. In line with this observation, an increase in the time spent with the cue was present between the control cue and the first presentation of the social odour in *WT* ($n = 13$, Simple effect: $t(1, 13) = 10.80$, $P < 0.001$), in the *Nlgn3*$^{+/-}$ ($n = 13$, Simple effect: $t(1, 13) = 5.50$, $P < 0.01$), in the *Cyfip1*$^{+/-}$ ($n = 14$, Simple effect: $t(1, 14) = 4.82$, $P = 0.021$), and in the *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ mice ($n = 14$, Simple effect: $t(1, 14) = 5.44$, $P < 0.01$, Figure 20 A). A decrease between the first and second presentation of the social odour, characteristic of habituation was also observed in *Nlgn3*$^{+/-}$ mice ($t(1, 13) = 4.80$, $P = 0.024$) and in the *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ mice ($t(1, 14) = 6.39$, $P < 0.01$). The interest in the social odour was reduced in the *Cyfip1*$^{+/-}$ females compared to the *WT* females (One-way ANOVA: main effect of genotype $F(3, 50) = 2.93$, $P = 0.043$, Tukey's HSD: $t(1, 50) = -2.68$, $P = 0.047$), indicating that while the discrimination between odours was unimpaired, there might be a deficit in the interest in social odours (Figure 20 B).

A



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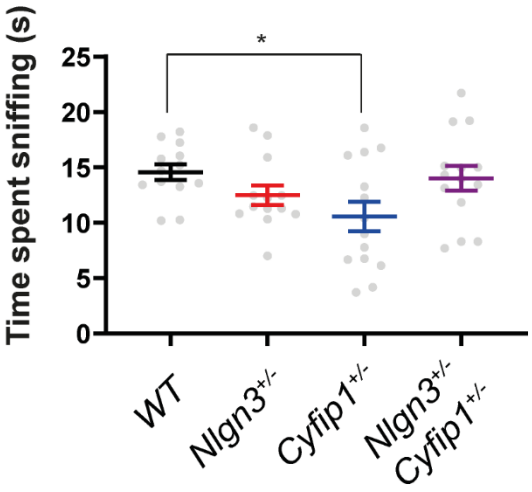


Figure 20 ***Cyfip1*$^{+/-}$ females showed reduced interest in social odours.** **A** Time spent sniffing the olfactory cue, for *WT*, *Nlgn3*$^{+/-}$, *Cyfip1*$^{+/-}$, and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ mice. All mice spent more time sniffing the social cue compared to the control cue. **B** Time spent sniffing during the first presentation of social olfactory cue depending on the genotype by female mice. The *Cyfip1*$^{+/-}$ females spent less time with the social olfactory cue than their *WT* littermates. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

4.3.7. Ultrasonic vocalisations and interaction during courtship were similar between *WT* and mutant male mice

The sociability of male mice was evaluated in the context of courtship. The number and duration of ultrasonic vocalisations in response to a female in oestrus were investigated as well as the time spent interacting with the female. The presence of the *Thy1-EGFP* transgene did not affect the number or the duration of the ultrasonic vocalizations emitted by the mice nor did it impact the time spent interacting with the female. The genotype of the mice did not influence the number or duration of the vocalizations emitted during courtship or the time spent interacting with the female, which suggests that all the males exhibited *WT*-level sociability (Figure 21 A-C). This observation was confirmed by re-analysis involving separating *Nlgn3* absence and *Cyfip1* absence as independent factors.

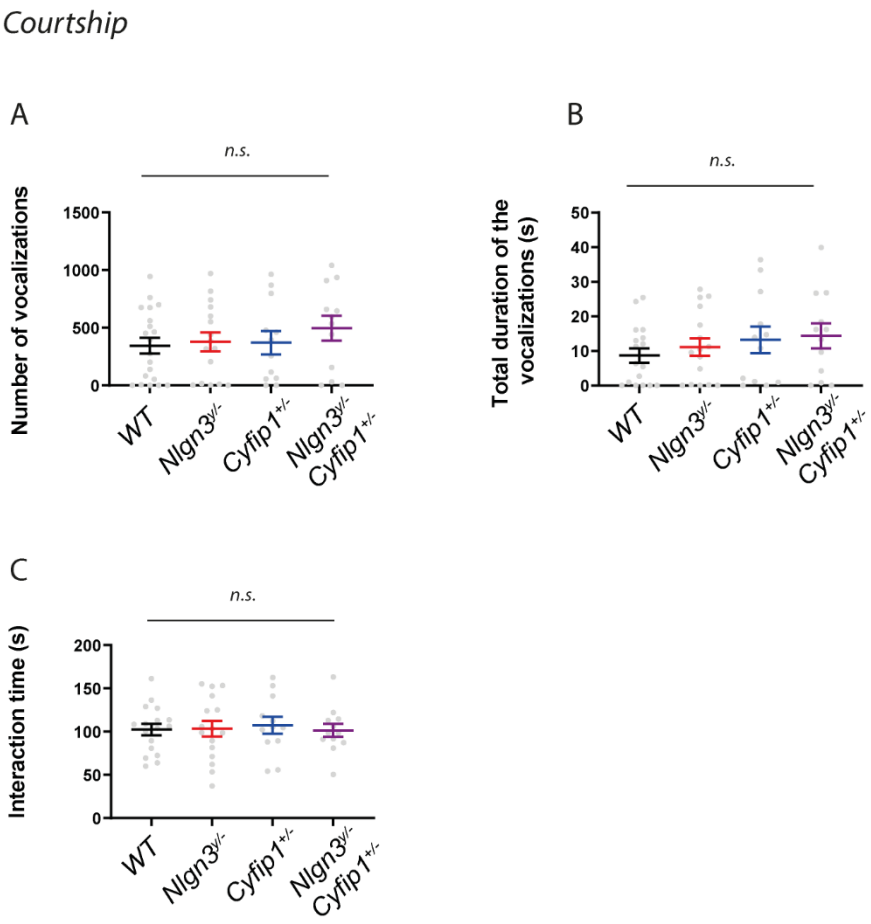


Figure 21 ***Nlgn3* deletion and *Cyfip1* haploinsuficiency had no effect on courtship behaviour in male mice. A** Number of vocalisations emitted in response to a female in oestrus by *WT*, *Nlgn3*$^{y/-}$, *Cyfip1*$^{+/-}$, and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice. All the males vocalised to the same degree. **B** Duration of vocalisations emitted in response to a female in oestrus. **C** Interaction time with a female in oestrus. All the males interacted with the female to the same degree. *n.s.* = not significantly different.

4.3.8. There was a subtle effect of social environment on the behaviour of *WT* littermates

Fewer vocalisations were emitted during courtship in the *WT* littermates than previously reported (Kalbassi *et al.* 2017). To determine if this difference might arise as a result of the social environment these mice were exposed to, the *WT* littermates housed with *Nlgn3*$^{y/-}$, *Cyfip1*$^{+/-}$, and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ male mice (MGH, mixed genotype housing) were compared to a cohort of *WT* mice that were only housed with other *WT* littermates (SGH, single genotype housing). All the previously conducted behavioural paradigms were included.

In the open field, the distance travelled was significantly higher in the MGH *WT* male mice than in the SGH *WT* mice across the two days of testing (Mixed ANOVA, the main effect of housing: $F(1, 35) = 10.30$, $P < 0.001$, Figure 22 A). There was no effect of housing on the time spent in the centre of the open field.

There was no overall effect of housing on the time spent on the rotarod. There was however an effect of the day of testing as well as trial, averaged over the two housing conditions (Mixed ANOVA, main effect of day: $F(1, 977) = 102.89$, the main effect of trial: $P < 0.001$, $F(9, 977) = 5.34$, $P < 0.001$). The effect of the day of testing on the latency to fall off the rod differed depending on the housing condition ($F(1, 977) = 102.89$, $P = 0.033$, Figure 22 C). This indicates that mice from different housing conditions may learn at a different pace. To investigate this further a simple effects analysis was used. Both MGH *WT* and SGH *WT* mice showed improvement in their performance across the days (Day 1 vs Day 2: $t(1, 17) = 3.91$, $P < 0.049$, Day 1 vs Day 3: $t(1, 17) = 7.56$, $P < 0.001$, Day 2 vs Day 3: $t(1, 17) = 3.66$, $P < 0.023$; Day 1 vs Day 2: $t(1, 15) = 6.58$, $P < 0.001$, Day 1 vs Day 3: $t(1, 15) = 9.21$, $P < 0.001$ respectively, Figure 22 D). Thus, even though there might be a subtle difference in the rate of learning, both groups of mice were able to acquire the knowledge of the new motor routine.

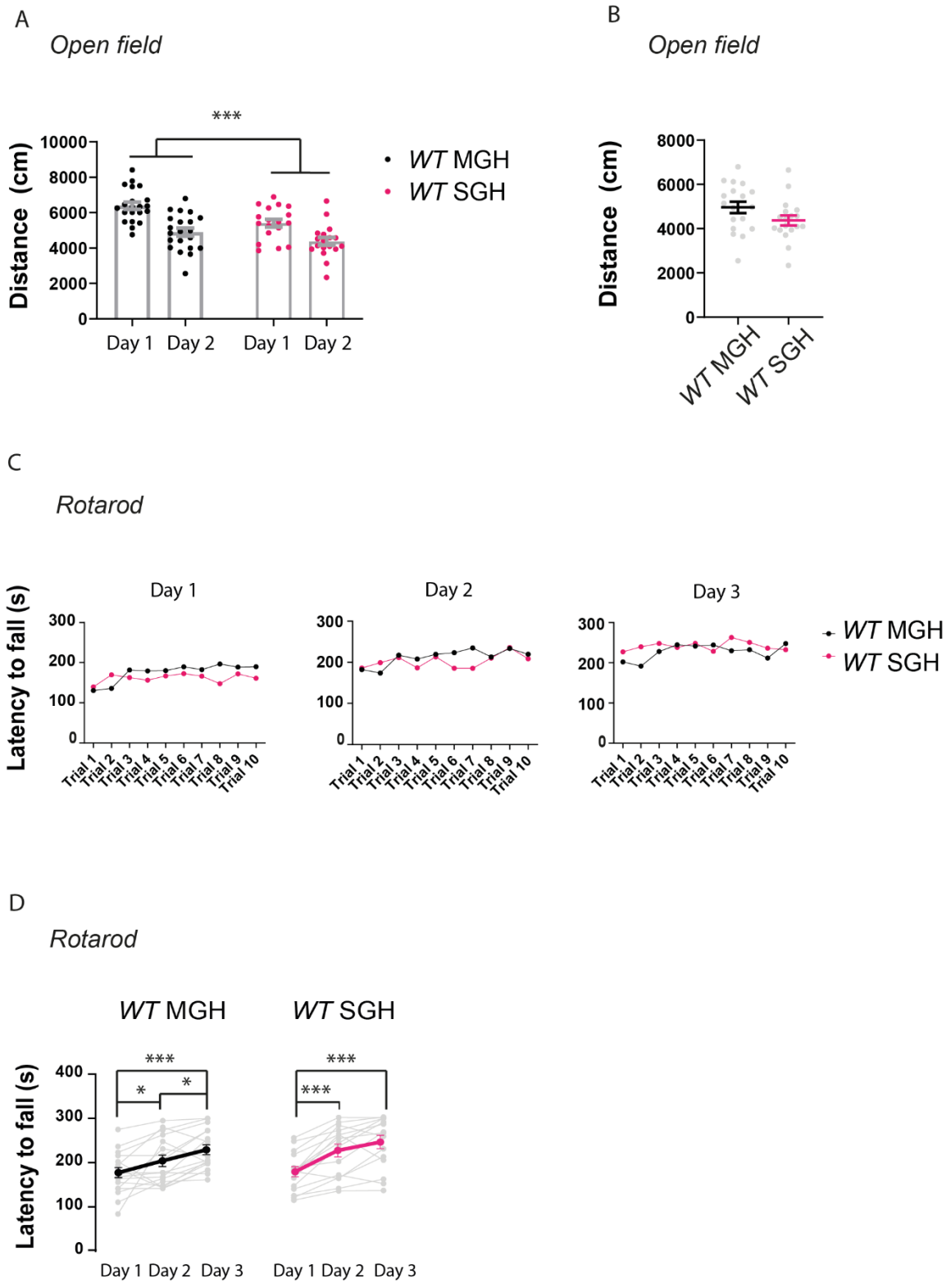


Figure 22 ***WT* MGH males showed hyperactivity in the open field compared to *WT* SGH males. A** Distance travelled in the open field by *WT* MGH and *WT* SGH males. The *WT* MGH males were hyperactive. **B** Distance travelled in the open field by *WT* MGH and *WT* SGH males on the second day of testing only. **C** Latency to fall off rotarod on individual trial for *WT* MGH and *WT* SGH males. **D** Latency to fall off rotarod on each day of testing. Both *WT* MGH and *WT* SGH males improved in their ability to stay on the rod across days. * = $P < 0.05$, *** = $P < 0.001$.

The housing also impacted on social olfaction, regardless of type of olfactory cue (Non-parametric Mixed ANOVA, main effect of housing: $F(1, 30) = 56.63$, $P < 0.001$, Figure 23 A). The type of cue also affected the time spent sniffing, averaged over the two housing conditions (Non-parametric Mixed ANOVA, main effect of cue: $F(2, 60) = 29.84$, $P < 0.001$). However, the effect of type of cue on the time spent sniffing did not differ between the two housing conditions suggesting the pattern of response was similar between the two. A simple effects analysis was conducted to confirm that the pattern of responses to the different cues was the same between the two housing conditions. An increase in the time spent sniffing was observed between the control cue and the first presentation of a social odour both in the MGH *WT* ($t(1, 13) = 2.70$, $P = 0.037$) and SGH *WT* mice ($t(1, 17) = 8.39$, $P < 0.001$). However, an increase in the time spent with the cue between the control and the second presentation of the social odour was only seen in the SGH *WT* mice ($t(1, 17) = 6.96$, $P < 0.001$). Similarly, a significant decrease in the time spent sniffing between the first and second presentation of the social odour, characteristic of habituation, was only observed in the SGH *WT* mice ($t(1, 17) = 13.00$, $P < 0.001$). We also compared the interest in the social olfactory cue between the two housing conditions. We found that MGH *WT* mice spent longer with the cue than SGH *WT* animals (Mann-Whitney test: $W(1,15) = 226$, $P < 0.001$). Thus, the responses the olfactory cues were subtly different between the males from the two housing conditions.

The housing had no impact on the number or duration of vocalisations emitted in response to a female in oestrus (Figure 23 B-C). Neither did it have any effect on the time spent interacting with the female (Figure 23 D).

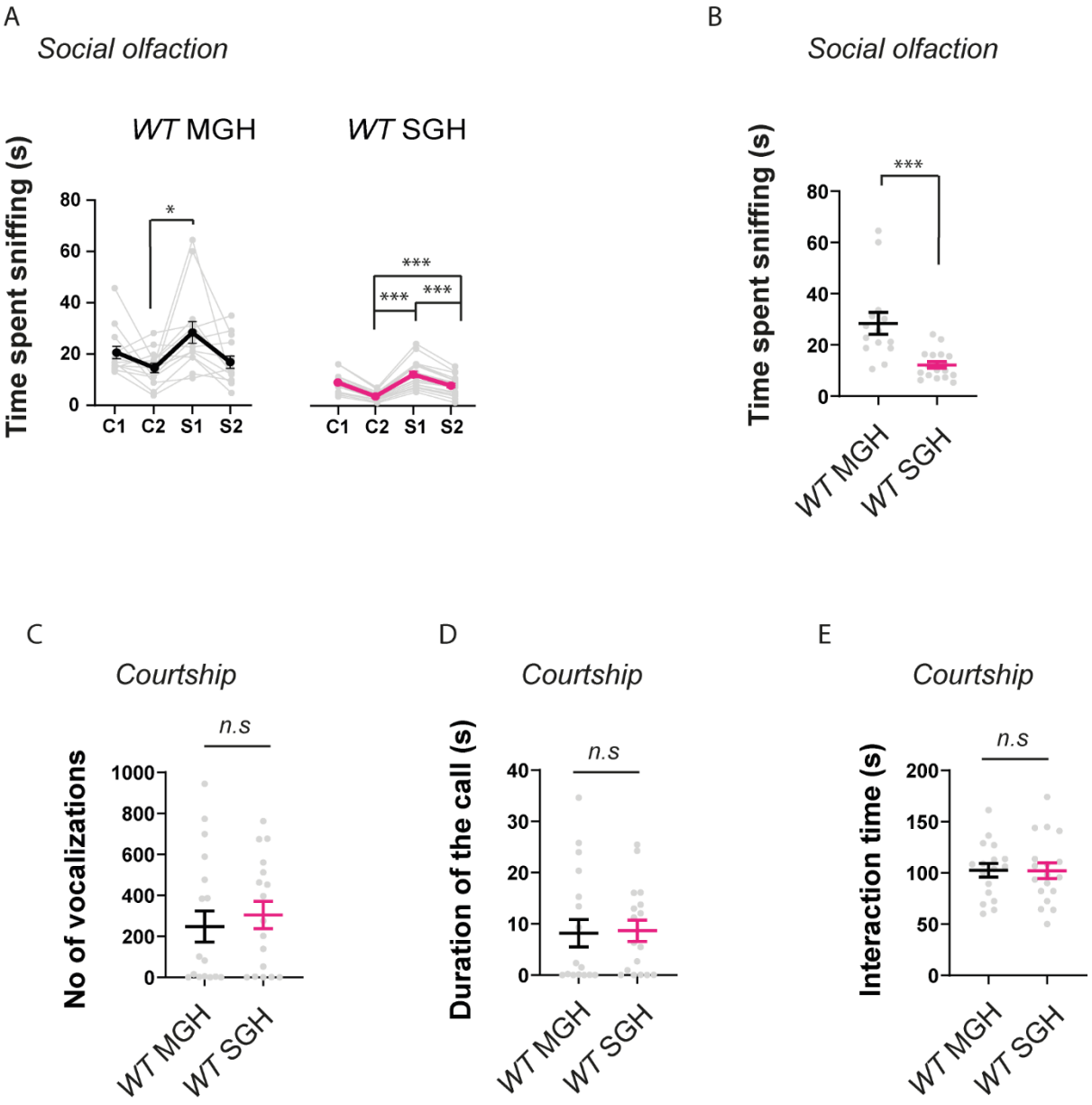


Figure 23 ***WT* SGH males showed less interests in odours than *WT* MGH males. A** Time spent sniffing olfactory cues by *WT* SGH and *WT* MGH males. Overall, the *WT* SGH males showed less interest in the olfactory cues, regardless of the type of cue. Both groups showed more interest in the social odour compared to the control. **B** Time spent with the social odour by *WT* SGH and *WT* MGH males. The *WT* MGH males showed more interest in the cue. **C** Number of vocalisations during courtship by *WT* SGH and *WT* MGH males. **D** Duration of vocalisations during courtship by *WT* SGH and *WT* MGH males. **E** Time spent interacting with the female in the context of courtship by *WT* SGH and *WT* MGH males. No differences in courtship behaviour were detected between these two groups of males. * = $P < 0.05$, *** = $P < 0.001$, *n.s.* = not significantly different.

Table 10 Descriptive statistics for the behaviour of male mice.

Test	***WT***		***Nlgn3$^{y/-}$***		***Cyfip1$^{+/-}$***		***Nlgn3$^{y/-}$Cyfip1$^{+/-}$***		***WT* SGH**	
	Mean	*SE*	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*SE*
Open field distance Day1 (cm)	6515.19	219.51	6845.64	198.83	5940.08	430.82	7503.05	326.34	5403.43	219.24
Open field distance Day2 (cm)	4962.81	258.24	6307.63	256.25	5227.60	345.70	6597.82	420.93	4374.01	223.90
Open field time in the centre Day 1 (s)	152.00	14.00	137.73	7.68	142.22	14.94	171.22	23.13	149.03	15.18
Open field time in the centre Day 2 (s)	137.00	13.60	136.03	8.97	131.16	18.71	134.17	14.83	138.09	11.11
Rotarod latency to fall Day 1 (s)	175.52	5.51	165.58	7.04	188.40	7.35	183.24	7.18	223.90	4.93
Rotarod latency to fall Day 2 (s)	202.25	5.88	244.34	6.07	209.23	7.31	223.25	7.12	212.51	5.39
Rotarod latency to fall Day 3 (s)	227.31	5.59	273.88	3.94	219.04	6.76	239.48	6.23	241.11	4.90
Social olfaction time spent sniffing C2 (s)	14.8	2.08	11.1	1.82	12.43	1.72	11.4	2.45	3.73	0.36
Social olfaction time spent sniffing S1 (s)	22.8	2.07	22.4	3.14	23.57	1.65	20.8	2.33	12.23	1.26
Social olfaction time spent sniffing S2 (s)	16.7	2.56	14.8	2.34	18.40	1.84	17.1	1.92	7.90	0.90
Courtship number of USVs	304.06	66.70	378.63	81.16	370.83	101.07	495.92	108.30	247.56	75.87
Courtship duration of USVs (s)	8.67	2.09	11.13	2.54	13.18	3.83	14.34	3.59	8.17	2.66
Courtship interaction with the female (s)	102.67	6.68	103.34	9.20	107.25	9.79	101.45	7.77	102.61	6.68

Table 11 Descriptive statistics for the behaviour of female mice.

Test	***WT***		***Nlgn3*$^{+/-}$**		***Cyfip1*$^{+/-}$**		***Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$**	
	Mean	*SE*	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*SE*
Open field distance Day1 (cm)	6576.72	324.63	7144.14	291.08	5791.98	411.07	6376.07	233.64
Open field distance Day2 (cm)	5138.13	317.12	6129.85	301.30	4550.05	309.99	5059.01	274.50
Open field time in the centre Day 1 (s)	135.49	14.63	132.25	12.42	149.88	17.90	134.33	8.16
Open field time in the centre Day 2 (s)	143.35	16.98	152.26	12.53	133.90	28.21	133.48	16.55
Rotarod latency to fall Day 1 (s)	192.47	5.95	193.95	5.77	213.10	6.02	244.73	5.39
Rotarod latency to fall Day 2 (s)	226.10	5.80	225.24	5.76	238.69	5.39	244.73	5.39
Rotarod latency to fall Day 3 (s)	242.14	5.58	245.34	5.22	250.60	5.37	249.50	5.27
Social olfaction time spent sniffing C2 (s)	6.37	1.15	6.71	1.13	5.96	0.69	7.18	1.23
Social olfaction time spent sniffing S1 (s)	14.57	0.70	12.50	0.89	10.57	1.33	14.01	1.12
Social olfaction time spent sniffing S2 (s)	9.28	2.39	8.34	1.08	9.28	1.27	10.10	1.40

4.4. Discussion

In this chapter, the impact of the *Nlgn3/Cyfip1* interaction on exploratory behaviour, motor learning, and social behaviour in mice was investigated. The exploratory behaviour of male mice lacking *Nlgn3* and heterozygous for *Cyfip1* was comparable to that of males with *Nlgn3* deletion. On the other hand, the motor learning deficit present in males heterozygous for *Cyfip1* was normalised by deleting *Nlgn3* in the double mutant mice. While a deficit in motor learning was observed in male mice heterozygous for *Cyfip1*, it was absent in females of the same genotype. In addition to the effect of sex, the social environment had a subtle effect on the behaviour of *WT* mice.

Some of the behavioural phenotypes associated with the lack of *Nlgn3* or *Cyfip1* haploinsufficiency were replicated here. Males lacking *Nlgn3* were hyperactive in the open field, in line with previous reports (Radyushkin and Hammerschmidt 2009; Rothwell *et al.* 2014). However, males heterozygous for *Cyfip1* did not show an expected reduction in their activity in the open field (Bozdagi *et al.* 2012). This is the second time *WT*-level activity in these mice was reported (Bachmann *et al.* 2019). A previously reported deficit in motor learning in these males (Bachmann *et al.* 2019) was replicated here. There was also an indication that motor learning was superior in males lacking *Nlgn3*, in line with previous reports (Rothwell *et al.* 2014). Perhaps most notably, no profound deficits in social behaviour in any of the mutants were found contradicting multiple previous results (Bachmann *et al.* 2019; Dere *et al.* 2018; Fischer and Hammerschmidt 2011; Radyushkin *et al.* 2009). This failure of replication could potentially be explained by differences in protocols used between the current and previous research. However, we observed a lower level of vocalisation in the context of courtship in the *WT* males than previously reported (Fischer and Hammerschmidt 2011; Kalbassi *et al.* 2017). This alerted us to the possibility that the behaviour of the *WT* might have been influenced by the social environment, masking an existing deficit in the mutant animals.

The only example of a deficit present in all: males with *Nlgn3* deletion, heterozygous for *Cyfip1* and the double mutants, was absence of habituation to the open field. While their *WT* littermates decreased their exploration on the second day of testing, this effect was absent in these mice. This was only true for the males, with all of the females showing habituation. Habituation in the open field in mice might have a genetic components as a recent study of different background strains showed significant variation in this measure (Bolivar *et al.* 2000). Additionally, for some strains, this effect was further modulated by the sex of an animal. This finding is in line with a proposition that lack of habituation to the sensory input might be as issue in children with ASD (Cheng and Jin 2019). This could lead to overstimulation, stress and perceiving the environment as unpredictable. However, a vast variation in habitation to

sensory stimuli was detected in a group of 40 children (Schoen *et al.* 2008). Thus, the absence of habituation might only be characteristic for certain groups of individuals with ASD.

