An investigation into the effect of the social environment on the phenotypes of mice lacking Nlgn3 and their wild-type littermates

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A thesis submitted to Cardiff University in accordance with the requirements for the degree of Doctor of Philosophy in the discipline of Neuroscience

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April 2019

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In memory of my Grandad

Acknowledgements

Firstly, I would like to thank my supervisor, Stéphane. I am grateful for the opportunity to study for a PhD in your lab, for all your enthusiasm and support, and for all the advice that you have given me over the years that has not only helped me through my PhD, but that will continue to help me throughout my career. Thank you for being a great supervisor.

This work was funded through the Welsh government's Sêr Cymru programme. I would like to thank them for making this work possible.

I would like to extend my gratitude to those who helped to make the work for my PhD possible. I am grateful to the JBOIS staff for the care and maintenance of my mice. I am also grateful to Angela Marchbank of the Genome Hub and Robert Andrew of the Data Hub, for their expert knowledge and assistance with the RNA sequencing and analysis.

Next, I would like to thank my colleagues, including Professor Yves Barde and his lab, Dr Isabel Martinez Garay and her lab, and all former lab members. I would like to thank you all not only for the intellectually stimulating lab environment that has helped me to grow as a researcher, but also for a lab environment which was enjoyable, inspirational, and unforgettable. In particular, I would like to thank my office-mates and comrades in the adventure that is the PhD: Ellen, Jess, and Natalia. I couldn't have asked for more wonderful colleagues.

A special thanks goes to my partner, Lee, for being a source of encouragement and motivation over the course of my PhD; all the little things you have done to cheer me on are much appreciated. I would like to thank Poe for bringing me joy. And finally, I would like to thank my parents and my brother for all their inspiration, support, and for always believing in me.

Abstract

The main focus of this thesis was to investigate if the social environment may be a factor leading to aberrant behavioural changes in the *Nlgn3* knockout mouse, a model of autism spectrum disorder (ASD). Initial observations revealed that mixed genotype housed male *Nlgn3* knockout mice and wild-type mice displayed deficits in their social behaviours and anxiety-related behaviours compared to male *Nlgn3* knockout mice and wild-type mice in single genotype housed conditions. Selective re-expression of *Nlgn3* in parvalbumin-expressing cells in transgenic mice rescued their social behaviour and alleviated the phenotypes of their wild-type littermates, thus further indicating that the social behaviour of *Nlgn3* knockout mice has a direct and measurable impact on their wild-type littermates' behaviour. Additionally, the social environment was confirmed to alter the transcriptome profile of the striatum and the hippocampus, as assessed by RNA sequencing. Following this, the question of the sex-specific sensitivity to the social environment was assessed by studying female *Nlgn3* knockout mice and their littermates. This revealed that the social environment is a factor influencing the behaviour of female *Nlgn3* knockout and wild-type mice. Finally, the potential generalization of the findings to other models for ASD was addressed, by assessing if Neuroligin-3 interacts with other proteins that are known risk factors for ASD. Together, these results demonstrate that *Nlgn3* knockout male and female mice show behavioural and physiological changes dependent on the social environment and that this is mediated by parvalbumin-expressing cells. Furthermore, the interaction of Neuroligin-3 with other ASD-related proteins suggest that other mouse models of ASD may also show this sensitivity to the social environment.

Abbreviations

ANOVA	Analysis of variance
ASD	Autism spectrum disorder
BCA	Bicinchoninic acid protein assay
bp	Base pair
Cb	Cerebellum
cDNA	Complementary deoxyribonucleic acid
cm	Centimetres
CT	Threshold cycle
Cx	Cortex
CYFIP1	Cytoplasmic FMRP interacting protein 1
C3	Complement component 3
C4	Complement component 4
df	Degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
F	F-statistic
FMRP	Fragile X mental retardation protein
g	G-force
GWAS	Genome wide association study
HRP	Horseradish peroxidase
Hz	Hertz
IRES	Internal ribosome entry site
LDS	Lithium dodecyl sulfate
MeCP2	Methyl-CpG-binding protein 2
MGH	Mixed genotype housing
mGluR	Metabotropic glutamate receptor
mPFC	Medial prefrontal cortex
mPOA	Medial preoptic area
mRNA	Messenger ribonucleic acid
ms	Millisecond/s

NAc	Nucleus accumbens
NCKAP1	Non-catalytic region of tyrosine kinase adaptor protein 1 associated protein 1
NMDAR	N-Methyl-D-aspartic acid receptor
P	P-value
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PC1/2	Principal Component 1/2
PFC	Prefrontal cortex
Pvalb	Parvalbumin
PV-MGH	MGH housing of mice expressing cre under the *Pvalb* promotor
PD(1-100)	Postnatal days (1-100)
qPCR	Quantitative real-time polymerase chain reaction
M	Mole
mg	Milligram/s
ml	Millilitre/s
mM	Millimole/s
ms	Millisecond/s
ng	Nanogram/s
Nlgn3$^{+/-}$ (H-KO)	*Nlgn3*$^{+/-}$ (heterozygous with knockout (*Nlgn3*$^{-/-}$))
Nlgn3$^{+/-}$ (H-WT)	*Nlgn3*$^{+/-}$ (heterozygous with wild-type (*Nlgn3*$^{+/+}$))
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
S	Second/s
SE	Standard error of the mean
SGH	Single genotype housing
Str	Striatum
USV	Ultrasonic vocalisation
VPA	Valporic acid
VTA	Ventral tegmental area

WAVE1	Wiskott-Aldrich syndrome protein family verprolin homologous protein 1
18S	18S ribosomal RNA
3’UTR	Three prime untranslated region
µl	Microlitre/s
µM	Micromole/s

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Chapter 1: Introduction

1.1. Nlgn3 knockout mice as a model of Autism Spectrum Disorder

Autism spectrum disorders (ASDs) lead to abnormal and impaired social interactions. ASD has been understood to have genetic and environmental influences, and from this knowledge, animal models have been developed to study its pathophysiology. These include animal models that have missing ASD-related genes, such as *Nlgn3*. In this section, the literature relating to ASD and *Nlgn3* knockout mice will be reviewed. Finally, outstanding questions and factors that need to be considered in the assessment *Nlgn3* knockout mice will be discussed.

1.1.1. Autism Spectrum Disorders

ASDs are characterized by a core triad of behavioural symptoms including impaired communication, restricted and repetitive behaviour, and impaired social interactions, however additional signs and symptoms may be present such as sleep disturbances, irritability, and cognitive problems (Tanguay et al 1998, Frazier et al 2012, American Psychiatric Association 2013). Symptoms of ASD become apparent during the first few years of development, with the child with ASD either never developing social skills and speech, or developing normally before undergoing a period of regression and losing the social skills and speech initially developed (reviewed in: Yirmiya and Charman 2010). It is robustly reported that there is a gender difference in the diagnosis of ASD, with its diagnosis ranging from a 4:1 ratio of diagnoses of males to females in those with normal intellectual quotients, to as high as 9:1 in high functioning ASD, or as low as 1.3:1 in intellectual disability (Lord, Schopler and Revicki 1982, Tsai and Beisler 1983, Fombonne 2003, Fombonne 2005, Baird et al 2006, Mandy et al 2011, Brugha et al 2011).

ASD has been demonstrated to be a highly genetic disorder; the concordance rate of ASD in monozygotic twins is higher (77.0-95.7%) than in dizygotic twins (21.5-33.0%), revealing that while the environment of the children is assumed to be equal, the genetics contribute in majority to the onset of ASD (Ritvo et al 1985, Ronald et al 2006, Hallmayer et al 2011). Mutations and deletions of genes have been associated with the onset of ASD. Penetrance of ASD associated genes has been shown to vary, from fully penetrant forms that may have associated symptoms and syndromes, sometimes referred to as being ‘syndromic’, to high to

low penetrance genes that are 'non-syndromic'. Syndromic forms include Rett syndrome or fragile X syndrome, which are diagnosed early in development and have many other symptoms including intellectual disability and physical changes (Fu et al 1991, Amir et al 1999). Genetic risks for ASD are typically identified through individual case studies or genome wide association studies (GWAS) (Jamain et al 2003, Durand et al 2007, Weiss et al 2009, Glessner et al 2009, Jackson et al 2009, Chaste et al 2015). Finally, in rarer cases, chromosomal deletions, duplications and copy number variants have also been associated with the onset of ASD (reviewed in: Devlin and Scherer et al 2012). While the genetics contribute in majority to the onset of ASD, environmental risk factors for developing ASD have also been identified, such as infection, antidepressant use, or exposure to pollutants during pregnancy (Atladóttir et al 2010, Croen et al 2011, Raz et al 2014). From this knowledge of the genetics and the environmental risk factors for ASD, mouse models of ASD have been developed to better understand the pathophysiology underlying the disorder.

1.1.2. Neuroligin-3

One gene that has been associated with ASD in humans, and subsequently has had mouse models developed to study it, is *NLGN3*. *NLGN3* an X-linked gene, located on the Xq13 locus, and encodes the protein Neuroligin-3. Both mutations and deletions of *NLGN3* have been associated with ASD in humans. It was initially observed that two brothers, one diagnosed with autism, and the other diagnosed with Asperger's, had a R451C mutation in Neuroligin-3 (Jamain et al 2003). The mutation was later shown to lead to endoplasmic retention of Neuroligin-3, leading to a 90% decrease in expression of Neuroligin-3 at the synapse (Comoletti et al 2004). This association of Neuroligin-3 mutations and ASD has also been observed in a GWAS of a Chinese Han cohort. It was observed that there was an enrichment of common variants of *NLGN3* in male, but not female, participants with ASD (Yu et al 2011). Furthermore, the association of *NLGN3* with ASD is not limited to mutations; lack of expression of *NLGN3* has also been shown to be associated with ASD in humans (Levy et al 2011, Sanders et al 2011, Gilman et al 2011).

Neuroligin-3 has been shown to be expressed within the brain, but not peripheral tissues (Ichtchenko et al 1996, Budreck et al 2007). In the brain, Neuroligin-3 has been shown to be expressed post-synaptically and not pre-synaptically at the synapses of both excitatory and inhibitory neurones (Budreck et al 2007). mRNA for *Nlgn3* has been observed in glia cells, some gliaomas have been shown to excrete Neuroligin-3, and cultured astrocytes of wild-

type, but not *Nlgn3* knockout mice, have been shown to secrete Neuroligin-3, indicating that Neuroligin-3 is present within glia cells (Gilbert et al 2001, Venkatesh et al 2015, Venkatesh et al 2017, Li et al 2018).

Altered dendritic and synaptic morphology has been observed in post-mortem studies of brains from humans with ASD or ASD-related syndromes (Hinton et al 1991, Belichenko et al 1994, Irwin et al 2001, Chapleau et al 2009, Broek et al 2014), and many mouse models of ASD also show dendritic and synaptic abnormalities (Comery et al 1997, Auerbach et al 2011, Chapleau et al 2012, Pathania et al 2014, Tang et al 2014). Consistent with the studies of the synaptic properties of post-mortem brain tissue of humans with ASD, and the synaptic properties of animal models of ASD, Neuroligin-3 has been shown to be important in regulating synaptic properties. At the synapse, Neuroligin-3 has been shown to function as a dimer, and experiments using mutated Neuroligin-3 that is unable to form dimers at the synapse leads to abnormal synaptic morphology, including altered spine turnover, decreased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor mediated currents, and altered expression of synaptic proteins (Shipman et al 2012). Over-expression of Neuroligin-3, but not of the mutated form of Neuroligin-3, led to increased synaptic puncta within cultured rat hippocampal neurones (Chih et al 2004), further showing that Neuroligin-3 regulates synaptic properties.

This has been further demonstrated through deletion of *Nlgn3*. Deletion of *Nlgn3* has been shown to disrupt endocannabinoid signalling leading to aberrant GABAergic signalling of hippocampal cholecystokinin expressing interneurons (Földy et al 2013). Additionally, specific knockout of *Nlgn3* within dopamine receptor 1 expressing neurones of the nucleus accumbens (NAc) leads to a reduction in inhibitory post-synaptic potentials (Rothwell et al 2014). Deletion of *Nlgn3* within *Pvalb*-expressing cells of the hippocampus leads to a reduction of post-synaptic N-Methyl-D-aspartic acid receptor (NMDAR) -mediated synaptic transmission and increased pre-synaptic glutamate release due to aberrant pre-synaptic metabotropic glutamate receptor 3 (mGluR3) signalling, together causing an abnormal theta and gamma rhythm, both of which have been associated with ASD (Dickinson et al 2015, Maxwell et al 2015, Larrain-Valenzuela et al 2017, Polepalli et al 2017). Furthermore, mGluR1 and mGluR5 dysfunction and, consequently, long term synaptic depression, has also been observed in many mouse models of ASD (reviewed in D'Antoni et al 2014). Consistent with this, there is a deficit in mGluR1 dependent long-term depression within the cerebellum of

Nlgn3$^{y/-}$ mice (Baudouin et al 2012). These studies show that *Nlgn3* mediates neuronal connectivity and electrophysiological properties of neurones.

The pathophysiology underlying mutations and deletions of Neuroligin-3 likely share some similar pathophysiological mechanisms associated with other ASD-related genes, as Neuroligin-3 has been shown to interact with other ASD-related proteins. Using coimmunoprecipitation experiments, Neuroligin-3 has been shown to associate with three transcripts of the ASD-related protein, Neurexin (Ichtchenko et al 1996, reviewed in Südhof 2008). Furthermore, it has been shown that Fragile X mental retardation protein (FMRP) binds to *Nlgn3* mRNA, and in mice lacking FMRP, levels of Neuroligin-3 have been shown to be increased (Chmielewska et al 2018). These studies show that Neuroligin-3 shares a common pathway, and likely a common mechanism of pathology, with other ASD-related proteins.

1.1.3. Behavioural analysis of Nlgn3 knockout mice

Many mouse models of ASD successfully capture the social behavioural abnormalities seen in ASD. These include changes in both juvenile and adult social interactions and in both male and female mice (Cheh et al 2006, DeLorey et al 2008, Nakatani et al 2009, Yang et al 2012, Bariselli et al 2016). Some behavioural analysis of *Nlgn3* knockout mice has been completed, predominantly in adult male mice.

Adult male *Nlgn3*$^{y/-}$ mice that have been compared to their *Nlgn3*$^{y/+}$ littermates show decreased levels of courtship ultrasonic vocalisation (USV) and increased latency to call when presented to an unfamiliar female in oestrous, indicating a deficit in courtship behaviours (Radyushkin et al 2009, Fischer and Hammerschmidt 2011). Similarly, *Nlgn3*$^{y/-}$ mice spent less time with female bedding compared to *Nlgn3*$^{y/+}$ mice (Dere et al 2018). Another social behaviour abnormality is that *Nlgn3*$^{y/-}$ mice show decreased time spent with a novel mouse on the three-chamber test when compared to their *Nlgn3*$^{y/+}$ littermates (Radyushkin et al 2009). Additionally, *Nlgn3*$^{y/-}$ mice show decreased socially conditioned place preference and decreased interaction with juvenile male mice (Bariselli et al 2018). Together, this indicates that *Nlgn3*$^{y/-}$ mice have both social behaviour and courtship behaviour abnormality.

The decreased USV and social interest could be explained by these not being encoded as being salient. However, the sucrose preference of *Nlgn3*$^{y/-}$ mice and their *Nlgn3*$^{y/+}$ littermates was observed to be similar (Radyushkin et al 2009), suggesting that there may not be a

difference in their perception and motivation for salient stimuli. The lack of interest for the novel mouse and the conditioned place preference could be explained by a lack of memory, but the learning on the morris water maze of *Nlgn3*$^{y/-}$ mice and their *Nlgn3*$^{y/+}$ littermates appears to be largely the same (Radyushkin et al 2009). Similarly, novel object recognition does not appear to be altered in *Nlgn3*$^{y/-}$ mice (Bariselli et al 2018). These results indicate that the deficits in memory and novelty appear to be social specific. Interestingly, contextual fear conditioning revealed that *Nlgn3*$^{y/-}$ mice spent less time freezing than their *Nlgn3*$^{y/+}$ littermates, however, as will be discussed, *Nlgn3*$^{y/-}$ mice have been shown to be hyperactive compared to their *Nlgn3*$^{y/+}$ littermates, likely indicating that the decreased freezing is not due to memory problems.

In the open field arena, *Nlgn3*$^{y/-}$ mice show hyperactivity compared to their *Nlgn3*$^{y/+}$ littermates (Radyushkin et al 2009, Rothwell et al 2014). Additionally, the anxiety levels of *Nlgn3*$^{y/-}$ mice compared to their *Nlgn3*$^{y/+}$ littermates has been assessed by quantifying thigmotaxic behaviour in the open field arena. Both studies reported that *Nlgn3*$^{y/-}$ mice do not show altered thigmotaxic behaviour compared to their *Nlgn3*$^{y/+}$ littermates (Radyushkin et al 2009, Rothwell et al 2014). Similarly, it was reported that elevated plus maze open arm exploration was not different between *Nlgn3*$^{y/-}$ mice and their *Nlgn3*$^{y/+}$ littermates, suggesting anxiety as measured on the elevated plus maze was also not different, however, nose poke behaviour was increased indicating a possible decreased anxiety (Radyushkin et al 2009). Finally, *Nlgn3*$^{y/-}$ mice were reported to show decreased digging for a food reward (Radyushkin et al 2009). The authors of this work speculate that this may be indicative of an olfactory deficit, however, it is worth noting that digging behaviour of mice can be impacted by anxiety and can also be a measure of repetitive behaviours. These behaviours are summarised in **figure 1.1**.

1.1.4. Behavioural phenotypes of Nlgn3$^{y/-}$ *mice with characterised neuronal correlates*

Some studies have investigated the impact of *Nlgn3* deletion within specific neuronal networks, and its impact on the behaviours of mice. It has been shown that Neuroligin-3 knockout mice have deficits in their long-term depression in the cerebellum that are associated with increased time for steps on the Erasmus ladder. Re-expression of Neuroligin-3 in the Purkinje cells of the cerebellum restores these phenotypes. Furthermore, deletion of Neuroligin-3 leads to abnormal purkinje cell projections which are also restored when *Nlgn3* is re-expressed in Purkinje cells (Baudouin et al 2012). Neuroligin-3 deletion in Purkinje cells

only also leads to hyperactivity in mice while *Nlgn3* deletion with all parvalbumin (*Pvalb*)-expressing cells, including the Purkinje cells, led to decreased activity within the open field arena, indicating that purkinje cells control specific aspects of the phenotypes of the mice (Rothwell et al 2014). Furthermore, it has been shown that *Nlgn3* deletion within *Pvalb*-expressing cells of the CA1 region of the hippocampus leads to impaired contextual fear extinction, but not fear conditioning (Polepalli et al 2017). These data show that *Pvalb*-expressing cells are key in the control of some of the phenotypes of *Nlgn3* knockout mice. The striatal circuitry has also been implicated in the behaviours of *Nlgn3* knockout mice. Deletion of Neuroligin-3 in the dopamine receptor 1 expressing cells of the nucleus accumbens (NAc) leads to enhanced rotorod performance (Rothwell et al 2014). Similarly, knockdown of *Nlgn3* within dopaminergic neurones of the ventral tegmental area (VTA) leads specifically to decreased social interactions with novel and non-novel mice, and decreased interest for social novelty as measured by the three-chamber test (Bariselli et al 2018). These data demonstrate that *Nlgn3* expression within multiple neuronal populations contributes to the phenotypes of the mice. The brain regions associated with phenotypes in *Nlgn3*$^{y/-}$ mice are summarised in **figure 1.1**.

Behavioural phenotypes of *Nlgn3*$^{y/-}$ mice relative to their *Nlgn3*$^{y/+}$ littermates

- Impaired social memory in the three-chamber test
- Decreased social conditioned place preference
- Decreased courtship ultrasonic vocalisations
- Decreased preference for female scented bedding
- Decreased digging for buried food
- No change in sucrose preference
- No change in novel object recognition and morris water maze learning
- No change in elevated plus maze or thigmotaxis behaviour
- Increased nose poking behaviour in the hole board test
- Decreased freezing in contextual fear conditioning task
- Hyperactivity in the open field arena
- Altered motoric action on the Erasmus ladder and enhanced motor learning on the rotarod

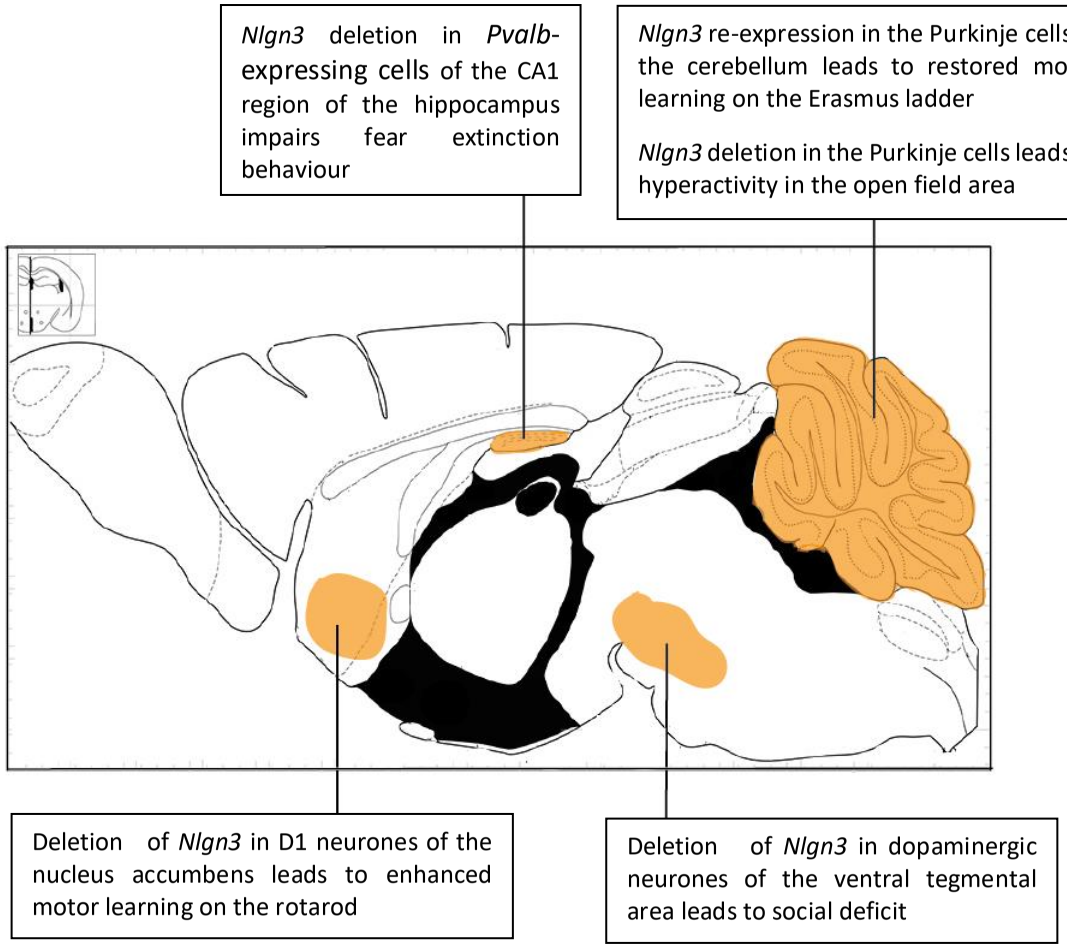


Figure 1.1. A summary of the known behavioural phenotypes of *Nlgn3*$^{y/-}$ mice, and, where known, their neuronal correlates. Image adapted from The Mouse Brain Atlas in Stereotaxic coordinates by Paxinos and Franklin, second edition, elsevier academic press.