We investigated the possibility that social environment might affect the behaviour of the *WT* males used as a control in these experiments. Previous research demonstrated that a mouse model of ASD with reduced sociability when reared with a highly sociable strain of mice, showed an improvement in their social behaviour (Yang *et al.* 2015; Yang *et al.* 2011). The reverse was demonstrated in males lacking *Nlgn3,* where *WT* males reared with their mutant littermates showed impairment in their social behaviour, including low number and duration of vocalisations in response to a female in oestrus and a reduced interest in social odours (Kalbassi *et al.* 2017). However, the differences in the behaviour of MGH and SGH *WT* males in the present study were minimal, including altered activity in the open field, potential changes in the rate of motor learning, and the level of preference for social odours. This indicated that an effect of social environment might be present. Since the changes are subtle, it would be interesting to extend the analysis beyond behaviour. One possibility would be to investigate the effect of social environment on molecular changes such as altered RNA expression.

The behavioural results suggest that the nature of the functional interaction between Neuroligin3 and CYFIP1 might be inhibitory. The hyperactivity characteristic of the males lacking *Nlgn3* was not remedied by lowering the levels of CYFIP1 in the double mutants, indicating the CYFIP1 had no influence on Neuroligin3 in the neuronal population regulating exploratory behaviour. The alternative explanation, however, is that a mere reduction in the level of CYFIP1 was not sufficient to affect exploratory behaviour, while the complete deletion of *Nlgn3* was. In contrast, the motor learning deficit associated with *Cyfip1* haploinsufficiency in the males was normalised by the deletion of *Nlgn3.* This finding is consistent with the interpretation that once Neuroligin3*,* which under physiological conditions might have had the capacity to inhibit CYFIP1, was deleted, the remaining CYFIP1 present in the double mutant could perform its function leading to the restoration of motor learning. Thus, in the neuronal population regulating behaviour, Neuroligin3 might inhibit CYFIP1. It is necessary to confirm if this is also the case for other phenotypes associated with ASD. The relationship between Neuroligin3 and CYFIP1 is discussed in more details in Section 7.2.

We replicated some of the behavioural findings in female mice. In the previous report, female mice heterozygous for *Nlgn3* were no different from their *WT* littermates. This was also observed in the present study. However, females heterozygous for *Nlgn3* were hyperactive in the open field in relation to their littermates heterozygous for *Cyfip1*. This indicated that there might a subtle phenotype in the females heterozygous for *Nlgn3* and it raised the possibility

that the females heterozygous for *Cyfip1* might be hypoactive in the open field. A reduction in the interest in social olfactory cues was also observed in females heterozygous for *Cyfip1.* These possible phenotypes should be further interrogated in a larger sample.

A sex difference in motor learning indicated a male-specific phenotype. While males heterozygous for *Cyfip1* showed no improvement on rotarod across days, females with the same genotype were able to learn. Sex differences in ASD have been frequently reported in the human population, which affects males and females at 4:1 ratio (Halladay *et al.* 2015). One possible explanation of this sex difference is a female protective effect. According to this theory, females require a more substantial disruption of the genetic network and the associated biological pathways for the manifestation of ASD symptoms (Ferri *et al.* 2019). In support of this theory, several genes associated with ASD are X-linked, resulting in females having two copies of the gene and the males having only one. Thus, a deletion of a single copy is likely to have more profound consequences in males. While *Cyfip1* is located on chromosome 7 in mice, it interacts with two X-linked genes: *Nlgn3* and *Fmr1.* As CYFIP1 was found to interact both with Neuroligin3 and FMRP, deletion of an allele of *Cyfip1* might affect males and females differently. This however might be complicated by X-inactivation. Sex differences observed in this thesis are further discussed in Section 7.3.

4.5. Conclusions

The pathway involving *Nlgn3* and *Cyfip1* regulates behaviour in male mice. While the exploratory behaviour is primarily affected by lack of *Nlgn3,* both *Nlgn3* and *Cyfip1* regulate motor learning. The restoration of motor learning in the double mutant mice suggests that *Nlgn3* might functionally inhibit *Cyfip1.* The effect of these genes on behaviour is further modulated by social environment and sex. *WT* animals housed with their mutant littermates showed subtle behavioural changes indicative of the effect of housing on behaviour. Sex further modulated the mouse behaviour with females often displaying no deficits.

Chapter 5: The effect of *Nlgn3* deletion and *Cyfip1* haploinsufficiency on cortical dendritic spine density in a mouse model

5.1. Introduction

In the previous chapter, the impact of the *Nlgn3/Cyfip1* interaction on mouse behaviour was evaluated demonstrating its effect on motor learning. In this chapter, we investigated the role of the *Nlgn3/Cyfip1* interaction on dendritic spine density, another trait often altered in ASD.

Dendritic spines constitute the postsynaptic docking site of a large number of excitatory synapses in the brain. Their density, morphology, and turnover is influenced by a number of stimuli and is dependent on actin polymerisation and local protein translation (Martínez-Cerdeño 2016). Increased density of dendritic spines is considered a hallmark symptom of Fragile X Syndrome, caused by mutations in *FMR1*, a form of syndromic ASD. This phenotype was observed in cortical and hippocampal neurons in post-mortem studies of individuals with Fragile X Syndrome and in the mouse model of *FMR1* deletion, using Golgi staining (Antar *et al.* 2006; Comery *et al.* 1997; Dolen *et al.* 2007; Galvez and Greenough 2005; Gross *et al.* 2010; Grossman *et al.* 2006; Hayashi *et al.* 2007; Liu *et al.* 2011; Mckinney *et al.* 2005; Padmashri *et al.* 2013; Su *et al.* 2011; Swanger *et al.* 2011). However, it is important to note that more recent studies employing two-photon imaging in the developing cortex of mice lacking *FMR1* failed to show any differences in dendritic spine density (Cruz-Martín *et al.* 2010; Pan *et al.* 2010). As discussed in Chapter 3, among the targets of FMRP are CYFIP1 and Neuroligin3, which interact with each other. In line with this observation, CYFIP1 and Neuroligin3 are also likely to impact on the density of dendritic spines. CYFIP1 was observed in the heads of dendritic spines and dendritic shafts (Hsiao, Harony-Nicolas, *et al.* 2016; Pathania *et al.* 2014). Unlike in mice lacking *Fmr1*, *Cyfip1* haploinsufficiency resulted in a decrease in spine density in the motor cortex and the olfactory bulb (Abekhoukh, H Bahar Sahin, *et al.* 2017; Bachmann *et al.* 2019). The relationship between dendritic spine density and deletion of Neuroligin3 is less clear. While triple deletion of *Nlgn1*, *-2*, and *-3*, resulted in a reduction of synaptic contacts *in vivo* (Varoqueaux *et al.* 2006) and lower spine number *in vitro* (Chih *et al.* 2004), the number of dendritic spines in the Neuroligin3 R451C model was unaltered (Isshiki *et al.* 2014). A recent study showed that hippocampal neurons transfected with different isoforms of *NLGN3* did not differ in spine density from controls (Uchigashima *et al.* 2020). However, the impact of *Nlgn3* deletion on spine density *in vivo* has not been verified yet.

In addition to differences in dendritic spine density, changes in morphology and turnover of dendritic spines are present in ASD models. In particular, mice heterozygous for *Cyfip1* phenocopied the increase in filamentous spines present in the mouse model of Fragile X Syndrome (Abekhoukh *et al.* 2017; De Rubeis *et al.* 2013; Pathania *et al.* 2014). This finding was replicated when *Cyfip1* was selectively deleted in excitatory neurons of the forebrain (Davenport *et al.* 2019). This phenotype was restored *in vitro* by transfecting *Cyfip1,* validating the role of *Cyfip1* in dendritic spine development (De Rubeis *et al.* 2013). Filamentous spines are often considered to be immature. An increase in those spines in mice heterozygous for *Cyfip1* could potentially be explained by an increased turnover of spines observed in these animals (Bachmann *et al.* 2019). This effect seems to be common in ASD models, including the Neuroligin3 R451C model, in which the turnover of dendritic spines in PSD-95 and gephyrin positive cells was increased (Isshiki *et al.* 2014). Information about the maturity of spines and spine turnover is missing for the *Nlgn3* deletion.

In this chapter, we evaluated the impact of the Neuroligin3 and CYFIP1 interaction on dendritic spine density. In order to visualise the dendritic spines, EGFP expressed under the *Thy* promoter was used, sparsely labelling dendrites in the cortex (Feng *et al.* 2000). The motor and visual cortices were selected as regions of interest, as the levels of CYFIP1 were shown to be reliably reduced in these areas in mice heterozygous for *Cyfip1* (Bachmann *et al.* 2019).

5.2. Aims and objectives

1. To verify the previously reported decrease of dendritic spine density in the motor cortex of *Cyfip1*$^{+/-}$ male mice.
2. To investigate the impact of the Neuroligin3/CYFIP1 interaction on dendritic spine density in the cortex.
3. To determine if a sex difference exists in dendritic spine density in the cortex.

5.3. Results

5.3.1. Spine density was reduced in the motor cortex of *Cyfip1*$^{+/-}$ males

Initially, we aimed to confirm the decrease in spine density in the motor cortex in *Cyfip1*$^{+/-}$ males, recently reported in Bachmann *et al.* (2019). For this purpose, data collected by Sophie Waldron, a former student in the laboratory, was re-analysed. The repetition of the analysis was performed to allow comparison between datasets and exclude the influence of experimenter as a factor. The density of dendritic spines was calculated as the number of spines per 10 µm of dendrite in the motor cortex, visual cortex, and the hippocampus of *Cyfip1*$^{+/-}$ males and their *WT* male littermates. EGFP was expressed under the *Thy1* promoter

in those mice to allow for visualisation of the spines. The mean number of dendritic spines in each condition and the associated standard error can be found in Table 12.

In the motor cortex, *Cyfip1*$^{+/-}$ males (n = 4) had a significantly lower density of spines than *WT* males (n = 4, independent t-test, $t(1, 59) = -4.17$, $P < 0.001$, Figure 24 A), as reported in Bachmann *et al.* (2019). There were no significant differences in any other brain region, suggesting regional specificity of the effect (Figure 24 B – D) in line with the previous findings (Bachmann *et al.* 2019).

Table 12 Mean and standard error of the number of dendritic spines in *Cyfip1*$^{+/-}$ and *WT* males.[4]

Number of dendritic spines per 10 µm dendrite	***WT***		***Cyfip1*$^{+/-}$**	
	Mean	*SE*	*Mean*	*SE*
Area M1	6.42	0.31	4.81	0.23
Area V1	6.33	0.37	6.95	0.43
Area CA1	7.26	0.35	7.79	0.30
Area CA3	7.13	0.31	7.02	0.38

[4] *SE* = standard error

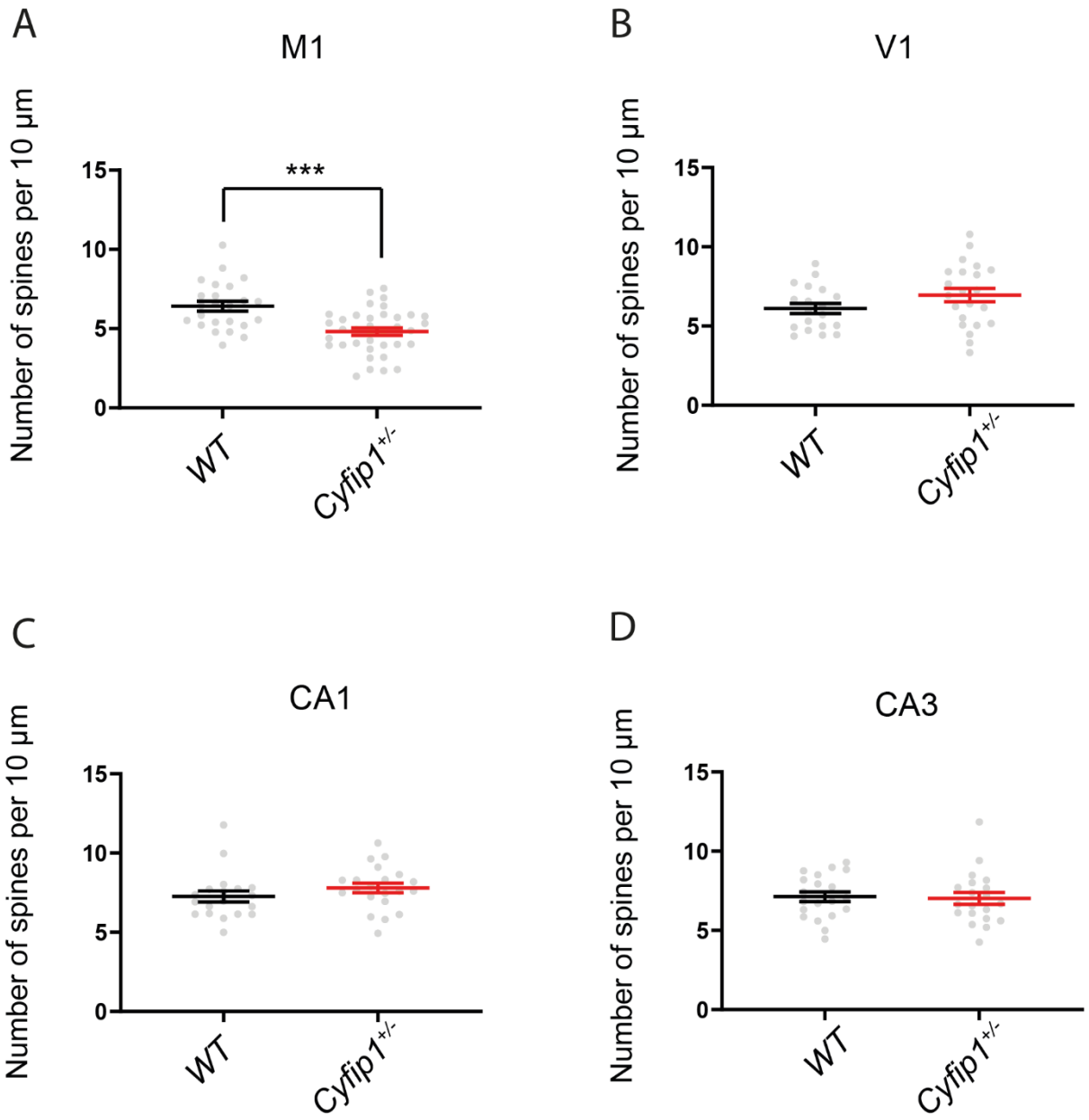


Figure 24 ***Cyfip1*$^{+/-}$ males showed a decrease in dendritic spine density in the motor cortex compared to their *WT* littermates.** **A** Number of dendritic spines per 10 µm of dendrite in area M1 of the motor cortex. *Cyfip1*$^{+/-}$ males had a lower dendritic spine density than their *WT* littermates. **B** Number of dendritic spines per 10 µm of dendrite in area V1 of the visual cortex. There were no differences between *Cyfip1*$^{+/-}$ and *WT* males. **C** Number of dendritic spines per 10 µm of dendrite in area CA1 of hippocampus. **D** Number of dendritic spines per 10 µm of dendrite in the area CA3 of hippocampus. *** = $P < 0.001$.

5.3.2. Spine density in the motor cortex was altered in *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* mice

We investigated the combined impact of *Nlgn3* and *Cyfip1* on dendritic spine density in the cortex. For this purpose, we used the *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* males and their single mutant and *WT* littermates. These mice in addition to a mutation in *Nlgn3* and/or *Cyfip1,* also contained the *Thy1-EGFP* transgene to allow for the visualisation of the spines. These were the same mice which have undergone behavioural testing as described in Chapter 4. All of the mice experienced the same assays and in the same order and were sacrificed shortly after the end of behavioural testing (approximately P100). The mean dendritic spine numbers per 10 µm dendrite and the associated standard error for each condition is available in Table 13.

Overall, the numbers of dendritic spines in this cohort were lower than in the previous experiment, which is further discussed in Section 5.6. As previously, only potential changes were in the motor cortex. The comparison between *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* males (n = 3) and *WT* (n = 4) males approached significance ($t(1, 11) = 1.50$, $P = 0.16$), with *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* males having more dendritic spines in the motor cortex (Figure 25 A). None of the other comparisons were significant in this area. In the visual cortex, there were no significant differences in the dendritic spine density between any of the groups (Figure 25 B).

Table 13 Mean and standard error of the number of dendritic spines in *WT, Cyfip1*$^{+/-}$, *Nlgn3*$^{y/-}$, and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ males. [5]

Number of dendritic spines per 10 µm dendrite	***WT***		***Nlgn3***$^{y/-}$		***Cyfip1***$^{+/-}$		***Nlgn3***$^{y/-}$ ***Cyfip1***$^{+/-}$	
	Mean	*SE*	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*SE*
Area M1	1.66	0.13	1.69	0.09	2.02	0.13	2.27	0.12
Area V1	2.01	0.13	2.05	0.18	2.29	0.17	2.40	0.18

[5] *SE* = standard error

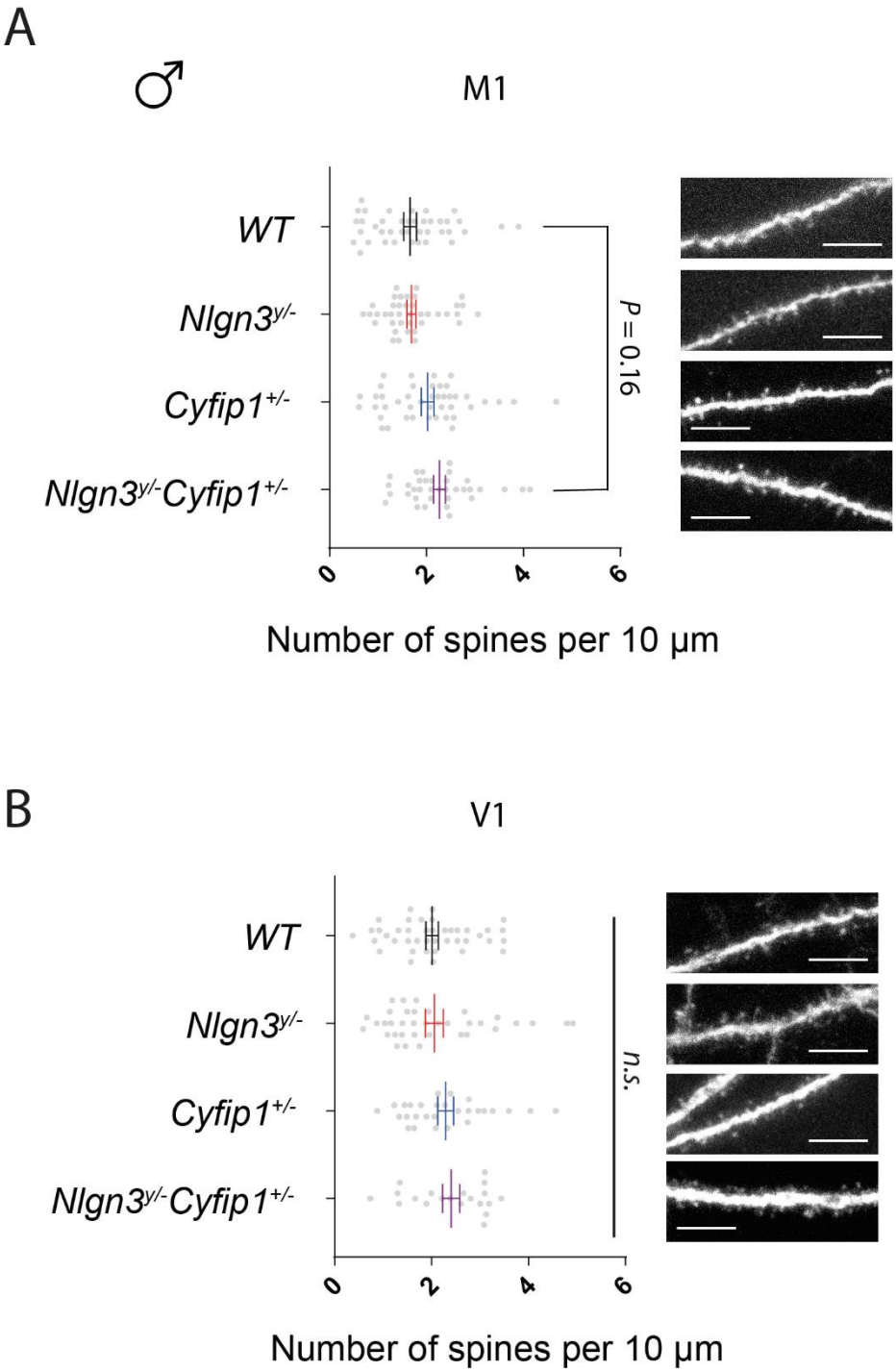


Figure 25 **Dendritic spine density was increased in *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* males. A** Number of dendritic spines per 10 µm of dendrite in area M1 of the motor cortex in *WT.* Dendritic spine density was only increased in the *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* males. **B** Number of dendritic spines per 10 µm of dendrite in area V1 of visual cortex. The dendritic spine density was unaltered in any of the models. Scales bars are 10 µm. *n.s.* = not significantly different.

5.3.3. There were no differences in dendritic spine density in female mice

In order to determine if there were any changes in dendritic spine density in female mice, we included samples from *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ females (*n* = 4) and their *WT* (*n* = 4), *Nlgn3*$^{+/-}$ (*n = 4*), *Cyfip1*$^{+/-}$ (*n* = 4) littermates. However, there were no differences in dendritic spine density in either the motor or visual cortex between the different genotypes (Table 14, Figure 26 A – B).

Interestingly, the overall number of dendritic spines seemed to vary between male and female mice. In order to confirm this observation, a direct comparison of dendritic spine density was conducted between *Cyfip1*$^{+/-}$ and *WT* male and female mice. *Nlgn3*$^{+/-}$ females, *Nlgn3*$^{y/-}$ males as well as the double mutants of either sex were excluded from the analysis as they differed in the *Nlgn3* gene dosage in addition to sex, making it impossible to compare them directly. Females were found to have on average more dendritic spines in the cortex than males, regardless of cortex region or genotype (GLM, the main effect of sex: $t(1,11) = -7.27$, $P < 0.001$, Figure 27).

Table 14 Mean and standard error of the number of dendritic spines in *WT, Cyfip1*$^{+/-}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ females. [6]

Number of dendritic spines per 10 µm dendrite	***WT***		***Nlgn3*$^{+/-}$**		***Cyfip1*$^{+/-}$**		***Nlgn3*$^{+/-}$ *Cyfip1*$^{+/-}$**	
	Mean	*SE*	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*SE*
Area M1	3.25	0.14	3.27	0.16	3.29	0.20	3.28	0.15
Area V1	3.23	0.25	2.91	0.14	3.53	0.19	3.22	0.14

[6] *SE* = standard error

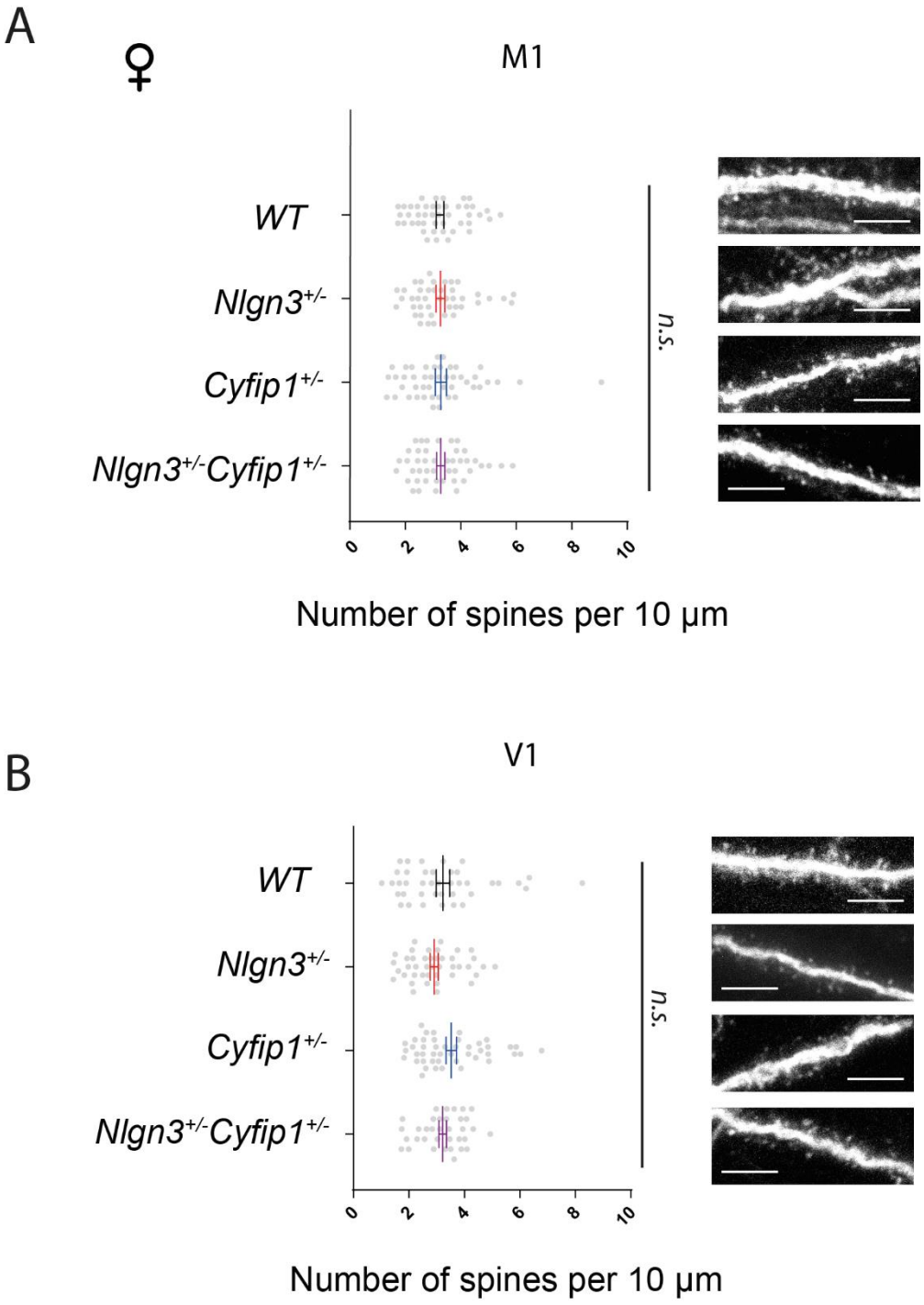


Figure 26 ***Nlgn3*** **deletion and** ***Cyfip1*** **haploinsufficiency had no impact on dendritic spine density in the cortex of female mice. A** Number of dendritic spines per 10 µm of dendrite in area M1 of the motor cortex in females. There were no differences depending on the genotype of the mice. **B** Number of dendritic spines per 10 µm of dendrite in area V1 of visual cortex in females. There were no differences depending on the genotype. Scales bars are 10 µm. *n.s.* = not significantly different.

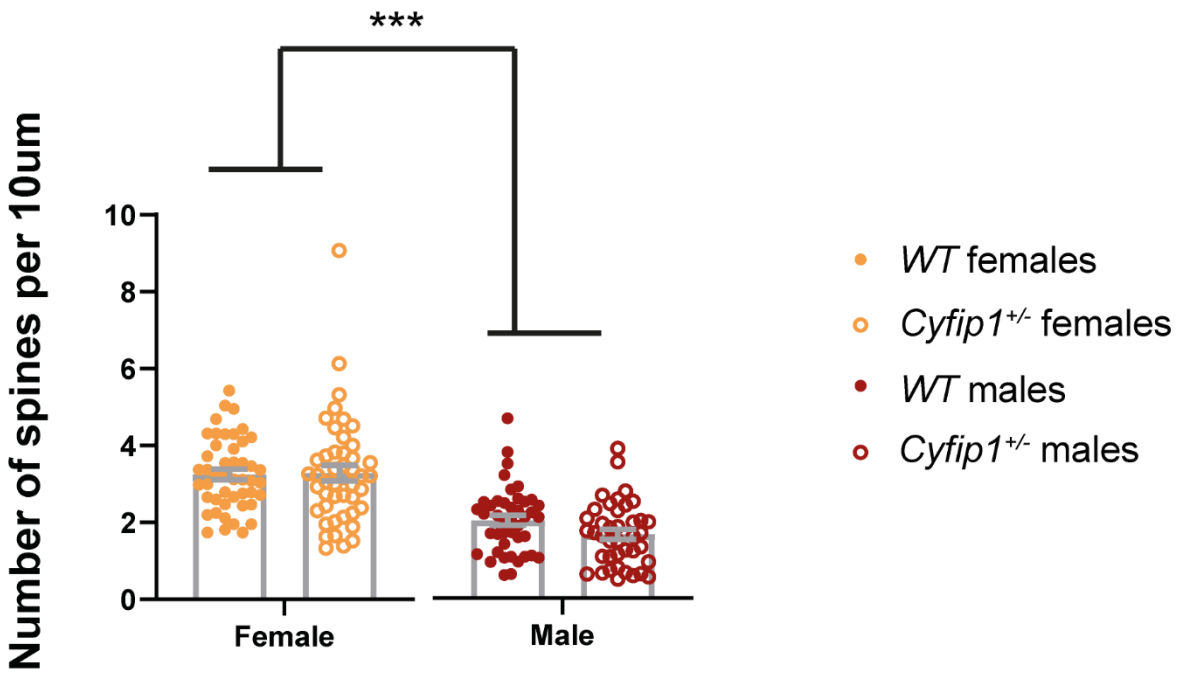


Figure 27 **Female mice had greater spine density than male mice in the cortex.** Number of dendritic spines per 10 µm of dendrite, averaged over the region of interest in male and female mice. *** = $P < 0.001$

5.4. Discussion

A decrease in dendritic spine density in males heterozygous for *Cyfip1* was found. The effect was specific to the motor cortex and did not extend to the visual cortex. There was also a suggestion there might be an increase in dendritic spine density in the double mutant males which lacked *Nlgn3* and were heterozygous for *Cyfip1,* in separate cohort of animals. As previously, this effect was only present in the motor cortex. There was a sex difference in the dendritic spine density, where females had markedly more spines in the cortex than males.

A decrease in dendritic spines in the motor cortex in males heterozygous for *Cyfip1* in the existing data was reported in Bachmann *et al.* (2019). This effect on dendritic spine density in this mouse model was also previously described by other groups (Pathania *et al.* 2014; Bagni and Greenough 2005). However, there was no evident decrease in males heterozygous for *Cyfip1* in the cohort containing double mutant animals. Both cohorts of mice came from the same breeding line and the images were analysed in the same manner and by the same experimenter, suggesting that the failure of replication is unlikely to be a technical issue. The lack of reproducibility of the effect was accompanied by a surprisingly low count of spines in all of the male mice in the second cohort, with only 1 – 5 spines per 10 µm, while the numerous previous studies reported a density of 5 – 15 spines per 10 µm both in the *Cyfip1*$^{+/-}$ and *WT* males (Pathania *et al.* 2014; De Rubeis *et al.* 2014; Abekhoukh *et al.* 2017). The possible explanation for the lack of a decrease in dendritic spine density in males heterozygous for *Cyfip1* is that the spine numbers in the *WT* littermates were exceptionally low in the second cohort masking the effect of *Cyfip1* haploinsufficiency on the dendritic spine density. Low count of dendritic spines in the *WT* males could be a result of the impact of social environment. The presence of littermates lacking *Nlgn3* in the cage during rearing was found to affect the behaviour of the *WT* males (Kalbassi *et al.* 2017). A similar phenomenon was described in Chapter 4 in *WT* males sharing the cage with their littermates either with a deletion of *Nlgn3* or heterozygous for *Cyfip1* or both. The low number of spines observed in the *WT* males in this experiment suggests that the effect of littermates lacking *Nlgn3* could potentially extend to the dendritic spine density. The effect of mutant littermates, however, does not seem to extend to the males heterozygous for *Cyfip1*, as the number of spines in the first cohort of mice falls within the previously reported range.

The potential effect of the social environment, which was not accounted for in this experiment, complicates the interpretation of the dendritic spine density increase observed in the double mutant mice. Mice lacking *Nlgn3* and heterozygous for *Cyfip1* were found to have greater spine density than their *WT* littermates as well as the littermates lacking *Nlgn3.* This observation suggested that increasing the genetic load to include two mutations led to greater

dendritic spine density, while the individual mutations were not always sufficient to result in an alteration. Increased spine density has been previously reported in ASD (Martínez-Cerdeño 2016). However, if the *WT* littermates had abnormally low spine numbers, it might be that after accounting for the social environment effect, double mutant mice might show *WT*-level number of spines. This result would suggest that another of the phenotypes associated with *Cyfip1* can be corrected by deleting *Nlgn3,* analogous to the motor behaviour phenotype described in Chapter 4. However, this possibility needs to be verified by conducting dendritic spine analysis in a cohort of SGH *WT* mice, which was not possible to include in this thesis due to practical constrains.

Interestingly, any changes in dendritic spine density observed in this chapter were limited to area M1 of the motor cortex. A deficit in motor learning in males heterozygous for *Cyfip1* was reported in Chapter 4 as well as in the literature (Bachmann *et al.* 2019), raising the question of the connection between an M1-specific deficit in dendritic spine number and motor learning. The role of dendritic spine plasticity in learning and memory has been extensively investigated (Gipson and Olive 2017). The recent view is that changes in the morphology of individual spines are more relevant for the learning than the density of spines (van der Zee 2015). When mice were trained on the rotarod, formation of new dendritic spines was observed in the primary motor cortex (Yang *et al.* 2009). However, only a small percentage of these spines persisted for a longer period of time, most of them being eliminated, likely due to exposure to other novel experiences. This study suggests that motor learning might have a short-term effect on dendritic spine density, while the turnover of dendritic spines might be a better correlate of motor learning. Males heterozygous for *Cyfip1* showed an increase in the number of newly formed spines in response to rotarod training, despite their lack of improvement across trials (Bachmann *et al.* 2019). However, there was an equivalent increase in the elimination of dendritic spines, supporting the idea that motor learning is more likely associated with changes in the turnover of spines rather than dendritic spine density.

The Neuroligin3/CYFIP1 interaction had no impact on dendritic spine density in the females. This finding is analogous to the results reported in Chapter 4, where the motor learning deficit was only present in the males. This raises the possibility that the female protective effect described in Chapter 4 extends to other phenotypes associated with ASD such as a change in dendritic spine density. Another possibility is that there is a correlation between dendritic spine density and motor behaviour and only of one of these parameters is affected by the female protective effect. Interestingly, there was an overall difference in dendritic spine density in the cortex, with females having more spines on average than males.

This observation could be the result of the social environment effect on *WT* males. A previous report showed no impact of the social environment on the behaviour of *WT* female mice (Kalbassi *et al.* 2017), suggesting that the social environment might also not influence the dendritic spine density in the females. Thus, the dendritic spine density might be lower both in males heterozygous for *Cyfip1* and their *WT* littermates compared to the females. On the other hand, there is some evidence that spine turnover in females is affected by the variation in oestrogen levels that accompanies the oestrus cycle (Hyer *et al.* 2018). However, this variation is unlikely to play a role in the sex difference observed in the current experiment, as the females were perfused for this experiment at random timepoints in relation to their cycle. Thus, the distribution of the different phases of the oestrus cycle among the groups of females was likely to be equivalent.

Additionally, changes in spine morphology are often reported in models of ASD as described in Section 5.1 of this chapter. These alterations in morphology might also allow for establishing a link between the investigation of dendritic spines and mouse behaviour. Thus, the changes in dendritic spine density described in this chapter might be accompanied by alterations in morphology. The categorisation of spines can be unreliable without the use of Stimulated Emission Depletion Microscopy (STED) (Wijetunge *et al.* 2014), so ideally this method should be used in establishing possible morphological changes.

5.5. Conclusions

Changes in dendritic spine density have been observed in a number of models of ASD. A decrease in dendritic spine density in males heterozygous for *Cyfip1* was previously reported. However, this decrease was not observed when these animals were housed with their littermates lacking *Nlgn3.* Instead, an increase in dendritic spine density was observed in males heterozygous for *Cyfip1* and lacking *Nlgn3.* This inconsistency could potentially be explained by the effect of males with *Nlgn3* deletion on the dendritic spine density in their littermates. However, this possibility needs to be further examined before a firm conclusion can be reached. Females mice showed the same dendritic spine density regardless of their genotype, which might be the result of combined female protective effect and selective impact of social environment.

Chapter 6: The effect of housing conditions and genotype on the transcriptome of mice with *Nlgn3* deletion and heterozygous for *Cyfip1*

6.1. Introduction

An interaction between Neuroligin3 and CYFIP1 was observed in neurons, as described in Chapter 3. The consequences of this association extended to the exploratory and motor behaviour of male mice as well as potentially to the dendritic spine density in the motor cortex as explained in Chapters 4 and 5. Next, we aimed to investigate if the interaction between Neuroligin3 and CYFIP1 might affect RNA expression.

Variations in RNA expression could be effectively used to distinguish between patients with ASD and controls (Voineagu *et al.* 2011), indicating fundamental differences in their transcriptomes. RNA expression regulation was also highlighted as one of the biological processes affected by multiple genes linked to ASD (Luo *et al.* 2012; De Rubeis *et al.* 2014). Neuroligin3 and CYFIP1 might indirectly impact on transcription regulation, via their interaction with FMRP as described in Chapter 3. However, the influence of *Nlgn3* deletion or *Cyfip1* haploinsufficiency on RNA expression has not yet been investigated.

The hippocampus was selected as an area of interest for this experiment. First, levels of CYFIP1 were shown to be reliably lowered in the hippocampus of *Cyfip1*$^{+/-}$ male mice (Bachmann *et al.* 2019) making it a suitable brain region for investigating the effect of *Cyfip1* haploinsufficiency on RNA expression. Also, expression of *Nlgn3* in areas adjacent to the hippocampus that are likely to be included in the dissection of this area, had an effect on mouse behaviour and physiology. Specifically, learning motor routines was influenced by *Nlgn3* expression in the nucleus accumbens (Rothwell *et al.* 2014), while social behaviour was affected by the deletion of *Nlgn3* in the ventral tegmental area (Bariselli *et al.* 2018). Deleting *Nlgn3* in the hippocampus was also found to affect hippocampal-dependent learning (Polepalli *et al.* 2017). Interestingly, a recent study showed that the different isoforms of *NLGN3* also had a differential effect on the inhibitory synaptic transmission in hippocampal neurons (Uchigashima *et al.* 2020). Finally, previous analysis of one of the datasets presented here indicated there might be some changes in the transcriptome in the hippocampus due to *Nlgn3* deletion. Thus, the hippocampus is an area sensitive to *Nlgn3* expression making it an interesting target for investigating the impact of *Nlgn3* deletion in shaping the transcription profile.

A subtle effect of the social environment on the behaviour of *WT* littermates of mutant mice was demonstrated in Chapter 4. In addition, a possibility exists that its influence might

extend to other phenotypes associated with ASD, as described in Chapter 5. However, attempts to investigate the impact of the social environment on mouse behaviour are inherently limited by the large variability in the resulting data. There is likely to be more random variation between individuals in their behaviour than in their RNA expression. Previously, experiments showed that the social environment experienced by mice in their early life, the instability and the position in the social hierarchy all have the capacity to alter the expression of mRNAs (Horii *et al.* 2017; Nesher *et al.* 2015; So *et al.* 2015). Therefore, we decided to investigate the effect of the social environment on RNA expression in addition to the influence of the Neuroligin3/CYFIP1 interaction in the hippocampus.

In this chapter, RNA expression in the hippocampus of male mice with *Nlgn3* deletion, or heterozygous for *Cyfip1*, or both was investigated. *WT* littermates housed with the mutant animals as well as a cohort of *WT* males housed exclusively with other *WT* animals were both included as controls and to account for the effect of the social environment.

6.2. Aims and objectives

1. To investigate if *Nlgn3* deletion, *Cyfip1* haploinsufficiency, or the combination of both affect the transcription in the hippocampus.
2. To determine if the social housing of mice with different genotypes impacts on the transcription in the hippocampus.

6.3. Results

6.3.1. *Nlgn3* could impact on transcription in the hippocampus

RNA expression in *Nlgn3*$^{y/-}$ (*n* = 3), *Cyfip1*$^{+/-}$ (*n* = 3), *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ (*n* = 3), as well as MGH *WT* (*n* = 6) and SGH *WT* (*n* = 3) adult male mice was investigated using RNA sequencing. *Nlgn3* was downregulated in the *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ animals, while the expression of *Cyfip1*$^{+/-}$ was decreased in *Cyfip1*$^{+/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ mice, as expected (Table 15). Initially, genes differentially expressed between pairs of conditions were considered (Figure 28). There were very few differentially expressed genes between mutant mice and MGH *WT* controls (MGH *WT* vs *Cyfip1*$^{+/-}$: 2 upregulated, 0 downregulated; MGH *WT* vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: 3 upregulated, 0 downregulated; MGH *WT* vs *Nlgn3*$^{y/-}$: 3 upregulated, 2 downregulated). The differences between SGH *WT* controls and *Nlgn3*$^{y/-}$ were more substantial (SGH *WT* vs *Nlgn3*$^{y/-}$: 12 upregulated, 5 downregulated). Similarly, the number of differentially expressed genes between SGH *WT* controls and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ double mutants was higher (SGH *WT* and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: 21 upregulated, 2 downregulated). There was also a lot of overlap in terms of the identity of the genes with changed expression in these two comparisons, suggesting that the changes observed are likely due to the deletion of *Nlgn3.* However, the differences between SGH *WT* animals and the *Cyfip1*$^{+/-}$ were negligible (SGH

WT vs *Cyfip1*$^{+/-}$: 2 upregulated, 1 downregulated). These findings indicated that while *Nlgn3* might influence RNA expression to a certain extent, *Cyfip1* has little effect on it. This was however not supported by hardly any differences found between *Cyfip1*$^{+/-}$ and *Nlgn3*$^{y/-}$ males (1 upregulated 1 downregulated) or *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ males (1 upregulated).

Table 15 Genes with altered expression depending on genotype and housing condition.[7]

MGH *WT* vs *Nlgn3*$^{y/-}$	MGH *WT* vs *Cyfip1*$^{+/-}$	MGH *WT* vs *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$	SGH *WT* vs *Nlgn3*$^{y/-}$	SGH *WT* vs *Cyfip1*$^{+/-}$	SGH *WT* vs *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$	SGH *WT* vs MGH *WT*	*Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$	*Nlgn3*$^{y/-}$ vs *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$	*Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$
Nlgn3	Cyfip1	Cyfip1	Nlgn3	Cyfip1	Cyfip1	Shox2	Cyfip1	Cyfip1	Nlgn3
C4b	C4b	Nlgn3	Shox2	Cecr2	Nlgn3	CF7L2	Nlgn3		
Gm34567		Nefm	CF7L2	Shox2	Shox2	SYNPO2			
Sv2c			SYNPO2		CF7L2	Tnnt1			
Fndc1			Kcnj10		SYNPO2	Kcnj10			
			Plcb4		Tnnt1	Plcb4			
			Cecr2		Kcnj10	Cpne9			
			Rgs8		Cpne9	Gata3			
			Gpnmb		Rgs8	AC1490901			
			C4b		Nefm	Rassf4			
			Lrrc55		Gpnmb	Vipr2			
			Foxo6		Srxn1	Ndnf			
			Sspo		Htr2a	Tafa4			
			Rora		Zdhhc22	IRX2			
			Cbfa2t3		Gm14569	Egr4			
			Fibcd1		Adarb1	Acan			
			Inhbb		Sparc	Slitrk6			
					Igfbp4	Strc			
					Endou				
					Vav3				
					Nefh				
					Paqr8				
					Acvr1c				

[7] Every instance of a change in *Nlgn3* expression is indicated with a blue background and a change in *Cyfip1* expression is marked with a pink background. Genes, which expression is changed in more than one condition is indicated with a yellow background. Genes written in red were downregulated and in black were upregulated in the given comparisons.

6.3.2. Social environment shaped the transcriptome in the hippocampus

We directly compared SGH *WT* and MGH *WT* males to determine if the social environment influenced RNA expression. There were 15 upregulated and 3 downregulated genes in this comparison, a magnitude comparable to the difference between *Nlgn3*$^{y/-}$ and SGH *WT* males (Figure 28). This finding suggested that social environment might impact on the transcriptional profile. There was also some overlap in the identity of the changed genes in this comparison and the comparisons between SGH *WT* and *Nlgn3*$^{y/-}$ or *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$. This observation supported the idea that the MGH *WT* animals were similar to their mutant littermates.

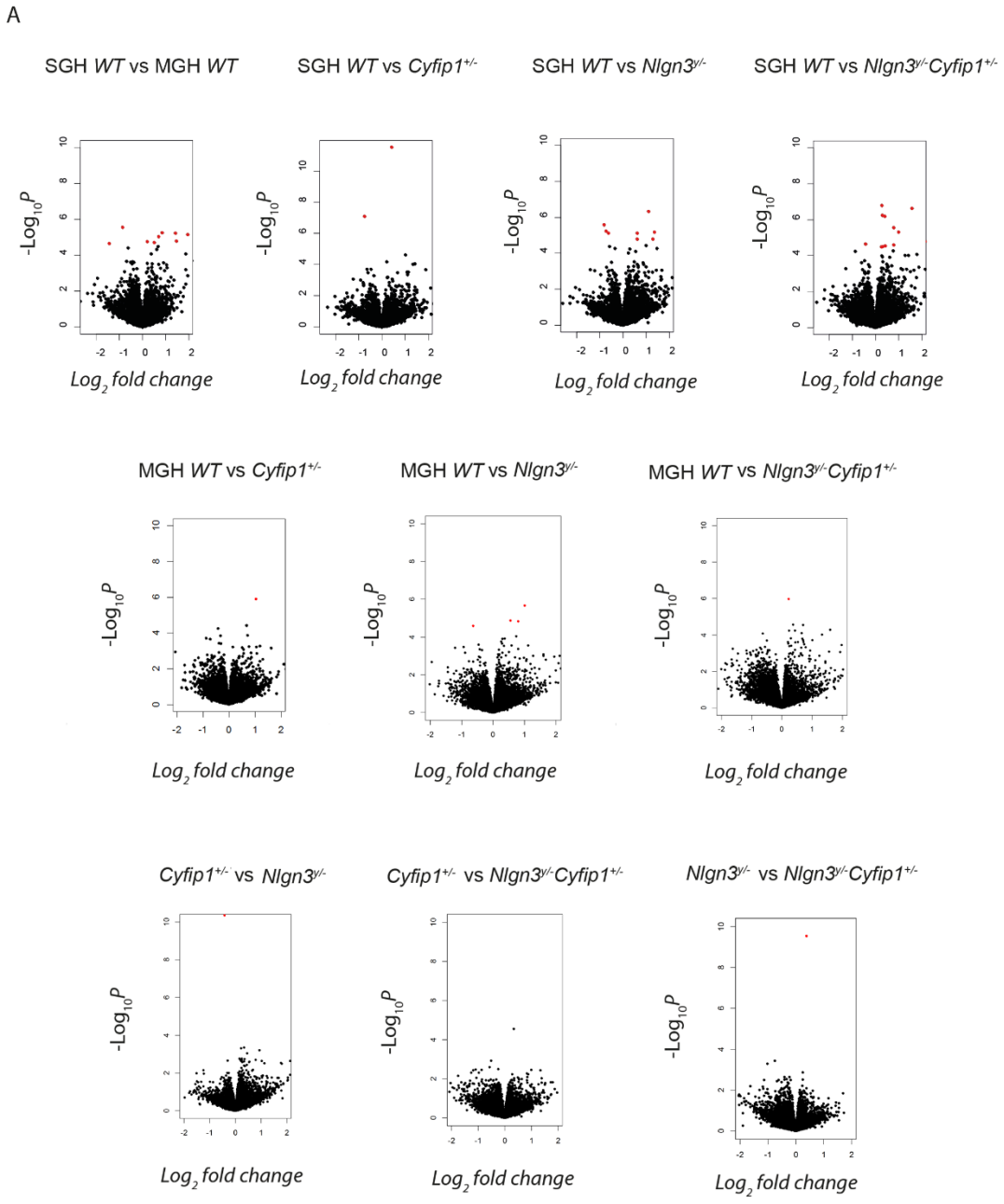


Figure 28 ***Nlgn3*** **deletion and social environment affected gene expression in the hippocampus.** Volcano plots showing differentially expressed genes between pairs of different housing or genotype conditions. The most pronounced differences were observed between SGH *WT* and MGH *WT* males. Please note the discrepancy in the scale between graphs. Some points might be missing due to axis formatting.

6.3.3. *Nlgn3* deletion and *Cyfip1* haploinsufficiency as well as social housing influenced the correlated gene networks

During the analysis of differentially expressed genes, a wealth of information about co-regulation between the different genes was lost due the use of an arbitrary p-value level. An alternative was to employ techniques which provide results on the whole dataset or utilise different thresholds. We attempted to move beyond the differentially expressed gene analysis to examine the pattern of RNA expression between the samples. First, we conducted hierarchical clustering based on either all genes or the top 20 genes with the greatest fold change (Figure 29 A-B). There was no clear separation between samples based on either genotype or social environment. Next, Principal Component Analysis (PCA) on the top 100 genes with the greatest fold change was conducted (Figure 30 A – B). Following the PCA the separation between conditions became more apparent, with SGH *WT* and MGH *WT* samples showing little overlap. There was also no overlap between MGH *WT* or SGH *WT* mice and the mutant samples.

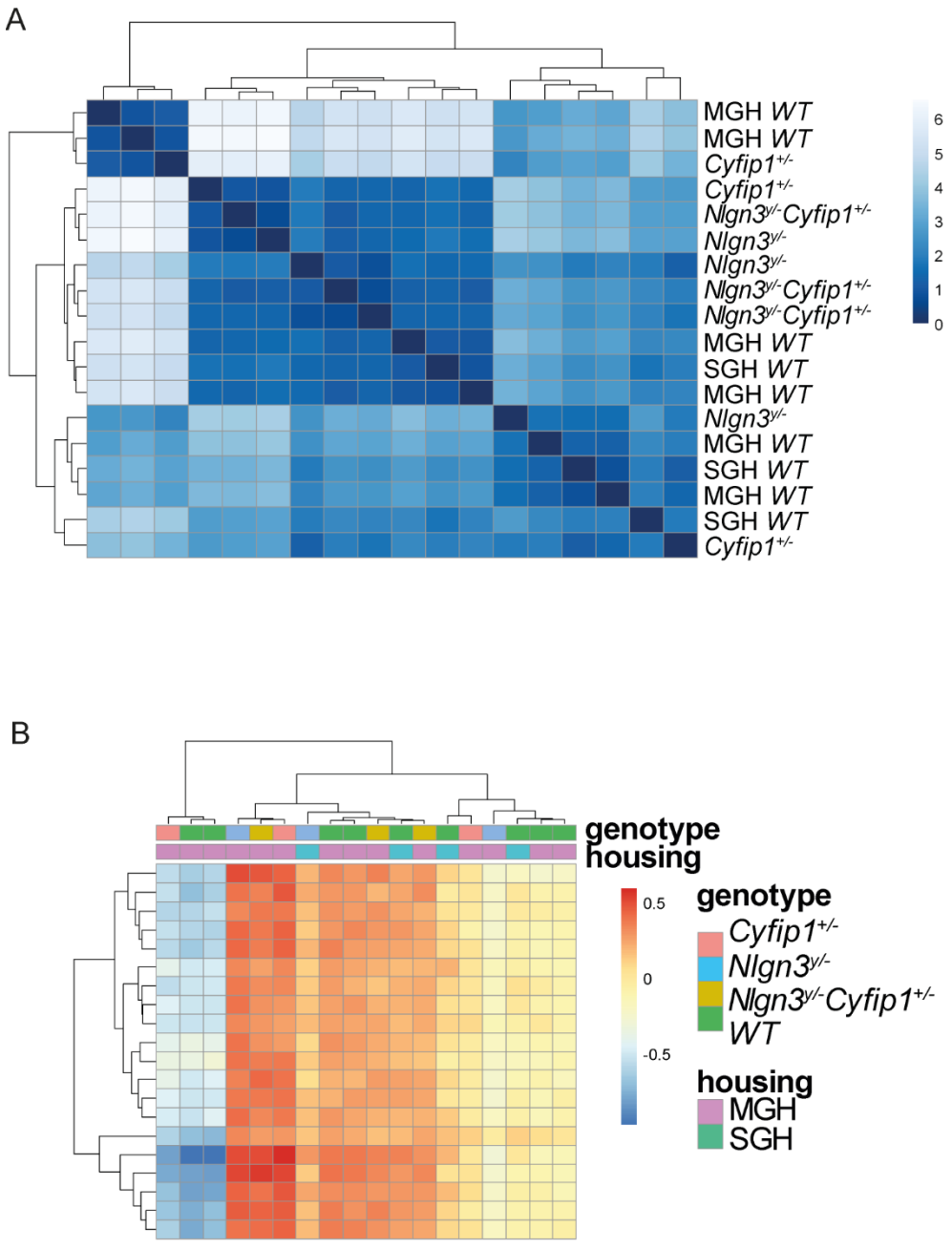


Figure 29 **Hierarchical clustering of genes expressed in the hippocampus did not allow for a distinction based on genotype or social housing. A** Hierarchical clustering of all genes following thresholding and normalisation procedures. There was no clear separation between different conditions. **B** Hierarchical clustering of top 20 genes with the largest fold change. There was no clear separation depending on the genotype or the housing conditions of the mice.