1.1.5. Behavioural phenotypes of female Nlgn3 knockout mice

Finally, the behavioural phenotypes of *Nlgn3*$^{+/-}$ mice and *Nlgn3*$^{-/-}$ mice remained largely unassessed or not assessed at all, respectively. One study characterised the preference of *Nlgn3*$^{+/-}$ mice compared to *Nlgn3*$^{+/+}$ mice to bedding taken from either male cages or clean bedding. This work revealed that *Nlgn3*$^{+/-}$ mice do not show a preference to spend time with the bedding from male cages, while *Nlgn3*$^{+/+}$ mice do, suggesting a phenotype relating to social behaviour or social olfaction may exist (Dere et al 2018). However, the behaviours of *Nlgn3* knockout female mice need further characterisation.

1.1.6. Considerations when investigating Nlgn3 knockout mice

Given the behavioural and synaptic properties, *Nlgn3* knockout mice seem like an ideal model to study ASD-related behaviours. However, some considerations need to be taken into account. Like in the human condition, sex differences in behaviours of mice are observed in female mouse models of ASD. For example, male but not female 16p11.2 deletion mice show sleep disturbances, and male *Ehmt1* mice but not female *Ehmt1* mice show decreased social play at 28-32 days old (Balemans et al 2010, Angelakos et al 2017). A similar observation has been made in environmental models of ASD; two studies looking at the effect of letrozole or valproic acid (VPA) exposure in utero in rats observed a sexual dimorphism in the social behaviours of the rats (Schneider et al 2008, Xu et al 2015). In *Nlgn3* knockout mice this is further complicated by *Nlgn3* being X-linked, meaning that while male mice are either *Nlgn3*$^{y/+}$ or *Nlgn3*$^{y/-}$, females can either be *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, or *Nlgn3*$^{-/-}$. This means that female *Nlgn3*$^{+/-}$ mice have altered gene dosage that is further complicated by the x-inactivation of one of the x chromosomes. The X-linked nature of the gene is important to consider, given that it has been theorised that X-linkage of ASD-related genes have been associated with the sex difference seen in ASD (Reviewed in Baron-Cohen et al 2011). These studies highlight the importance of assessing the behaviours of both male and female models of ASD.

Furthermore, it is frequently found that mouse models of ASD lead to consequences on behaviours that can be interpreted as being related to social hierarchy. The social hierarchy is defined as individual mice within a social group occupying dominant, subdominant, and submissive positions (Lindzey et al 1961, Wang et al 2011, Van den Berg et al 2015, So et al 2015). The position within the social hierarchy impacts the behaviours and physiology of mice; this will be discussed at greater length in **section 1.2.4**. More dominant mice show

increased numbers of agonistic displays, increased courtship USV, and increased numbers of wins in the tube test, a test in which the submissive mouse gets pushed out of a tube by the dominant mouse (Lindzey et al 1961, Wang et al 2011, Van den Berg et al 2015, Stagkourakis et al 2018). Mouse and rat models of ASD have been shown to have phenotypes relating to social submission, such as decreased aggression, decreased courtship USV, and decreased numbers of wins in the tube test (Spencer et al 2005, Cheh et al 2006, Jamain et al 2008, Wöhr et al 2011, Yang et al 2015, Saxena et al 2018). These results indicate that social dominance behaviours of rodent models of ASD may be commonly disrupted. Consistent with this, it was observed that *Nlgn3*$^{y/-}$ mice have decreased courtship USV (Radyushkin et al 2009, Fischer and Hammerschmidt 2011), possibly indicating that they are socially submissive. Furthermore, given that the hierarchy involves the interaction of mice within that social environment, it is important to consider how the presence of mouse models with socially submissive phenotypes could impact the group.

1.1.7. Summary and outstanding questions

Nlgn3 knockout mice share many of the core pathologies seen in other mouse models and in those with ASD, indicating that *Nlgn3* mice successfully recapitulate aspects of the disorder. While the behavioural analyses of *Nlgn3*$^{y/-}$ mice gives an insight into the impact of *Nlgn3* deletion on these phenotypes, some questions remain outstanding. The analysis was only thoroughly complete in male mice, however, given the sex bias seen in ASD, and the sexual dimorphisms seen in some animal models of ASD, it is important to assess how deletion of *Nlgn3* impact the behaviours of female mice. Additionally, the decreased levels of USV of *Nlgn3*$^{y/-}$ mice could indicate that the *Nlgn3*$^{y/-}$ mice have a socially submissive phenotype, however other measures of social hierarchy have not been taken to confirm this. Given the changes in the social and, possibly dominance, behaviours of *Nlgn3* knockout mice, it is of interest to know whether *Nlgn3* knockout mice may be impacting the behaviours of their wild-type littermates. As will be discussed in the next section, the social environment is key in shaping the behaviours and physiology of mice. Therefore, it is possible that the inclusion of *Nlgn3* knockout mice could lead to disruption of the behaviours of their littermates.

1.2. The influence of the social environment on behaviour and physiology

Mice are social animals. They form social groups where they interact and react to each other. The social environment is an important factor to consider when looking at the behaviour and physiology of mice, as mice show alterations in their behaviour and physiology dependent upon their social experiences. This is particularly important within the laboratory setting, where laboratory mice are typically housed in small social environments, and therefore the social environment could possibly be influencing factors relating to experimental outcomes. In this section, the literature relating to how to the social environment can be an important factor in mediating the behaviours and physiology of mice will be reviewed, with some attention being given to other species where necessary.

1.2.1. The effect of early life social manipulations

One of the most effective ways to show that social housing is key in mediating the behaviours and physiology of mice is through social isolation and social enrichment paradigms. The effects of the early life social environment can be explored through early life maternal separation or communal nesting. This will be discussed in this section.

Early in development, mice are dependent upon the dam for warmth and nutrition. Manipulations of the pup-dam relationship have demonstrated that social interaction with the dam can lead to long term behavioural, biochemical, and morphological changes. Paradigms utilising maternal separation demonstrate that maternally separated pups show decreased hippocampal dependent learning and memory, increased depressive behaviours, increased locomotion, aberrant stress response, and increased adult subordinate behaviours (Romeo et al 2003, MacQueen et al 2003, Macrì et al 2004, Millstein and Holmes 2007, Rice et al 2008, Curley et al 2008, George et al 2010, Benner et al 2014). Similarly, in male rats, maternal separation has been shown to have long-lasting effects on their behaviours, including altered stress response, and in addition to this, a correlation between maternal care behaviours, such as licking, and adult anxiety of the pups has been observed (Caldji et al 1998, Huot et al 2004, Menard et al 2004).

In contrast to early life separation, early life enrichment can also be used to assess how the early life social environment leads to changes in behaviour and physiology of mice. In one such experiment, the effect of communal housing compared to normal housing of pups was assessed. Communal housing consists of housing numerous dams and their pups together,

compared to the 'standard' condition, of one dam and her litter only. This work only assessed the behaviours of the male mice, in adulthood. It was observed that mice from communal housing conditions went on to develop, as adult mice, more 'pro-social' behaviours of social investigation, allogrooming, and allosniffing, furthermore the social hierarchy was established more rapidly (D'Andrea et al 2007, Branchi, D'Andrea, Fiore et al 2006). Moreover, it has been observed that adult male mice that were raised in communal housing conditions during development showed increased depressive like behaviours, increased anxiety behaviours, and altered hippocampus neurogenesis, giving further evidence to the notion that these changes are affecting the emotional reactivity of the mice (Branchi and Alleva 2006, Branchi, D'andrea, Sietzema et al 2006). These studies show that early life social enrichment can influence the behaviours and social structures of the mice in adulthood. These early life manipulations are important as they demonstrate how small alterations of the early life social environment can shape the future behaviours and physiology of the mice in adulthood.

1.2.2. Social isolation and instability in adolescent and adult mice

Social environment manipulations can also be applied later in development, either post-weaning in adolescence or in adulthood. The benefit of utilising social environment manipulations in adolescence and adulthood is to determine if the social environment influences the behaviours of adult mice.

Adult male mice that were socially isolated following weaning show increased levels of social interaction with the novel mouse in the three-chamber paradigm (Naert et al 2011). This would suggest that either the social interest or the social memory of these mice has been impacted by the isolation. Post-weaning social isolation has also been shown to lead to increased levels of aggressive behaviours of mice (Ibi et al 2008), indicating that the social environment also shapes aggressive behaviours. Furthermore, the study of Naert et al (2011) also looked at the locomotive behaviour of the mice over 23 hours and observed alterations of the socially isolated mice in comparison to group housed mice, in particular in the dark phase. A similar finding of increased open field activity has been observed in socially isolated male rats (Ashby et al 2010, Schiavone et al 2009, Schiavone et al 2012).

Changes relating to emotional processing are also observed in socially isolated male mice. Adult male mice were socially housed or socially isolated for 7 weeks, in either 'enriched' or 'impoverished' conditions to assess how social housing impacts emotional reactivity to the

material environment. Enriched conditions included having additional objects, such as tubes and dens, while 'impoverished' conditions included only a nest. It was observed that environmental enrichment leads to reduced anxiety, as measured by thigmotaxis behaviour, in the socially housed but not socially isolated mice (Chourbaji et al 2005). This suggests that the social environment is an important factor in mediating the behavioural response of mice to external stimuli.

Anatomically, social isolation of adult male mice leads to decreased myelination within the prefrontal cortex (PFC) that corresponds to decreased levels of social interaction. When the myelination was reversed in these mice using pharmacological intervention, the decreased levels of social interaction were shown to be reversed (Liu, Dupree, Gacias et al 2016). In other studies, in socially isolated adult male and female mice, it was revealed that the structural connectivity changes in brain regions in male and female mice correlated with behavioural abnormalities in fear conditioning and open field locomotive behaviours (Liu, Li, Edwards et al 2016). These studies show that social isolation leads to neurological change that corresponds with behavioural change.

Furthermore, the social environment of mice can have profound effects on other pathophysiological processes. It has been demonstrated that incision wound healing was delayed in two species of mice that had been socially isolated and had stress induced, compared to those that are stressed and in group housed conditions. This shows that housing impacts more general pathophysiological processes (Glasper et al 2005). Similarly, social housing has also been shown to lead to increased recovery from traumatic head injury in mice (Doulames et al 2015). Importantly, investigation into how the quality of social housing can impact healing has been explored. It has been demonstrated that in adult male mice that had been given an artery occlusion as a model of stroke, mice that were co-housed with a healthy partner had significantly increased recovery rates compared to isolated mice. Interestingly, mice that were co-housed with a partner that had also received an artery occlusion did not recover as well as mice that were co-housed with a healthy partner, suggesting that individual factors relating to the partner mouse can lead to different outcomes for the subject (Venna et al 2014). These studies again indicate that not only the presence of the social environment is important, but the manifestation of the social environment itself also seems to be key in regulating the outcomes of the mice within that group.

The manifestation of the social environment in adolescent male mice has been explored. A social instability paradigm has been shown to lead to alterations in behaviour and physiology; the social instability paradigm consists of alternating the mice within a specific social environment, ensuring any added mice are always novel. It was observed that mice subjected to the social instability paradigm demonstrate alteration to factors that mediate the stress response, such as a decrease in hippocampal levels of mineralocorticoid receptor and glucocorticoid receptor mRNAs, receptors that mediate the stress response and changes in the adrenal and thymus glands, and corticosterone levels. Consistent with this is the observation that the mice that were subjected to the social instability paradigm also show decreased time spent in the open arms of the elevated plus maze and increased levels of novelty supressed feeding, indicating an increase of anxiety (Schmidt et al 2007, Sterlemann et al 2008). These studies are of importance, as they suggest that the manifestation of the social environment may be as influential on the behaviours and physiology of the mice as the impact of social isolation.

These social isolation paradigms in adolescent and adult mice and rats inform us that there is no critical window for general behavioural and physiological change due to social housing in mice. They further demonstrate that the social environment is key in regulating normal physiological and behavioural processing of the mice. Furthermore, the experiments looking at the effect of the changing social environment, and the housing of the two stroke mice together, reveal that when it comes to the effect of social housing, the quality, and not just the quantity, of the social interactions appear to be important. Given the vast array of outcomes of the altered social environment, from behaviour, physiology and pathophysiology, and anatomical changes, it appears that social housing is a key factor in the assessment of experimental outcomes.

1.2.3. Sexually dimorphic responses to the social environment.

An important question that needs to be addressed is if the social environment impacts male and female mice equally. As will be discussed later, male mice form social hierarchies that impact the individuals within the groups. Male mice show higher levels of aggression towards unfamiliar males and show aggressive displays to other mice within their social hierarchies. In contrast to this, aggression in female laboratory mice is uncommon, typically only appearing during lactation (Uhrich et al 1938, Kudryavtseva 1991, Matsumoto et al 2005, Wang et al 2011). This suggests that the fundamental social strategies utilised by female mice

are different to male mice, and therefore female mice may not react in the same manner as male mice to the social environment.

A sexually dimorphic response to the relationship between maternal care and the behaviour of the pups has been observed. Maternal separation studies have shown that maternally separated male mice show increased anxiety in the open field arena and elevated plus maze, however only maternally separated female mice, when in diestrous, show increased anxiety in the open field arena (Romeo et al 2003). Regarding social behaviours, maternally separated female, but not male mice show increased time spent with a social partner in the three-chamber test and decreased time in the open arms of the elevated plus maze (Bondar et al 2018). A similar observation of increased social interaction in maternally separated female, but not male, rats has been observed (Farrell et al 2016). Furthermore, maternally separated male mice have been shown to have an increased latency to attack an unfamiliar male mouse, whilst, when lactating, maternally separated female mice have a decreased duration to attack an unfamiliar male mouse (Veenema et al 2007). Locomotion and memory processing have also been observed to have a sexually dimorphic response to maternal care. The offspring of female mice that are heterozygous for the gene *Peg3*, leading to defective maternal behaviours, have been assessed. It was observed that female, but not male mice, show altered locomotive reactivity to the open field arena as well as decreased reaction to the novel object in the novel object recognition test (Curley et al 2008).

Social isolation and social instability have also been shown to lead to a sexually dimorphic response. Socially isolated male and female mice have been assessed, revealing that there are decreased exploratory behaviours and increased locomotion of female, but not male mice (Palanza et al 2001). As previously discussed, a social instability paradigm was developed and used in adolescent male mice (Schmidt et al 2007, Sterlemann et al 2008). This paradigm was later applied to female mice, and the data were compared with previous studies. Some similarities to the observations made in the male mice were observed; like the males, the females showed increased corticosterone levels, increased adrenal and thymus weight, and increased anxiety, as measured by novelty supressed feeding. Some sexually dimorphic changes were observed; males showed unchanged levels of corticotropin-releasing hormone in the paraventricular nucleus of the hypothalamus, while in females this was decreased, and the males showed increased levels of vasopressin, decreased time in the open arms of the elevated plus maze, and decreased glucocorticoid receptor, in the females

these were unchanged (Schmidt et al 2010). These data suggest that female mice respond differently to the social environment, however, these experiments were conducted years apart, and the study of Schmidt et al (2010) does not contain male mice data as a control.

In rats, the impact of social isolation and crowding has been shown to have a sexually dimorphic response. Crowding leads to reduced corticosterone in female, but not male rats, while isolated male rats have decreased corticosterone, and isolated female rats have increased levels of corticosterone (Brown et al 1995). In a social instability paradigm, both male and female rats showed increased weight, decreased thymus size, unchanged corticosterone levels, while only females showed increased adrenal weight (Haller et al 1999). Behaviourally, sexually dimorphic changes in anxiety levels as measured by the elevated plus maze and adrenocorticotropic hormone following acoustic startle in socially isolated male and female rats have been recorded (Weiss et al 2004). Finally, these differential impacts of socially isolated male and female rats lead to altered longevity of bromodeoxyuridine staining, suggesting that neurogenesis in response to the social environment may also be sexually dimorphic (Westenbroek et al 2004).

All together, these studies demonstrate that male and female mice and rats can respond differently to the social environment, both behaviourally and physiologically. Therefore, when assessing the impact of the social environment, it is important to characterise its effect in females as well as males.

1.2.4. Impact of hierarchy on behaviours and physiology

A major factor in how the social environment can lead to a sexually dimorphic impact on the behaviour of mice is the formation of social hierarchies. Social hierarchies are a social structure seen across many species: humans, apes, pigs, mice, fish, birds, cockroaches, and lobsters, to name a few, all show versions of social hierarchy (Fielder et al 1965, Meese et al 1973, Bell et al 1978, Ejike et al 1980, Marler et al 1995, Sapolsky et al 2005, Zink et al 2008). Social hierarchies are well defined in male wild and laboratory mice, in which the individual mice take up positions as either the dominant, subdominant, and the submissive (Lindzey et al 1961, Van Loo et al 2001, Wang et al 2011, So et al 2015, Williamson, Lee and Curley, 2016, Williamson, Franks and Curley 2016, Van den Berg et al 2015, Horii et al 2017). Behaviourally, dominant mice show increased numbers of agonistic displays of chasing, and more rarely attacking, towards their littermates, especially when given new bedding possibly indicating a territorial behaviour (Wang et al 2011). Furthermore, they overgroom and pluck the whiskers

of their submissive littermates, known as the Dalia effect (Warne 1947, Wang et al 2011, Kalueff et al 2006).

Many assessments in mice, and other species, have revealed that depending on the position within the social hierarchy, numerous characteristics of the individual can be altered. Therefore, when understanding how the social environment contributes to the development of the characteristics of the individuals of that group, it is important to determine what role social hierarchy may be playing. Finally, before the discussion of the impact of hierarchy on the individuals, it is important to clarify that social hierarchy is different to social defeat paradigms utilised in some experiments. Social hierarchy is defined by the interactions of animals of a group known to each other, while social defeat typically involves interaction with an unfamiliar, and often aggressive, partner.

Behaviourally, dominant lab mice have been shown to have increased 'patrols' compared to submissive mice while submissive mice show avoidance behaviours of dominant mice (Crowcroft and Rowe 1963, Ely et al 1978) and dominant mice show increased territorial urination (Desjardins et al 1973, Wang et al 2011). Position within the social hierarchy impacts the distribution of time spent on activities, with dominant mice spending more time on a wheel, a rewarding stimulus for mice (Vargas-Pérez et al 2008, Vargas-Pérez et al 2009). Furthermore, dominant mice have priority over resources (Merlot et al 2004, Wang et al 2011), and show increased courtship behaviour towards females (Wang et al 2011).

The position in the social hierarchy has been shown to influence how mice react to threat from unfamiliar mice not from their own social hierarchy. In one study of mice that are assessed for dominance in the tube test, it has been shown that prior to social defeat, dominant mice showed higher levels of anxiety, and no changes in the locomotion, as measured by the elevated plus maze and thigmotaxis in the open field arena. Following chronic social defeat, both the dominant and submissive mice showed similar anxiety profiles, while the dominant mice showed a more pronounced increase in body weight. Baseline and post-chronic social defeat NAc metabolism were also observed to be dimorphic between dominant and submissive mice (Larrieu et al 2017). Of note, however, is that there are contrasting reports on whether dominant mice show increased anxiety. Another report shows that it is the submissive mice that spends less time within the open arms of the elevated plus maze, while no difference in open field thigmotaxis is observed between the dominant and submissive, and additionally the submissive shows increased helplessness in

the forced swim test compared to dominant male mice (Horii et al 2017). Consistent with the work of Larrieu et al (2017) is that locomotion within the open field activity arena has been shown to remain unchanged dependent upon hierachy (Horii et al 2017). The work of Larrieu et al (2017) assessed dominance of mice, that were group housed at 6 weeks old, using the tube test, while the work of Horii et al (2017) assessed the aggressive behaviours of mice that were group housed with each other at 6 weeks of age, possibly leading to the discrepancy observed in the results. The aggression could possibly be the submissive mouse challenging the dominant mouse in this case, and other factors such as food and territory size could also lead to this difference. Regardless, there appears to be a split in the behaviours of mice dependent upon if they are dominant or submissive.