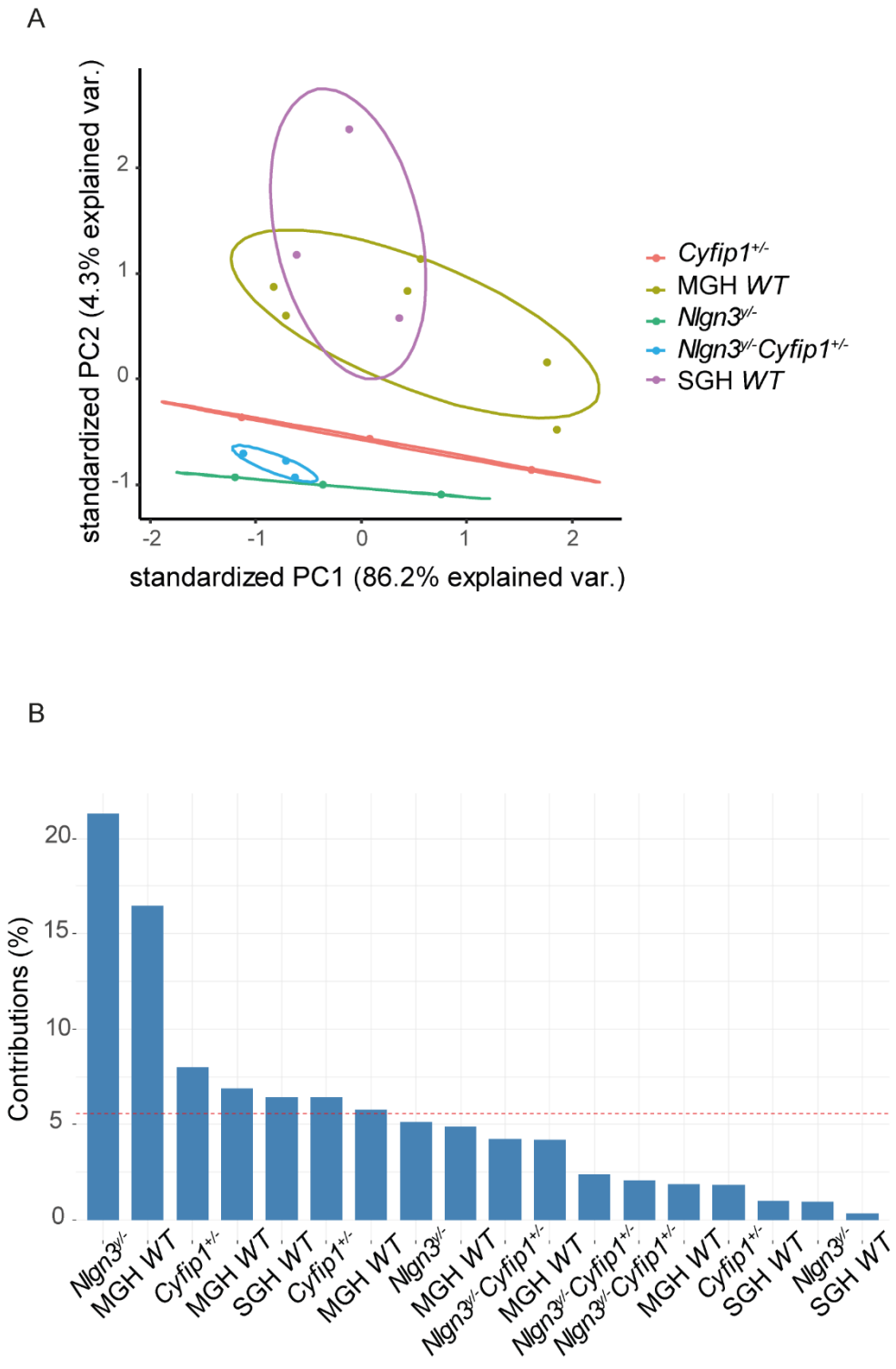


Figure 30 **Principal component analysis of the genes expressed in the hippocampus showed a separation based on the genotype and housing condition of mice. A** Position of the different samples in the space made up from Principal Component 1 and Principal Component 2, based on the top 100 genes. There was little overlap between the different conditions. **B** Percentage contribution of individual samples to the Principal Component 1 and 2.

In order to investigate the connections between the genes in more depth, we used a weighted gene correlation network analysis (WGCNA). The aim of this analysis is to cluster genes into modules which then can be related to another variable such as expression of *Nlgn3, Cyfip1* or the condition of social environment. In the course of this analysis, gene co-expression similarity measure was calculated for each pair of genes, which allowed for a construction of an adjacency matrix. The matrix was constructed using a power function to assess the strength of the connection between different genes. Then genes were clustered into modules using topological overlap measure. Each gene within a module was assigned a eigengene, which were then correlated with the traits of interest. Clustering of the data into modules reduced the problem of multiple comparisons present in the traditional differential expression gene analysis. The input for this examination was all genes remaining after thresholding and normalisation. The threshold was set at the level of expression of *Nlgn3* in the $Nlgn3^{y/-}$ samples. As no expression of *Nlgn3* was expected in these samples, this expression level was considered noise. Variance stabilising transformation was also used on the data. Following these procedures, the remaining genes (n = 17806) were used for the WGCNA. Considering the size of the dataset, the minimum module size was set to 200 genes. A co-expression network containing 20 modules was constructed (Figure 31).

The association between the different genetic and housing conditions and the gene modules was evaluated. The value of the correlation between the module and trait and the q-value following False Discovery Rate adjustment are given here. Lack of *Nlgn3* was found to be significantly associated with module 11 (R^2 = -0.42, FDR q = 0.09, Figure 31), while a reduced level of *Cyfip1* was not linked to any of the modules. The combination of the two mutations, however, resulted in the $Nlgn3^{y/-}Cyfip1^{+/-}$ genotype being associated with modules 11 (R^2 = 0.66, FDR q = 0.003), module 12 (R^2 = -0.42, FDR q = 0.08), and module 14 (R^2 = -0.43, FDR q = 0.08). This suggests that while each mutation had low impact on these correlated gene networks on their own, there was an additive effect when both mutations were combined. When the effect of housing was considered, module 8 was found to be upregulated in the SGH animals compared to the MGH animals (R^2 = 0.75, FDR q < 0.01), while module 17 was downregulated (R^2 = -0.58, FDR q = 0.01).

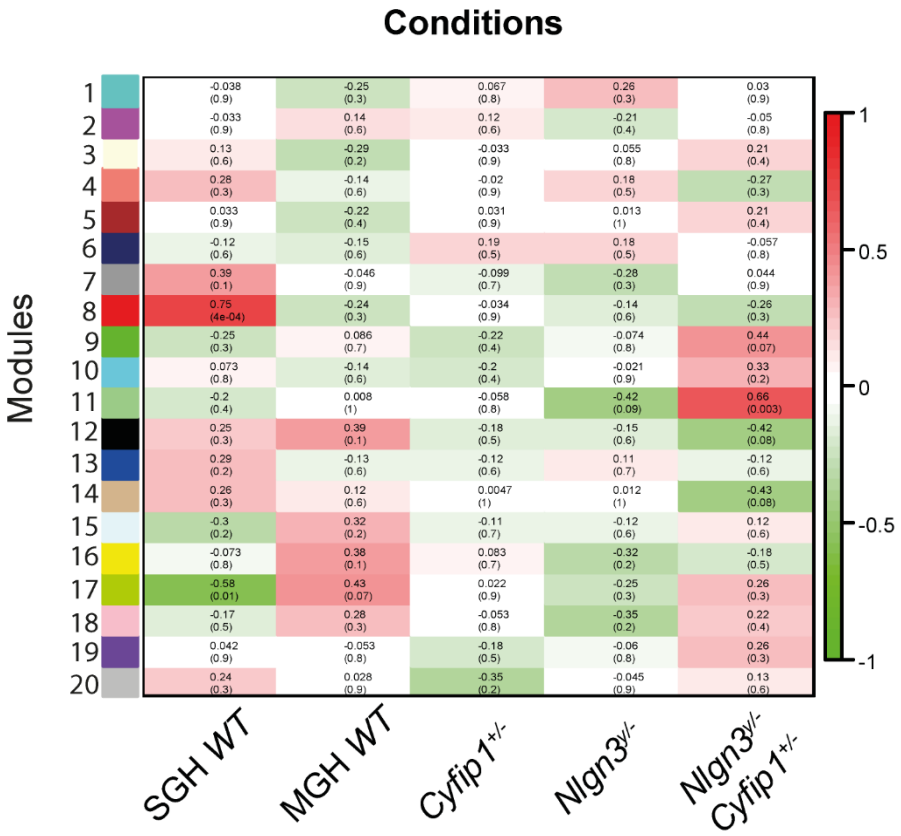


Figure 31 **Weighted gene correlation network analysis showed an effect of *Nlgn3* deletion and social housing on the gene co-expression.** The corrections between different genotype and housing conditions and the modules identified through WGCNA. Red indicated positive correlation between the expression of the genes in the module and the given housing/genotype combination and green signified the negative correlation between a module and a trait. The value of the correlation was given in the top of each segment while the False Discovery Rate adjusted q-values was given below.

Next, we conducted Gene Ontology (GO) Enrichment analysis to identify the biological pathways associated with the different modules within the gene correlation network. We focused on modules 8 and 17, as these two modules were linked to differences in housing. The genes in module 8 were found to be associated with nervous system development (Figure 32 A). It was also linked to synaptic transmission and cerebellar cell proliferation and signalling. Meanwhile, module 17 was associated with a range of metabolic processes (Figure 32 B).

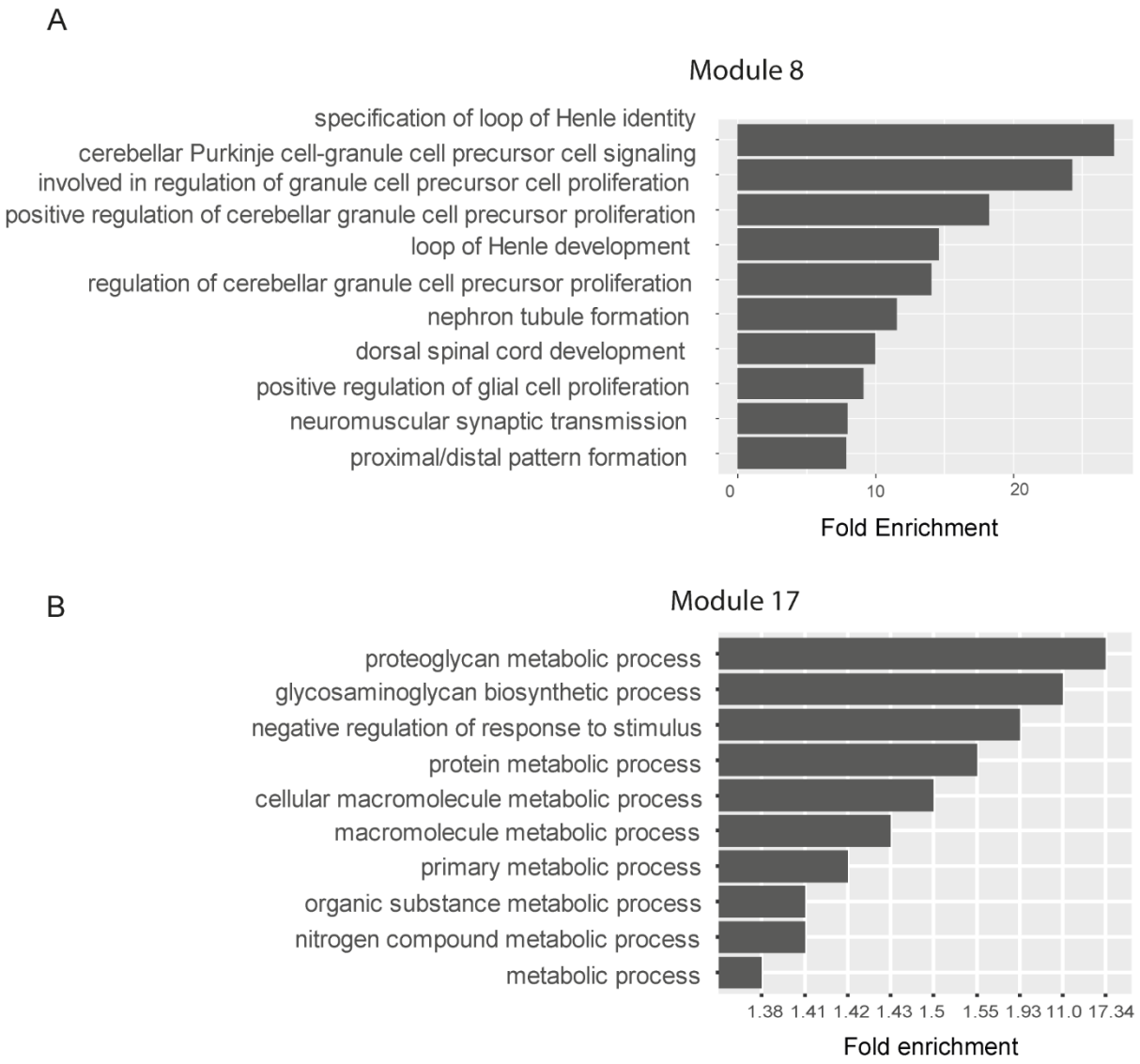


Figure 32 **Single genotype housing of *WT* mice was associated with changes in genes linked to development and metabolic processes. A** Top ten GO terms for the genes in module 8. **B** Top ten GO terms for the genes in module 17.

6.3.4. Social environment impacted on the transcription profile of *WT* as well as *Nlgn3$^{y/-}$* mice

In the previous set of experiments, the SGH *WT* mice originated from a separate breeding line than the *Nlgn3$^{y/-}$Cyfip1$^{+/-}$* double mutant males and their littermates. This raises the possibility that biological differences between SGH and MGH *WT* mice could affect RNA expression beyond the influence of the social environment. In order to confirm that the effect on the transcriptome was exclusively due to the social environment, we took advantage of an existing dataset collected by Dr. Shireene Kalbassi, a previous member of the research group, and we conducted further analysis on those data. In this dataset, the parents of MGH and SGH mice were taken from *Nlgn3* colony, suggesting that they might be more genetically homogenous. The dataset consisted of adult SGH *WT* (*n* = 3) and MGH *WT* (*n* = 3) males as well as SGH *Nlgn3$^{y/-}$* (*n* = 3) and MGH *Nlgn3$^{y/-}$* (*n* = 3) animals. The SGH *Nlgn3$^{y/-}$* males were included to investigate if the effect of social housing extended to the mutant animals.

As expected *Nlgn3* was downregulated in all of the comparisons involving *Nlgn3$^{y/-}$* mice (Table 16). Similar to the previous experiment, there was a number of differentially expressed genes between SGH and MGH *WT* males (23 upregulated, 34 downregulated, Figure 33). This confirms the previous result, suggesting that the social environment had an impact on RNA expression. Housing also affected the differentially expressed genes in the *Nlgn3$^{y/-}$* animals. There were 6 upregulated and 14 downregulated genes in SGH *Nlgn3$^{y/-}$* as compared to MGH *Nlgn3$^{y/-}$* males. There were also several differentially expressed genes in SGH *WT* as compared to SGH *Nlgn3$^{y/-}$* males (39 upregulated and 18 downregulated) that were never housed with an animal of a different genotype. Meanwhile, there were very few differentially expressed genes in MGH *WT* as compared to MGH *Nlgn3$^{y/-}$* that were housed together (1 upregulated, 7 downregulated). This finding suggests that while there might be a small effect of *Nlgn3* on RNA expression, there is also a pronounced effect of housing. This was observed both in *WT* and potentially to a smaller degree in the *Nlgn3$^{y/-}$* animals. While, as in the previous cohort, differences were observed in the RNA expression depending on the presence of *Nlgn3* and on housing conditions, the identity of the significantly changed genes was different from the previous cohort.

Table 16 Genes with altered expression depending on the presence of *Nlgn3* and housing.[8]

SGH *WT* vs MGH *WT*	**SGH *Nlgn3*$^{y/-}$ vs MGH *Nlgn3*$^{y/-}$**	**SGH *WT* vs SGH *Nlgn3*$^{y/-}$**	**MGH *WT* vs MGH *Nlgn3*$^{y/-}$**	**MGH *WT* vs SGH *Nlgn3*$^{y/-}$**
Cdhr1	Pitx2	Nlgn3	Nlgn3	Nlgn3
Dnaic2	Fstl5	Gm21986	Gm21986	Gm21986
Sytl1	Cabp1	Fstl5	Cdhr1	Dnaic2
Lcn2	Lamp5	Sytl1	Pitx2	Gm2115
Tnxb	C130074G19Rik	Cabp1	Magel2	Fmo2
AC149090.1	Ighm	Lamp5	Xlr3a	Tnxb
Rnf122	Car10	Gm2115	Irx6	Cd6
Gpr17	Neu2	C130074G19Rik	Barhl2	Adora2a
Tnc	Adam33	Ighm		Rpe65
Adora2a	Ighg2c	Fmo2		Gsg1l
Rbm3	Cd6	Lcn2		Fbn2
Top2a	Adora2a	Car10		Hspa12a
Gm44677	Rpe65	Neu2		Sln
Pisd-ps1	Gsg1l	Adam33		Peak1
Krt2	Stard8	Ighg2c		Ryr3
Gpx8	Cd4	AC149090.1		Slc9a2
Syndig1l	Ptprv	Rnf122		Man1a
Nde1	Mapk4	Gpr17		Ppp4r4
Casp6	Ngef	Tnc		Trpc4
Gm26906	Drd1	Cd6		Chrna5
Mir5125		Rbm3		Nrp1
Tcn2		Top2a		Cacnb2
Gm27627		Rpe65		Thsd4
Hspb1		Gsg1l		Pcsk9
Hist1h1c		Gm27646		Raver2
Malat1		Hnrnpa0		Kit
Ntn5		Krt9		Slc44a5
Gm15852		Spag6		Pcdh19
Hist1h2bc		Tnnt2		Slco2a1
Lrrc51		P4ha1		T2
Hist1h2be		Arhgef26		Pde11a
Ucp2		Mir762		Gpr161
Cdc25b		Loxl1		Ndst4
Gm37376		Krt12		A830012C17Rik
Cat		Nos1		
Snhg18		Agmat		
Ccdc190		Gm26995		
Alox12b		Emp2		
Dnaic1		Prss16		
Plce1		Cirbp		
Spaca6		Mei1		
Sec1		Ciart		
Sfswap		4930524O07Rik		
Gm3764		Epop		
Ptpro		Tcf19		
Slc26a8		Pde8b		
Gm973		Parvb		
Gpr149		E2f1		
Prelp		Lamb1		
Gm15851		Gm13375		
Muc3a		Zbtb46		
Plppr1		Aqp4		
Ak3		1810041L15Rik		
Lrrc2		Cd38		
		Pla2g4e		
		Casp1		
		Mylk3		

[8] Every instance of a change in *Nlgn3* expression is indicated with a blue background and of *Cyfip1* with a pink background. Genes, which expression is changed in more than one condition is indicated with a yellow background. Genes written in red were downregulated.

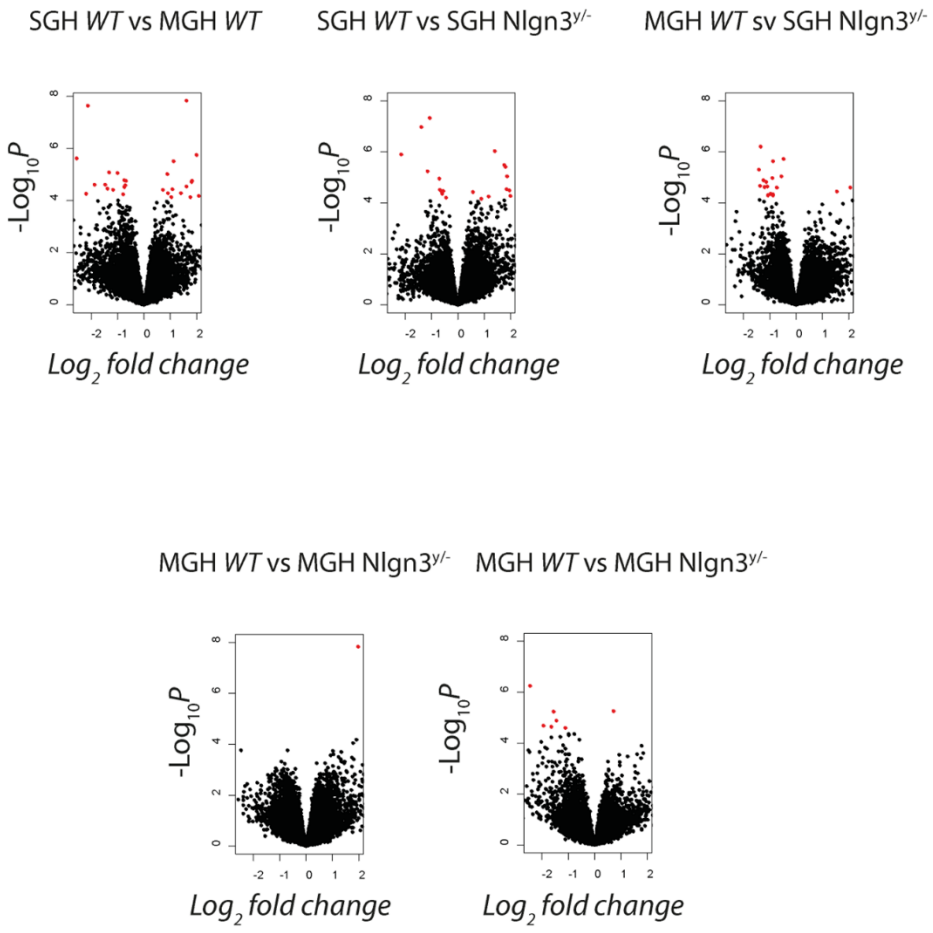
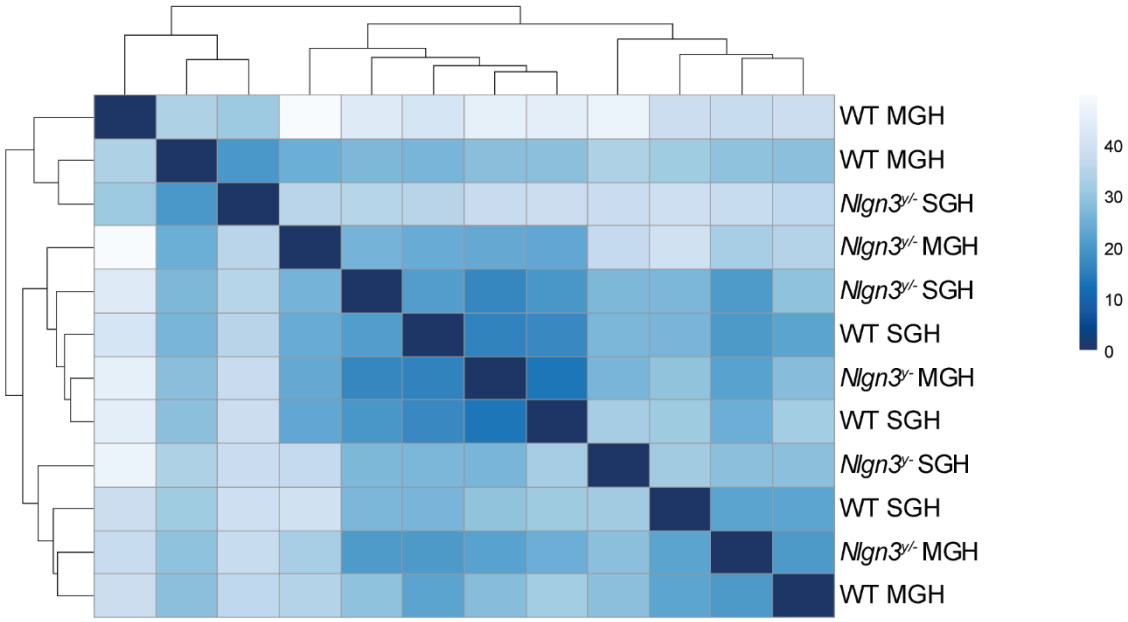


Figure 33 **Social environment affected the differential expression of genes in *WT* and *Nlgn3$^{y/-}$* mice.** Differentially expressed genes between pairs of different housing or genotype conditions. The most pronounced differences were between SGH and MGH males. Some points might be missing due to axis formatting.

However, following hierarchical clustering, there was no clear separation based on housing (Figure 34 A – B). A degree of separation was present following Principal Component Analysis, where SGH *WT* and MGH *WT* samples occupied partially non-overlapping space (Figure 35 A- B). The input for WGCNA was 19906 genes following thresholding and normalisation. As previously, the minimum module size was set to 200 genes, resulting in a network containing 20 modules (Figure 36). In SGH *WT* animals, modules 9 and 10 were significantly upregulated (R^2 = 0.5, FDR q = 0.09), and modules 14 (R^2 = -0.53, FDR q = 0.08), 16 (R^2 = -0.61, FDR q = 0.04), and 18 (R^2 = 0.68, FDR q = 0.02) were significantly downregulated (Figure 36). In SGH *Nlgn3*$^{y/-}$ males, module 14 (R^2 = 0.60, FDR q = 0.04) was significantly upregulated and module 2 was significantly downregulated (R^2 = -0.75, FDR q = 0.005). In contrast, in the MGH *WT* animals, modules 18 and 20 were significantly upregulated (R^2 = 0.55, FDR q = 0.06; R^2 = 0.71, FDR q = 0.009 respectively), and in the MGH *Nlgn3*$^{y/-}$ module 1 was upregulated (R^2 = 0.51, FDR q = 0.09). Different modules being associated with different genotype and housing conditions suggest that both might influence the transcriptome profiles in these mice. Next, we conducted a GO terms analysis for the SGH *WT* males, as for the previous dataset. We found that module 18 contained genes primarily responsible for cell cycle processes and chromatin regulation (Figure 37 A). Meanwhile, module 16 contained genes associated with RNA regulation (Figure 37 B).

A



B

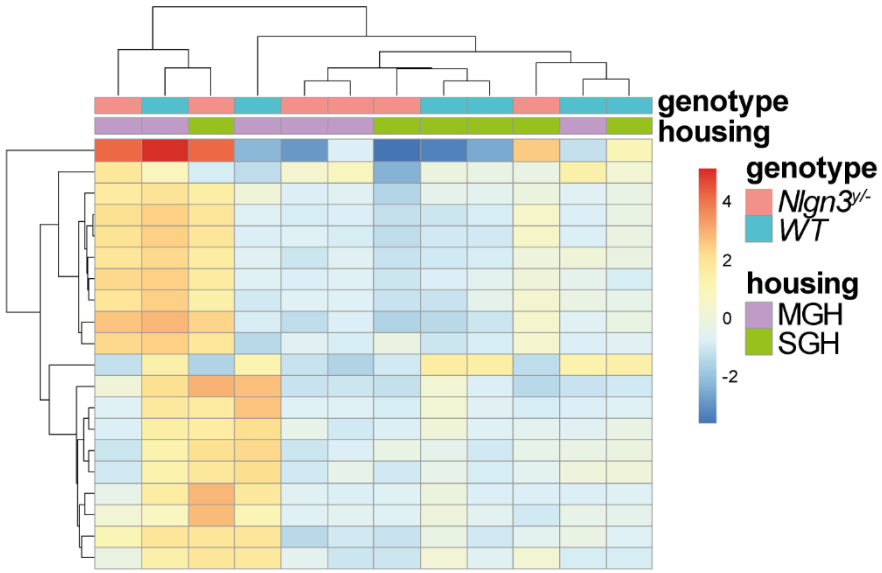


Figure 34 **There was no clear separation based on hierarchical clustering of genes expressed in the hippocampus. A** Hierarchical clustering of all genes following thresholding and normalisation procedures. No clear separation based on genotype or housing conditions was observed. **B** Hierarchical clustering of top 20 genes with the largest fold change. Some separation based on genotype or housing conditions was observed.

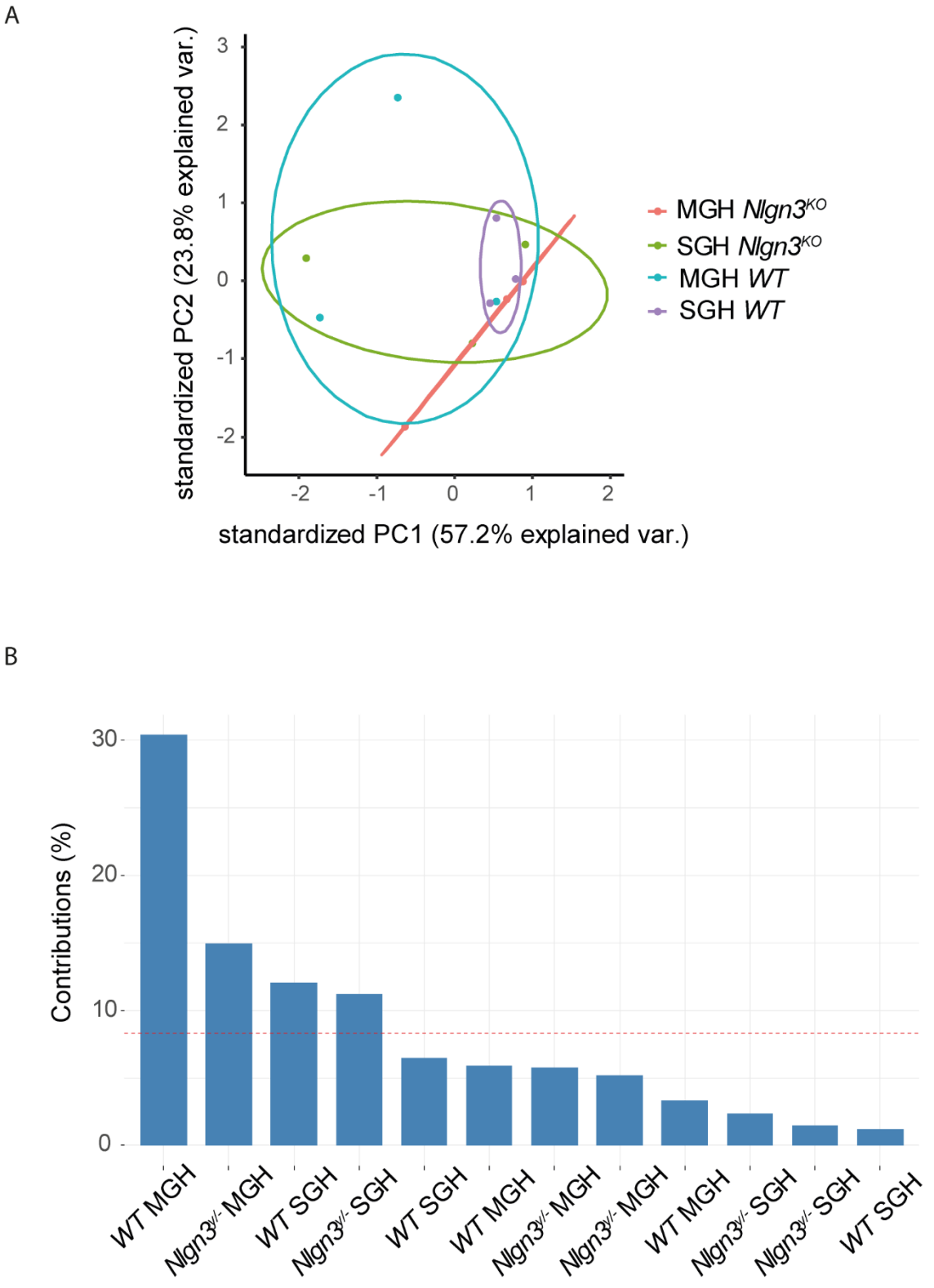


Figure 35 **There was no separation based on genotype and housing conditions following the Principal Component Analysis of the genes expressed in the hippocampus. A** Position of the different samples in the space made up from Principal Component 1 and Principal Component 2, based on the top 100 genes with the greatest fold change. The space occupied by the different groups of mice was partially over-lapping. **B** Percentage contribution of individual samples to the Principal Component 1 and 2.

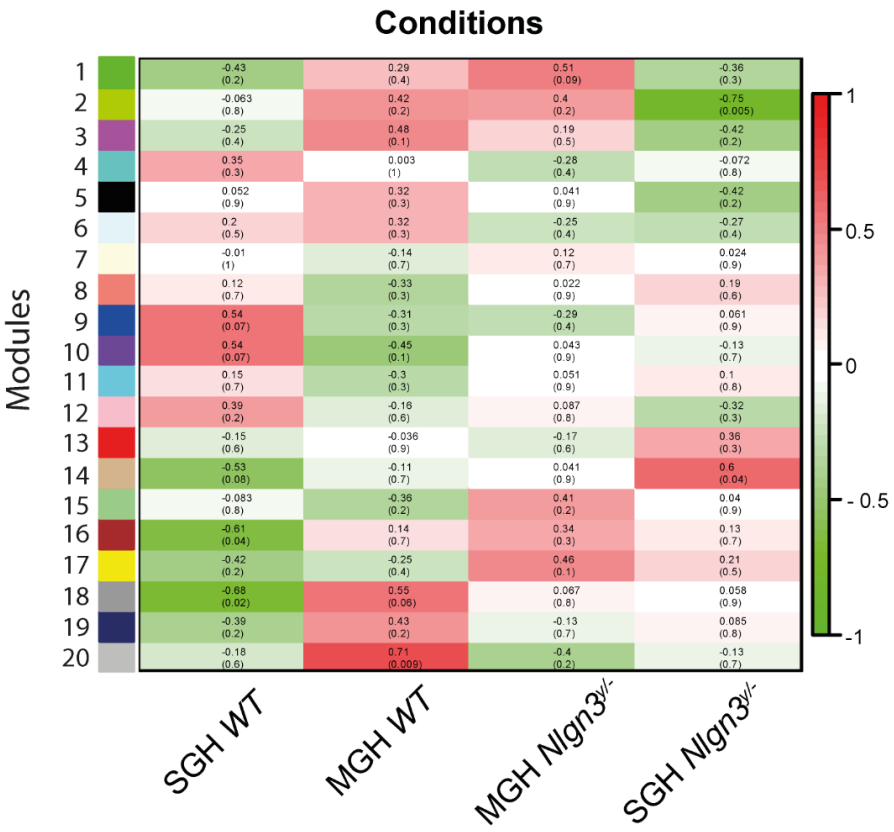
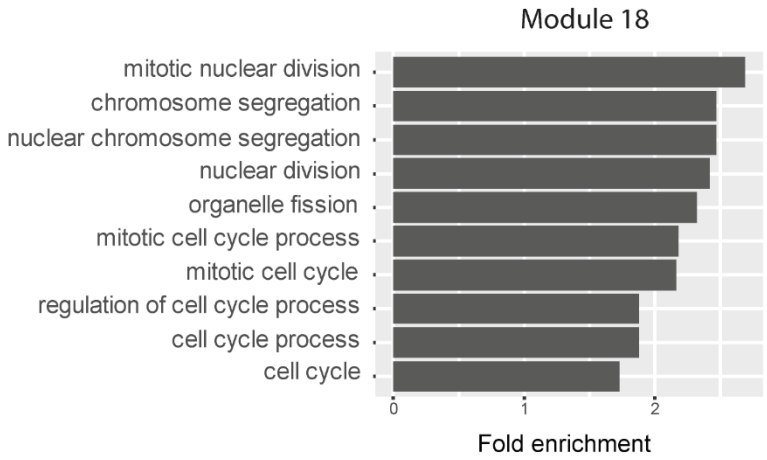


Figure 36 **Different modules of correlated genes expressed in the hippocampus were associated with different genotype and housing conditions.** The corrections between different genotype and housing conditions and the modules identified through WGCNA. Red indicated positive correlation between the expression of the genes in the module and the given housing/genotype combination and green signified the negative correlation between a module and a trait. The value of the correlation was given in the top of each segment while the False Discovery Rate adjusted q-values was given below.

A



B

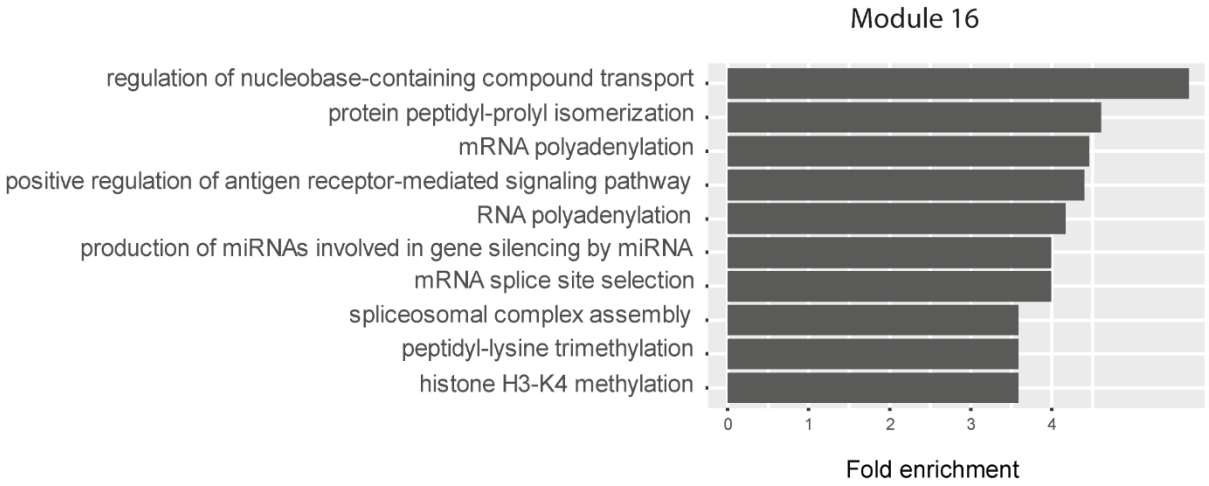


Figure 37 **Single genotype housing of *WT* males was associated with changes in genes responsible for cell cycle processes and RNA regulation. A** Top ten GO terms for the genes in module 18. **B** Top ten GO terms for the genes in module 16.

6.4. Discussion

We have found differentially expressed genes between males with and without *Nlgn3* as well as coming from different housing conditions, suggesting that both *Nlgn3* deletion and social housing might affect RNA expression in the hippocampus. The differences were most pronounced between SGH and MGH males, indicating that the effect of the social environment could extend beyond behaviour into molecular events or that the changes in behaviour due to social environment resulted in altered transcription. The effect of housing was evident both in the *WT* animals as well as, to a smaller extent, in the *Nlgn3* knockout animals. Network analysis showed that different modules of correlated genes were associated with a lack of *Nlgn3* and single genotype housing. The modules associated with single genotype housing contained genes involved in the development of the CNS, synaptic transmission, metabolic processes, chromatin regulation, and RNA regulation.

Some differences were observed in RNA expression between mice with and without *Nlgn3* deletion. However, the changes in males heterozygous for *Cyfip1* were negligible. This discrepancy might arise from the fact that while a complete deletion of a gene might lead to some transcriptional changes, merely reducing the level of CYFIP1 was not sufficient to impact on transcriptional regulation. Additionally, there is a possibility that a form of compensation occurred in the males heterozygous for *Cyfip1.* Transcriptional changes associated with *Nlgn3* deletion have not previously been reported and therefore the mechanism leading to these changes is currently unclear. Neuroligin3 plays a role in synaptic transmission as detailed in Chapter 1, which can then affect RNA expression (Yap and Greenberg 2018). Thus, deletion of *Nlgn3* might result in an altered transcription profile through this route. One gene which was consistently downregulated in the samples lacking *Nlgn3* was *Shox2,* a homeobox gene containing a DNA binding domain. These genes are transcriptional regulators involved in development (Rappold *et al.* 2012). Interestingly, a number of genetic disorders is caused by mutations in the homeobox genes. These samples also expressed less *SYNPO2*, which has the ability to bind actin and induce actin polymerisation. This seems to be in line with the observation that Neuroligin3 interacts with CYFIP1, which regulates actin dynamics. SYNPO2 has also been implicated in autophagy, a process that might be dysregulated in ASD (Dana *et al.* 2020). *Nlgn3* deletion was also associated with a decrease in *Kcnj10* expression, a potassium channel characteristic of glial cells. Mutations in this gene have been linked with epilepsy, which is in line with the dysregulation of synaptic transmission associated with *Nlgn3* deletion (Reichold *et al.* 2010). It is important to note that the number of differentially expressed genes, especially in the first cohort, was not large. This might be due to depth of sequencing chosen or due to the heterogeneity of the starting material. The depth of sequencing is defined as a ratio of the number of bases obtained and the size of target genome

(Jiang *et al.* 2019). It impacts on a number of parameters including the completeness of the genome assembly as well as the number or level of expressed genes. This interpretation was supported by similar number of differentially expressed genes across the two cohorts, which have used material originating from the same brain region and the same depth of sequencing.

The greatest changes in RNA expression were observed between MGH and SGH *WT* animals, which were confirmed through differential gene expression analysis, PCA, and WGCNA. These findings were also replicated in two cohorts of animals. The first cohort of SGH males originated from a different breeding line, suggesting that there might have been unaccounted for biological differences between those males and the mutant males, resulting in differential RNA expression. However, the results were replicated in the second cohort of animals, where SGH and MGH *WT* animals had the same parents. One advantage of using SGH and MGH *WT* animals that share parents is accounting for any possible mothering effects. Thus, the effect of the social environment on RNA expression appears to be independent of the influence of the parents.

The effect of social environment on behaviour of male mice was previously reported in Kalbassi *et al.* (2017), as well as described in Chapter 4. Here, we demonstrated this influence extended beyond behaviour, to the molecular events associated with transcription regulation. Replication of this effect in a cohort of MGH animals raised with littermates lacking *Nlgn3*, as well as in males with littermates lacking *Nlgn3,* or heterozygous for *Cyfip1*, or both, suggested that this effect was not limited to *Nlgn3*. Interestingly, the social environment seemed to also impact on the transcriptome of mice lacking *Nlgn3* as well as their *WT* littermates. This finding mimicked the behavioural results, where the behaviour of mice lacking *Nlgn3* was more similar to their MGH *WT* littermates, rather than SGH males lacking *Nlgn3* (Kalbassi *et al.* 2017). These results suggested that housing mice together led to a form of homogenisation of behaviour, that might also affect molecular events such as RNA expression. According to this idea, when mice with different levels of sociability, due to their genetic make-up, are placed in a common social context, a homogenisation of behaviour occurs until a common level of sociability is established. In line with this idea, the social systems are highly plastic and susceptible to small changes. These changes in behaviour might, in turn, be accompanied by changes in transcriptional regulation.

In this chapter WGCNA was used to investigate the correlations between different genes, construct a network of modules of correlated genes and investigate the relationship of these modules to expression of *Nlgn3*, *Cyfip1* and social environment conditions. However, this analysis comes with certain caveats. Correlations between genes usually reflect functional relationships, however this is not always true, as correlation can be a result of technical

artefacts. Sometimes the correlations might also reflect structural properties of the sample rather than functional properties of the genes. For example, genes expressed in inhibitory neurons might cluster together. Additionally, WGCNA works best with a larger number of samples. Here 15 samples were utilised in the first data set and 12 in the second, thus the results should be interpreted with caution.

As the effect of the social environment on the transcriptome was investigated in two cohorts of animals, it led to the construction of two networks of correlated genes. While there were gene modules associated with SGH *WT* animals in both cases, the identity of the genes making up the modules differed between the two cohorts. While in the first cohort the SGH *WT* was associated with synaptic transmission and metabolic processes, in the second one, the same trait was associated with cell cycle processes and RNA regulation. These differences might arise from the fact that the two cohorts of animals originated from different breeding lines, introducing unaccounted for biological factors which increased the variability. Furthermore, the adaptation of the behaviour to a given social environment might require an array of subtle changes that might differ depending on the precise circumstance. As RNA sequencing allows only for a snapshot of RNA expression in a particular moment, it might not illustrate well the variety of different subtle changes in response to the social environment.

6.5. Conclusions

RNA expression profiles differ between individuals with ASD and controls. We investigated the impact of the *Nlgn3/Cyfip1* interaction on the transcriptome in the mouse hippocampus, while accounting for the effect of the social environment on the *WT* controls as well as males lacking *Nlgn3.* Although the RNA expression varied between the males with and without *Nlgn3, Cyfip1* haploinsufficiency did not affect the transcriptome. The social environment was found to impact on the RNA expression both in *WT* mice, in two separate cohorts, and to a smaller degree, in males lacking *Nlgn3.* However, the precise nature of changes in RNA expression evoked by different social environments remains elusive.

Chapter 7: General discussion

7.1. Summary of results

ASD affects 1% of the population, however the understanding of the biology underlaying these conditions is still limited. The aetiology of ASD is complex, with many candidate genes involved. While there might be convergence in the biological pathways these genes impact on, the mechanistic understanding of them is lacking. In this thesis we considered the combined effect of two genes associated with ASD: *Nlgn3* and *Cyfip1.*

The impact of the Neuroligin3/CYFIP1 interaction on a range of phenotypes associated with ASD was evaluated. Initially, the interactome of Neuroligin3 in neurons, glial cells and heterogenous brain samples was investigated using co-immunoprecipitation, followed by either mass-spectrometry or Western Blot. The interaction partners of Neuroligin3 largely differed between neuronal and glial cell populations. Among the identified interactors of Neuroligin3 both in neurons and in the heterogeneous cellular population originating from the brain was CYFIP1. Neuroligin3 also interacted with two known interactors of CYFIP1: FMRP and WAVE1. In addition, Neuroligin3 was found to interact with several other proteins originating from genes associated with ASD.

The impact of the interaction of Neuroligin3 and CYFIP1 on mouse behaviour, dendritic spine density in the cortex, and the hippocampal transcriptome was then investigated (Table 17). Males lacking *Nlgn3* and males both lacking *Nlgn3* and heterozygous for *Cyfip1,* but not males only heterozygous for *Cyfip1,* were found to be hyperactive in the open field, suggesting the double mutant phenocopied the mice with *Nlgn3* deletion. The motor learning was found to be impaired only in the males heterozygous for *Cyfip1*, implicating that the deletion of *Nlgn3* in these mice restores motor learning. There was also some indication that deletion of *Nlgn3* was associated with an improved rotarod performance. There were no deficits in social behaviour in any of the males compared to their littermates. Neither deletion of *Nlgn3* nor *Cyfip1* haploinsufficiency affected the dendritic spine density in the cortex. However, the two mutations combined resulted in a possible increase in the dendritic spine density in the motor, but not the visual cortex. When the transcriptome was investigated, the deletion of *Nlgn3* had a minor effect, however, the *Cyfip1* haploinsufficiency did not produce a significant change. In line with this observation, the impact of the two mutations combined had a similar effect on the transcriptome as the deletion of *Nlgn3* only.

Table 17 Summary of results for male mice.

Assay	***Nlgn3$^{y/-}$* males**	***Cyfip1$^{+/-}$* males**	***Nlgn3$^{y/-}$Cyfip1$^{+/-}$* males**	***WT* SGH**
Behaviour				
Open field (distance)	Increased ↑	Unchanged	Increased ↑	Decreased ↓
Open field (habituation)	Absent ↓	Absent ↓	Absent ↓	Present
Open field (time in the centre)	Unchanged	Unchanged	Unchanged	Unchanged
Rotarod	Unchanged/ increased ↑	Decreased ↓	Unchanged	Unchanged
Interest in olfactory cues	Unchanged	Unchanged	Unchanged	Decreased ↓
Courtship	Unchanged	Unchanged	Unchanged	Unchanged
Density of dendritic spines				
Motor cortex	Unchanged	Unchanged/ Decreased ↓	Unchanged/ Increased ↑	Unknown
Visual cortex	Unchanged	Unchanged	Unchanged	Unknown
RNA expression	3 downregulated ↓and 2 upregulated ↑	2 downregulated genes ↓	3 downregulated genes ↓	15 upregulated ↑ and 3 downregulated genes ↓

Sex and social environment modulated the effect of *Nlgn3* and *Cyfip1* on the phenotypes associated with ASD. In contrast to the males, the females showed very few behavioural impairments (Table 18). The only notable exception was that females heterozygous for *Nlgn3* were hyperactive in the open filed in comparison to their littermates heterozygous for *Cyfip1*. Consequently, there was a sex difference in the ability to learn new motor routines, where the males heterozygous for *Cyfip1* showed an impairment, while females did not. Additionally, a group of single genotype housed *WT* was included to investigate the effect of the social environment on behaviour and the transcriptome in the hippocampus. There were subtle differences between the *WT* males housed with their mutant littermates and other *WT* males. SGH *WT* males tended to travel less in the open field compared to MGH *WT* males. They also showed less overall interest in odours. There were some differences in the transcriptome in the hippocampus between these two groups. The differentially expressed genes were associated with development, synaptic transmission, cell cycle, and RNA regulation.

Table 18 Summary of results for female mice.

Assay	***Nlgn3*$^{+/-}$ females**	***Cyfip1*$^{+/-}$ females**	***Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ females**
Behaviour			
Open field (distance)	Increased ↑	Decreased	Unchanged/increased ↑
Open field (habituation)	Unchanged	Unchanged	Unchanged
Open field (time in the centre)	Unchanged	Unchanged	Unchanged
Rotarod	Unchanged	Unchanged	Unchanged
Interest in olfactory cues	Unchanged	Decreased ↓	Unchanged
Density of dendritic spines			
Motor Cortex	Unchanged	Unchanged	Unchanged
Visual Cortex	Unchanged	Unchanged	Unchanged

Overall, Neuroligin3 interacts with several other proteins associated with ASD, including CYFIP1. The interaction between Neuroligin3 and CYFIP1 affected some of the phenotypes associated with ASD. These two proteins together had an impact on activity, motor learning, dendritic spine density, and RNA expression in mice. Sex and social environment further modulated this effect.