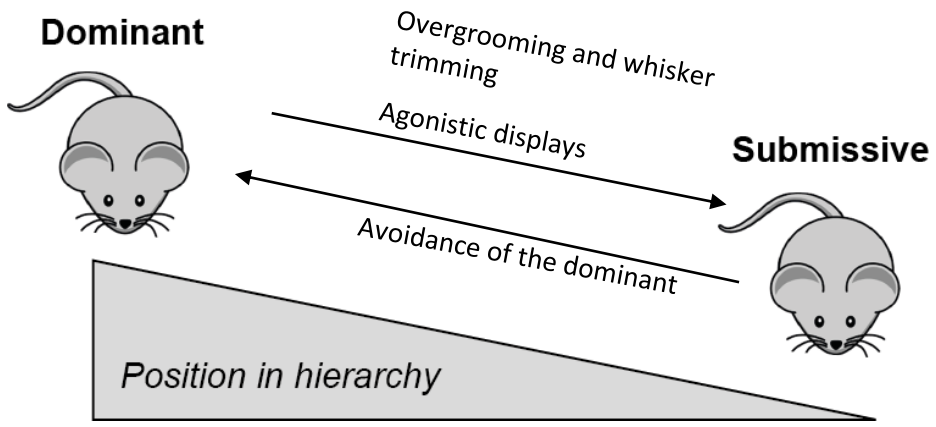
The position of the social hierarchy has been shown to impact the expression of mRNAs in the brain. In mice that were determined to be dominant or submissive through computerised tracking of behavioural interactions, quantitative real-time polymerase chain reaction (qPCR) was used to assess levels of mRNA dependent upon position within the social hierarchy. It was observed that levels of corticotropin-releasing hormone mRNA of the amygdala and medial preoptic area (mPOA), and glucocorticoid receptor mRNA and brain-derived neurotrophic factor mRNA levels of the hippocampus were all found to be increased in dominant mice (So et al 2015). Similarly, qPCR analysis of the hippocampus of dominant and submissive mice revealed that mRNAs for many serotonin receptors, tropomyosin receptor kinase B (TrkB), and cAMP responsive element binding protein 1 (Creb1) were all elevated in dominant mice, corticotropin-releasing hormone mRNA is decreased in the hypothalamus of dominant mice, and in both the hippocampus and striatum of submissive mice levels of synapsin IIb mRNA and synapsin IIb protein are increased (Horii et al 2017, Nesher et al 2015). Interestingly, as discussed in **section 1.2.1**, the early life social environment was shown to impact corticotropin-releasing hormone, glucocorticoid receptor and brain-derived neurotrophic factor levels of the mice in adulthood. Together, this suggests that like the early life environment, the position within the social hierarchy also leads to altered transcriptomes of the mice, implicating stress response and synaptic functioning in the mice, dependent upon position within the social hierarchy.

The position within the social hierarchy impacts physiology of animals. Dominant mice have altered properties relating to dopaminergic and adrenalin physiology, and they showed decreased levels of corticosterone when stressed. Similarly, submissive mice have been

observed to have increased corticosterone levels, that are comparable to the levels seen in socially isolated mice (Ely et al 1978, Doulames et al 2015). The impact of the position within the social hierarchy on the neurophysiology of primates has also been assessed. In macaques it has been observed that low position within the social hierarchy, as determined through aggression and grooming behaviours, leads to decreased occupancy of the dopamine 2 receptor, accompanied by increased cocaine self-administration (Morgan et al 2002). Similarly, in rhesus monkeys, it was observed that social rank influences the sedative effects of diazepam, with submissive monkeys showing increased sedative effects from diazepam. The social rank was determined by behaviours including aggression, food access, and retreating behaviours (Delgado et al 1976). A summary of the influence of hierarchy on the behaviours and physiology of mice can be found in **figure 1.2.**

While females of many species do show the existence of social hierarchies, in female laboratory mice these are much less well defined than in males. Female laboratory mice and wild-derived mice rarely fight with each other and typically only show aggression during lactation (Uhrich et al 1938, Hurst et al 1987, Hasan et al 2005, König et al 2012). By assessing behaviours of albino Han:NMRI female mice, such as pushing, climbing, and grooming, and correlating these behaviours with levels of corticosterone, it was observed that there was a correlation of dominance levels and corticosterone, with dominant mice having lower corticosterone levels. This is consistent with what is observed in male mice, indicating that female mice have social hierarchies (Schuhr et al 1987, Doulames et al 2015). Sniffing behaviour has indeed been related to social dominance within the social hierarchy of male mice, suggesting potentially some preservation of social structure between the two sexes (So et al 2015). In contrast to the work of Schuhr et al (1987), another study characterised the social hierarchies of wild-derived female mice, by scoring within cage agonistic behaviours. Out of the 10 groups observed, only 4 showed the presence of a consistently dominant female, with the majority of the females showing similar dominance levels to each other. In this study, no link was found between corticosterone levels and numbers of agonistic displays (Weidt et al 2018). This difference could potentially be explained by the different methods used to score hierarchy in the mice, and the different background of the mice used. Finally, in another study, the tube test determined that female mice, like male mice, have linear hierarchies, however, some differences in how the hierarchies manifest were observed (Van den Berg et al 2015). Altogether, these data do indicate that female mice may have social hierarchies or some type of group inequality.

Together, these studies demonstrate that social hierarchy mediates individual response at both a baseline level and when in superficial conditions. As the social hierarchy is a manifestation of the social environment, any altered social behaviours of individuals that could impact the hierarchy may have behavioural and physiological consequence on other members of that hierarchy. Many studies overlook the impact of the potential disruption to the social hierarchy of the mice. Therefore, when assessing the group behaviours of mice, the consequence of disruption to the social hierarchy needs to be considered given that social hierarchy has such a strong effect on behaviour and physiology.



Dominant Mice:

- ↑Increased courtship ultrasonic vocalisations towards female and territorial marking
- ↑ Increased access to resources
- ↑ Increased participation in rewarding behaviours (wheel running)
- ↑Increased glucocorticoid receptor mRNA and brain-derived neurotropic factor mRNA (hippocampus)
- ↓ Decreased corticotropin-releasing hormone mRNA (hypothalamus)

Submissive Mice:

- ↓ Decreased TrkB mRNA, serotonin receptors mRNA, and Creb1 mRNA (hypothalamus)
- ↑ Increased expression synapsin IIb mRNA and protein (hippocampus and striatum)
- ↓ Decreased corticotropin-releasing hormone mRNA (amygdala and medial preoptic area)
- ↑Increased corticosterone levels

Conflicting reports on impact of hierarchy on anxiety levels

Dominant Monkeys:

- ↑ Increased dopamine receptor 2 availability, and decreased addiction

Submissive Monkeys:

- ↑ Increased sedative effects of diazepam

Figure 1.2. Position within the social hierarchy impacts both behaviour and physiology.

1.2.5. Socially defeated mice

Social defeat typically involves interaction with a partner chosen due to high levels of aggressive behaviours, or interaction with a partner in an environment manipulated in order to induce aggressive behaviours. Social defeat is not equated to hierarchy, unless the relationship between the two mice is maintained allowing a hierarchy to be established. Nonetheless, social defeat is a useful tool in demonstrating how the interactions between mice can shape their behaviour and physiology. Male mice that have been socially defeated by another aggressive mouse show a long-lasting social withdrawal from unfamiliar mice, an effect that was mediated by brain-derived neurotrophic factor activity within the VTA and NAc (Berton et al 2006). Similarly, it has been demonstrated the mesolimbic dopaminergic system responds to social defeat in mice and rats (Tidey et al 1996, Krishnan et al 2007, Anstrom et al 2009, Cao et al 2010). These studies show further that the interactions of mice shape the behaviour and physiology and implicate the striatum as region that may be important in mediating the response to the social environment. Other brain regions have also been shown to have changes following social defeat in rats and mice, including the hippocampus, cortex, medial PFC (mPFC), and brainstem (Kang et al 1991, Krugers et al 1993, Wohleb et al 2011). Interestingly, RNA sequencing (RNAseq) analysis of socially defeated mice has shown that many autism related genes show altered expression within the hippocampus, striatum, raphe nucleus, and hypothalamus. Interestingly, *Nlgn3* was observed to be decreased in the hypothalamus of defeated mice (Kudryavtseva et al 2017). However, this work was only done on three mice per group, and the results were not validated in a larger sample size. Altogether, these studies demonstrate that even brief interactions with intruders are sufficient to lead to behavioural and physiological responses in mice and rats.

1.2.6. Transfer of emotional state in rodents

Some studies have assessed the transfer of emotion in rodents. It has been shown that mice are sensitive to the pain of peers, showing increased pain sensitivity when watching another mouse in pain (Langford et al 2006). It has also been demonstrated that when 'observer' C57BL/6 mice are conditioned to tones that precede the distress of another mouse, there is an observable increase in heart rate and freezing behaviour of the observer C57BL/6 mice upon hearing those tones again (Chen et al 2009). In rats, when a subject rat was foot-shocked and then housed with a rat 'observer' that had not received nor seen the foot-shock, the observer showed increased levels of c-fos in the amygdala that were comparable to those

of the foot shocked rat, and both had significantly elevated levels of amygdala c-fos levels compared to control non-foot shocked rats and their 'observer' rats (Knapska et al 2006). In similar studies, it has been observed that the behaviour of the 'observer' rats are also impacted, with the observer showing increased social exploration, and freezing behaviour (Knapska et al 2010, Bruchey et al 2010).

Studies in mice and rats also show that following a stressor, social housing can ameliorate some of the stress response. In mice, social housing after fear conditioning impacts the consolidation of the fear memory (Bredy and Barad 2009). Similarly, in rats that have been exposed to a foot shock, being housed with an unfamiliar rat alleviates some of the autonomic response, while being housed with a familiar rat alleviates both the autonomic and behavioural responses (Kiyokawa et al 2007).

These studies are important as they demonstrate that the emotional state of one animal within a social environment can impact the emotional state of another. Given that many models of neurodevelopmental disorders show behavioural change, including altered anxiety behaviour, it seems plausible that there could be a transfer of these phenotypes to other animals within that social environment.

1.2.7. The impact of mixed genotype housing on the behaviours of mice

Given that the social environment is key in shaping the behaviours of the rodents, it is important to consider how the presence of mice with altered behaviours impact the social behaviours of the mice within the group. Some studies have demonstrated that the genotype of the littermates does influence the phenotypes of the mice. It has been demonstrated that the cross fostering of genetically variable mice with genetically uniform mice leads to behavioural changes, known as indirect genetic effects, on the suckling behaviours of the genetically uniform mice (Ashbrook, Sharmin, Hager 2017). This is of importance as it demonstrates how the phenotypic expression of an individual's genome can lead to altered phenotypic expression in mice that have a different genome to themselves. That is to say, when mice are housed with other mice with the same genome as themselves, they may have different phenotypes compared to when these same mice are housed with mice with different genomes as themselves. This is an important factor to consider, as variations in genetic expression of mice could influence behavioural outcomes in the littermates that could have important implications in the analysis of all behaviours of animal's form that group.

Similar effects have been seen in mice that have phenotypes that are of interest to the neuroscience community. BTBR mice are an inbred mouse strain with social deficits and memory deficit and are used to model ASD. In one study, BTBR mice were either housed with a C57BL/6 mouse or BTBR mice. It was observed that BTBR mice housed with C57BL/6 showed increased sociability, but not normalised grooming behaviours, compared to BTBR mice housed with other BTBR mice (Yang et al 2011). Similarly, another study demonstrated that BTBR mice that were housed with C57BL/6 mice and subsequently underwent 'co-learning' with C57BL/6 mice showed improved performance on the novel object recognition task compared to BTBR mice that were trained alone or underwent co-learning with another BTBR mouse (Lipina et al 2013).

In another study, mice with estrogen receptor α (ERα) knockout were investigated. The mice were either housed as wild-types only, knockouts with wild-types, or knockouts only. At weaning, the mice were housed only with other mice the same genotype as themselves. Wild-type male mice from mixed genotype conditions showed significantly more aggression when faced with an unfamiliar mouse, while knockout male mice were not affected. Female knockout mice raised with other female knockout mice showed lower levels of social interaction (Crews et al 2009). These demonstrate that early life experiences with mice with altered social behaviours can impact the adult behaviours of the mice. Another example is that in male 16p11.2 deletion mice that were weaned into either wild-type only housing, mixed genotype housing, or heterozygous only housing, it was observed that the 16p11.2 deletion mice in mixed genotype housing showed courtship USV deficits, while 16p11.2 deletion mice in single genotype housing showed normal courtship USV (Yang et al 2015).

The impact of the presence of genetic models with altered dominance behaviours on the behaviours of their littermates has been assessed. Adult male *Cdkn1c*^{BACx1} mice have increased dominance when tested against unfamiliar mice (McNamara et al 2017). It has been shown that when *Cdkn1c*^{BACx1} mice are housed with wild-type littermates there is increased hierarchy destabilisation and increased fighting compared to cages of wild-type mice that are not housed with *Cdkn1c*^{BACx1} littermates (McNamara et al 2018). This shows that the presence of littermates with aberrant dominance behaviour can lead to disruption of the hierarchy of the group. Furthermore, when *Fmr1* knockout rats that are housed with wild-type littermates were tested in the tube test against unfamiliar wild-type rats, they were submissive, while *Fmr1* knockout rats that were housed with other *Fmr1* knockout rats were

dominant over unfamiliar wild-type rats (Saxena et al 2018). This demonstrates that the wild-type littermates can also influence the genetic model.

These studies demonstrate that it is possible that the behaviours of a genetic animal model can influence, and can be influenced by, the behaviours of other members of the group. It is important to consider how widespread this effect is as many models of neurodevelopmental and neuropsychiatric disorders have altered social and dominance-related behaviours.

1.2.8. Conclusion and outstanding questions

The literature reviewed in this section demonstrates that the social environment is key in shaping the behaviours and physiology of mice and rats. From deprivation of maternal behaviours to early life social enrichment, the studies in juvenile mice demonstrate how the social interactions during development shape the future of the behaviours and physiology of the mice in adulthood. In adolescence and in adulthood, isolation and an unstable social environment have shown to lead to behavioural and physiological changes in mice. Furthermore, it has been shown the response of the mice to the social environment are not necessarily the same in male and female mice. As discussed, this may be attributed to the clearly defined and reinforced social hierarchy in male mice, while hierarchies in females are less overt. Finally, some evidence suggests the presence of mice of a different genotype could influence the behaviours of the mouse of the different genotype. All these studies demonstrate that when assessing the behaviours of mice, the social environment needs to be considered. This is important as many studies in mouse models do not consider how the mouse model could be impacting the wild-type littermates. Indeed, this is of particular significance as many mouse models of neuropsychiatric and developmental disorders often manifest with social deficit, that could therefore impact their peers. Therefore, how the presence of mice with social deficits could impact their littermates is an important question that needs addressing.

1.3. Pvalb-expressing cells

Parvalbumin, encoded by the gene *Pvalb*, is expressed in inhibitory interneurons throughout the central nervous system. Levels of parvalbumin and properties of *Pvalb*-expressing cells have been shown to be altered in ASD in humans, animal models of ASD, as well as being influenced by the social environment. In this section, the literature relating to the properties of *Pvalb*-expressing cells and their relationship with social behaviours and the social environment will be explored.

1.3.1. The localisation and properties of Pvalb-expressing cells

Pvalb-expressing cells are found throughout the central nervous system. *Pvalb*-expressing cells are GABAergic interneurons, that regulate the activity of other neurones through perisomatic and dendritic inhibition (Booker et al 2013). They are typically fast spiking and control the levels of gamma and theta oscillations (Vreugdenhil et al 2003, Sohal et al 2009, Wulff et al 2009, Carlen et al 2012). *Pvalb*-expressing cells are chandelier and basket interneurons of the cortex (Gabbott et al 1996, Rudy et al 2011), the basket cells and inhibitory interneurones of the hippocampus (Klausberger et al 2005, Katsumaru et al 1988), Purkinje cells, and molecular layer basket and stellate interneurons of the cerebellum (Zilla et al 1985, Kosaka et al 1993, Schwaller et al 2002), and they are found in the thalamus (Arai et al 1994), the striatum (Cowan et al 1990, Kita et al 1990, Teramoto et al 2003), the brainstem (Bennett-Clarke et al 1992, Lohmann et al 1996) and in the olfactory bulb of the mouse (Miyamichi et al 2013).

1.3.2. Social behaviours and Pvalb-expressing cells

Pvalb-expressing cell dysfunction has been associated with disorders affecting social behaviours in humans. Decreased numbers of *Pvalb*-expressing cells have been observed in the brains of those who had ASD, and in the brains of those that had schizophrenia (Zikopoulos et al 2013, Stoner et al 2014, Berridge et al 2013). Furthermore, individuals with ASD have altered levels of gamma and theta frequency oscillations, which are controlled by *Pvalb*-expressing cells (Dickinson et al 2015, Maxwell et al 2015, Larrain-Valenzuela et al 2017).

Studies characterising mouse models for ASD have observed decreased levels of parvalbumin protein within the brain (Peñagarikano et al 2011, Martins et al 2011, Selby et al 2007, Dong et al 2016). These studies suggest that there is either a decreased number of *Pvalb*-expressing

cells or a decreased level of parvalbumin protein. In the *Nlgn3* R451C mutant model, it has been observed that there are fewer *Pvalb*-expressing cells (Gogolla et al 2009). Similarly, in a *Pten* mutant mouse, *Pvalb*-expressing cells were shown to have altered projections, with ectopic projections in some cortical layers, due to overgrowth of the *Pvalb*-expressing cells (Vogt et al 2015).

These changes in parvalbumin levels are not limited to genetic models of ASD; they have also been seen in environmental models. In mice that have been exposed to valproic acid in utero, it has been observed that there are decreased levels of parvalbumin protein in the cortex and the striatum (Gogolla et al 2009, Lauber et al 2016). Therefore, both genetic and environmental models of ASD in mice lead to changes in levels of parvalbumin protein or impact the numbers and properties of *Pvalb*-expressing cells, suggesting a common pathology.

The association of *Pvalb*-expressing cells with autism related phenotypes have been explored further by using conditional knockout of genes of interest, to spatially restrict the expression within, or exclude from, *Pvalb*-expressing cells. Functionally, conditional knockout of the Rett syndrome associated gene, *MECP2,* within *Pvalb*-expressing cells of mice leads to changes in motor coordination, learning and memory, and social behaviours (Ito-Ishida et al 2015). *Cox10* encodes part of the respiratory chain, and deletion of *Cox10* within mitochondria of *Pvalb*-expressing cells leads to impaired sociability as measured by the three-chamber test (Inan et al 2016). Deletion of *Pvalb* itself is sufficient to induce behavioural phenotypes related to ASD, including decreased social interest, impaired communication, and repetitive behaviours in mice (Wöhr et al 2015). Furthermore, optogenetically increasing the activity of *Pvalb*-expressing cells in the mPFC in the *Cntnap2* knockout mice led to normalised levels of social interaction in a social dyadic pairing test (Selimbeyoglu et al 2017). A similar observation was made in the *Nlgn3* R451C mutant knock-in mice, in which optogenetic modulation of the *Pvalb*-expressing cells restored the social deficit observed in the three-chamber test (Cao et al 2018). Together, these studies demonstrate that the activity of *Pvalb*-expressing cells is important in the control of social behaviours of mice.

Interestingly, there may be a sexually dimorphic control of ASD-related behaviours by *Pvalb*-expressing cells. When *Pvalb*-expressing cells and cholinergic interneurons of the dorsal striatum were ablated it was observed that male, but not female mice showed social deficit in the three-chamber test, increased anxiety, and increased stereotypy (Rapanelli et al 2017).

Of note is that the authors did not look at sex-specific effects of *Pvalb*-expressing cell ablation only, meaning that it is not clear if *Pvalb*-expressing cells only are mediating the sexually dimorphic response seen. In *mGlur5* knockout mice, the numbers of *Pvalb*-expressing cells in the striatum were more severely impacted in male *mGlur5* knockout mice than female *mGlur5* knockout mice; this was consistent with the male mice showing more pronounced repetitive behaviours and increased paired pule inhibition phenotypes (Barnes et al 2015). An additional study showed that *Pvalb* heterozygous mice treated with estradiol have increased levels of parvalbumin protein, and a small improvement of their social behaviours compared to *Pvalb* heterozygous mice not treated with estradiol (Filice et al 2018). This study suggests that circulating sex-hormones may lead to a sexually dimorphic response in how genetic or environmental insults may impact *Pvalb*-expressing cells. Taken together, these studies suggest that *Pvalb*-expressing cells may be controlled in a sexually dimorphic manner, and therefore male and female mouse models of ASD that have phenotypes mediated by *Pvalb*-expressing cells may not necessarily show the same phenotypes. Given that many models of ASD show an impact on their *Pvalb*-expressing cell, and have phenotypes mediated by *Pvalb*-expressing cells, it seems that sexual dimorphism of *Pvalb*-expressing cells is a factor to consider when looking at the behaviours of male and female mice.

1.3.3. The impact of the social environment on Pvalb-expressing cells

Social isolation in mice has been shown to lead to decreased levels of parvalbumin protein, and morphological changes of *Pvalb*-expressing cells, particularly within the hippocampus (Ueno et al 2017). Furthermore, in a mouse model of schizophrenia, it has been observed that *Pvalb*-expressing cells are at increased risk of oxidative damage induced by social isolation (Jiang et al 2013). In adult male and female rats that have been socially isolated, there is a decreased detection of parvalbumin immunoreactive cells in the hippocampus (Harte et al 2007, Filipović et al 2013, Filipović et al 2018). Decreased levels of parvalbumin immunoreactivity have also been observed in the PFC and the NAc of socially isolated male rats (Schiavone et al 2009, Schiavone et al 2012). Similarly, in rats that were separated from the dam during development, the rats showed decreased levels of parvalbumin within the PFC (Brenhouse and Andersen 2011). These studies show that levels of parvalbumin protein and properties of *Pvalb*-expressing cells are influenced by the social environment. Interestingly, there may be a sexual dimorphism of the impact of the social environment on *Pvalb*-expressing cells. In a model of early life maternal separation in rats, the maternally

separated juvenile female, but not male rats showed decreased levels of parvalbumin protein and decreased sociability. The inverse was seen in adolescents, with the maternally separated male, but not female rats showing decreased levels of parvalbumin protein and decreased sociability (Holland et al 2014). This again indicates that *Pvalb*-expressing cell property may be regulated in a sexually dimorphic manner.

Taken together these data demonstrate that levels of parvalbumin protein and properties of *Pvalb*-expressing cells are impacted by the social environment. In the majority of the studies, it is unclear whether or not the levels of parvalbumin protein itself is decreased, or if there is a loss of *Pvalb*-expressing cells themselves. Nonetheless, it is clear that levels of parvalbumin protein, and *Pvalb*-expressing cells themselves, are sensitive to the social environment.

1.3.4. Conclusions and outstanding questions

Post-mortem studies and animal models revealed that dysregulation of *Pvalb*-expressing cells is associated with ASD. Furthermore, the social environment led to aberrant levels of parvalbumin protein and altered properties of *Pvalb*-expressing cells. Therefore, when investigating aberrant social behaviour associated with ASD, *Pvalb*-expressing cells are an ideal population to explore further. Finally, some evidence indicates that *Pvalb*-expressing cells may be regulated in a sexually dimorphic manner; this should be considered when investigating the role of *Pvalb*-expressing cells in ASD-related phenotypes of mice.