7.2. The relationship between Neuroligin3 and CYFIP1

Neuroligin3 was found to interact with CYFIP1, in neurons, *in vivo*. A number of proteins containing the WIRS binding domain, which can bind WRC, were previously reported to exist (Chen *et al.* 2014). These proteins were able to bind to a surface created by CYFIP1 and ABI1, two members of the WRC. Neuroligin4, another member of the neuroligin family was confirmed to bind the WRC via the WIRS domain (Chen *et al.* 2014). Neuroligin3 also contains the WIRS domain however its binding to the WRC has not been confirmed before. Here, an interaction between Neuroligin3 and CYFIP1 in neurons in the mouse striatum and cerebellum was detected. This interaction could potentially occur via the WIRS binding domain. In order to determine if this is indeed the case, it would be necessary to investigate the effect of the deletion of this binding domain on the interaction with CYFIP1 in a model system. For example, a *Nlgn3* construct with and without the WIRS binding domain could be transfected into HEK293 or COS7 cells, and the binding with either endogenous or transfected CYFIP1 could be investigated using co-immunoprecipitation and Western Blot. The cytoplasmic tail of Neuroligin3 also contains two other known binding domains: a PDZ-binding domain (Irie *et al.* 1997), and a gephyrin-binding domain (Poulopoulos *et al.* 2009). Ideally, control conditions in which Neuroligin3 lacks only the PDZ-binding domain or the gephyrin-binding domain should be included to account for the possibility that the interaction with CYFIP1 occurs via one of these binding domains or is indirect. It would also be interesting to verify if the interaction between Neuroligin3 and CYFIP1 is direct or indirect using Förster Resonance Energy Transfer (FRET) assay. However, this assay relies on antibodies allowing for effective visualisation of both Neuroligin3 and CYFIP1, which are currently not available. Provided that the binding between Neruroligin3 and CYFIP1 is found to depend on WIRS domain, the consequences of this interaction could be investigated in neurons. Neurons could be derived from human inducted pluripotent stem cells (iPSC) or mouse embryonic stem cells (ESC), corresponding to the two mammalian systems ASD is usually considered in. However, this would involve the use of gene editing techniques such as clustered regularly interspaced short palindromic repeats (CRISPR) Cas9 or zinc finger nuclease to create a new cell line, in which Neuroligin3 lacks the WIRS binding domain. In neurons, the effect of Neuroligin3 binding CYFIP1 could be investigated on the cellular processes such as protein translation or actin polymerisation.

The interaction between Neurolgin3 and CYFIP1 was found to affect mouse behaviour, dendritic spine density, and RNA expression (Figure 38 A). Findings from these experiments could form a base for speculation about the functional relationship between these two proteins. Males without *Nlgn3* were shown to be hyperactive in the open field, to acquire motor routines at a similar or perhaps higher rate as their *WT* littermates, while the dendritic spine density was not altered in this model. On the other hand, males heterozygous for *Cyfip1* showed no changes in their levels of activity or dendritic spine density, but a deficit in motor learning. The double mutant mice which both lacked *Nlgn3* and were heterozygous for *Cyfip1* were characterised by an enhanced activity in the open field and possibly increased dendritic spine density in the motor cortex, but *WT*-level motor learning ability. There are three possibilities that can explain these results fully or at least partially.

The first possibility is that Neuroligin3 inhibits CYFIP1 (Figure 38 B). As a result, *Nlgn3* deletion would be accompanied by an increased availability of CYFIP1, as Neuroligin3 would not be present to bind some of the CYFIP1. This increase in the available CYFIP1 could then lead to the hyperactivity observed in the open field. This hypothesis is, however, contradicted by a study in which human *CYFIP1* was overexpressed in mice that did not show hyperactivity (Fricano-kugler *et al.* 2019). According to this model, in mice heterozygous for *Cyfip1*, Neuroligin3 would still be available to bind most of the already depleted pool of CYFIP1. The decrease in available CYFIP1 could then result in motor learning impairment that could be associated with a decrease in dendritic spine density in the motor cortex that was previously observed in these mice (Bachmann *et al.* 2019). However, it is important to note that we were unable to replicate this decrease here. Finally, in the double mutant mice, due to the deletion in *Nlgn3*, there would be no Neuroligin3 inhibiting the function of the remaining CYFIP1. Even though the levels of CYFIP1 are reduced, the deletion of Neuroligin3 might leave enough CYFIP1 available to restore motor learning. Interestingly, the double mutation is associated with hyperactivity and an increase in dendritic spine density which are not easily explained. Overall, an inhibitory relationship between Neuroligin3 and CYFIP1 would explain the effect on motor learning, although it would not account for the hyperactivity.

The second possibility is that Neurolgin3 and CYFIP1 form a complex, which then affects certain phenotypes (Figure 38 C). According to this model, mice lacking *Nlgn3* would be unable to form the Neurolgin3/CYFIP1 complex, leading to hyperactivity. In mice heterozygous for *Cyfip1* the complex could still be formed, leading to a *WT*-level of activity. However, the reduction in CYFIP1 still seems to result in a deficit in motor learning. On the other hand, in the double mutant mice, the level of CYFIP1 would be reduced and the complex between Neuroligin3 and CYFIP1 would not be formed. Similar to the *Nlgn3* deletion, the lack

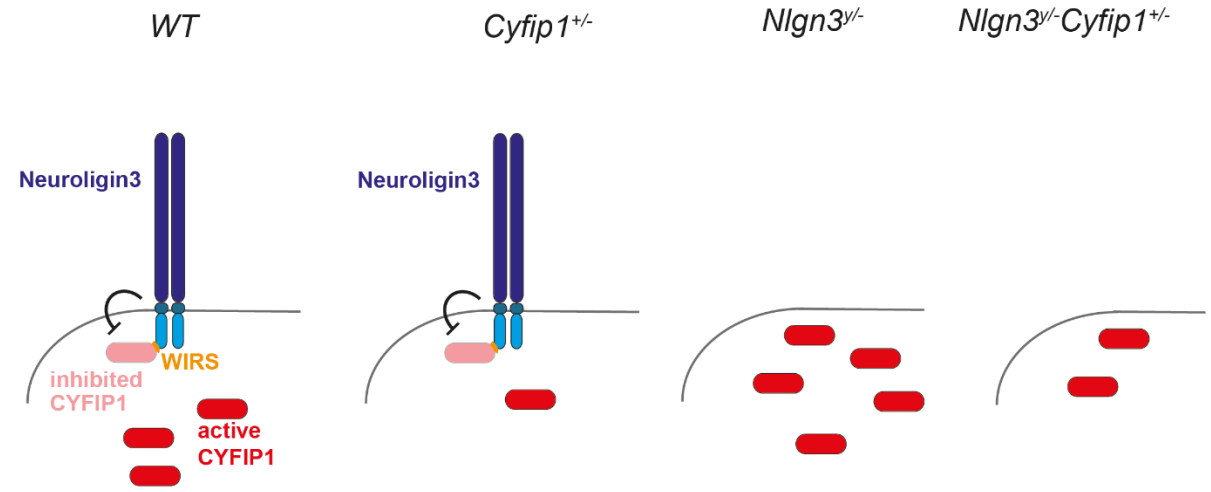
of Neuroligin3/CYFIP1 complex might lead to hyperactivity. However, the motor learning in this model is restored to *WT*-level, which is difficult to account for, considering that the levels of CYFIP1 would be reduced just like in the mice heterozygous for *Cyfip1.* Additionally, this model does not explain why the increase in dendritic spine density is present only in the double mutants. Overall, this option accounts reasonably well for the hyperactivity seen in some of these models but not for phenotypes associated with motor learning and dendritic spine density.

Finally, there is a possibility that the effects of Neuroligin3 and CYFIP1 are exerted through separate downstream pathways and the interaction between them is inconsequential (Figure 38 D). In this case, the deletion of *Nlgn3* would be associated with hyperactivity and *Cyfip1* haploinsufficiency would be associated with a motor deficit and potentially a decrease in dendritic spine density. While this model explains well the hyperactivity present in the double mutants it does not account for the restoration of motor learning or the increase in dendritic spine density in the motor cortex in this model.

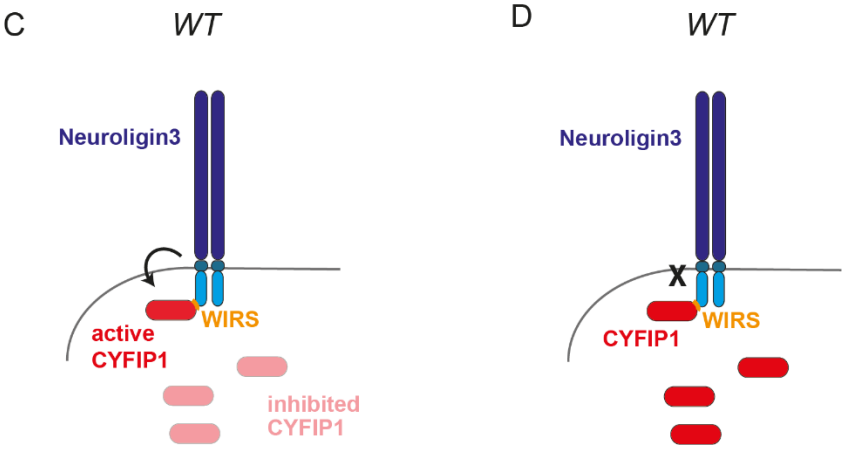
A

	Cyfip1$^{+/-}$	*Nlgn3*$^{y/-}$	*Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$
Activity	Unaffected	Increased	Increased
Motor learning	Deficit	Unaffected	Unaffected
Spine density	Unaffected	Unaffected	Increased

B



C



D

Figure 38 Neuroligin3 is likely to inhibit CYFIP1. A Summary of the impact of *Nlgn3* and *Cyfip1* on behaviour and dendritic spine density in the cortex. **B** Neuroligin3 might inhibit CYFIP1 in *WT* mice. In mice heterozygous for *Cyfip1*, Neuroligin3 might bind most of the available CYFIP1. In mice with *Nlgn3* deletion, a lack of inhibition from Neuroligin3 might increase the availability of CYFIP1. In the double mutant the pool of CYFIP1 could reduced as a result of *Cyfip1* haploinsufficiency but at the same time the absence of Neuroligin3 could increase the available CYFIP1. **C** Another possibility is that Neuroligin3 and CYFIP1 form a complex in the *WT* animals, and that the behavioural outcomes depend on its formation. **D** The interaction between Neuroligin3 and CYFIP1 might also have no functional consequences on the behaviour and dendritic spine density.

Overall, it seems that there is a functional relationship between Neuroligin3 and CYFIP1 that regulates phenotypes associated with ASD. There is a possibility that Neuroligin3 and CYFIP1 impact on certain phenotypes individually and the interaction between them is only relevant for some processes. For example, Neuroligin3 might regulate exploratory behaviour, and when it comes in contact with CYFIP1, it might also reflect on motor learning. It is also important to consider the possibility that the effect of the interaction between Neuroligin3 and CYFIP1 might be specific to different cellular populations. This idea is supported by the observation that the interactors of Neuroligin3 varied depending on the cellular population considered, as described in Chapter 3. As neurons forming different circuits affect different behaviours, the variation in molecular interactions depending on the neuronal population might impact on behaviour as well. However, there are inherent limitations to trying to infer the nature of molecular interactions between proteins by observing their effects on behaviour. Ideally, a mechanism by which these proteins exert their effect on behaviour should be investigated. Similarly, the molecular interactions might impact on the dendritic spine density.

One possibility is that the effect on behaviour and dendritic spine density resulting from the deletion of *Nlgn3* or *Cyfip1* haploinsufficiency occurs via regulation of actin polymerisation and protein translation. CYFIP1 regulates protein translation via its interaction with FMRP and actin polymerisation via its participation in the WAVE regulatory complex, as described in Chapter 1. Interestingly, Neuroligin3 might also affect these two processes, likely via its interaction with CYFIP1. In *Drosophila,* there are three orthologs of mammalian neuroligins, dNlg1, dNlg2, and dNlg3, which can bind an ortholog of neurexins (Banovic *et al.* 2010; Biswas *et al.* 2010; Sun *et al.* 2011). A reduction in the levels of these proteins was associated with a reduction in the amount of polymerised actin. This was not observed when the WIRS binding domain was deleted. Only the construct containing this domain was able to rescue the deficits associated with dNlg1 reduction. Thus, Neuroligin3 might affect actin polymerisation via its interaction with CYFIP1. However, to verify this possibility, the levels of polymerised actin would need to be verified in a model system of *Nlgn3* deletion. This experiment could be conducted either in HEK293 or COS7 cells transfected with a full-length *Nlgn3* construct or a construct lacking the different binding domains or in neurons with a *Nlgn3* deletion. The levels of polymerised actin could then be verified by staining with phalloidin, a marker for polymerised actin. Ideally the experiment could be extended to include neurons from primary cell culture or arising from iPSC or ESC differentiation and the analysis of dendritic spine density and morphology *in vitro* should be conducted.

The possible impact of Neuroligin3 on protein translation is less well investigated. An inhibitor of protein translation, FMRP, was shown to interact with both Neuroligin3 and CYFIP1 as described in Chapters 1 and 3. Additionally, a recent study demonstrated that FMRP regulates the levels of neuroligins, including Neuroligin3 at the synapse (Chmielewska *et al.* 2018). In mice lacking *Fmr1*, *Nlgn3* mRNA was increased and so was the incorporation of Neuroligin3 into the membrane. Thus, there is evidence for a regulation of Neuroligin3 by FMRP. However, it is unclear if Neuroligin3 can, in turn, modulate the function of FMRP, for example through its interaction with CYFIP1, a FMRP inhibitor (De Rubeis *et al.* 2014). This possibility could be verified by evaluating the bulk protein translation levels *in vitro* in cells lacking *Nlgn3* or transfected with *Nlgn3* without key binding domains, using puromycin incorporation or a similar method. Puromycin is an analogue of tRNAs and as such can be incorporated into newly synthesised amino acid chains (Goodman and Hornberger 2015). Following this with labelling using a fluorescent tag or an antibody would provide a measure of bulk protein translation. This analysis could also be done *in vivo*, being of particular interest for the study of mice with *Nlgn3* deletion.

7.3. Sex differences

There were sex differences in the way *Nlgn3* deletion and *Cyfip1* haploinsufficiency affected phenotypes associated with ASD. Specifically, a difference was observed in motor learning and the dendritic spine density in the motor cortex. In both cases a deficit was observed in males, while females remained unaffected. It is important to note that only the *WT* females and those heterozygous for *Cyfip1* were considered in this comparison. It was not possible to compare females heterozygous for *Nlgn3* with males with a complete deletion of *Nlgn3*, as they had a different gene dosage.

Female mice have historically been rarely included in studies of mouse models of ASD. However, *Nlgn3* deletion is one of the models where sex differences were investigated (Kalbassi *et al.* 2017). While females with *Nlgn3* deletion phenocopied the males with *Nlgn3* deletion, females heterozygous for *Nlgn3* were found to phenocopy their *WT* littermates. As *Nlgn3* is an X-linked gene, *WT* females have two alleles of the gene available while *WT* males have only one. Therefore, when one of the alleles is deleted in the females, the remaining allele might be sufficient to result in *WT*-level behaviour as observed in Kalbassi *et al.* (2017). The males on the other hand showed a deficit upon losing their only allele of the gene. While it was not possible to compare females heterozygous for *Nlgn3* with males with *Nlgn3* deletion directly, it is interesting to consider the comparison made between different groups of females. Females heterozygous for *Nlgn3* showed no deficit in their behaviour in relation to their *WT* littermates, in line with the previous study (Kalbassi *et al.* 2017). This picture could potentially

be complicated by X-inactivation. Thus, in females heterozygous for *Nlgn3,* around 50% percent of the cells would express the *Nlgn3,* at random. In some females there might therefore be more *Nlgn3* positive cells in certain brain regions, than in others. This is further complicated by the fact that that the X-inactivation in girls with ASD is skewed towards inactivating the X chromosome with genetic mutations, as well as certain genes escaping X-inactivation altogether (Talebizadeh *et al.* 2005). Therefore, a possibility exists that in females heterozygous for *Nlgn3,* majority of the cells expresses the X chromosome with functional *Nlgn3.*

However, females heterozygous for *Nlgn3* were hyperactive in relation to females heterozygous for *Cyfip1.* This finding indicates that there might be some subtle changes in the behaviour of the females heterozygous for *Nlgn3,* or those heterozygous for *Cyfip1,* or both. One possible explanation for subtle behavioural changes in the *Nlgn3* heterozygous females could be cellular interference. This is a phenomenon arising as a result of X-inactivation, where *WT* and mutant cells fail to cooperate, resulting in a deficit. This phenomenon was demonstrated for Neuroligin1, where the dendritic density in the cortex depended on the level of Neuroligin1 present in the neighbouring neurons (Kwon *et al.* 2012). Similarly, in mice heterozygous for *Nlgn3,* X-inactivation could lead to cellular interference between *WT* cells and those lacking *Nlgn3*, leading to subtle behavioural deficits. However, it is important note that X-inactivation in these animals might not be random as discussed above. This explanation also would not account for a possible deficit in the females heterozygous for *Cyfip1.*

While many of the genes associated with ASD are X-linked, including *Nlgn3, Cyfip1* is not. Therefore, location on the X chromosome alone cannot explain the sex differences observed in the behaviour and dendritic spine density in mice heterozygous for *Cyfip1.* On the other hand, as described in Chapter 3, CYFIP1 interacts with FMPR and Neuroligin3, which are both located on the X chromosome. Thus, these interactions might have a different effect on males and females, although the precise mechanism remains unknown. The sex differences in ASD are often explained in the context of the female protective effect. According to this theory, females require a greater genetic load for the phenotypes associated with ASD to manifest. This theory is supported by a greater number of mutations present in female individuals diagnosed with ASD (Jacquemont *et al.* 2014; Zhang *et al.* 2020). However, it is unlikely for a single genetic locus located on the X-chromosome to accounts for this effect (Gockley *et al.* 2015). There might be other biological factors that distinguish males from females, such as oestrogen and testosterone levels that might account for the female protective effect (Ferri *et al.* 2019). The alternative explanation is that ASD presents differently in females and thus is underdiagnosed, for which there is also support (Hull 2020).

The sex difference in dendritic spine density could be explained in terms of hormonal differences. It has previously been reported that dendritic spine density in the hippocampus was increased in females in proestrus, when oestrogen levels are high (Shors *et al.* 2001). These females were shown to have an increased number of dendritic spines in comparison to males. However, it is important to note that in the experiment described in Chapter 5, the females were randomly selected, regardless of the stage of oestrus they were in. Thus, the levels of oestrogen should be randomly distributed throughout the sample. The alternative explanation could be that the levels of testosterone in males might have affected the results, however little is known about the correlation between spine density and the fluctuations in testosterone levels in males.

A further explanation is that spine density is affected by the social environment. The social environment was shown to affect male and female mice differently (Kalbassi *et al.* 2017). Specifically, while the behaviour of *WT* males was affected by being housed with their littermates lacking *Nlgn3,* there were no differences in behaviour between *WT* females housed with *WT* littermates or littermates lacking *Nlgn3.* This suggests that males might be more susceptible to the effects of social environment, which might extend to dendritic spine regulation. Social environment as a modulating variable is further discussed in Section 7.4.

7.4. The impact of social environment

Social environment was found to impact the behaviour and RNA expression of *WT* mice. *WT* littermates that were housed with their mutant littermates were used as a control throughout (mixed genotype housing, MGH). However, unexpectedly, low interest in social odours, a small number and short duration of ultrasonic vocalisation during courtship and low density of dendritic spines in the cortex were observed in these *WT* males. As a result, a control group of *WT* males that have only ever been housed with their *WT* littermates was included (single genotype housing, SGH). The MGH males were found to be hyperactive in the open field and more interested in odours than the SGH males. There were also differences in RNA expression between these two groups.

Social environment has been shown to affect both behaviour and certain aspects of the physiology of mice as discussed in Chapter 1. Here, the behaviour of *WT* littermates was found to be affected by being housed with their mutant littermates. Being housed with males lacking *Nlgn3* was previously found to affect the behaviour of *WT* littermates (Kalbassi *et al.* 2017). In this instance, the MGH *WT* males were housed not only with mice lacking *Nlgn3,* but also with mice heterozygous for *Cyfip1* or carrying both mutations. Interestingly, the effect on behaviour seemed to extend to both the double mutants and the mice heterozygous for *Cyfip1*.

It is important to note that the pattern of results did not correspond perfectly to the findings in Kalbassi *et al.* (2017). In the current study, the deficits observed were much more subtle. This could be because the presence of males heterozygous for *Cyfip1* affected the behaviour differently than those with *Nlgn3* deletion or that while deletion of *Nlgn3* affected the behaviour of the littermates, *Cyfip1* haploinsufficiency did not. To distinguish between the two possibilities, the behaviour of *WT* littermates housed exclusively with males lacking *Nlgn3* should be compared to those housed with males heterozygous for *Cyfip1.* It would be ideal to use males entirely lacking *Cyfip1* in this experiment. While ubiquitous deletion of *Cyfip1* is embryonic lethal, a conditional knockout of *Cyfip1* has recently become available. A promising area to delete *Cyfip1* in could be the prefrontal cortex, as it has been shown to be associated with social hierarchy (Stagkourakis *et al.* 2018; Wang *et al.* 2011; Zhou *et al.* 2017). There is also an intriguing possibility that the mice with *Nlgn3* deletion might impact on the behaviour of the mice heterozygous for *Cyfip1* or vice versa. This unfortunately was outside the scope of this thesis due to large number of mice required.

The impact of social housing on the behaviour of *WT* littermates could be explained in two ways. Firstly, lack of *Nlgn3* and *Cyfip1* haploinsufficiency is linked with deficits in social behaviour. As discussed previously in Chapter 1, often only *WT* littermates can restore the behaviour or physiological processes of mutant animals and the behaviour of animals is negatively affected by destabilising social hierarchy. Thus, when *WT* littermates are forced to interact with their mutant littermates that are less sociable, it might lead to destabilizing the existing social hierarchy or the inability to form a stable one. As a result, the behaviour of the *WT* might be affected. Interestingly, the influence of being housed with mutant littermates extends to altered RNA expression. This is consistent with the literature showing the impact of social environment on the expression of certain genes as described in Chapter 1. The other possible explanation is that, the mutations in *Nlgn3,* and potentially in *Cyfip1,* affect pheromone production. Social environment was shown to affect the olfactory receptors in the VNO, olfactory epithelium and olfactory bulb of mice (van der Linden *et al.* 2018), as well as in *Drosophila* (Kent *et al.* 2008). Altered pheromones emitted by the mutant mice could be difficult to interpret by *WT* littermates. As a result, the behaviour of *WT* littermates of the mutant mice could change. In agreement with this theory, the results showed the overall difference in the interest in social odours in the *WT* mouse in the SGH and MGH housing conditions.

Differences in RNA expression between SGH and MGH *WT* animals were also found. Although the number of up- and down-regulated genes was low, this might be due to the depth of sequencing, as the effect of housing on the transcriptome was replicated in two independent cohorts. One of them came from the C57BL/6 line, ordered from an external vendor (Charles

River). The other was generated by crossing *WT* mice originating from the *Nlgn3* colony. Therefore, the MGH and SGH *WT* mice from the second cohort shared the same pool of parents, limiting the possibility that other biological factors or mothering effects led to the difference between them. The differences in RNA expression depending on housing might also be related to the formation of social hierarchy. Previous studies showed that social structure can alter the expression of certain mRNAs in the hippocampus and the expression of some of them depends on the position in the social hierarchy (Horii *et al.* 2017; Nesher *et al.* 2015; Schmidt *et al.* 2007; So *et al.* 2015). This theory could be tested by employing tube test, a behavioural assay allowing for determination of the social hierarchy within the cage prior to RNA sequencing. Additionally, the identity of the mRNAs with differential expression between MGH and SGH *WT* males should be confirmed through qPCR, which has not been achieves within this thesis due to practical concerns.

7.5. Convergence in ASD

While there are many genes associated with ASD as described in Chapter 1, the condition is diagnosed based on only two core behavioural symptoms. One theory to explain this phenomenon is that there is a convergence in the function of the genes associated with ASD. As a result, they impact on the same set of biological pathways leading to a limited set of behavioural phenotypes. Evidence for this theory had historically come from an analysis of the pre-described functions of genes linked to ASD, through gene ontology and similar analyses. Here we showed that Neuroligin3 interacts with several other proteins associated with ASD and that the interaction between Neuroligin3 and CYFIP1 affected certain traits. However, the interactions between Neuroligin3 and other proteins linked to ASD might impact on these phenotypes as well (Figure 39).

Another process associated with ASD that Neuroligin3 and its interactors might impact on is metabotropic glutamate receptor (mGluR) mediated long term depression (LTD). Group-1 mGluRs (mGluR1 and mGluR5) are located at the excitatory synapses and have been shown to influence the function of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDAR) (Connor *et al.* 2014). Some proteins associated with ASD were shown to scaffold these mGluRs and support their function. A deletion of *Nlgn3* in cerebellar slices was shown to lead to a deficit in mGluR – dependent LTD (Baudouin *et al.* 2012). This deficit could be reversed by the re-expression of *Nlgn3* in parvalbumin-expressing cells. Similarly, the expression of *Nlgn3* in a mouse with an *Nlgn1* deletion was found to affect LTD (Dang *et al.* 2018), and the R451C mutation in Neuroligin3 impacted on LTD in the striatum (Martella *et al.* 2018). In addition, Neuroligin3 was shown to interact with FMRP. Interestingly, deletion of *Fmr1* was also associated with

changes in mGluR-dependent LTD. However, while the deletion of *Nlgn3* was linked to a deficit in LTD, the deletion of *Fmr1* was shown to enhance LTD (Barnes *et al.* 2012; Nosyreva *et al.* 2006; Hou *et al.* 2006; Huber *et al.* 2000). A possible explanation for these effects could be the role of FMRP, a protein synthesis inhibitor, and its interactor Neuroligin3 in the protein synthesis required for LTD. However, the effect on LTD was found to be independent of protein synthesis (Barnes *et al.* 2012; Nosyreva *et al.* 2006; Hou *et al.* 2006; Huber *et al.* 2000). Instead the enhancement of LTD was attributed to increased internalisation of GluR1 in this model (Nakamoto *et al.* 2007). Interestingly, mice heterozygous for *Cyfip1* also showed an enhanced LTD, that is independent of protein synthesis (Bozdagi *et al.* 2012). Thus, while deletion of *Nlgn3* seems to result in an absence of LTD, the LTD was more pronounced as a result of *Fmr1* deletion and *Cyfip1* haploinsufficiency. The associated proteins might affect LTD via participation in the same pathway. However, to verify this hypothesis LTD should be investigated in double mutant mice with deletion of *Nlgn3* and heterozygous for *Cyfip1.* If the double mutation leads to a restoration of normal LTD, it might indicate that the interaction between Neuroligin3 and CYFIP1 impacts on LTD.

Neuroligin3 was also found to interact with Homer, as described in Chapter 3. Homer is thought to orchestrate the connection between phosphoinositide 3-kinase (PI3K) and mGluRs. Interestingly, Neuroligin3 and its interactor FRMP might also have the capacity to influence this pathway (Venkatesh *et al.* 2015). Deletion of *Fmr1* was found to be associated with a reduction in PI3K, which had consequences for dendritic spine density and marble burying behaviour (Gross *et al.* 2015). Therefore, Neuroligin3 might regulate the PI3K pathway via its interaction with FRMP and Homer. This pathway was shown to impact on other phenotypes associated with ASD such as dendritic spine density alterations and changes in mGluR - dependent LTD (Gross *et al.* 2010; Gross *et al.* 2015). It would be interesting to investigate the impact of this pathway further by deleting members of the pathway *in vitro* and combining it with a deletion of *Nlgn3* to check for the combined effect of these manipulations on the dendritic spine density or LTD.

The interaction between Neuroligin3 and PSD95 might also have an impact on dendritic spine morphology and LTD. PSD95 plays a role in AMPAR function and regulation of long-term potentiation (LTP) and LTD (Carlisle *et al.* 2008). A decrease in PSD95 levels was associated with a reduction of the magnitude of dendritic spine density increase that usually results from LTP (Ehrlich *et al.* 2007). The effect of Neuroligin3 on LTD might occur via its interaction with PSD95. Among the interactors of PSD95, was BRAG1 encoded by *Iqsec2*, which also plays a role in the removal of AMPARs (Brown *et al.* 2015). The effect on synaptic function and dendritic spine density might also occur via Syngap1, another interactor

of PSD95 (Aceti *et al.* 2015; Walkup *et al.* 2016). However, the exact involvement of Neuroligin3 in this pathway needs to be further clarified.

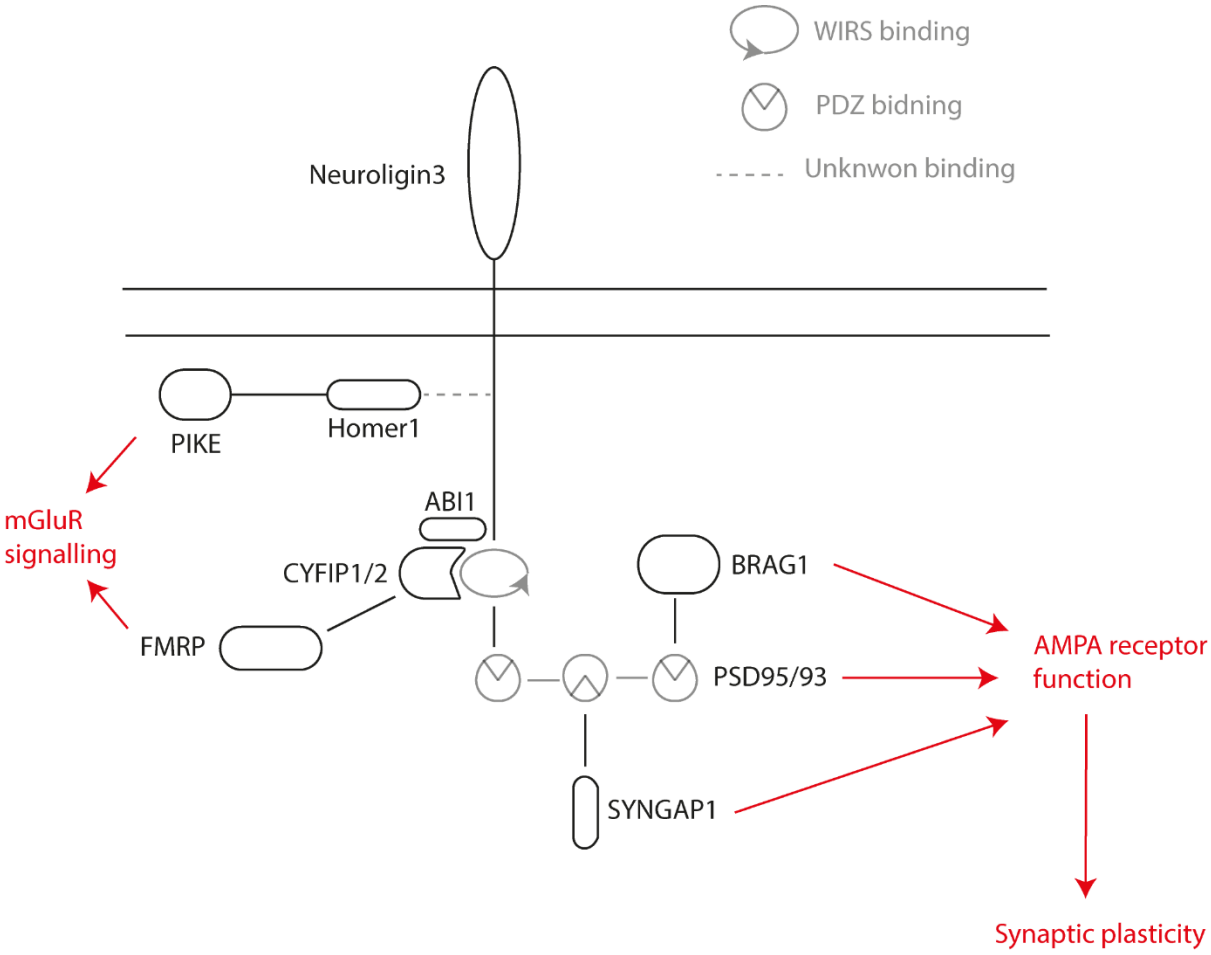


Figure 39 **Neuroligin3 might indirectly impact on mGluR signalling and synaptic plasticity**. PIKE and FMRP have been shown to influence mGluR signalling. Neuroligin3 might also have an effect on this system through its interactions with Homer1 and CYFIP1. Similarly, it might influence AMPA receptor function and synaptic plasticity through its interactions with PSD95/93, SYNGAP1 and BRAG1.

7.6. Application to the human population

Mouse models are used in an attempt to investigate the biological mechanisms underpinning ASD, which might also apply to the human population. However, the translation of results from mouse models to humans is not straightforward. Mouse models have the advantage over certain other model organisms such as *Drosophila*, in that they constitute a mammalian model. The construct validity of the models used in this thesis is discussed in Chapter 1. The results obtained here reflected further on the face validity of these models.

Changes in activity levels, motor learning, dendritic spine density, and the transcriptome were described here in relation to deletion of *Nlgn3* and *Cyfip1* haploinsufficiency. While ASD in the human population is diagnosed based on deficits in social communication and behaviour and repetitive behaviour, there is some variation in the behavioural symptoms present in ASD. A systematic review showed that individuals with ASD show impairment in motor learning on a variety of tasks (de Moraes *et al.* 2017). ASD is often diagnosed alongside ADHD, suggesting that changes in overall activity levels in these individuals are common (Scandurra *et al.* 2019). However, the face validity of the models investigated here, is undermined by the fact that few deficits in social behaviour were observed. It is important to note that a limited array of tests was employed here to investigate these deficits, which might manifest differently than in the human population. Assessment of the behaviour in these models could be extended to include an evaluation in the home cage environment, using an automatized system to observe a more naturalistic behaviour. The lack of observed impairment might also arise from the impact of the social environment as a modulating variable as discussed in Section 7.4. Interestingly, the social environment was also shown to impact on the behaviour of individuals with ASD, where the presence of siblings alleviated the symptoms of these individuals (Ben-Itzchak *et al.* 2019). In turn, social support was shown to modulate the behaviour of siblings of children with ASD (Hastings 2003). These findings suggest that much like in the mouse models, the social environment is a modulating variable in the human population.

Changes in dendritic spine density also occur both in the mouse models and the human population. Most of the information on humans comes from post-mortem studies, primarily of the cortex and hippocampus. While changes were observed in dendritic spine density, some of the studies showed an increase and others a decrease (Martínez-Cerdeño 2016). Similarly, studies of some mouse models show either increase or a decrease in dendritic spine density. While dendritic spine density might be an important biomarker in both animal and human populations, other factors affecting dendritic spine development might need to be considered. Here we discussed the possibility that the different genetic mutations might

affect dendritic spine density differently and that additional factors such as sex and the social environment might modulate these effects.

Interestingly, a sex difference in behaviour and dendritic spine density was observed in the mouse models discussed here. Gender discrepancy is frequently reported in the diagnosis of ASD, where as much as 4-5 times more boys are diagnosed with ASD in high-functioning individuals and twice as much when ASD is comorbid with intellectual disability (Fombonne 2005; Baird *et al.* 2006). One possibility is that there is biological basis for this effect. This idea is reflected in the fact that female mice heterozygous for *Nlgn3* or *Cyfip1* showed very mild if any deficits, while the males with a deletion of *Nlgn3* or heterozygous for *Cyfip1* showed more profound impairments. These differences might arise due to a higher threshold for females to become symptomatic. According to this theory girls and women diagnosed with ASD should carry higher mutation load and thus their relatives should also be at a higher risk of ASD. In line with this idea, a large study of 9000 dizygotic twin pairs showed that sibling of girls with ASD show greater impairments than those of siblings of boys diagnosed with ASD (Robinson *et al.* 2013). However, another possibility is that, in the human population, girls are rarely diagnosed with ASD due to a bias in diagnostic criteria. Girls were less likely to meet the criteria for ASD, even when they have scored highly on ASD-like traits, indicating a possible male bias in the diagnostic criteria (Dworzynski *et al.* 2012). This bias might arise simply from the female profile not being accurately captured by the current criteria (Kirkovski *et al.* 2013). Or it might reflect the tendency of girls to engage in more coping behaviours which camouflage their symptoms (Attwood 2006). Likely the gender bias observed in ASD arise through a complex interaction of biological and social factors.

Some differences in behaviour and RNA expression in the hippocampus depending on social environment were observed here. This finding is in line with the tendency of the social environment to impact on the outcomes of individuals with ASD. Children diagnosed with autism who have typically developing siblings tend to score better on social competence measures (McHale *et al.* 2016). On the other hand, there is some indication that the typically developing siblings of children diagnosed with ASD tend to display more cognitive, social and communication impairments (Georgiades *et al.* 2013). Thus there seems to be a parallel between this finding and the observation that mice with mutations associated with ASD can affect their wild type littermates.

Although parallels exist between the mouse models of ASD and the patient population, there are inherent limitations to studying human neurodevelopmental disorders with the aid of mouse models. While both are mammals with 6-layered cortex, the human neocortex is larger, more complex and follows a longer developmental trajectory (Zhao and Bhattacharyya 2018).

Alternative splicing of some of the genes involved in neurodevelopment is also unique to humans (Zhao and Bhattacharyya 2018). As many of the neurodevelopmental disorders are diagnosed based on behavioural deficits, it is important to note that mouse models cannot recapitulate language and some cognitive abilities present in the human population. Further studies translating the manipulations which might alleviate the symptoms into the human population are necessary to verify the predictive validity of these models.

7.7. Conclusions

The aim of this thesis was to investigate the impact of an interaction between Neuroligin3 and CYFIP1 on phenotypes associated with ASD. Neuroligin3 was found to interact with a number of other proteins linked to ASD, in neurons. These interactions were likely to influence phenotypes associated with ASD such as changes in dendritic spine development, morphology and plasticity. Accordingly, the interaction between Neuroligin3 and CYFIP1 was found to impact on motor learning, activity levels, dendritic spines density, and the transcriptome. Together these results indicate that there might be convergence in the biological processes affected by the different genetic mutations linked to ASD. The genotype-phenotype relationships are further modulated by factors such as sex and social environment. These findings increase the understanding of the role of protein interactions in biological processes underlying neurodevelopmental disorders and highlight the importance of studying the effects of sex and social environment.

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Appendix

Appendix 1

Figure	Normality	Equality of variances	Group differences	Sample size
No figure Distance in open field in males	Shapiro-Wilk test $W = 0.99$ $P = 0.60$	No heteroscedasticity	Mix Model ANOVA, main effect of *Thy1-EGFP* transgene: $F(1, 65) = 0.40$, $P = 0.53$	*Thy-EGFP n* = 43 No *Thy-EGFP n* = 23
Figure 11 A	Shapiro-Wilk test $W = 0.99$ $P = 0.53$	No heteroscedasticity	Mix Model ANOVA, main effect of genotype: $F(3, 62) = 6.45$, $P < 0.001$, main effect of day: $F(1, 62) = 35.54$, $P < 0 .001$, interaction of the effect of genotype and day: $F(3, 62) = 2.41$, $P = 0.08$ Tukey HSD: *WT* Day 1 vs *WT* Day 2 $t(1, 62) = 5.29$, $P < 0.001$ *Nlgn3*$^{y/-}$ Day 1 vs *Nlgn3*$^{y/-}$ Day 2 $t(1, 62) = 2.21$, $P = 0.42$ *Cyfip1*$^{+/-}$ Day 1 vs *Cyfip1*$^{+/-}$ Day 2 $t(1, 62) = 1.99$, $P = 0.50$ *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ Day 1 vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ Day 2 $t(1, 62) = 2.52$, $P = 0.21$ *WT* vs *Cyfip1*$^{+/-}$ $t(1, 62) = 0.42$, $P = 0.98$ *WT* vs *Nlgn3*$^{y/-}$ $t(1, 62) = -2.70$, $P = 0.043$ *WT* vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ $t(1, 62) = -3.53$, $P < 0.01$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$ $t(1, 62) = -2.82$, $P = 0.031$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ $t(1, 62) = -3.61$, $P < 0.01$ *Nlgn3*$^{y/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ $t(1, 62) = -1.35$, $P = 0.54$	*WT n* = 18 *Nlgn3*$^{y/-}$ *n* = 24 *Cyfip1*$^{+/-}$ *n*= 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12

No figure Time spent in the centre of the open field in males	Shapiro-Wilk test $W = 0.97$ $P < 0.01$	No heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of *Thy1-EGFP* transgene: $F(1, 63) = 0.78$ $P = 0.38$	*Thy-EGFP n* = 42 No *Thy-EGFP n* = 22
Figure 11 B	Shapiro-Wilk test $W = 0.98$ $P = 0.04$	No heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of genotype: $F(3, 60) = 0.25$, $P = 0.83$, main effect of day: $F(1, 63) = 2.91$, $P = 0.09$, interaction of the effect of genotype and day: $F(3, 60) = 0.29$, $P = 0.83$	*WT n* = 16 *Nlgn3*$^{y/-}$ *n* = 24 *Cyfip1*$^{+/-}$ *n*= 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12
Figure 12 A	Shapiro-Wilk test $W = 0.99$ $P = 0.91$	No heteroscedasticity	One-way ANOVA, main effect of genotype: $F(3, 62) = 6.55$ $P < 0.001$ Tukey HSD: *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$ $t(1, 65) = -2.46$, $P = 0.08$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/--}$ $t(1, 65) = -2.70$, $P = 0.043$ *Cyfip1*$^{+/-}$ vs *WT* $t(1, 65) = 0.57$, $P = 0.94$ *Nlgn3*$^{y/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ $t(1, 65) = -0.66$, $P = 0.91$ *Nlgn3*$^{y/-}$ vs *WT* $t(1, 65) = 3.47$, $P < 0.01$ *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ vs *WT* $t(1, 65) = 3.53$, $P < 0.01$	*WT n* = 18 *Nlgn3*$^{y/-}$ *n* = 24 *Cyfip1*$^{+/-}$ *n*= 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12
Figure 12 B	Shapiro-Wilk test $W = 0.96$ $P = 0.02$	Heteroscedasticity	Kruskal-Wallis, main effect of genotype: $\chi^2(3, 60) = 3.39$, $P = 0.94$	*WT n* = 16 *Nlgn3*$^{y/-}$ *n* = 24 *Cyfip1*$^{+/-}$ *n*= 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12

No figure Distance in the open field in males Re-analysis	Shapiro-Wilk test *W* = 0.99 *P* = 0.53	No heteroscedasticity	Mix Model ANOVA, main effect of day: *F*(1, 62) = 35.54, *P* < 0.0001, main effect of *Nlgn3* absence *F*(1, 62) = 16.99, *P* < 0.0001, main effect of *Cyfip1* absence *F*(1, 62) = 0.11, *P* = 0.75.	*WT n* = 18 *Nlgn3*$^{y/-}$ *n* = 24 *Cyfip1*$^{+/-}$ *n*= 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12
No figure Time in the centre in the open field in males Re-analysis	Shapiro-Wilk test *W* = 0.98 *P* = 0.038	No heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of day: *F*(1, 60) = 2.91, *P* = 0.08, main effect of *Nlgn3* absence *F*(1, 60) = 0.08, *P* = 0.77, main effect of *Cyfip1* absence *F*(1, 60) = 0.02, *P* = 0.90.	*WT n* = 16 *Nlgn3*$^{y/-}$ *n* = 24 *Cyfip1*$^{+/-}$ *n*= 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12
No figure Distance in the open field in females	Shapiro-Wilk test *W* = 0.99 *P* = 0.25	No heteroscedasticity	Mix Model ANOVA, main effect of *Thy1-EGFP* transgene: *F*(1, 62) = 1.09, *P* = 0.28	*Thy-EGFP n* = 38 No *Thy-EGFP n* = 25
Figure 13 A	Shapiro-Wilk test *W* = 0.99 *P* = 0.22	No heteroscedasticity	Mix Model ANOVA, main effect of genotype: *F*(3, 59) = 4.51, *P* < 0.01, main effect of day: *F*(1, 59) = 92.13, *P* = < 0.001, interaction of the effect of genotype and day: *F*(3, 59) = 0.56, *P* = 0.65. Tukey HSD: *WT* Day 1 vs *WT* Day 2 *t*(1, 59) = 5.61, *P* < 0.001 *Nlgn3*$^{+/-}$ Day 1 vs *Nlgn3*$^{+/-}$ Day 2 *t*(1, 59) = 4.42, *P* < 0.001 *Cyfip1*$^{+/-}$ Day 1 vs *Cyfip1*$^{+/-}$ Day 2 *t*(1, 59) = 3.83, *P* < 0.01 *Nlgn3*$^{+/-}$ *Cyfip1*$^{+/-}$ Day 1 vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ Day 2 *t*(1, 59) = 5.30, *P* < 0.001 *WT* vs *Cyfip1*$^{+/-}$ *t*(1, 59) = 1.54, *P* = 0.42 *WT* vs *Nlgn3*$^{+/-}$ *t*(1, 59) = -2.10, *P* = 0.16 *WT* vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *t*(1, 59) = 0.36, *P* = 0.98 *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{+/-}$ *t*(1, 59) = -3.42, *P* < 0.01	*WT n* = 16 *Nlgn3*$^{+/-}$ *n* = 20 *Cyfip1*$^{+/-}$ *n*= 10 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 17

			Cyfip1$^{+/-}$ vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ $t(1, 59) = -1.24$, $P = 0.60$ *Nlgn3*$^{+/-}$ vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ $t(1, 59) = 2.52$, $P = 0.07$	
No figure Time in the centre of open field in females	Shapiro-Wilk test $W = 0.99$ $P = 0.25$	No heteroscedasticity	Mix Model ANOVA, main effect of *Thy1-EGFP* transgene: $F(1, 62) = 0.20$, $P = 0.66$	*Thy-EGFP n* = 38 No *Thy-EGFP n* = 25
Figure 13 B	Shapiro-Wilk test $W = 0.99$ $P = 0.27$	No heteroscedasticity	Mix Model ANOVA, main effect of genotype: $F(3, 59) = 0.13$, $P = 0.94$, main effect of day: $F(1, 59) = 0.52$, $P = 0.47$, interaction of the effect of genotype and day: $F(3, 59) = 0.84$, $P = 0.48$	*WT n* = 16 *Nlgn3*$^{+/-}$ *n* = 20 *Cyfip1*$^{+/-}$ *n*= 10 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 17
Figure 14 A	Shapiro-Wilk test $W = 0.97$ $P = 0.12$	No heteroscedasticity	One-way ANOVA, main effect of genotype: $F(3, 59) = 4.64$, $P < 0.01$ Tukey HSD: *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{+/-}$ $t(1, 59) = -3.34$, $P < 0.01$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/--}$ $t(1, 59) = -1.05$, $P = 0.72$ *Cyfip1*$^{+/-}$ vs *WT* $t(1, 59) = -1.20$, $P = 0.63$ *Nlgn3*$^{+/-}$ vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ $t(1, 59) = 2.66$, $P < 0.05$ *Nlgn3*$^{+/-}$ vs *WT* $t(1, 59) = 2.42$, $P = 0.08$ *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ vs *WT* $t(1, 59) = -0.19$, $P = 1.00$	*WT n* = 16 *Nlgn3*$^{+/-}$ *n* = 20 *Cyfip1*$^{+/-}$ *n*= 10 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 17
Figure 14 B	Shapiro-Wilk test $W = 0.96$ $P = 0.03$	Heteroscedasticity	Kruskal-Wallis, main effect of genotype: $\chi^2(3, 59) = 2.23$, $P = 0.53$	*WT n* = 16 *Nlgn3*$^{+/-}$ *n* = 20 *Cyfip1*$^{+/-}$ *n*= 10 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 17
No figure Distance in the open	Shapiro-Wilk test $W = 0.99$ $P = 0.23$	No heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of day: $F(1, 59) = 92.13$, $P < 0.0001$, main effect of *Nlgn3* absence $F(1, 59) = 5.78$, $P = 0.019$, main effect of *Cyfip1* absence $F(1, 59) = 7.58$,	*WT n* = 16 *Nlgn3*$^{+/-}$ *n* = 20

field in females Re-analysis			*P* = 0.008.	*Cyfip1*$^{+/-}$ *n*= 10 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 17
No figure Time in the centre in the open field in females Re-analysis	Shapiro-Wilk test *W* = 0.99 *P* = 0.27	No heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of day: *F*(1, 59) = 0.52, *P* = 0.47, main effect of *Nlgn3* absence *F*(1, 59) = 0.17, *P* = 0.69, main effect of *Cyfip1* absence *F*(1, 59) = 0.01, *P* = 0.91.	*WT n* = 16 *Nlgn3*$^{+/-}$ *n* = 20 *Cyfip1*$^{+/-}$ *n*= 10 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 17
Figure 15 B	Shapiro-Wilk test *W* = 0.91 *P* < 0.001	No heteroscedasticity	Scheirer–Ray–Hare test, main effect of genotype: *H(1, 52)* = 1.48, *P* = 0.22, main effect of sex: *H(1, 52)* = 0.18 , *P* = 0.67, interaction between the effect of genotype and sex: *H(1, 52)* = 0.01, *P* = 0.93.	*Males:* *WT n* = 18 *Cyfip1*$^{+/-}$ *n* = 12 *Females:* *WT n* = 16 *Cyfip1*$^{+/-}$ *n* = 10
No figure Latency to fall off rotarod in males	Shapiro-Wilk test *W* = 0.99 *P* < 0.001	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of *Thy1-EGFP* transgene: *F*(1, 53) = 2.83, *P* = 0.09	*Thy-EGFP n* = 37 No *Thy-EGFP n* = 20
Figure 16	Shapiro-Wilk test *W* = 0.99 *P* < 0.001	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of genotype: *F(3, 53)* = 1.90, *P* = 0.13 ,main effect of day: *F(1, 1577)* = 57.36, *P* < 0.001, main effect of trial: *F(9, 1577)* = 2.68, *P* < 0.01, interaction of the effect of genotype and day: *F(3, 1577)* = 4.61, *P* < 0.01. Simple effects: *WT* Day1 vs Day 2: *t*(1, 16) = 2.12, *P* = 0.049	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12