1.4. Aims and objectives of this thesis

The aim of this PhD was to understand how *Nlgn3* deletion impacts individual and group behaviours of mice, and to identify a neuronal population mediating this effect. This was achieved firstly by further characterising the social behaviours of *Nlgn3*$^{y/-}$ mice, and by characterising the social behaviours of *Nlgn3*$^{+/-}$ mice and *Nlgn3*$^{-/-}$ mice. This was followed by a comparison of mice from mixed genotype housed conditions with mice from single genotype housed conditions, to determine if the presence of mice with *Nlgn3* knockout disrupts their littermates and to determine if the wild-type littermates also impact the knockout mice. Additionally, *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{-/-}$ mice that have *Nlgn3* re-expressed in *Pvalb*-expressing cells were assessed to observe if individual and group behaviours were restored by re-expression of *Nlgn3* in parvalbumin expressing cells. As the social environment has been shown to impact anxiety-related behaviours and the transcription of different

genes, these factors were also investigated to better understand the consequence of the presence of *Nlgn3* knockout mice on the behaviour and physiology of the group.

Chapter 2: Materials and methods

2.1. Husbandry and legislation

All animal husbandry and experiments were performed in compliance with the UK Animals (Scientific Procedures) Act 1986 (ASPA, Home Office 1986), as amended in accordance with the Cardiff University Animal Care Committee's regulations. The mice were identified using ear notching at PD19 (postnatal day 19). Weaning took place at PD30, this allowed juvenile behaviour to be measured without the confounding stress caused by weaning. After weaning, mice were kept in same sex cages with their littermates, consisting of 2-5 mice per cage, and the littermates were never separated, nor were cage compositions rearranged. Females aged p60+ were used for breeding. Sires were separated from the female during pregnancy. All mice used had been backcrossed to a C57BL/6 background for at least eight generations. The mice had free access to food and water. For environmental enrichment mice had nesting material, a wooden chew stick and a cardboard tube. The mice were kept in cages of 16 cm x 48 cm, with a height of 14 cm and with a sawdust bedding that was changed once per week. The temperature was maintained between 18°C-22°C. The mice were kept on a 12-hour light/ dark cycle, at 06:00 the lights were turned on and at 18:00 the lights were turned off. All behavioural analysis was conducted in the light cycle. The mice were habituated to the testing room for at least 30 minutes before testing. The mice were handled regularly with minimal restraint to ensure that the mice were habituated to being handled prior to experiments, this was to avoid confounding factors caused by being handled with restraint (Hurst and West 2010, Ghosal et al 2015, Gouveia and Hurst 2017, Clarkson et al 2018). Handling of the mice began on PD19 onwards over the course of 2-3 days. Initially the carboard tube was used at first to guide the animals into walking onto the gloved hand. The cardboard tube was used to allow the mice to begin to explore the gloved hand under their own duress, without being lifted by the tail and placed onto the hand which could cause stress to the animal. The mice were then given time to become comfortable with sitting and walking on the gloved hand without becoming distressed or agitated. Importantly, this involved minimal restraint, such as tail holding. Once the mice were comfortable with being transported on the hand with no restraint, the mice were considered habituated to handling and experiments could begin.

2.2. Experimental animals

2.2.1. Nlgn3 knockout mice

Nlgn3 knockout mice contain a premature STOP-tetO sequence within the promotor region of the *Nlgn3* gene (Tanaka et al 2010). The STOP-tetO sequence has loxP sites on either side, to allow for excision by Cre for re-expression of *Nlgn3* (**figure 2.1.A**).

2.2.2. Mice expressing Cre under the parvalbumin promotor

Mice expressing Cre under the parvalbumin promotor were used. The mice originated from JAX (JAX:017320). The construct consists of an internal ribosome entry site (IRES) site, Cre, and a polyadenylation sequence expressed in the 3' UTR of exon 5 of the parvalbumin locus. When crossed with mice expressing the STOP-tetO codon, the Cre excises the STOP codon, and *Nlgn3* is re-expressed in *Pvalb*-expressing cells only (**figure 2.1.B**). Additionally, it was shown that $Nlgn3^{+/+}Pvalb^{cre/+}$ mice compared to $Nlgn3^{+/+}$ mice do not have altered social behaviour, activity, or anxiety related behaviour (**appendix 1.0**). This indicates that the presence of the $Pvalb^{cre/+}$ construct alone is not a confounding factor in the behavioural measurements.

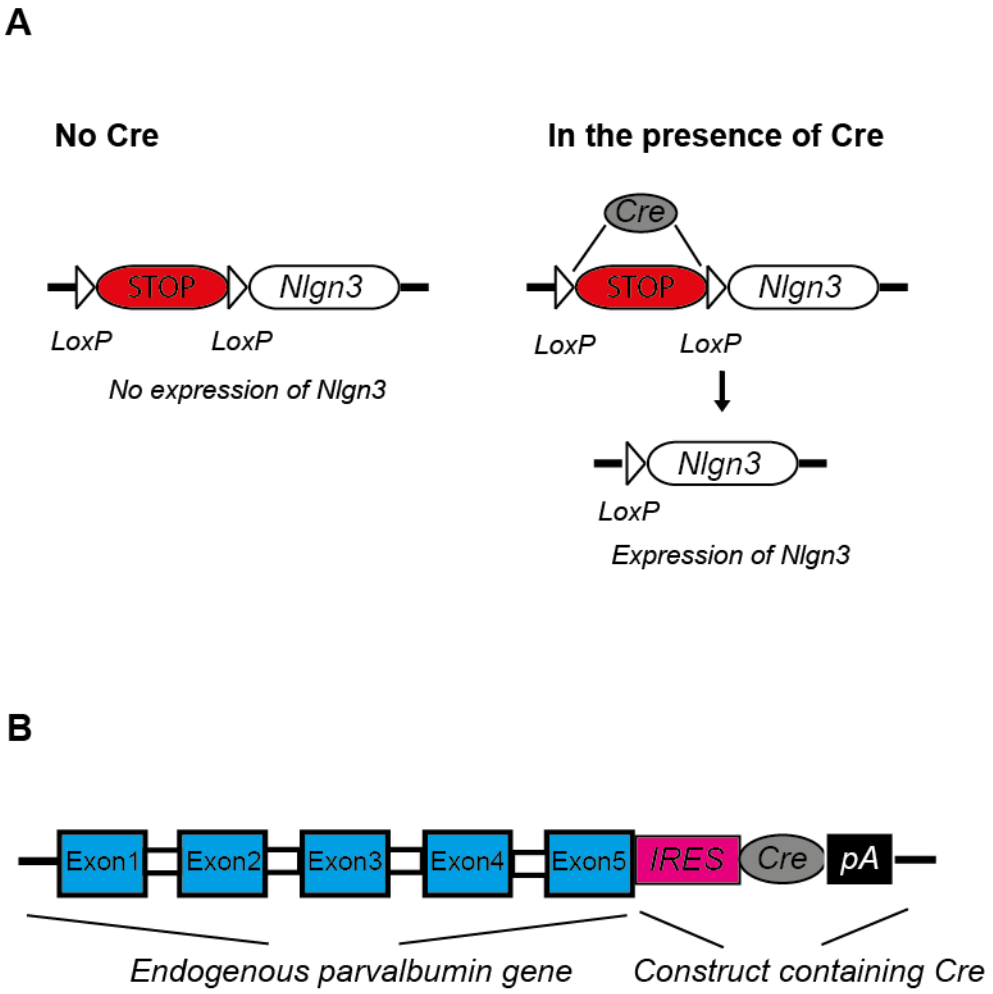


Figure 2.1. A schematic of the *Nlgn3* knockout construct and re-expression with Cre. ***A***, a premature STOP-tetO codon is inserted into the promotor region of the gene surrounded by LoxP sites, preventing the transcription of the gene. In the presence of Cre, the STOP codon is excised, and the *Nlgn3* gene is re-expressed. ***B***, a schematic of the construct in mice expressing the *Pvalb*$^{Cre/+}$ construct. The construct is inserted into the three prime untranslated region (3'-UTR) of exon 5 of the endogenous parvalbumin gene. An internal ribosome entry site (IRES) prevents disruption of the endogenous parvalbumin protein expression. Therefore, when mice with the knockout of *Nlgn3* are crossed with mice expressed Cre under the parvalbumin promotor, *Nlgn3* is expressed within *Pvalb*-expressing cells only.

2.3. Genotyping

In order to determine the genotype of the mice, ear notch samples were processed in order to extract DNA. These were taken on PD19 onwards and then again following death.

2.3.1. DNA extraction and preparation

Ear notch samples were placed into an Eppendorf tube, and the DNA was extracted on the same day. 150 µl of 0.5 M NaOH was added to the samples, which were then heated for 1 hour at 90°C. The samples were then placed on ice for 5 minutes, following which, 50 µl of TrisEDTA (Tris 1 M, EDTA 4 mM pH 7.5) was added. The samples were vortexed, and then frozen at -20°C until used for polymerase chain reaction (PCR) confirmation of the presence of the genetic construct.

2.3.2. Nlgn3 genotyping

Detection of the *Nlgn3* STOP-tetO construct was determined using the following primers, sense: 5' TCCGTGGGCACATACACATTCAGA 3' and antisense: 5' AGCAGAGCTCGTTTAGTGAACCGT 3'. Running this on a 2% agarose gel with ethidium bromide gave a 700 base pair (bp) product corresponding to detection of *Nlgn3* STOP-tetO construct, indicating that that there is a knockout allele present. The mice were also genotyped for the wild-type allele. The sequences for these are; sense: 5' TCCGTGGGCACATACACATTCAGA 3' and antisense: 5' GGGCTGGATGTTGCAATTGGAGTT 3'. Running this on a 1% agarose gel with ethidium bromide reveals a 1000 bp product corresponding to the presence of the wild-type allele (see **table 2.1**). The combination of both genotyping primers made it possible to genotype *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice, and, importantly, to differentiate between *Nlgn3*$^{-/-}$ and *Nlgn3*$^{+/-}$ mice.

2.3.3. Cre genotyping

In order to detect the presence of Cre the following primers were used, sense: 5' GGTTATGCGGCGGATCCGAAAAGAAA and antisense: 5': ACCCGGCAAAACAGGTAGTTATTCGGATCA. Running this on a 2% agarose gel with ethidium bromide gives a 381 bp product that corresponds to the presence of the construct for Cre (see **table 2.1**).

Table 2.1. **A summary of the primers, reagents, and polymerase chain reaction sequence used for genotyping**. Abbreviations: base-pair (bp), deoxyribonucleotide triphosphate (dNTP), dimethyl sulfoxide (DMSO).

	Primers 5' to 3'	**Reagents**	**Product size**	**Cycle**
STOP-tetO for genotyping *Nlgn3* knockout	Sense: TCCGTGGG CACATACA CATTCAGA Antisense: AGCAGAGC TCGTTTAGT GAACCGT	2.5 µl New England Biolabs buffer 0.5 µl 10 mM dNTP 0.5 µl each primer (10 mM) 0.12 µl New England Biolabs taq polymerase 1µl DNA 19.88 µl water	700 bp	1. 2 minutes at 95°C 2. (30 seconds at 95°C, 30 seconds at 61°C, 30 seconds at 72°C) x 29 3. 5 minutes at 72°C 4. Hold at 4°C.
Nlgn3 wild-type allele	Sense: TCCGTGGG CACATACA CATTCAGA Antisense: GGGCTGGA TGTTGCAA TTGGAGTT	2.0 µl New England Biolabs buffer 0.5 µl 10 mM dNTP 0.4 µl DMSO 1.0 µl each primer (10 mM) 0.36 µl New England Biolabs taq polymerase 1 µl DNA 12.24 µl water	1000 bp	1. 5 minutes at 95°C 2. (30 seconds at 95°C, 30 seconds at 57°C, 1 minute at 68°C) x35 3. 5 minutes at 72°C 4. Hold at 4°C.
Cre	Sense: GGTTATGC GGCGGATC CGAAAAGA AA Antisense: ACCCGGCA AAACAGGT AGTTATTC GGATCA.	2.5 µl New England Biolabs buffer 0.5 µl 10 mM dNTP 0.2 µl each primer (10 mM) 0.12 µl New England Biolabs taq polymerase 1 µl DNA 20.48 µl water	381 bp	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.

2.4. Breeding Schemes and housing conditions

The work presented in this thesis differentiates between housing conditions containing different genotypes. In all breeding schemes, the mice were in mixed sex conditions, until weaning, when they were split into separate sex housing conditions. All breeding schemes are summarised in **table 2.2**.

2.4.1. Single genotype housed (SGH) mice

Both wild-type and knockout mice are kept in single genotype housed conditions (SGH) for these experiments. For the wild-type only mice, this means that the groups consist entirely of only *Nlgn3*$^{y/+}$ or *Nlgn3*$^{+/+}$ mice. For the knockout only mice, these groups consist entirely of only *Nlgn3*$^{y/-}$ or *Nlgn3*$^{-/-}$ mice. In order to generate litters of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{+/+}$ mice only, *Nlgn3*$^{+/+}$ females were crossed with *Nlgn3*$^{y/+}$ males. In order to generate litters of *Nlgn3*$^{y/-}$ and *Nlgn3*$^{-/-}$ mice only, *Nlgn3*$^{-/-}$ females were crossed with *Nlgn3*$^{y/-}$ males. See **table 2.2** for a summary of the crossings and the genotypes of the litters

2.4.2. Mixed genotype housed (MGH) mice

Mixed genotype housed mice can be generated in two ways. In order to generate litters of *Nlgn3*$^{y/-}$, *Nlgn3*$^{y/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{+/+}$ mice, *Nlgn3*$^{+/-}$ females were crossed with *Nlgn3*$^{y/+}$ males. In this case, the *Nlgn3*$^{+/-}$ mice are referred to as *Nlgn3*$^{+/-}$ (H-WT) mice (heterozygous with wild-type), as they are housed with *Nlgn3*$^{+/+}$ littermates. This is the breeding scheme used in the assessment of the behaviours of male mice from MGH. See **table 2.2.**

Importantly, as *Nlgn3* is an X-linked gene, it is not possible to generate litters of both *Nlgn3*$^{+/+}$ females and *Nlgn3*$^{-/-}$ females. A separated breeding strategy was needed to generate *Nlgn3*$^{-/-}$ mice that are housed with *Nlgn3*$^{+/-}$ mice. *Nlgn3*$^{+/-}$ females were crossed with *Nlgn3*$^{y/-}$ males, to give litters of *Nlgn3*$^{y/-}$, *Nlgn3*$^{y/+}$, *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ females. In this case, the *Nlgn3*$^{+/-}$ mice are referred to as *Nlgn3*$^{+/-}$ (H-KO) mice (heterozygous with knockouts) as they are housed with *Nlgn3*$^{-/-}$ littermates. See **table 2.2.**

2.4.3. Re-expression of Nlgn3 under the parvalbumin promotor

This work also focuses on using selective re-expression of *Nlgn3* within *Pvalb*-expressing cells. To explore the effect of this on *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, two different breeding schemes were used. *Nlgn3*$^{+/-}$ mice were crossed with *Nlgn3*$^{y/+}$*Pvalb*$^{cre/cre}$ mice to produce litters of *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ *Nlgn3*$^{+/-}$*Pvalb*$^{cre/+}$ and *Nlgn3*$^{+/+}$*Pvalb*$^{cre/+}$

mice. As these mice are in mixed genotype housed conditions, they are referred to as PV-MGH. See **table 2.2.**

Nlgn3$^{y/-}$*Pvalb*$^{cre/+}$ mice could not be used to generate litters, as parvalbumin is expressed in sperm, leading to unwanted germline re-expression. Therefore, for the generation of *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, *Nlgn3*$^{+/-}$*Pvalb*$^{cre/+}$ mice were crossed with *Nlgn3*$^{y/-}$ mice. This led to litters of *Nlgn3*$^{+/-}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{y/-}$, *Nlgn3*$^{y/+}$, *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice, however, due to experimental considerations litters containing *Nlgn3*$^{-/-}$ mice were not analysed. As these mice are in mixed genotype housed conditions, they are referred to as PV-MGH. See **table 2.2.**

Table 2.2. A summary of the breeding schemes used. Abbreviations used: mixed genotype housing (MGH), single genotype housing (SGH), mixed genotype housing of mice expressing *Pvalb*$^{cre/+}$ (PV-MGH).

Breeding Schemes		
Parents genotype	**Genotype of litter**	**Acronyms used**
Nlgn3$^{+/-}$ *and Nlgn3*$^{y/+}$	*Nlgn3*$^{y/+,}$ *Nlgn3*$^{y/-}$ *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$	MGH *Nlgn3*$^{+/-}$(H-WT)
Nlgn3$^{+/-}$ and *Nlgn3*$^{y/-}$	*Nlgn3*$^{y/+}$, *Nlgn3*$^{y/-}$, *Nlgn3*$^{-/-}$, *Nlgn3* $^{+/-}$	MGH *Nlgn3*$^{+/-}$(H-KO)
Nlgn3$^{y/+}$ and *Nlgn3*$^{+/+}$	*Nlgn3*$^{y/+}$, *Nlgn3*$^{+/+}$	SGH
Nlgn3$^{y/-}$ and *Nlgn3*$^{-/-}$	*Nlgn3*$^{y/-}$, *Nlgn3*$^{-/-}$	SGH
Nlgn3$^{+/-}$ and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/cre}$	*Nlgn3*$^{y/+}$*Pvalb* $^{cre/+}$, *Nlgn3*$^{+/-}$*Pvalb* $^{cre/+}$, *Nlgn3*$^{y/-}$*Pvalb* $^{cre/+}$, *Nlgn3*$^{+/+}$*Pvalb* $^{cre/+}$,	PV-MGH
Nlgn3$^{+/-}$*Pvalb*$^{cre/+}$ *and* *Nlgn3*$^{y/-}$	*Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{+/-}$, *Nlgn3*$^{+/-}$ *Pvalb*$^{cre/+}$, *Nlgn3*$^{y/+}$, *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{y/-}$, *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ (litters containing *Nlgn3*$^{-/-}$ mice were not analysed)	PV-MGH

2.5. Behavioural Assays

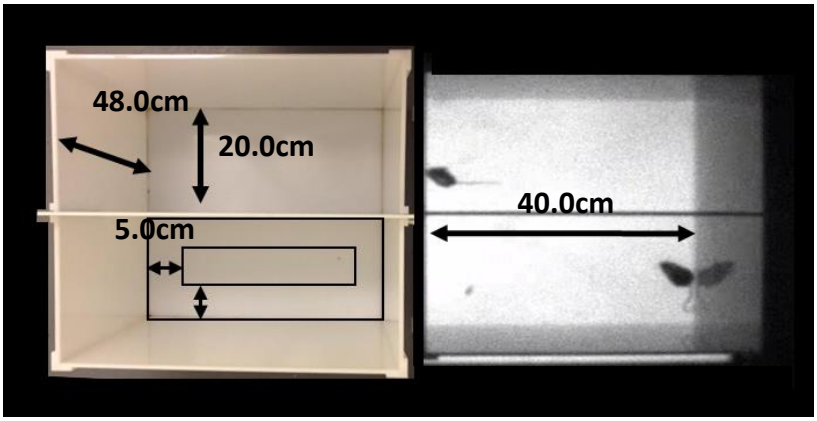
2.5.1. Open field activity

Spontaneous activity of the mice was tested in an open field arena, with a floor space of 40 cm x 20 cm and a height of 48 cm, for 20 minutes in the dark. The mice were able to freely move and explore the environment during the trial. The arena was illuminated from the bottom by an infrared light source, for detection of the mice. The behaviour was recorded by a computer linked video camera located above the arena. Tracking software (EthoVision XT, Noldus) was used to define the outside of the area (the area contained within 5 cm away from arena walls) and the centre of the arena (area that is 5 cm or more from the walls), see **figure 2.2.A**. The software was used to track the total distance travelled (cm) in the arena. Thigmotaxis behaviour of the mice was defined by either time spent in the centre of the open field arena (seconds) or as the ratio of distance travelled in the centre of the arena/ total distance travelled in the arena.

2.5.2. Elevated plus maze

The elevated plus maze is used to assess anxiety, as more anxious mice spend decreased time in the open arms (Lister 1987, Walf and Frye 2007). The elevated plus maze has two open arms and two closed arms. Each arm of the elevated plus maze measures 40 cm by 7 cm. The sides of the open arms are 1 cm high and made of clear plastic, while the sides of the closed arms are 16 cm and made of black plastic. The centre of the elevated plus maze is 6 cm by 6 cm. The total duration of the trial was recorded by a camera above the arena. A tracking software (EthoVision XT, Noldus) was used to define the different arms of the arena (open or closed). Open arm quantification did not include the centre, see **figure 2.2.B**. One mouse was placed on the elevated plus maze per trial. The trials last for 5 minutes each and were conducted in a well-lit room. The activity of the mouse was tracked by the automated tracking system, and the data was automatically quantified. The software quantified the duration of time that the mice spent in each arm. The summation of time that the mice spent in the two open arms was taken as the final measure of activity in the open arm.

A



B

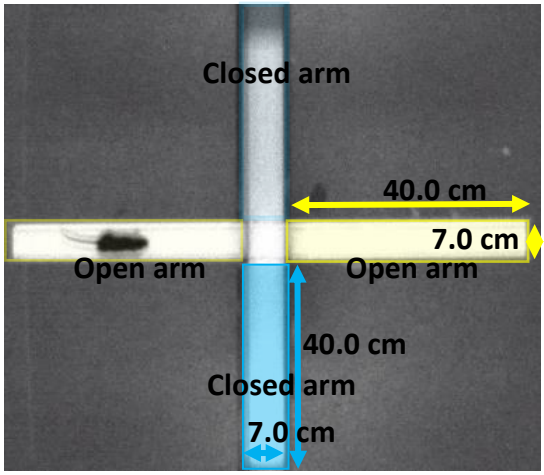


Figure 2.2. The open field arena and the elevated plus maze. ***A*,** the open field arena. Prior to the behavioural recording of the mice, the parameters if the open field arena are defined on the software. The total size of the open field arena in 40 cm x 20 cm, and the 'outside' is defined at 5 cm from the wall. The boxes have an infrared light source illuminating the boxes, and the software tracks the movement of the mice within the open field, recording total distance travelled, and time in the centre and outside. The images on the left show the activity boxes, while the images on the right show the boxes with the infrared light source, as seen by the camera and detection software. ***B***, the elevated plus maze. The open and closed arms and the centre of the elevated plus maze are predefined on the software. The arms are 40 cm in length. The behaviour is conducted over 5 minutes in the light and the movement of the mouse is tracked by the software. The time spent in the open arms is quantified.