			Day1 vs Day 3: $t(1, 16) = 6.28$, $P < 0.001$ Day2 vs Day 3: $t(1, 16) = 2.86$, $P = 0.023$ *Nlgn3*$^{y/-}$ Day1 vs Day 2: $t(1, 15) = 6.16$, $P < 0.001$ Day1 vs Day 3: $t(1, 15) = 9.14$, $P < 0.001$ Day2 vs Day 3: $t(1, 15) = 3.02$, $P < 0.01$ *Cyfip1*$^{+/-}$ Day1 vs Day 2: $t(1, 11) = 1.10$, $P = 0.50$ Day1 vs Day 3: $t(1, 11) = 1.54$, $P = 0.39$ Day2 vs Day 3: $t(1, 11) = 1.11$, $P = 0.50$ *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ Day1 vs Day 2: $t(1, 11) = 5.05$, $P < 0.001$ Day1 vs Day 3: $t(1, 11) = 4.69$, $P < 0.001$ Day2 vs Day 3: $t(1, 11) = 1.73$, $P = 0.06$ Simple effets: Day 1 *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$: $t(1, 21) = 1.58$, $P = 0.69$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: $t(1, 21) = 0.32$, $P = 0.99$ *Cyfip1*$^{+/-}$ vs *WT* : $t(1, 21) = 0.90$, $P = 0.92$ *Nlgn3*$^{y/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: $t(1, 22) = 1.26$, $P = 0.81$ *Nlgn3*$^{y/-}$ vs *WT* : $t(1, 31) = 0.85$, $P = 0.93$ *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ vs *WT* : $t(1, 22) = 0.56$, $P = 0.98$ Day 2 *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$: $t(1, 21) = 2.43$, $P = 0.34$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: $t(1, 21) = 0.93$, $P = 0.91$ *Cyfip1*$^{+/-}$ vs *WT* : $t(1, 21) = 0.49$, $P = 0.99$ *Nlgn3*$^{y/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: $t(1, 22) = 1.51$, $P = 0.71$ *Nlgn3*$^{y/-}$ vs *WT* : $t(1, 31) = 3.19$, $P = 0.13$ *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ vs *WT* : $t(1, 22) = 1.51$, $P = 0.71$ Day 2 *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$: $t(1, 21) = 6.42$, $P < 0.001$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: $t(1, 21) = 1.92$, $P = 0.54$ *Cyfip1*$^{+/-}$ vs *WT* : $t(1, 21) = 0.78$, $P = 0.95$ *Nlgn3*$^{y/-}$ vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$: $t(1, 22) = 3.73$, $P = 0.071$ *Nlgn3*$^{y/-}$ vs *WT* : $t(1, 31) = 5.02$, $P < 0.01$	

			Nlgn3$^{y/-}$*Cyfip1*$^{+/-}$ vs *WT* : $t(1, 22) = 1.09$, $P = 0.87$	
No figure Latency to fall off rotarod in males Re-analysis	Shapiro-Wilk test $W = 0.99$ $P < 0.001$	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of *Nlgn3* absence: $F(1, 53) = 3.48$, $P = 0.062$,main effect of *Cyfip1* absence: $F(1, 53) = 0.14$, $P = 0.713$, main effect of day: $F(3, 1577) = 52.37$, $P < 0.0001$.	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 12
No figure Latency to fall off rotarod in females	Shapiro-Wilk test $W = 0.97$ $P < 0.001$	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of *Thy1-EGFP* transgene, $F(1, 52) = 0.01$, $P = 0.93$	*Thy-EGFP n* = 37 No *Thy-EGFP n* = 17
Figure 17	Shapiro-Wilk test $W = 0.98$ $P < 0.001$	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of genotype: $F(3, 50) = 2.88$, $P = 0.73$, main effect of day: $F(1, 1490) = 66.39$, $P < 0.001$, main effect of trial: $F(9, 1490) = 18.84$, $P < 0.001$, interaction of the effect of genotype and day: $F(3, 1577) = 4.45$, $P = 0.13$. Simple effects: *WT* Day1 vs Day 2: $t(1, 12) = 5.09$, $P < 0.01$ Day1 vs Day 3: $t(1, 12) = 5.85$, $P < 0.01$ Day2 vs Day 3: $t(1, 12) = 3.47$, $P = 0.07$ *Nlgn3*$^{+/-}$ Day1 vs Day 2: $t(1, 12) = 6.15$, $P < 0.01$ Day1 vs Day 3: $t(1, 12) = 8.06$, $P < 0.001$ Day2 vs Day 3: $t(1, 12) = 4.23$, $P = 0.028$ *Cyfip1*$^{+/-}$ Day1 vs Day 2: $t(1, 13) = 3.73$ $P = 0.05$ Day1 vs Day 3: $t(1, 13) = 5.95$, $P < 0.01$ Day2 vs Day 3: $t(1, 13) = 2.21$, $P = 0.29$ *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ Day1 vs Day 2: $t(1, 13) = 7.34$ $P < 0.001$	*WT n* = 13 *Nlgn3*$^{+/-}$ *n* = 13 *Cyfip1*$^{+/-}$ *n*= 14 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 14

			Day1 vs Day 3: $t(1, 13) = 7.41$, $P < 0.01$ Day2 vs Day 3: $t(1, 13) = 1.05$, $P = 0.74$	
No figure Latency to fall off rotarod in females Re-analysis	Shapiro-Wilk test $W = 0.98$ $P < 0.001$	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of *Nlgn3* absence: $F(1, 52) = 0.13$, $P = 0.723$,main effect of *Cyfip1* absence: $F(1, 52) = 0.97$, $P = 0.326$, main effect of day: $F(3, 1577) = 67.21$, $P < 0.0001$.	*WT n* = 13 *Nlgn3*$^{+/-}$ *n* = 13 *Cyfip1*$^{+/-}$ *n*= 14 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 14
Figure 18	Shapiro-Wilk test $W = 0.98$ $P < 0.001$	No heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of genotype: $F(1, 52) = 1.01$, $P = 0.32$, main effect of day: $F(1, 52) = 27.26$, $P < 0.001$, main effect of sex: $F(1, 52) = 4.88$, $P = 0.032$.	*Males:* *WT n* = 17 *Cyfip1*$^{+/-}$ *n* = 12 *Females:* *WT n* = 13 *Cyfip1*$^{+/-}$ *n* = 14
No figure Time spent sniffing in males	Shapiro-Wilk test $W = 0.93$ $P < 0.001$	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of *Thy1-EGFP* transgene, $F(1, 52)$ 1.01, $P = 0.32$.	*Thy-EGFP n* = 31 No *Thy-EGFP n* = 18
Figure 19 A	Shapiro-Wilk test $W = 0.98$ $P = 0.06$	Heteroscedasticity	Mix Model ANOVA, main effect of genotype: $F(3, 45) = 0.38$, $P = 0.77$, main effect of trial: $F(2, 90) = 36.37$, $P < 0.001$, interaction of the effect of genotype and trial: $F(6, 90) = 0.54$, $P = 0.78$. Simple effects: *WT* C1 vs C2: $t(1, 11) = 2.40$, $P = 0.37$ C1 vs S1: $t(1, 11) = 1.60$ $P = 0.67$ C1 vs S2: $t(1, 11) = 1.19$, $P = 0.84$ C2 vs S1: $t(1, 11) = 6.48$, $P = 0.0037$	*WT n* = 12 *Nlgn3*$^{y/-}$ *n* = 14 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 11

			C2 vs S2: $t(1, 11) = 0.85$, $P = 0.93$ S1 vs S2: $t(1, 11) = 3.39$, $P = 0.14$ *Nlgn3*$^{y/-}$ C1 vs C2: $t(1, 13) = 11.47$ $P < 0.001$ C1 vs S1: $t(1, 13) = 1.51$ $P = 0.71$ C1 vs S2: $t(1, 13) = 3.01$, $P = 0.19$ C2 vs S1: $t(1, 13) = 5.41$, $P < 0.01$ C2 vs S2: $t(1, 13) = 2.34$, $P = 0.38$ S1 vs S2: $t(1, 13) = 4.61$ $P = 0.03$ *Cyfip1*$^{+/-}$ C1 vs C2: $t(1, 11) = 4.04$ $P = 0.06$ C1 vs S1: $t(1, 11) = 1.08$ $P = 0.87$ C1 vs S2: $t(1, 11) = 1.30$, $P = 0.80$ C2 vs S1: $t(1, 11) = 8.74$, $P < 0.001$ C2 vs S2: $t(1, 11) = 4.69$, $P = 0.029$ S1 vs S2: $t(1, 11) = 3.37$, $P = 0.14$ *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ C1 vs C2: $t(1, 10) = 1.66$ $P = 0.67$ C1 vs S1: $t(1, 10) = 3.34$ $P = 0.15$ C1 vs S2: $t(1, 10) = 3.49$, $P = 0.13$ C2 vs S1: $t(1, 10) = 5.06$, $P = 0.02$ C2 vs S2: $t(1, 10) = 4.40$, $P = 0.046$ S1 vs S2: $t(1, 10) = 2.14$, $P = 0.35$	
Figure 19 B	Shapiro-Wilk test $W = 0.93$ $P < 0.01$	Heteroscedasticity	Kruskal-Wallis, main effect of genotype: $\chi^2(3, 53) = 0.73$, $P = 0.87$	*WT n* = 12 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12
No figure Time spent sniffing in males Re-analysis	Shapiro-Wilk test $W = 0.98$ $P = 0.06$	Heteroscedasticity	Mix Model ANOVA, main effect of *Cyfip1* absence: $F(1, 45) = 0.03$, $P = 0.86$, main effect of *Nlgn3* absence: $F(1, 45) = 1.11$, $P = 0.30$, main effect of trial: $F(2, 90) = 36.37$, $P < 0.0001$.	*WT n* = 12 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12

				Nlgn3$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12
No figure Time spent sniffing in females	Shapiro-Wilk test *W* = 0.92976 *P* < 0.001	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of *Thy1-EGFP* transgene, *F*(1, 52) = 0.46, *P* = 0.50.	*Thy-EGFP n* = 37 No *Thy-EGFP n* = 17
Figure 20 A	Shapiro-Wilk test *W* = 0.90 *P* < 0.001	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of genotype: *F(3, 50)* = 0.81, *P* = 0.48,main effect of trial: *F(2, 100)* = 41.07, *P* < 0.001, interaction of the effect of genotype and trial: *F(6, 100)* = 1.26 *P* = 0.28 Simple effects: *WT* C1 vs C2: *t*(1, 12) = 2.43, *P* = 0.36 C1 vs S1: *t*(1, 12) = 8.31 *P* < 0.001 C1 vs S2: *t*(1, 12) = 0.41, *P* = 0.99 C2 vs S1: *t*(1, 12) = 10.79, *P* < 0.001 C2 vs S2: *t*(1, 12) = 1.57, *P* = 0.69 S1 vs S2: *t*(1, 12) = 3.11, *P* = 0.18 *Nlgn3*$^{+/-}$ C1 vs C2: *t*(1, 12) = 2.46, *P* = 0.35 C1 vs S1: *t*(1, 12) = 2.62 *P* = 0.30 C1 vs S2: *t*(1, 12) = 1.01, *P* = 0.89 C2 vs S1: *t*(1, 12) = 5.50, *P* = 0.01 C2 vs S2: *t*(1, 12) = 1.73, *P* = 0.62 S1 vs S2: *t*(1, 12) = 4.79, *P* = 0.028 *Cyfip1*$^{+/-}$ C1 vs C2: *t*(1, 13) = 3.85, *P* = 0.07 C1 vs S1: *t*(1, 13) = 1.99 *P* = 0.52 C1 vs S2: *t*(1, 13) = 0.42, *P* = 0.99 C2 vs S1: *t*(1, 13) = 4.82, *P* = 0.029 C2 vs S2: *t*(1, 13) = 3.27, *P* = 0.15 S1 vs S2: *t*(1, 13) = 1.12, *P* = 0.86 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ C1 vs C2: *t*(1, 13) = 2.23, *P* = 0.42	*WT n* = 13 *Nlgn3*$^{+/-}$ *n* = 13 *Cyfip1*$^{+/-}$ *n* = 14 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 14

			C1 vs S1: $t(1, 13) = 5.95$ $P < 0.01$ C1 vs S2: $t(1, 13) = 0.97$, $P = 0.90$ C2 vs S1: $t(1, 13) = 5.44$, $P < 0.01$ C2 vs S2: $t(1, 13) = 2.38$, $P = 0.37$ S1 vs S2: $t(1, 13) = 6.39$, $P < 0.01$	
Figure 20 B	Shapiro-Wilk test $W = 0.97$ $P = 0.21$	No heteroscedasticity	One-way ANOVA, main effect of genotype: $F(3, 50) = 2.92$, $P = 0.043$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{+/-}$ $t(1, 50) = -1.29$, $P = 0.57$ *Cyfip1*$^{+/-}$ vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/--}$ $t(1, 50) = -2.35$, $P = 0.10$ *Cyfip1*$^{+/-}$ vs *WT* $t(1, 50) = -2.68$, $P = 0.047$ *Nlgn3*$^{+/-}$ vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ $t(1, 50) = -1.01$, $P = 0.74$ *Nlgn3*$^{+/-}$ vs *WT* $t(1, 50) = -1.36$, $P = 0.53$ *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ vs *WT* $t(1, 50) = -0.37$, $P = 0.98$	*WT n* = 13 *Nlgn3*$^{+/-}$ *n* = 13 *Cyfip1*$^{+/-}$ *n* = 14 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 14
No figure Time spent sniffing in females Re-analysis	Shapiro-Wilk test $W = 0.89$ $P < 0.0001$	Heteroscedasticity	Mix Model ANOVA, main effect of *Cyfip1* absence: $F(1, 50) = 0.03$, $P = 0.87$, main effect of *Nlgn3* absence: $F(1, 50) = 0.79$, $P = 0.37$, main effect of trial: $F(2, 100) = 41.07$, $P < 0.0001$.	*WT n* = 13 *Nlgn3*$^{+/-}$ *n* = 13 *Cyfip1*$^{+/-}$ *n* = 14 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 14
No figure Number of vocalisations	Shapiro-Wilk test $W = 0.93$ $P < 0.01$	Heteroscedasticity	Kruskal-Wallis, main effect of *Thy-EGFP* transgene, $\chi^2(1, 56) = 1.58$, $P = 0.21$	*Thy-EGFP n* = 20 No *Thy-EGFP n* = 37
Figure 21 A	Shapiro-Wilk test $W = 0.94$ $P < 0.01$	Heteroscedasticity	Kruskal-Wallis, main effect of genotype: $\chi^2(3, 56) = 2.04$, $P = 0.56$	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12

No figure Number of vocalisations Re-analysis	Shapiro-Wilk test $W = 0.94$ $P = 0.008$	Heteroscedasticity	Scheirer–Ray–Hare, main effect of *Cyfip1* absence: $H(1,53) = 0.83$, $P = 0.36$, main effect of *Nlgn3* absence: $H(1,53) = 1.17$, P = 0.28, absence of *Nlgn3* and *Cyfip1* interaction: $H(1,53) = 0.03$, $P = 0.86$.	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12
Duration of vocalisations	Shapiro-Wilk test $W = 0.93$ $P < 0.01$	Heteroscedasticity	Kruskal-Wallis, main effect of *Thy-EGFP* transgene χ^2 (1, 56) = 1.38 $P = 0.24$	*Thy-EGFP n* = 20 No *Thy-EGFP n* = 37
Figure 21 B	Shapiro-Wilk test $W = 0.94$ $P < 0.01$	Heteroscedasticity	Kruskal-Wallis, main effect of genotype: χ^2 (3, 52) = 1.98 $P = 0.57$	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12
No figure Duration of vocalisations Re-analysis	Shapiro-Wilk test $W = 0.94$ $P = 0.006$	Heteroscedasticity	Scheirer–Ray–Hare, main effect of *Cyfip1* absence: $H(1,53) = 1.51$, $P = 0.22$, main effect of *Nlgn3* absence: $H(1,53) = 0.43$, P = 0.51, absence of *Nlgn3* and *Cyfip1* interaction: $H(1,53) = 0.05$, $P = 0.83$.	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12
No figure Interaction time	Shapiro-Wilk test $W = 0.98$ $P = 0.46$	Heteroscedasticity	Welch's Mix Model ANOVA, main effect of *Thy-EGFP* transgene: $F(1, 56) = 1.19$, $P = 0.28$	*Thy-EGFP n* = 20 No *Thy-EGFP n* = 37
Figure 21 C	Shapiro-Wilk test $W = 0.99$ $P = 0.46$	Heteroscedasticity	One-way ANOVA, main effect of genotype: $F(3, 53) = 0.08$, $P = 0.97$	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12

No figure Interaction time Re-analysis	Shapiro-Wilk test *W* = 0.98 *P* = 0.46	No heteroscedasticity	Two-way ANOVA, main effect of *Cyfip1* absence: $F(1, 53) = 0.03$, $P = 0.869$, main effect of *Nlgn3* absence: $F(1, 53) = 0.06$, $P = 0.81$, absence of *Nlgn3* and *Cyfip1* interaction: $H(1,53) = 0.15$, $P = 0.704$.	*WT n* = 17 *Nlgn3*$^{y/-}$ *n* = 16 *Cyfip1*$^{+/-}$ *n* = 12 *Nlgn3*$^{y/-}$ *Cyfip1*$^{+/-}$ *n* = 12
Figure 22 A	Shapiro-Wilk test *W* = 0.98 *P* = 0.26	No heteroscedasticity	Mix Model ANOVA, main effect of housing, $F(1, 35) = 10.30$, $P < 0.01$, main effect of day, $F(1, 35) = 46.26$, $P < 0.001$, interaction of the effect of housing and day $F(1, 35) = 1.90$, $P < 0.001$	*WT* MGH *n* = 18 *WT* SGH *n* = 18
No figure Time in the centre of open field	Shapiro-Wilk test *W* = 0.96 *P* = 0.035	No heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of housing: $F(1, 35) = 0.55$, $P = 0.46$, main effect of day: $F(1, 35) = 1.02$, $P = 0.32$, interaction of the effect of housing and day: $F(1, 35) = 0.01$, $P = 0.91$.	*WT* MGH *n* = 18 *WT* SGH *n* = 18
Figure 22 B	Shapiro-Wilk test *W* = 0.99 *P* = 0.99	Heteroscedasticity	Welch's One-way ANOVA, main effect of housing $F(1,34) = 2.97$, $P = 0.09$	*WT* MGH *n* = 18 *WT* SGH *n* = 18
No figure Time in the centre of open field Day 2 only	Shapiro-Wilk test *W* = 0.91 *P* < 0.001	No heteroscedasticity	Kruskal-Wallis, main effect of housing, $\chi^2(1,34) = 0.51$, $P = 0.48$.	*WT* MGH *n* = 18 *WT* SGH *n* = 18
Figure 22 D	Shapiro-Wilk test *W* = 0.99 *P* = 0.12	Heteroscedasticity	Mix Model ANOVA, main effect of housing: $F(1, 33) = 0.59$, $P = 0.45$, main effect of day: $F(1, 1490) = 102.89$, $P < 0.001$, main effect of trial: $F(9, 1490) = 5.34$, $P < 0.001$, interaction of the effect of housing and day: $F(3, 1577) = 4.54$, $P = 0.03$ Simple effects: *WT* MGH Day1 vs Day 2: $t(1, 17) = 3.91$, $P = 0.049$ Day1 vs Day 3: $t(1, 17) = 7.56$, $P < 0.001$ Day2 vs Day 3: $t(1, 17) = 3.66$, $P < 0.0226$ *WT* SGH Day 1 vs Day 2: $t(1, 15) = 6.58$, $P < 0.001$	*WT* MGH *n* = 18 *WT* SGH n = 16

			Day 1 vs Day 3: $t(1, 15) = 9.21$, $P < 0.001$ Day2 vs Day 3: $t(1, 17) = 2.06$, $P = 0.06$	
Figure 23 A	Shapiro-Wilk test $W = 0.85$ $P < 0.001$	Heteroscedasticity	Non-parametric Mix Model ANOVA, main effect of housing: $F(1, 30) = 56.63$, $P < 0.001$, main effect of trial: $F(2, 60) = 29.84$, $P < 0.001$, interaction of the effect of housing and trial: $F(2, 60) = 2.95$, $P = 0.06$. *Simple effect* *WT* MGH C1 vs C2: $t(1, 13) = 3.07$, $P = 0.18$ C1 vs S1: $t(1, 13) = 2.70$ $P = 0.27$ C1 vs S2: $t(1, 13) = 1.60$, $P = 0.68$ C2 vs S1: $t(1, 13) = 4.60$, $P = 0.0037$ C2 vs S2: $t(1, 13) = 1.14$, $P = 0.85$ S1 vs S2: $t(1, 13) = 3.68$, $P = 0.09$ *WT* SGH C1 vs C2: $t(1, 17) = 16.22$, $P < 0.001$ C1 vs S1: $t(1, 17) = 8.39$, $P < 0.001$ C1 vs S2: $t(1, 17) = 6.96$, $P < 0.001$ C2 vs S1: $t(1, 17) = 13.07$, $P < 0.001$ C2 vs S2: $t(1, 17) = 10.75$, $P < 0.001$ S1 vs S2: $t(1, 17) = 13.00$, $P < 0.001$	*WT* MGH $n = 14$ *WT* SGH $n = 18$
Figure 23 B	Shapiro-Wilk test $W = 0.78$ $P < 0.001$	No heteroscedasticity	Mann-Whitney test, main effect of housing: $W(1,15) = 226$, $P < 0.001$	*WT* MGH $n = 14$ *WT* SGH $n = 18$
Figure 23 C	Shapiro-Wilk test $W = 0.84$ $P < 0.001$	Heteroscedasticity	Kruskal-Wallis, main effect of housing: $\chi^2(1, 53) = 0.02$, $P = 0.88$	*WT* MGH $n = 17$ *WT* SGH $n = 18$
Figure 23 D	Shapiro-Wilk test $W = 0.86$ $P < 0.001$	Heteroscedasticity	Kruskal-Wallis, main effect of housing: $\chi^2(1, 53) = 0.13$, $P = 0.72$	*WT* MGH $n = 17$ *WT* SGH $n = 18$

Figure 23 E	Shapiro-Wilk test *W* = 0.97 *P* = 0.53	Heteroscedasticity	One-way ANOVA, main effect of housing: $F(1,33) = 0.01$, $P = 0.95$	*WT* MGH *n* = 17 *WT* SGH *n* = 18
Figure 24 A	Shaprio-Wilk test *W* = 0.99 *P* = 0.70	No heteroscedasticity	Independent t-test: $t(1, 59) = -4.17$, $P < 0.001$	*WT n* = 24 *Cyfip1*$^{+/-}$ *n* = 37
Figure 24 B	Shaprio-Wilk test *W* = 0.97 *P* = 0.31	No heteroscedasticity	Independent t-test: $t(1, 40) = 1.09$, $P = 0.28$	*WT n* = 19 *Cyfip1*$^{+/-}$ *n* = 22
Figure 24 C	Shaprio-Wilk test *W* = 0.96, *P* = 0.13	No heteroscedasticity	Independent t-test: $t(1, 38) = 1.15$, $P = 0.26$	*WT n* = 19 *Cyfip1*$^{+/-}$ *n* = 21
Figure 24 D	Shaprio-Wilk test *W* = 0.97 *P* = 0.40	No heteroscedasticity	Independent t-test: $t(1, 38) = -0.21$, $P = 0.83$	*WT n* = 20 *Cyfip1*$^{+/-}$ *n* = 20
Figure 25 A	N/A	N/A	Generalised linear model *WT* vs *Nlgn3*$^{y/-}$ t(1, 11) = 0.02, *P* = 0.99 *WT* vs *Cyfip1*$^{+/-}$ *t*(1, 11) = 0.83, *P* = 0.42 *WT* vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *t*(1, 11) = 1.50 , *P* = 0.16	*WT n* = 4 *Nlgn3*$^{y/-}$ *n* = 4 *Cyfip1*$^{+/-}$ *n* = 4 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 3
Figure 25 B	N/A	N/A	Generalised linear model *WT* vs *Nlgn3*$^{y/-}$ t(1, 11) = 0.02, *P* = 0.99 *WT* vs *Cyfip1*$^{+/-}$ *t*(1, 11) = 0.42, *P* = 0.68 *WT* vs *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *t*(1, 11) = 0.76 , *P* = 0.47	*WT n* = 4 *Nlgn3*$^{y/-}$ *n* = 4 *Cyfip1*$^{+/-}$ *n* = 4 *Nlgn3*$^{y/-}$*Cyfip1*$^{+/-}$ *n* = 3

Figure 26 A	N/A	N/A	Generalised linear model *WT* vs *Nlgn3*$^{+/-}$ t(1, 12) = 0.09, *P* = 0.93 *WT* vs *Cyfip1*$^{+/-}$ *t*(1, 12) = 0.09 , *P* = 0.93 *WT* vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *t*(1, 12) = 0.02 , *P* = 0.99	*WT n* = 4 *Nlgn3*$^{+/-}$ *n* = 4 *Cyfip1*$^{+/-}$ *n* = 4 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 4
Figure 26 B	N/A	N/A	Generalised linear model *WT* vs *Nlgn3*$^{+/-}$ t(1, 12) = -0.75, *P* = 0.47 *WT* vs *Cyfip1*$^{+/-}$ *t*(1, 12) = 0.33 , *P* = 0.75 *WT* vs *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *t*(1, 12) = -0.20 , *P* = 0.84	*WT n* = 4 *Nlgn3*$^{+/-}$ *n* = 4 *Cyfip1*$^{+/-}$ *n* = 4 *Nlgn3*$^{+/-}$*Cyfip1*$^{+/-}$ *n* = 4
Figure 27	N/A	N/A	Generalised linear model, main effect of sex: *t*(1,11) = -7.27 , *P* < 0.001, main effect of genotype: *t*(1,11) = -0.99, *P* = 0.32, sex and ge notype interaction: *t*(1,11) = -0.51, *P* = 0.61 Tukey HSD: *Cyfip1*$^{+/-}$ F vs *Cyfip1*$^{+/-}$ M *t*(1, 326) = 7.28, *P* < 0.001 *Cyfip1*$^{+/-}$ F vs *WT* F *t*(1, 326) = 0.10, *P* = 0.75 *Cyfip1*$^{+/-}$ F vs *WT* M *t*(1, 326) = 9.26, *P* < 0.001 *Cyfip1*$^{+/-}$ M vs *WT* F *t*(1, 326) = -6.27, *P* < 0.001 *Cyfip1*$^{+/-}$ M vs *WT* M *t*(1, 326) = 1.61, *P* = 0.37 *WT* F vs *WT*$^{-}$ M *t*(1, 326) = 8.20, *P* < 0.001	*Males:* *WT n* = 4 *Cyfip1*$^{+/-}$ *n* = 4 Females: *WT n* = 4 *Cyfip1*$^{+/-}$ *n* = 4