2.5.3. Social interaction

Interaction with female mice was tested by exposing the subject mouse to an unfamiliar adult female known to be in oestrous (**section 2.5.5**). These interactions were tested in the same boxes as those used in the open field arena (**section 2.5.1**, **figure 2.2.A**). Prior to the social interaction, the subject mouse was habituated to the open field arena for 3 minutes. Following this, the female was added to the arena. The mice were allowed to freely interact with each other for 3 minutes and then the trial was ended. An experimenter blind to the genotype scored the interaction of the mice. When mice were within 2 cm of each other, this was scored as social interaction.

2.5.4. Ultrasonic vocalisation (USV)

During courtship, adult male mice emit courtship ultrasonic vocalisations (USV) towards females. These can be recorded and quantified (Holy and Guo 2005). It has been shown that the USV is from the male towards the female, and not the other way around (Whitney et al 1973). Male mice were habituated to the open field arena (**section 2.5.1**, **figure 2.2.A**) for 3 minutes. A female mouse in oestrous (**section 2.5.5**) was added to the open field arena and the mice were allowed to freely interact with each other for 3 minutes. In order to determine levels of USV, a microphone (UltraSoundGate 416H preamplifier connected UltraSoundGate CM16 microphone, Aviosoft Bioacoustics) was added to the top of the wall of the arena, its elevated position (~48 cm) prevented the mice from being distracted by it. Levels of USV emitted were quantified (SASLabPro software, Aviosoft Bioacoustics). Two different parameters of USV were assessed; the number of calls, and the total duration spent calling over the 3 minutes. For the total duration calling over the 3 minutes, the duration of time of all sounds within the frequency range of 30 to 200 hertz (Hz) were quantified. When characterising the number of individual calls over the 3 minutes, a call was defined as an event in the frequency range of 30 to 200 Hz that lasted longer than 5 ms (milliseconds) and that occurred at least 500 ms after the previous call (Holy and Guo 2005). In addition, video footage of the trials were recorded by an overhead camera. As before for the social interaction, an experimenter blind to the genotype scored the interaction of the mice, using the same parameter of the mice being within 2 cm of each other being classified as social interaction.

2.5.5. Confirmation of oestrous

The female was confirmed to be in oestrous by taking vaginal smears using saline. The smears were fixed and then stained using blue modified Giemsa solution (fixative, blue/azure dye and xanthene dye, Polysciences Inc.). The stains were examined under the microscope, and the morphology of the cells was used to indicate what stage of the oestrous cycle the mice were in (Caligioni et al 2009).

2.5.6. The tube test

Social dominance was assessed using the tube test apparatus (Lindzey et al 1961, Wang et al 2011). The tube test is a clear acrylic tube (length: 30 cm, internal diameter 3.5 cm) with automated doors at either entrance and in the centre (**figure 2.3.A**). Before assessment of hierarchy, all mice were trained to the same standard, whereby they would wait at the automatic doors until they open, before moving through the tube in one direction. This training took place over four days. Initial training involved the mouse walking through the tube whilst being blocked from moving backwards. During this initial training period, all doors were kept open. Once the mouse had learnt to walk through the tube without stopping or retreating, the doors were then used. The mouse had to be habituated to the doors in such a way that it would not flinch or retreat when the door opened in front of them. Additionally, when the mouse encountered the middle door, it was trained to wait by the door until it opened, and once it opened the mouse should then continue moving forwards through the tube test.

The mice were not tested on the day after cleaning the cage, to avoid disruption of the hierarchy or dominance caused by the removal of olfactory information. During the trial, the mice were placed at opposite ends of the tube and released to enter the tube. Once the mice meet in the centre of the tube, the middle door opens, and the mice are challenged to push their opponent out of the tube. The mouse that gets pushed out of the tube is declared the submissive mouse of that trial. Two versions of the tube test were used in these experiments.

2.5.6.1. Determining within cage hierarchy

By using pairings of mice from the same group and testing them in a round robin fashion the dominance of each individual from the pair and the hierarchy of the cage can be determined. Therefore, when assessing the dominance and hierarchy of each cage, the mice were tested in pairs in a round robin design, ensuring all pairings of mice had been tested. The sides of

entry for the mice were alternated to avoid bias. The first mouse of the pairing to push the other mouse out twice was the victor of the pairing. This provided a measure of the hierarchy of the mice within the cage. This was repeated over three days, and the measures of hierarchy and dominance on the last day were taken (**figure 2.3.B**).

2.5.6.2. Determining relative dominance

In the second version of the tube test, the tube test was used to assess the dominance behaviour of the subject mouse against an unfamiliar male mouse. Prior to testing, all the unfamiliar wild-type male mice used in these experiments were determined to have a 43% win rate against other unfamiliar wild-type male mice.

The subject mice were matched with three different unfamiliar wild-type males. The sides in which the mice were placed were alternated to avoid bias. All three trials were not conducted directly after each other, to avoid an influence of the outcome of the previous trial on the trial that came after it. The percentage of victories for the individual mice was calculated. This allowed the assessment of the relative dominance of different groups of mice that are not housed together (**figure 2.3.C**).

2.5.7. Pup retrieval

In order to assess the maternal behaviours of the mice, pup retrieval behaviour was completed at p7. During this behaviour, the pups and their nest were carefully removed from the home cage (Floor space: 16 cm x 48 cm, with a height of 14 cm) for 3 minutes and placed into a clean home in their nest and with their littermates. After three minutes of separation, the nest is replaced in its usual location in the home cage, and the pups are placed in the opposite side of the cage. The retrieval behaviour of the dam is recorded by an overhead camera. The time for the dam to sniff the pups and then to retrieve 3 pups were measured.

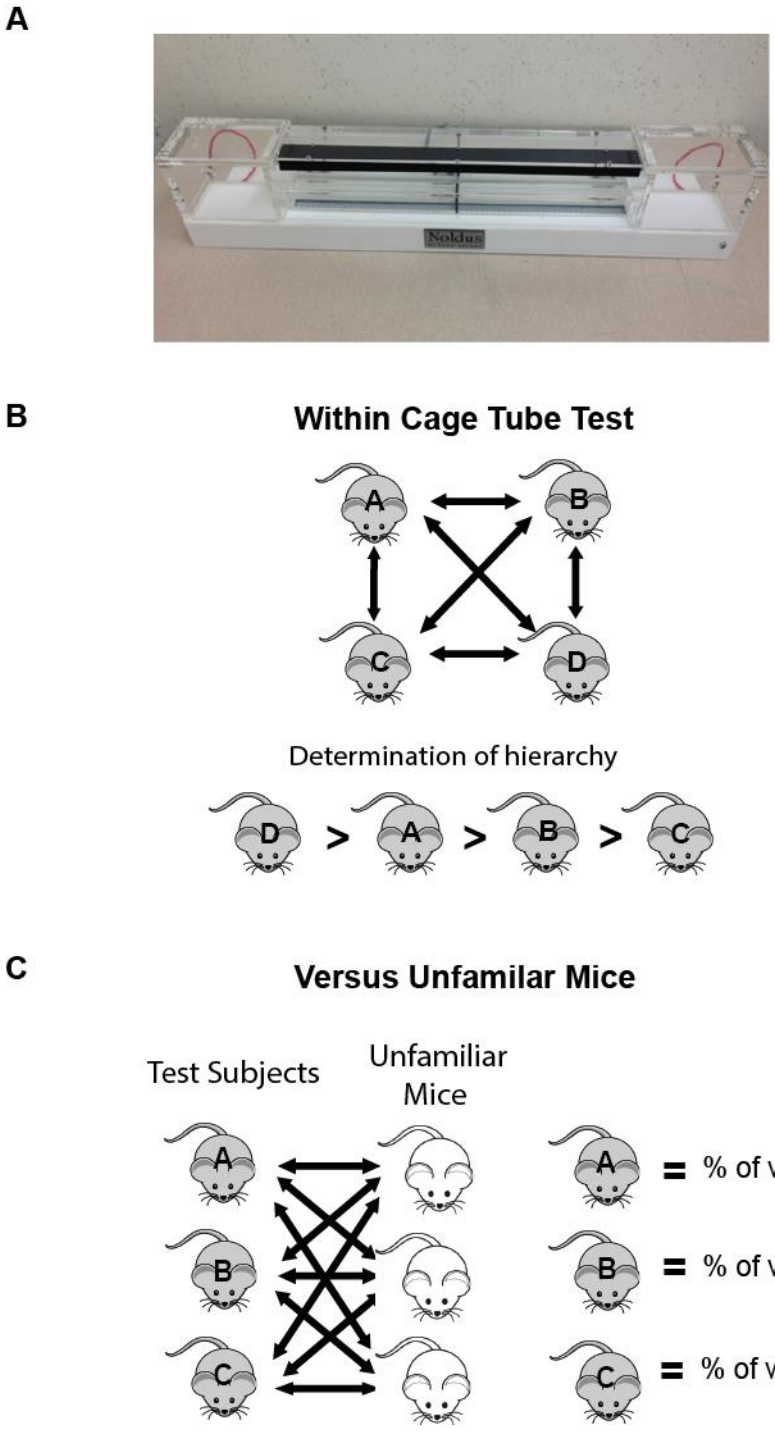


Figure 2.3. Testing for dominance using the tube test. ***A***, a photograph of the tube test apparatus. ***B***, a schematic of the within cage tube test. All mice that are housed together are tested against each other to determine the dominance of the individuals within the cage and to determine the hierarchy. ***C***, a schematic of the process of testing mice against unfamiliar male mice. Each mouse is trialled against three different unfamiliar mice of known social ranking. The percentage of victories is calculated per mouse.

2.6. Biochemistry and molecular biology

2.6.1. Testosterone quantification

Urine was collected immediately after bladder voiding using an Eppendorf tube, frozen instantly on dry ice, and then stored at -20°C until use. Urinary testosterone levels were measured using an enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (Arbor Assays). In order to validate the sensitivity of the ELISA, the urine of adult *Nlgn3*$^{y/+}$ mice was compared to that of adult *Nlgn3*$^{+/+}$ mice, as males are known to have higher levels of urinary testosterone. Adult *Nlgn3*$^{y/+}$ mice were found to have higher levels of urinary testosterone compared to adult *Nlgn3*$^{+/+}$ mice, validating the sensitivity of the ELISA (**figure 2.4,** independent samples t-test, *P = 0.007, df=11*, *Nlgn3*$^{y/+}$ mice n = 6, *Nlgn3*$^{+/+}$ mice n = 7).

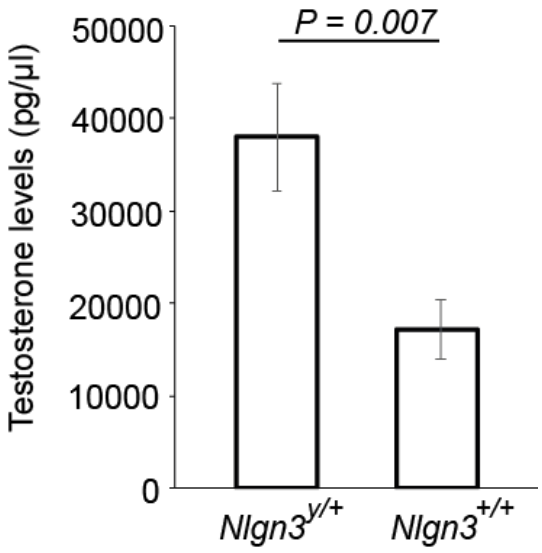


Figure 2.4. **Validation of the ELISA for determination of urinary testosterone levels.** Urinary testosterone levels of *Nlgn3$^{y/+}$* and *Nlgn3$^{+/+}$* mice. Both *Nlgn3$^{y/+}$* and *Nlgn3$^{+/+}$* mice are taken from single genotype housed conditions. The average urinary testosterone levels (pg/µl) and standard error of the mean (SE) for *Nlgn3$^{y/+}$* mice, 37959.4(SE:5780), and *Nlgn3$^{+/+}$* mice, 17182.2(SE:3159.9). An independent samples t-test revealed a significant difference between the sexes (*P = 0.007*).

2.6.2. Brain dissection

Mice were sacrificed using cervical dislocation. The brain was extracted and placed in an ice-cold brain mould (Electron Microscopy). The brain was then dissected into the appropriate regions of interest using blades (Electron Microscopy) and using the Mouse Brain Atlas in Stereotaxic coordinates (Paxinos and Franklin 2001) as a guide. See **figure 2.5** for an image of a brain in the brain mould and a schematic of the dissection of the brain into the different regions. In order to dissect the brain slices, the brain slices were placed onto an ice-cold metal block, with cold phosphate buffered saline (PBS; Thermo Fisher Scientific). For protein extraction for western blot, and for ribonucleic acid (RNA) extraction, the different brain regions were stored in Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at -80°C until further use (**section 2.6.3** and **section 2.6.8** respectively). For synaptosome preperation, the tissue was immediately used in downstream processing (**section 2.6.4**).

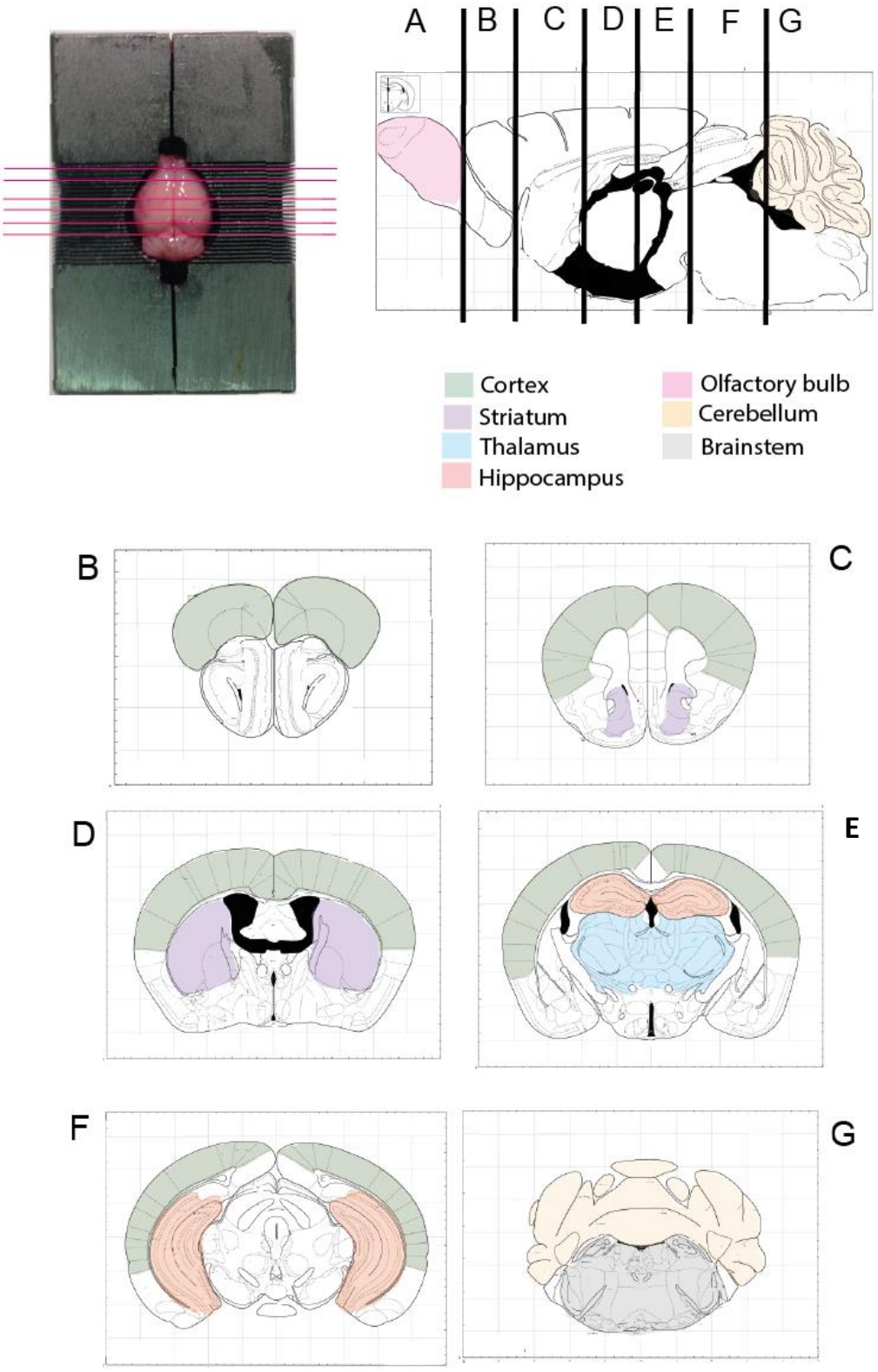


Figure 2.5. Dissection of the brain. The brain in the brain-mould, and a schematic of the dissection of the brain into regions of interest. These images are adapted from The Mouse Brain Atlas in Stereotaxic coordinates by Paxinos and Franklin, second edition, Elsevier Academic Press.

2.6.3. Protein extraction

The entire protocol was conducted on ice, except for the steps in the fridge and the centrifuge, for which a temperature of 4°C was used. Lysis buffer (50 mM Tris-HCl, 1 mM ethylenediamine tetra-acetic acid, 0.1% sodium dodecyl sulfate, 50 mM NaCl, 1% triton, 10 mM NaF, 1 mM NaVO4, 1 mM dithiothreitol, 10 µl per 1ml of complete protease inhibitor (Merck)) was added in a 10:1 volume to tissue ratio. The samples were homogenised with the lysis buffer. The samples were placed on a 4°C fridge rotator for 30 minutes. The samples were then centrifuged at 14,000 x g for 30 minutes. The supernatant containing the protein was removed, and frozen at -80°C, until needed for protein level determination and sample dilution as described in **section 2.6.5.**

2.6.4. Synaptosome extraction

The tissue used in this protocol was processed immediately following culling and dissection to avoid any potential corruption of the tissue due to the freeze-thaw process. SynPer (Thermo Fisher Scientific) with proteinase and phosphatase inhibitors added (10 mM NaF, 1 mM NaVO4, 10 µl per 1 ml of complete protease inhibitor (Merck)) was used to extract the synaptic proteins. The entire protocol was conducted on ice, except the centrifuge steps, that were conducted at 4°C. The tissue was homogenised in SynPer using a dounce tissue grinder. The sample was centrifuged at 1,200 x g for 10 minutes to give a pellet and the homogenate. A sample of the homogenate was saved, and then the sample was centrifuged at 15,000 x g for 20 minutes, to give the cytosol and the synaptosome. The synaptosome was resuspended in SynPer. If the sample was to be used for western blotting, the level of protein and appropriate dilution was carried out as described in **section 2.6.5**. If the sample was to be used for co-immunoprecipitation, then the sample was immediately used in this protocol (**section 2.6.6**).

2.6.5. Bicinchoninic acid protein assay (BCA)

Protein levels were determined using a Pierce™ bicinchoninic acid protein assay (BCA, Sigma) as per manufacturer instructions, using a standard curve of bovine serum albumin (BSA) ranging from 0.0 mg/ml to 2.0 mg/ml, diluted from the 20 mg/ml BSA provided from the kit. Following determination of protein levels, the samples were diluted to 2.5 mg/µl in lithium dodecyl sulfate buffer (LDS, 106 mM Tris-HCL, 141 mM Tris-base, 2% lithium dodecyl sulfate, 10% glycerol, 0.51 mM EDTA, 0.22 mM G250 Coommassie Blue, 0.175 mM Phenol Red, 10

mM Dithiothreitol; pH 8.5). These were stored at -20°C until use western blot analysis (**section 2.6.7**).

2.6.6. Co-immunoprecipitation

Immediately following synaptosome extraction, the samples were used for co-immunoprecipitation. Samples were kept on ice for the procedure, except for the centrifugation and rotation stage, which was done at 4°C. A sample of synaptosome was taken and added to LDS buffer, to be used as the input. The rest of the sample was then processed for use in the immunoprecipitation. Here the sample used for the immunoprecipitation was precleared with protein A sepharose beads (Abcam) and cold PBS (Thermo Fisher Scientific) and centrifuged for 2,000 x g for 2 minutes. After centrifugation, the sample was added to washed protein A sepharose beads (Abcam) and 2 µl of anti-Neuroligin-3 antibody (Synaptic Systems) was added. This was incubated for an hour at 4°C. The sample was then washed 3 times, and then the beads were eluted with 50 µl of LDS buffer. The sample was then incubated at 70°C for 10 minutes. The sample was then centrifuged to remove the beads. The supernatant, containing the proteins and LDS buffer, was used as the immunoprecipitate (IP). The sample is then stored at -20°C until used for western blot (**section 2.6.7**).

2.6.7. Western blot

Prior to loading the sample on the membrane, samples were heated for 5 minutes at 70°C, vortexed and then centrifuged. 10 µl per sample was loaded onto a Bis-Tris polyacrylamide gels (NuPAGE, Novex), in electrophoresis chambers of MES running buffer (**appendix 1.2**). This was run at 120 mv for 1 hour 45 minutes. Wet transfer in NuPage transfer buffer (**appendix 1.2**) was used to transfer the protein from the Bis-Tris gel to the nitrocellulose membrane (GE Healthcare). Following the transfer, the membranes were washed with TBS-T (**appendix 1.2**) for 3 x 5 minutes before being blocked in 5% milk in TBS-T for 1 hour. Primary antibodies were diluted in 5% milk in TBS-T and incubated on the membrane overnight (**table 2.3.A)**. The next morning, the membranes were washed 3x times with TBS-T, and then the secondary antibody diluted in 5% milk in TBS-T was incubated on the membrane for an hour (**table 2.3.B**). The blots were washed 3x time with TBS-T. If a horseradish peroxidase (HRP) antibody was used, then the enhanced chemiluminescence (ECL) reagent (Advansta) was prepared and distributed on the blot immediately prior to imaging. The blots were imaged

using imaging hardware (Bio-Rad) and image lab 5.0 software. The band sizes were quantified using the image lab software.

Tables 2.3. Use of antibodies for western blot analysis. *A*, primary antibodies used for western blotting. ***B*,** secondary antibodies used for western blotting.

A

Primary antibodies	Company	Epitope	Species	Concentration
Anti-Neuroligin-3	Abcam	Monoclonal Clone; EPR16158	Rabbit	1 in 2000
Anti-beta actin	Abcam	Polyclonal	Chicken	1 in 5000
Anti-beta III tubulin	Abcam	Monoclonal (Clone TUJ-1)	Mouse	1 in 5000
Anti-MeCP2	Sigma	Monoclonal: Clone; Men-8	Mouse	1 in 2000
Anit-HDAC2	Invitrogen	Polyclonal	Rabbit	1 in 2000
Anti-PSD95	Abcam	Polyclonal	Rabbit	1 in 2000
Anti-FMRP	Cell-signalling technologies	Polyclonal	Rabbit	1 in 2000
Anti-Neuroligin-2	Synaptic Systems	Monoclonal Clone; 5E6	Mouse	1 in 2000
Anti-PIR121-1/Sra-1 (CYFIP1) Antibody	Merkmillipore	Polyclonal	Rabbit	1 in 2000
Anti-Wave1	Merkmillipore	Monoclonal Clone; k91/36	Mouse	1 in 2000
Anti-Nckap1	Novus Biologicals	Polyclonal	Rabbit	1 in 2000

B

Secondary antibodies	Company	Concentration
Anti-rabbit IgG HRP	Promega	1 in 10000
Anti-mouse IgG HRP	Promega	1 in 10000
Anti-chicken IgG HRP	Abcam	1 in 10000

2.6.8. RNA extraction

All RNA processing was done in RNAase free conditions, established using RNAaseZap decontamination solution (Sigma) to clean a designated RNAase-free fume hood for RNA work. The dissected brain regions were weighed, and 1 ml of TRIzol Reagent (Invitrogen) per 50-100 mg of tissue was added. The tissue was homogenised and incubated for 5 minutes. The samples were then transferred to phasemaker tubes (Invitrogen). 0.2 ml of chloroform (Sigma) per 1 ml of TRIzol reagent used in the initial step was added, and the sample was vigorously shaken for 15 seconds. The samples were then incubated for 15 minutes at room temperature before centrifuging for 5 minutes at 14,000 x g at 4°C. The upper phase, containing the RNA, was transferred into new tubes, and 0.5 ml of isopropanol per 1 ml of TRIzol used was added. The samples were incubated for 1 hour at -80°C. The samples were then centrifuged for 10 minutes at 10,000 x g, the supernatant was removed, and the pellet containing the RNA was washed using 75% EtoH, and centrifuged again at 7,500 x g and the supernatant was discarded. The RNA pellet was air dried for 5-10 minutes prior to dissolving in 20µl RNAase free water. The RNA was incubated for 15 minutes at 55°C. The samples were treated with DNAse (Ambion), as per manufacturer instructions. By assessing the 260/280 ratio and 230/260 ratio using a nanodrop spectrophotometer (Eppendorf), the RNA checked to be of good quality with little contamination. Using the nanodrop spectrophotometer, the RNA was quantified. The RNA was then frozen prior to reverse transcription (**section 2.6.9**), or library preparation for RNA sequencing (**section 2.6.11**).

2.6.9. Reverse transcription

Reverse transcription was used to convert RNA to complementary DNA (cDNA). 1250 ng of RNA in 11 µl of dH_20 was incubated with 1 µl random primers (Promega) and 1 µl dNTPs (10 mM, Promega) for 5 minutes at 65°C, and then for 1 minute on ice. 4 µl of 5x buffer (Invitrogen), 1 µl Dithiothreitol (0.1 M, Invitrogen), 1 µl RNAsin plus (Promega), and 1µl of superscript III reverse transcriptase (Invitrogen) was added. This was incubated for 5 minutes at room temperature, and then for 2 hours at 50°C. The reaction was then inactivated by incubation at 70°C for 10 minutes, and stored at -80°C, until needed for quantitative real-time PCR (qPCR) (**section 2.6.10**).

2.6.10. Quantitative real-time PCR (qPCR)

Prior to the qPCR experiments, the primers used were tested to ensure an efficiency of 90-110%, and the melting curves were assessed to ensure one single product was being produced. Per reaction, 12.5 µl of SYBR Green master mix (Applied Biosystems), 0.625 µl of each primer (Sigma Aldrich), an appropriate volume of cDNA of the range of 60 ng - 180 ng, depending on the primers and brain region, and dH_20 was used to complete the volume at 25 µl. For complement component 3 (C3), the primers used were, forward: 5'-3', AGCAGGTCATCAAGTCAGGC, reverse: 5'-3', GATGTAGCTGGTTGTTGGGCT. For complement component 4 (C4), the primers used were, forwards: 5'-3', GACAAGGCACCTTCAGAACC, reverse: 5'-3', CAGCAGCTTAGTCAGGGTTACA. A control, 18S ribosomal RNA (18S) was used, the primers for 18S were, forward: 5'3, GTCTGTGATGCCCTTAGATG, reverse, 5'-3', AGCTTATGACCCGCACTTAC. The samples were loaded in duplicates into a 96 well plate. qPCR was conducted using a Real-Time PCR system (Thermo Fisher Scientific). The cycle was as follows: step one: 95°C for 10 minutes, step 2: 95°C for 15 minutes and 60°C for 1 minute. Step two was repeated 40 times. Step three, the melt curve analysis: 95°C, 15 minutes, 60°C, 1 minute, 95°C, 15 minutes. The delta threshold cycle (CT) of the genes were calculated as the average CT of the gene of interest (C3 or C4) – the average CT of the housekeeping gene (18S).

2.6.11. RNA sequencing

The cDNA library used for RNA sequencing (RNAseq) was prepared by Angela Marchbank of the Genome Hub, at Cardiff University. 500 ng of RNA per sample was used. Prior to their use, the RNA samples were confirmed to be of high quality, with minimal degradation, and low contamination by assessment using a Tape Station and Qubit. The libraries were prepared in accordance to manufacturer instructions (Illumina TruSeq). In brief, the RNA samples were purified with magnetic beads to remove ribosomal RNA and non-messenger RNAs. Following washing and then elution from the beads, the mRNA was then transferred for first strand cDNA synthesis, using superscript II. The second strand was synthesised, and the mRNA template was destroyed. Adapters are ligated on the cDNAs, for binding to the sequencing flowcell. The cDNA then goes through a short amplification step to enrich the libraries, and then a small amount of each sample is tested on a DNA chip, for quality control and for assessment of library size, prior to normalising the library sizes. The RNA sequencing was also done by Angela Marchbank, using the illumina NextSeq500 system in a (1x75) bp

cartridge, as per manufacturer instructions. In brief, a strand of cDNA is bound to a docking site of the flowcell. Fluorescently tagged dNTPs are added one at a time, emitting fluorescence. This is read, allowing all the cDNAs to be sequenced. The analysis was done by Robert Andrew, of the Data Hub, Cardiff University. The sequences were trimmed with Trimmomatic (Bolger, Lohse and Usadel 2014) and assessed for quality using FastQC. The reads were mapped to the mouse GRCm38 reference genome using STAR and assigned transcripts using FeatureCounts, both downloaded from Enssmbl FTP site (Dobin et al 2013, Liao, Smyth and Shi 2013). Differential gene expression analysis used to the DESeq2 package (Love, Huber, and Anders 2014). Normalised gene expression values (DESeq2) were used for principal component analysis in R using prcomp. The relative expression of the genes was also assessed, in an analysis that is weighted to include all social dominance environment and genotype conditions, per brain region, this was followed by a Benjamini-Hochberg correction. This yields the P-value and the $\log_2$ (fold change) for each individual gene per comparison.

2.7. Statistics

All data was analysed using SPSS software, version 23 (IBM). For the comparative analysis of *Nlgn3*$^{y/-}$ mice from MGH and their *Nlgn3*$^{y/+}$ littermates, with *Nlgn3*$^{y/+}$ mice from SGH, *Nlgn3*$^{y/-}$ mice from SGH, and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates, two-way ANOVAs were used. For the social dominance environment, three factors were used, mice being either MGH (indicating *Nlgn3*$^{y/+}$ mice from MGH and their littermates, *Nlgn3*$^{y/+}$ from MGH), SGH (indicating *Nlgn3*$^{y/+}$ mice from SGH or *Nlgn3*$^{y/-}$ mice from SGH), or PV-MGH, (indicating *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates). For genotype, two factors where used, mice were either *Nlgn3*$^{y/+}$ (for *Nlgn3*$^{y/+}$ mice from SGH, *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice), or *Nlgn3*$^{y/-}$ (for *Nlgn3*$^{y/-}$ mice from MGH, *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ mice, and *Nlgn3*$^{y/-}$ mice from SGH). In all other cases, simple comparisons were used.

The data was tested for normal distribution using a Shapiro-Wilk test, and by assessment of the Q-Q plot. If the result of the Shapiro-Wilk test was higher than 0.05, and the Q-Q test indicated normality, then the data was assumed to be normal. In the case of non-normal data, when applicable (independent samples t-test, one-way ANOVAs) a non-parametric test was used as an alternative (Mann-Whitney U test, Kruskal-Wallis test). As there is no non-normal equivalent of a two-way ANOVAs the data was first analysis with a Kruskal-Wallis test,

then analysed with a two-way ANOVA, and finally, a two-way ANOVA with transformed data was used to confirm any significant effects. As there is no non-parametric equivalent of a repeated measures two-way ANOVA, the data was analysed using a two-way repeated measures ANOVA, and then any significant interactions are confirmed using a two-way repeated measures ANOVA on transformed data. In addition, the data was tested for homogeneity of variance using a Levene's test; if the result of the Levene's test was 0.05 or higher then the data was assumed to have equality of variances. If the data did not have equality of variance any significant effects were confirmed on transformed data. For all significant results, the statistical output is presented in the text. In the supplementary tables, the details of the Shapiro-Wilk test, Levene's test, statistical outputs including any non-significant results can be found. In addition, the power of the statistical tests presented in the results can also be found in the supplementary tables. In the figure legends, the average value and standard error of the mean (SE) can be found. In the main text, and the supplementary information, the number of mice can be found, in all cases 'n' equals the number of mice used.

Chapter 3: The effect of the social dominance environment and *Nlgn3* re-expression on the social behaviours of *Nlgn3* knockout mice and their wild-type littermates

3.1. Introduction

Adult male mice form social hierarchies, consisting of dominant, subdominant and submissive mice. Among other behaviours, it has been shown that socially submissive mice have decreased courtship ultrasonic vocalisation (USV), and decreased numbers of victories in the tube test (Lindzey et al 1961, Wang et al 2011). Many rodent models of ASD have been shown to be socially submissive to their peers, as measured through the tube test (Spencer et al 2005, Saxena et al 2018), and by assessment of levels of courtship USV or assessment of agonistic and defensive behaviours (Cheh et al 2006, Jamain et al 2008, Wöhr et al 2011, Wang et al 2011, Yang et al 2015), indicating that this may be a phenotype commonly seen in models of ASD. Adult male mice missing *Nlgn3* have been shown to have decreased courtship USV towards females in oestrous, possibly indicating social submission (Radyushkin et al 2009, Fischer and Hammerschmidt 2011). One of the hypotheses investigated in this chapter is to determine if *Nlgn3*$^{y/-}$ mice are socially submissive. Additionally, female *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice had not yet been phenotyped, with the exception of a single experiment on *Nlgn3*$^{+/-}$ mice, revealing that they have altered social olfactory preference (Dere et al 2018). Another hypothesis investigated in this chapter is to assess if *Nlgn3*$^{-/-}$ and *Nlgn3*$^{+/-}$ mice have altered phenotypes relating to social behaviours.

The position within the social hierarchy and the manifestation of the social environment has been shown to impact social behaviours of mice (Ely et al 1978, Branchi, D'Andrea, Fiore et al 2006, D'Andrea et al 2007, Naert et al 2011). Therefore, another aim addressed in this chapter is to assess if the presence of *Nlgn3*$^{y/-}$, *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice influence the social behaviours of other members of the shared social environment.

Finally, cells expressing *Pvalb*, which encodes the protein product parvalbumin, have been shown to have altered properties in many mouse models of ASD (Peñagarikano et al 2011, Martins et al 2011, Selby et al 2007, Gogolla et al 2009, Vogt et al 2015), and studies specifically targeting *Pvalb*-expressing cells demonstrate manipulations of social phenotypes (Ito-Ishida et al 2015, Wöhr et al 2015, Inan et al 2016, Selimbeyoglu et al 2017, Cao et al 2018). Furthermore, *Nlgn3* is expressed in *Pvalb*-expressing cells (Rothwell et al 2014,

Polepalli et al 2017). Given that *Pvalb*-expressing cells control social behaviours, they may be a good target to selectively restore expression of *Nlgn3* in, firstly to see if this restores individual social behaviours, and secondly to see if this influences the social behaviours of the group. Therefore, the final hypothesis tested in this chapter is to assess if selective re-expression of *Nlgn3* within *Pvalb*-expressing cells restores the individual and group behaviours of the mice.

3.2. Aims and objectives

1. To identify if *Nlgn3*$^{y/-}$ mice have altered phenotypes relating to social hierarchy and if re-expression of *Nlgn3* within *Pvalb*-expressing cells of *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice restores hierarchy related behaviours.
2. To identify how the social behaviours of *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from mixed genotype housing (MGH) compare to the behaviours of *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from single genotype housing (SGH), and to *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates.
3. To identify if *Nlgn3*$^{+/-}$ mice and *Nlgn3*$^{-/-}$ mice have altered social behaviours, and:
    - To determine if these are impacted by the social environment,
    - To determine if re-expression of *Nlgn3* within *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice restores their social behaviours.
4. To establish if the social behaviours of *Nlgn3*$^{+/+}$ mice are impacted by MGH or SGH.

3.3. Results

3.3.1. The influence of social dominance environment and re-expression of Nlgn3 on the social behaviours of adult male Nlgn3$^{y/+}$ mice and Nlgn3$^{y/-}$ mice.

This work aims to determine what influence the *Nlgn3$^{y/-}$* mice could be having on their peers. As the social environment is influenced by dominance behaviours, and *Nlgn3$^{y/-}$* mice may have aberrant dominance behaviour, as will be explored in this chapter, the social environment is referred to the social dominance environment. To determine if MGH influences *Nlgn3$^{y/+}$* mice and their *Nlgn3$^{y/-}$* littermates, the phenotypes of *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH were compared with those of *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH. The work in the chapter also aims to assess if re-expression of *Nlgn3* within *Pvalb*-expressing cells of *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice restores the social behaviours in *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, and subsequently leads to altered behaviours in their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates. To that end, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates were characterised. As will be explored in section 3.3.1.1, and figure 3.1, the re-expression of *Nlgn3* in the *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* mice could impact the social dominance of the mice, and therefore could lead to an altered social dominance environment of the mice. Therefore, for all analysis, the MGH of *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates was classified as a social dominance environment of their own, referred to as PV-MGH. The housing conditions of the male mice used are summarised in **table 3.1.**

Table 3.1. The social dominance environment of the male mice. SGH stands for single genotype housing, MGH stands for mixed genotype housing, PV-MGH indicates mixed genotype housing of mice expressing *Pvalb*$^{cre/+}$.

Genotype of litter	***Acronym***
Nlgn3$^{y/+}$*, and Nlgn3*$^{y/-}$ (and *Nlgn3*$^{+/+}$*, Nlgn3*$^{+/-}$ until weaning)	*Nlgn3*$^{y/-}$ mice from MGH *Nlgn3*$^{y/+}$ mice from MGH
Nlgn3$^{y/+}$ (and *Nlgn3*$^{+/+}$ until weaning)	*Nlgn3*$^{y/+}$ mice from SGH
Nlgn3$^{y/-}$ (and *Nlgn3*$^{-/-}$ until weaning)	*Nlgn3*$^{y/-}$ mice from SGH
Nlgn3$^{y/+}$*Pvalb*$^{cre/+}$*, Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$*.* (and *Nlgn3*$^{+/-}$*Pvalb*$^{cre/+}$*,* and *Nlgn3*$^{+/+}$*Pvalb*$^{cre/+}$ until weaning)	PV-MGH

3.3.1.1. Within cage tube test dominance of Nlgn3$^{y/-}$ mice and their Nlgn3$^{y/+}$ littermates.

The tube test was used to determine if *Nlgn3$^{y/-}$* mice are submissive to their *Nlgn3$^{y/+}$* littermates (Lindzey et al 1961, Wang et al 2011). It was revealed that *Nlgn3$^{y/-}$* mice lost significantly more trials of the tube test than their *Nlgn3$^{y/+}$* littermates (**figure 3.1.A,** Mann-Whitney U test, *P = 0.025, Nlgn3$^{y/-}$* mice from MGH n = 19, *Nlgn3$^{y/+}$* mice from MGH n = 19). This shows that *Nlgn3$^{y/-}$* mice are socially submissive relative to their *Nlgn3$^{y/+}$* littermates demonstrating that *Nlgn3* expression within the brain mediates dominance behaviours in mice. Therefore, it is possible that re-expression of *Nlgn3* can restore this phenotype.

3.3.1.2. Re-expression of Nlgn3 under the parvalbumin promotor.

To determine if Neuroligin-3 is re-expressed in *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, the levels of Neuroligin-3 were determined using western blot analysis of brain lysate taken from adult male mice. Brain tissue from *Nlgn3$^{y/+}$* mice, *Nlgn3$^{y/-}$* mice, and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice were used. Neuroligin-3 was detected within the cerebellum, cortex, brainstem, thalamus and striatum of *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice but not in their hippocampus. Of note is that there is an upper band that does not correspond to Neuroligin-3 (**figure 3.1.B**) Quantification of additional western blots of lysate from *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice revealed a high level of Neuroligin-3 expression in the cerebellum, a lower expression in the cortex, brainstem and striatum, and no expression in the hippocampus (**figure 3.1.C**). The corresponding western blots can be found in the appendix (**figure A1.1**). Given that it was found that there is robust re-expression of Neuroligin-3 in the *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, it would appear that *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice are suitable for assessing if *Nlgn3* re-expression in *Pvalb*-expressing cells contributes to the phenotypes seen in the *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice.

3.3.1.3. Within cage tube test dominance of Nlgn3$^{y/-}$Pvalb$^{cre/+}$ mice and their Nlgn3$^{y/+}$Pvalb$^{cre/+}$ littermates

To assess if re-expression of *Nlgn3* in *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice may be sufficient to restore the social dominance of the mice, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice were tested against their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates, using the tube test. It was revealed that there is no significant difference between the two groups (**figure 3.1.D**, independent samples t-test, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice n = 9, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 8). This suggests that re-expression of *Nlgn3* in *Pvalb*-expressing cells of *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice is sufficient to restore a normal dominance relationship between *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice.

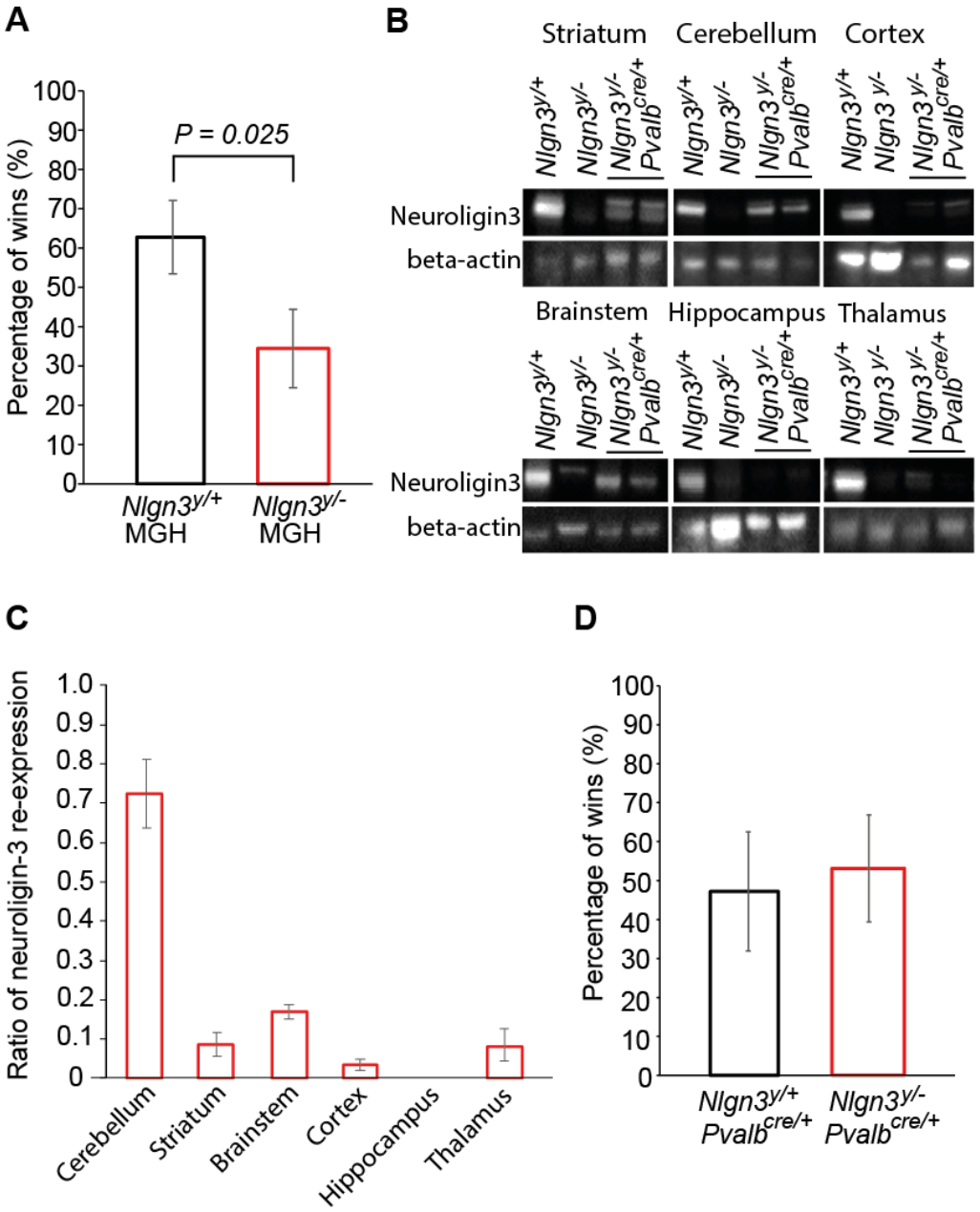


Figure 3.1. The within cage tube test of mice from MGH, re-expression of Neuroligin-3 in *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and the within cage tube test of *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice. *A*, the average percentage (%) of tube test wins and standard error of the mean (SE) of *Nlgn3$^{y/+}$* mice from MGH, 64.7(SE:9.27), *Nlgn3$^{y/-}$* mice from MGH, 32.6(SE:9.85). A Mann-Whitney U test revealed a significant difference between the groups, as shown on the figure. ***B,*** protein levels of Neuroligin-3 detected via western blot in different brain regions of, left to right, *Nlgn3$^{y/+}$* mice, *Nlgn3$^{y/-}$* mice, and two samples from *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice. ***C,*** quantification of levels of Neuroligin-3, relative to beta-actin and normalised to *Nlgn3$^{y/+}$* levels, cerebellum ~0.71, Striatum ~ 0.07, Brainstem ~1.50, Cortex ~0.04, Thalamus ~0.75. No signal detected in the hippocampus. ***D,*** the average percentage (%) of wins and standard error of the mean (SE) of *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 47.2(SE:13.7), and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 53.1(SE:15.3). An independent samples t-test revealed no significant difference between the groups.

3.3.1.4. The influence of the social dominance environment and re-expression on the tube test behaviour of Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice against unfamiliar male mice

To determine the effect of the social dominance environment on dominance behaviour, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates were tested against unfamiliar submissive wild-type mice from an independent C57BL/6 colony. A submissive mouse was selected when it lost 57% of its tube test trials against unfamiliar wild-type male mice. It was observed that *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH are more submissive against unfamiliar wild-type mice compared to *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates (**figure 3.2.A**, two-way ANOVA, social dominance environment: *P = 0.003, F = 6.2, df = 2,* Bonferroni post-hoc analysis, MGH and SGH: *P = 0.015*, MGH and *Pvalb: P = 0.015*, *Nlgn3$^{y/+}$* mice from MGH n = 16*, Nlgn3$^{y/-}$* mice from MGH n = 13, *Nlgn3$^{y/+}$* mice from SGH n = 15, *Nlgn3$^{y/-}$* mice from SGH n = 12, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 9, and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice n = 8). This suggests that the presence of *Nlgn3$^{y/-}$* mice could impact the relative tube test dominance of their *Nlgn3$^{y/+}$* littermates. Furthermore, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates show increased tube test victories compared to the mice in MGH, indicating that the normalisation of the within cage tube test dominance of the *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice leads to a restoration of the relative dominance behaviours of both *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates.

3.3.1.5. The influence of the social dominance environment and re-expression on urinary testosterone levels of adult Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

Some evidence exists to suggest that levels of testosterone are a marker of dominance related behaviours in male mice (Juntti et al 2010). Therefore, the urinary testosterone levels were assessed, revealing that *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH have lower testosterone than *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, consistent with the decreased tube test victories. Furthermore, it was observed that *Nlgn3$^{y/-}$* mice have decreased testosterone compared to *Nlgn3$^{y/+}$* mice when in SGH, and that the social dominance environment influences the testosterone levels of *Nlgn3$^{y/+}$* mice to a larger degree than *Nlgn3$^{y/-}$* mice (**figure 3.2.B,** two-way ANOVA*,* social dominance environment: *P = 0.0004, F = 9.1, df=2,* social dominance environment: *P = 0.021, F = 5.6, df=1,* Genotype x social dominance environment: *P = 0.026, F = 3.9, df =* 2. Bonferroni

post-hoc analysis, MGH and SGH: *P = 0.00008*, MGH and *Pvalb: P = 0.044*. Bonferroni adjusted pairwise analysis, *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH: *P = 0.00016*, *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/+}$ mice from MGH: *P = 9.36e-06, Nlgn3*$^{y/+}$ mice from MGH n = 10, *Nlgn3*$^{y/-}$ mice from MGH n = 8, *Nlgn3*$^{y/+}$ mice from SGH n = 15, *Nlgn3*$^{y/-}$ mice from SGH n = 12, *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice n = 9, *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice n = 6). Therefore, it appears that the mixed genotype of *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{y/+}$ mice leads to a reduction of testosterone levels, and single genotype housing and re-expression of *Nlgn3* within *Pvalb*-expressing cells increases this.

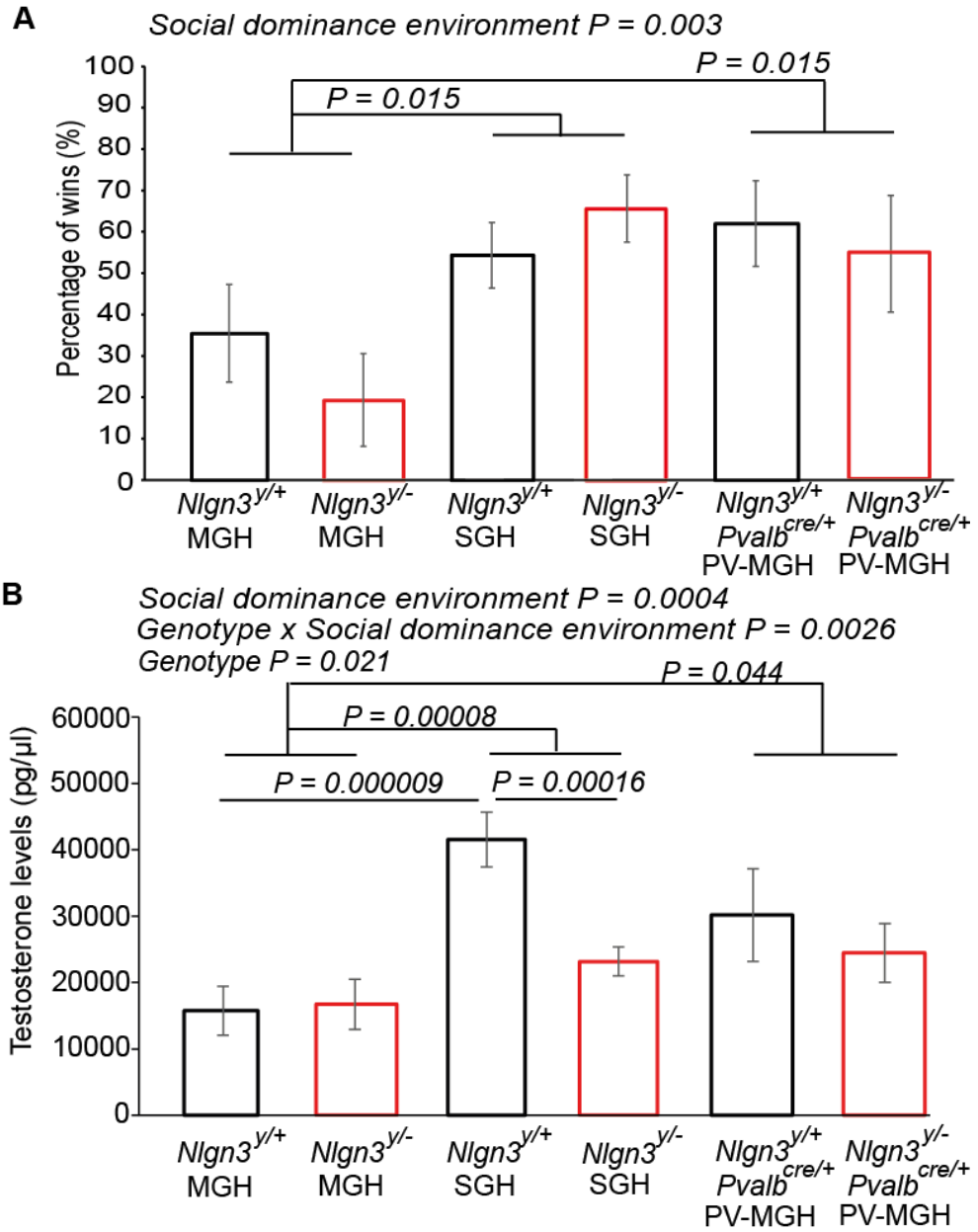


Figure 3.2. The impact of social dominance environment and re-expression on the tube test victories against unfamiliar male mice and urinary testosterone levels of adult *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice. ***A***, the average percentage (%) of wins and the standard error of the mean (SE) for *Nlgn3$^{y/+}$* mice from MGH, 33.3(SE:9.6), *Nlgn3$^{y/-}$* mice from MGH 25.6(SE:9.3), *Nlgn3$^{y/+}$*mice from SGH, 55.5(SE:8.0), *Nlgn3$^{y/-}$* mice from SGH, 66.7(SE:8.2), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 62.9(SE:11.7), and for *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 55.6(SE:14.6). A two-way ANOVA revealed a significant effect of social dominance environment (*P = 0.003*). Results of the Bonferroni post-hoc test are shown on the figure. ***B,*** the average urinary testosterone levels (pg/µl) and standard error of the mean (SE) for *Nlgn3$^{y/+}$* mice from MGH, 15737.7(SE:3686), *Nlgn3$^{y/-}$* mice from MGH, 16735.86(SE:3779), *Nlgn3$^{y/+}$* mice from SGH, 43970(SE:4115.4), *Nlgn3$^{y/-}$* mice from SGH, 23164.9 (SE:2184.9), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice , 30172.5(SE:6981) and for *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 24478.6 (SE:4418). A two-way ANOVA revealed a significant effect of social dominance environment (*P = 0.0004*) and genotype (*P = 0.021*, and genotype and social dominance environment (*P = 0.0026*). The results of the Bonferroni post-hoc analysis and Bonferroni adjust pairwise comparison are shown on the figure.

3.3.1.6. The influence of social dominance environment on the courtship ultrasonic vocalisations (USV) of adult Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

The levels of courtship USV were compared. Two different parameters of USV were assessed; the number of calls and the total duration spent calling over three minutes. It is important to assess both criteria as it is possible that the structure of the calls themselves could become corrupted, leading to incorrect numbers of calls being detected. *Nlgn3$^{y/-}$* mice, regardless of social dominance environment conditions or re-expression of *Nlgn3*, have decreased numbers of calls compared to *Nlgn3$^{y/+}$* mice, while social dominance environment did not influence the USV of *Nlgn3$^{y/+}$* or *Nlgn3$^{y/-}$* mice (**figure 3.3.A**, two-way ANOVA, Genotype: *P = 0.00002, F = 21.2, df= 1*, *Nlgn3$^{y/+}$* mice from MGH n = 11, *Nlgn3$^{y/-}$* mice from MGH n = 15, *Nlgn3$^{y/+}$* from SGH n = 15, *Nlgn3$^{y/-}$* from SGH n = 12, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 11 and *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 10). For the total duration calling, a similar result was observed in which *Nlgn3$^{y/-}$* mice regardless of social dominance environment and re-expression showed decreased total duration of USV compared to *Nlgn3$^{y/+}$* mice, and social dominance environment does not influence the calls of either *Nlgn3$^{y/-}$* or *Nlgn3$^{y/+}$* mice (**figure 3.3.B,** two-way ANOVA, Genotype: *P = 0.00008, F=21.2, df =1, Nlgn3$^{y/+}$* mice from MGH n = 11, *Nlgn3$^{y/-}$* mice from MGH n = 15, *Nlgn3$^{y/+}$* from SGH n = 15, *Nlgn3$^{y/-}$* from SGH n = 12, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 11 and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* n = 10). Together, these results are consistent with the previous observations of *Nlgn3$^{y/-}$* mice having decreased courtship USVs (Radyushkin et al 2009, Fischer and Hammerschmidt 2011). Furthermore, while it has previously been reported that social dominance in the tube test influences levels of courtship USV, this is not reflected in this data. Finally, it appears that *Nlgn3* expression in *Pvalb*-expressing cells does not mediate the USV courtship behaviour.

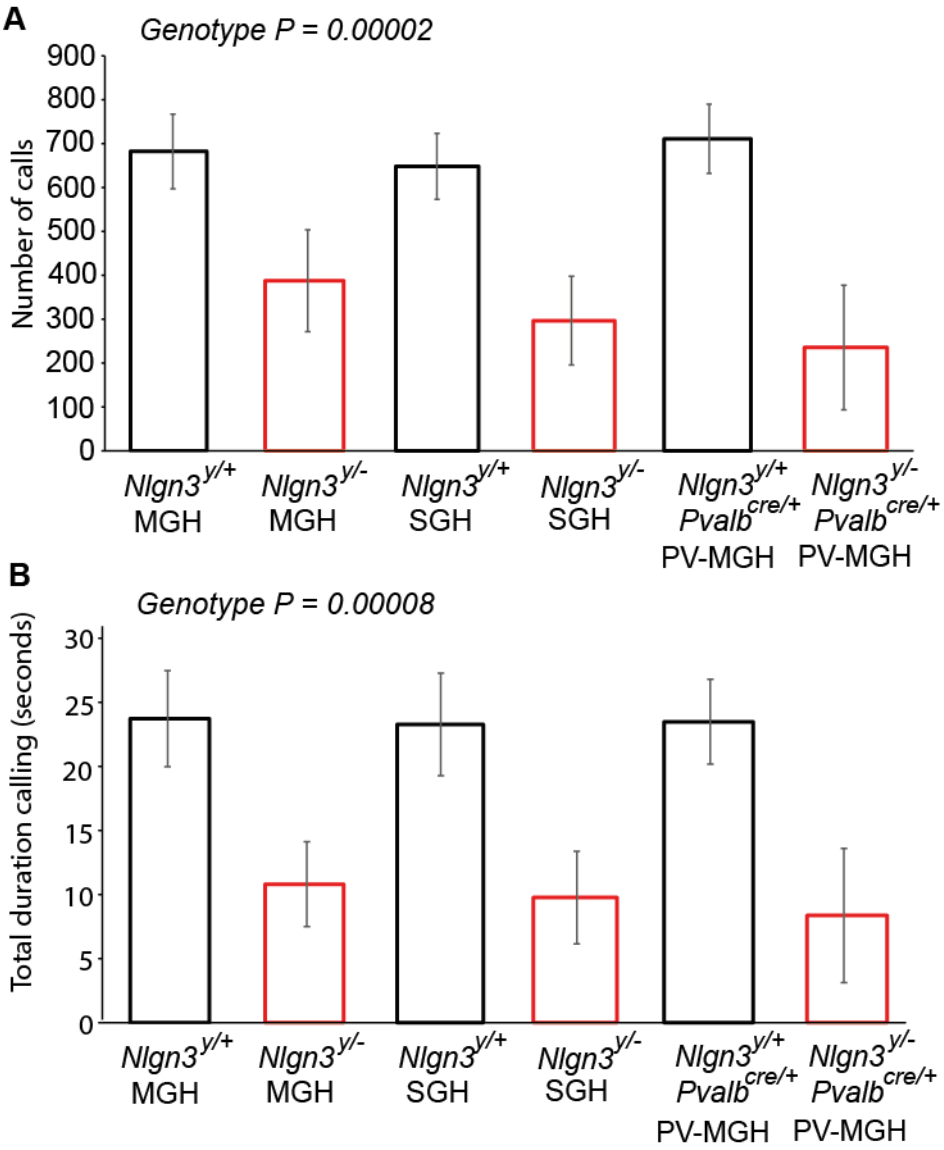


Figure 3.3. The impact of social dominance environment and re-expression on the courtship related behaviours of adult male *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice. *A*, average total number of calls and standard error of the mean (SE) for *Nlgn3$^{y/+}$* mice from MGH, 682.5(SE:84.8), *Nlgn3$^{y/-}$* mice from MGH, 387.7(SE:116.3), *Nlgn3$^{y/+}$* mice from SGH, 648.5(SE:75.5), *Nlgn3$^{y/-}$* mice from SGH, 296.7(SE:101.1), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 711.2(SE:78.7), and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 235.5(SE:141.9). A two-way ANOVA revealed a significant impact of genotype (*P = 0.00002*). ***B***, average total duration calling (seconds) and standard error of the mean (SE) for *Nlgn3$^{y/+}$* mice from MGH, 23.7(SE:3.7), *Nlgn3$^{y/-}$* mice from MGH, 10.8(SE:3.3), *Nlgn3$^{y/+}$* mice from SGH, 23.3(SE:4.0), *Nlgn3$^{y/-}$* mice from SGH, 9.7(SE:3.6), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 23.5(SE:3.3), and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$*mice, 8.4(SE:5.3). A two-way ANOVA revealed a significant impact of genotype (*P = 0.00008*).

The exploration of the USV does not differentiate between an inability to vocalise, and aberrant courtship behaviour. By exploring the interaction time with the female, it is possible to better determine if the courtship itself is altered. The interaction of the mice with the female in oestrous was scored, revealing that *Nlgn3$^{y/-}$* mice show decreased time in interaction with the female, and neither social dominance environment nor re-expression of *Nlgn3* influenced the interaction time of *Nlgn3$^{y/-}$* mice or *Nlgn3$^{y/+}$* mice (**figure 3.4.A,** two-way ANOVA, Genotype: *P = 0.00000001, F = 42.3, df= 1, Nlgn3$^{y/+}$* mice from MGH n = 15, *Nlgn3$^{y/-}$* mice from MGH n = 11, *Nlgn3$^{y/+}$* from SGH n = 15, *Nlgn3$^{y/-}$* from SGH n = 12, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 11, and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* n = 10). These results indicate that *Nlgn3$^{y/-}$* mice have decreased interest in the female mice, regardless of social dominance environment or re-expression, reflecting what is seen in the USV results.

Finally, as it has previously been shown that the position within the social hierarchy as tested by the tube test correlates with the numbers of ultrasonic vocalisation (Wang et al 2011), a correlation of the two measures was assessed. Cages of three mice from MGH conditions and cages of three *Nlgn3$^{y/+}$* mice from SGH conditions were assessed. It was observed that in cages of *Nlgn3$^{y/+}$* mice from SGH conditions, the mice with the highest numbers of USV were typically the dominant within the tube test, while in the mice from MGH conditions, this trend is not observed (**figure 3.4.B,** Pearson's rank test, *Nlgn3$^{y/+}$* mice in SGH *r^2 = 0.84*, MGH mice r^2 = *0.43*, number of cages *Nlgn3$^{y/+}$* mice SGH N = 7, number of cages MGH mice N = 6). This suggests that while in SGH the expected correlation of measures is observed, however in MGH this correlation is disrupted.

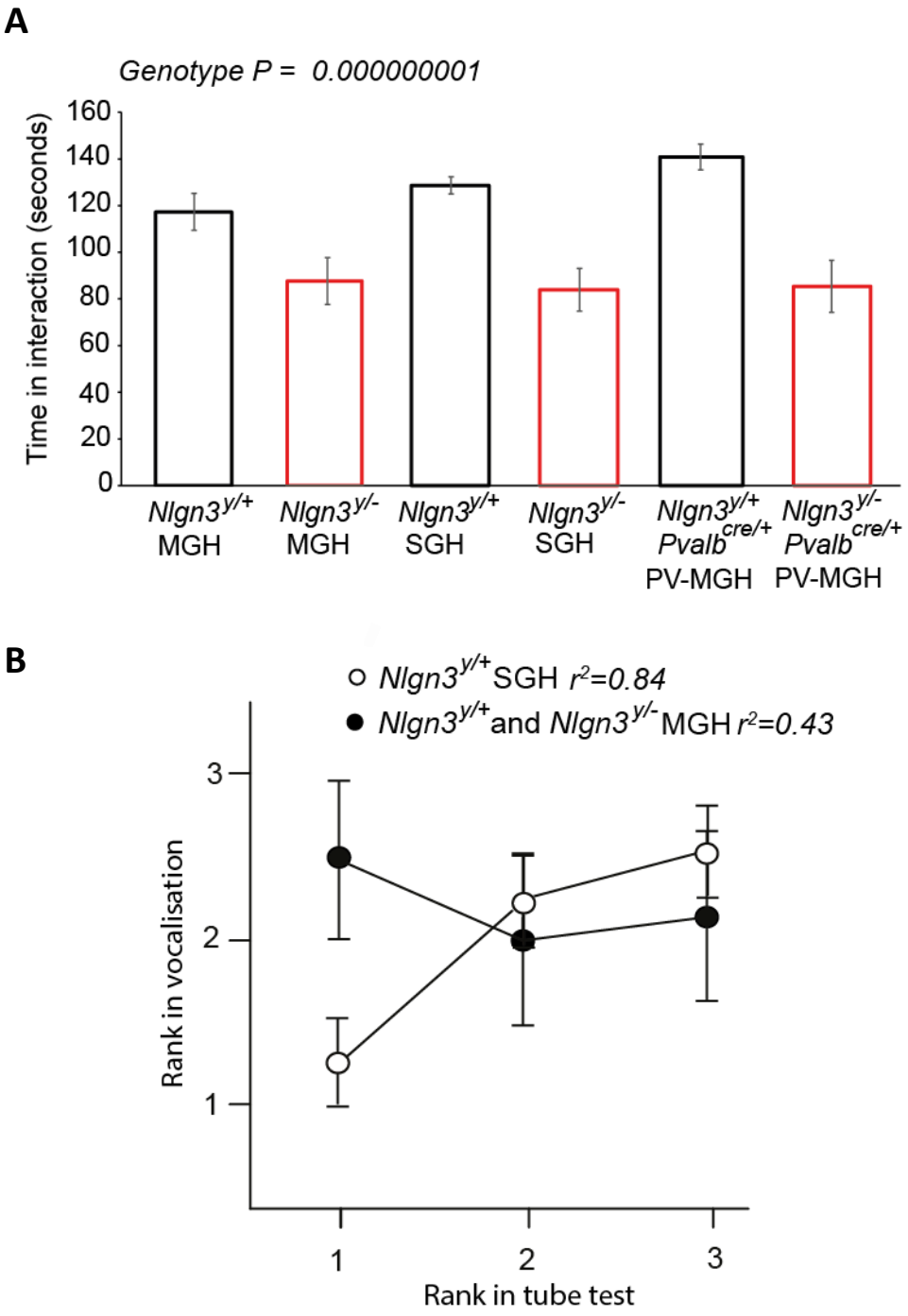


Figure 3.4. Interaction with female mice, and correlation of tube test and vocalisation. ***A***, average total duration interaction (seconds) and standard error of the mean (SE) of *Nlgn3$^{y/+}$* mice from MGH, 117.3(SE:7.9), *Nlgn3$^{y/-}$* mice from MGH, 87.7.3(SE:10.0), *Nlgn3$^{y/+}$* mice from SGH, 128.7(SE:3.6), *Nlgn3$^{y/-}$* mice from SGH, 83.9(SE:9.2), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 140.9(SE:5.2), and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 85.4(SE:11.1). A one-way ANOVA revealed a significant difference of genotype (*P = 0.000000001*). ***B,*** correlation of the average tube test and vocalisation and standard error of the mean (SE) of cages of three mice of either *Nlgn3$^{y/+}$* mice from SGH conditions, or MGH mice.

3.3.2. The impact of the social dominance environment and Nlgn3 re-expression on the social behaviours of adult female Nlgn3$^{+/+}$ mice, Nlgn3$^{-/-}$ mice, and Nlgn3$^{+/-}$ mice.

In this section, the behaviours of *Nlgn3*$^{+/+}$ mice, *Nlgn3*$^{+/-}$ mice, and *Nlgn3*$^{-/-}$ mice were assessed. Primarily, this was to determine if there is a phenotype relating to social behaviours in *Nlgn3*$^{+/-}$ mice and *Nlgn3*$^{-/-}$ mice relative to their littermates. The second aim was to determine the impact of social dominance environment on the behaviours of the mice. Given that this work focuses on multiple social dominance environment conditions, and as *Nlgn3* is X-linked, a targeted analytical approach was used to understand the impact of social dominance environment on each genotype. Initially, *Nlgn3*$^{+/+}$ mice, their *Nlgn3*$^{+/-}$ (H-WT) littermates, *Nlgn3*$^{-/-}$ mice, and their *Nlgn3*$^{+/-}$ (H-KO) littermates were compared. Following this initial characterisation, the impact of the social environment on the behaviours of *Nlgn3*$^{+/+}$ mice were assessed. *Nlgn3*$^{+/+}$ mice from MGH were compared to *Nlgn3*$^{+/+}$ mice from SGH. Importantly, the data from *Nlgn3*$^{+/+}$ mice from MGH is replotted from the initial analysis. Next, to determine if the behaviours of *Nlgn3*$^{-/-}$ mice are influenced by the social environment, *Nlgn3*$^{-/-}$ mice from MGH, *Nlgn3*$^{-/-}$ mice from SGH, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice were analysed; this allowed the effect of re-expression of *Nlgn3* in *Pvalb*-expressing cells of *Nlgn3*$^{-/-}$ mice to be explored. Importantly, the data from the *Nlgn3*$^{-/-}$ mice from MGH is replotted from the initial analysis. **Table 3.2** summarises the social dominance environment conditions used.

Table 3.2. The social dominance environment of the female mice. SGH stands for single genotype housed. MGH stands for mixed genotype housed. H-WT indicates that the *Nlgn3*$^{+/-}$ mice are housed with *Nlgn3*$^{+/+}$ littermates. H-KO indicates that the *Nlgn3*$^{+/-}$ mice are housed with *Nlgn3*$^{-/-}$ littermates, PV-MGH indicates mixed genotype housing of mice expressing *Pvalb*$^{cre/+}$.

Genotype of litter	**Acronym**
Nlgn3$^{+/+}$, *Nlgn3*$^{+/-}$ *(and Nlgn3*$^{y/+}$ *and Nlgn3*$^{y/-}$ *until weaning)*	*Nlgn3*$^{+/+}$ mice from MGH *Nlgn3*$^{+/-}$ *(H-WT)*
Nlgn3$^{-/-}$, *Nlgn3*$^{+/-}$ *(and Nlgn3*$^{y/+}$, *and Nlgn3*$^{y/-}$ *until weaning)*	*Nlgn3*$^{-/-}$ *mice from MGH Nlgn3*$^{+/-}$ *(H-KO)*
Nlgn3$^{-/-}$ *(and Nlgn3*$^{y/-}$ *until weaning)*	*Nlgn3*$^{-/-}$ mice from SGH
Nlgn3$^{+/+}$ *(and Nlgn3*$^{y/+}$ *until weaning)*	*Nlgn3*$^{+/+}$ mice from SGH
Nlgn3$^{-/-}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{+/-}$, *Nlgn3*$^{+/-}$*Pvalb*$^{cre/+}$ *(and Nlgn3*$^{y/+}$, *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$, *Nlgn3*$^{y/-}$, *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ *until weaning, and no litters with Nlgn3*$^{-/-}$ *littermates were used)*	PV-MGH

3.3.2.1. The influence of the social dominance environment on the social interaction behaviour of adult Nlgn3$^{+/+}$ mice, Nlgn3$^{-/-}$ mice, and their Nlgn3$^{+/-}$ littermates.

To assess if *Nlgn3*$^{-/-}$ mice may have changes in their social behaviour and may influence the social behaviours of their littermates, interaction with an unfamiliar female was assessed. It was observed that *Nlgn3*$^{-/-}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates have lower levels of social interaction than *Nlgn3*$^{+/+}$ mice from MGH and their *Ngn3*$^{+/-}$ (H-WT) littermates (**figure 3.5.A**, one-way ANOVA, *P = 0.0004, F = 7.9, df=3,* Tukey's post-hoc analysis, *Nlgn3*$^{+/+}$ mice and *Nlgn3*$^{+/-}$ (H-KO) mice: *P = 0.040*, *Nlgn3*$^{+/+}$ mice and *Nlgn3*$^{-/-}$ mice from MGH: *P = 0.006*, *Nlgn3*$^{+/-}$ (H-WT) mice and *Nlgn3*$^{+/-}$ (H-KO) mice: *P = 0.017*, *Nlgn3*$^{+/-}$ (H-WT) mice and *Nlgn3*$^{-/-}$ mice from MGH: *P = 0.002*, *Nlgn3*$^{+/+}$ mice from MGH n = 10, *Nlgn3*$^{+/-}$ (H-WT) mice n = 10, *Nlgn3*$^{-/-}$ mice from MGH n = 12, *Nlgn3*$^{+/-}$ (H-KO) mice n = 7). *Nlgn3*$^{+/-}$ mice have altered social interaction dependent upon if they are housed with either *Nlgn3*$^{+/+}$ or *Nlgn3*$^{-/-}$ littermates, demonstrating that the presence of *Nlgn3*$^{-/-}$ mice or *Nlgn3*$^{+/+}$ mice influences their social behaviours.

To explore how the social dominance environment impacts the behaviours of *Nlgn3*$^{+/+}$ mice, the social interaction of the *Nlgn3*$^{+/+}$ mice from MGH was compared to the social interaction of *Nlgn3*$^{+/+}$ mice from SGH. It was observed that there is no significant difference between the total time in interaction with an unfamiliar wild-type female of *Nlgn3*$^{+/+}$ mice from SGH and *Nlgn3*$^{+/+}$ mice from MGH (**figure 3.5.B,** independent samples t-test, *Nlgn3*$^{+/+}$ mice from MGH n= 10, *Nlgn3*$^{+/+}$ mice from SGH n = 11). These data suggest that adult *Nlgn3*$^{+/-}$ female mice do not influence the social interaction behaviour of their *Nlgn3*$^{+/+}$ littermates.

Finally, a comparison of the social behaviours of *Nlgn3*$^{-/-}$ mice from MGH, *Nlgn3*$^{-/-}$ mice from SGH, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice revealed that *Nlgn3*$^{-/-}$ mice from MGH have decreased social interaction. Furthermore, this decreased social interaction seen in MGH is restored in SGH conditions, and also by re-expression of *Nlgn3* within *Pvalb*-expressing cells (**figure 3.5.C,** one-way ANOVA, *P = 0.00001, F = 17.2, df=2.* Tukey's post-hoc analysis, MGH and SGH: *P = 0.0003*, MGH and *Pvalb*: *P = 0.00002*, *Nlgn3*$^{-/-}$ mice from MGH n = 12, *Nlgn3*$^{-/-}$ mice from SGH n = 11, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice n = 10). This suggests that the social dominance environment and re-expression of *Nlgn3* in *Pvalb*-expressing cells does lead to changes in the social behaviours of *Nlgn3*$^{-/-}$ mice.

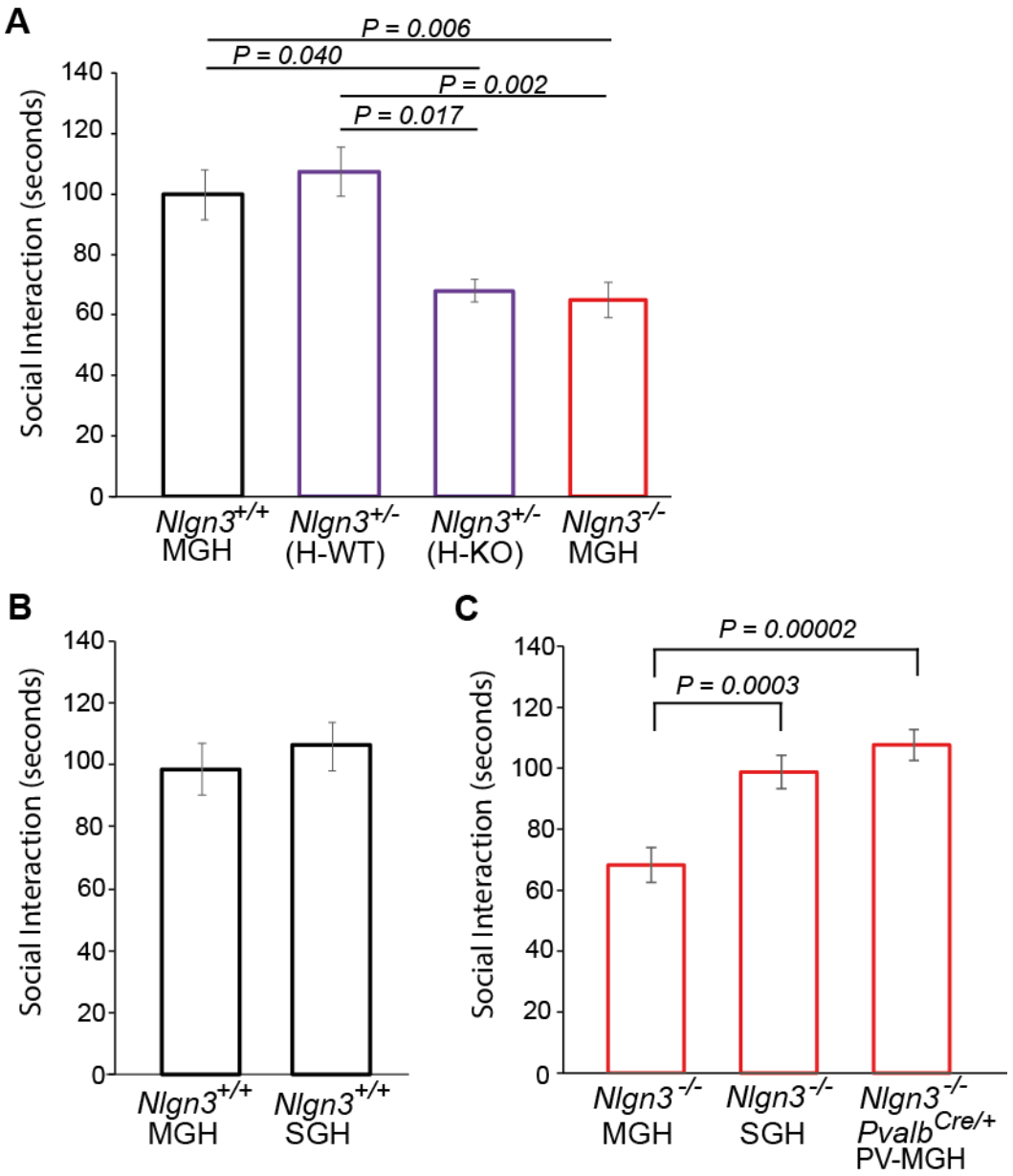


Figure 3.5. The impact of the social dominance environment on the time spent by adult *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice interacting with an unfamiliar mouse. ***A***, average time spent interacting with an unfamiliar female (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 99.8(SE: 8.4), *Nlgn3*$^{+/-}$ (H-WT) mice, 107.3(SE: 8.1), *Nlgn3*$^{+/-}$ (H-KO) mice, 68.0(SE:3.8), *Nlgn3*$^{-/-}$ mice from MGH, 64.9(SE:5.3). A one-way ANOVA revealed a significant difference between the groups (*P = 0.0004*), the results of the Bonferroni post-hoc are seen in the figure. ***B***, average time spent interacting with an unfamiliar female (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 99.8(SE:8.4), *Nlgn3*$^{+/+}$ mice from SGH, 105.8(SE:6.8). An independent samples t-test revealed no significant difference between the groups. ***C***, average time spent interacting with an unfamiliar female (seconds) and standard error of the mean (SE) of *Nlgn3*$^{-/-}$ mice from MGH, 64.93(SE:5.7), *Nlgn3*$^{-/-}$ mice from SGH, 98.7(SE:5.4), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$mice, 107.6(SE:5.0). A one-way ANOVA revealed a significant difference between the groups (*P = 0.00001*), the results of the Tukey's post-hoc analysis are seen in the figure.

3.3.2.2. Levels of male courtship USV towards Nlgn3$^{+/+}$ mice, Nlgn3$^{+/-}$ mice or Nlgn3$^{-/-}$ mice

To observe if *Nlgn3* knockout impacts the courtship behaviour of wild-type male mice towards females with *Nlgn3* knockout, the courtship USV of wild-type males towards females was assessed. It was observed that there is no difference in the duration of calls of male wild-type mice towards female *Nlgn3*$^{+/+}$ mice, *Nlgn3*$^{+/-}$ mice, and *Nlgn3*$^{-/-}$ mice (**figure 3.6.A**, one-way ANOVA, *Nlgn3*$^{+/+}$ mice n = 8, *Nlgn3*$^{+/-}$ mice n = 9, and *Nlgn3*$^{-/-}$ mice n = 8). Similarly, there is no difference in the number of calls towards female *Nlgn3*$^{+/+}$ mice, *Nlgn3*$^{+/-}$ mice, and *Nlgn3*$^{-/-}$ mice (**figure 3.6.B,** Kruskal-Wallis test, *Nlgn3*$^{+/+}$ mice n = 8, *Nlgn3*$^{+/-}$ mice n = 9, and *Nlgn3*$^{-/-}$ mice n = 8). These data show that there is no difference in courtship USV directed towards *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice, suggesting that the sexual behaviours of *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice are not different.

3.3.2.3. Characterisation of pup retrieval in Nlgn3$^{+/+}$, Nlgn3$^{+/-}$ and Nlgn3$^{-/-}$ mice

The maternal behaviours of female mice can be assessed through pup retrieval paradigms (Brown et al 1999). As different genotypes of female mice are used in the breedings for these experiments it is important to determine that this is no divergence in maternal care. Assessment of the maternal behaviours of *Nlgn3*$^{+/+}$ mice, *Nlgn3*$^{+/-}$ mice, and *Nlgn3*$^{-/-}$ mice revealed that there is no significant difference in the time that it takes for the pups to be returned to the nest, however *Nlgn3*$^{-/-}$ mice come into contact with the pups faster than *Nlgn3*$^{+/+}$ mice (**figure 3.6.C**, Kruskal-Wallis test, first contact: *P = 0.022,* Dunn's pairwise test, first contact, *Nlgn3*$^{-/-}$ mice and *Nlgn3*$^{+/+}$ mice: *P = 0.034*, *Nlgn3*$^{+/+}$ mice n = 13, *Nlgn3*$^{+/-}$ mice n = 9, *Nlgn3*$^{-/-}$ mice n = 9). These data suggest that while there is no difference in the time it takes to retrieve the pups to the nest, a slight discrepancy between the time to first contact the pups may exist in the *Nlgn3*$^{-/-}$ mice, indicating a faster response to the pups, but no overall change in the retrieval behaviour.

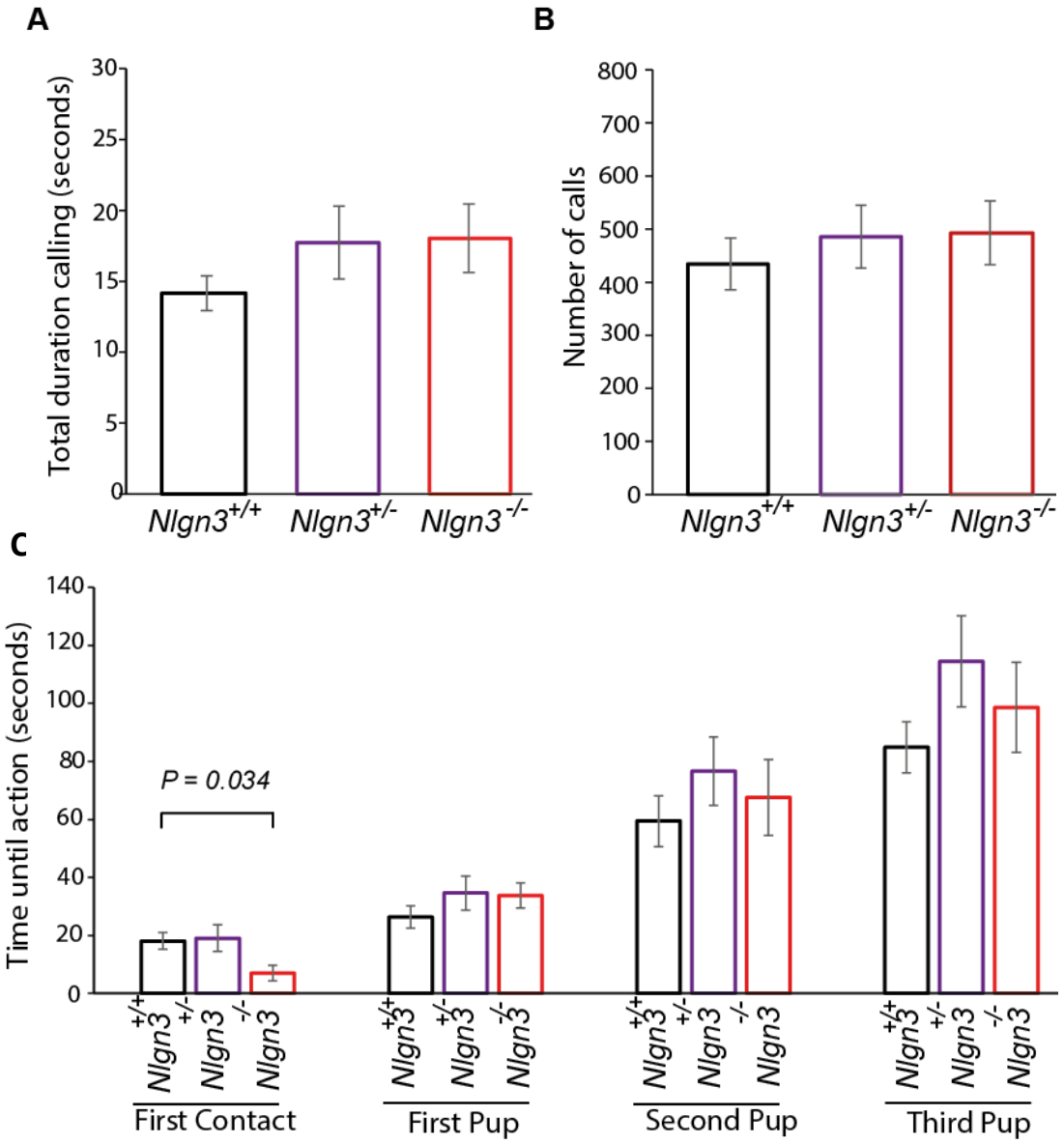


Figure 3.6, Courtship towards adult *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice and their maternal behaviours. ***A***, average total duration calling (seconds) and standard error of the mean (SE) of males to *Nlgn3*$^{+/+}$ mice, 14.2(SE:1.2), *Nlgn3*$^{+/-}$ mice, 17.7(SE:2.6), *Nlgn3*$^{-/-}$ mice, 18.0(SE:2.4). A one-way ANOVA revealed that there is no significant difference between the groups. ***B***, average total number of calls and standard error of the mean (SE) of males to *Nlgn3*$^{+/+}$ mice, 434.1(SE:48.7), *Nlgn3*$^{+/-}$ mice, 492.5(SE:60.1), *Nlgn3*$^{-/-}$ mice, 485.5(SE:59.3). A Kruskal-Wallis revealed that there is no significant difference between the groups. ***C***, average time taken for action (seconds) and standard error of the mean (SE) for *Nlgn3*$^{+/+}$ mice for the first contact, 18.1(SE:2.9), first pup, 26.3(SE:3.8), second pup, 59.4(SE:8.7), third pup, 84.9(SE:8.9). Time taken for *Nlgn3*$^{+/-}$ for the first contact 19.1(SE:4.4), first pup, 34.6(SE:5.5), second pup, 76.6(SE:10.8), third pup, 110.1(SE:15.1).Time taken for *Nlgn3*$^{-/-}$ mice for the first contact, 7.0(SE:2.7), first pup, 33.7(SE:4.3), second pup, 67.6(SE:13.1), third pup, 98.7(SE:15.6). A Kruskal-Wallis test revealed a significant difference for the first contact (*P = 0.022*). The results of the Dunns pairwise comparison is shown on the figure.

3.3.3. The influence of the social dominance environment and Nlgn3 re-expression on the social behaviours of juvenile male and female mice

In this section, the impact of *Nlgn3* knockout, the social dominance environment, and re-expression on the social behaviours of juvenile (aged p21-28) male and female mice are assessed. At this age, the mice are not separated from their opposite sex littermates (See **tables 3.1** and **3.2**). Juvenile male mice are social, but unlike adult male mice they show low levels of aggression with each other until around p35, indicating that the social dominance environment of juvenile male mice is different to that of adult male mice (Pellis et al 1999, Terranova et al 1998, Brown 1953). Juvenile C57BL/6 male and female mice also find social interaction rewarding (Panksepp and Lahvis 2007). As the juvenile mice are not separated from their opposite sex littermates, initial analysis on $Nlgn3^{+/+}$ mice, $Nlgn3^{y/+}$ mice, $Nlgn3^{-/-}$ mice and $Nlgn3^{y/-}$ mice was conducted to determine if there is an effect of sex on the behaviours of the juvenile mice. It was observed that there is an effect of social dominance environment, and of sex (**figure 3.7.A,** two-way ANOVA, social dominance environment: *P = 0.000086, F = 16.8, df = 1*, Sex: *P = 0.048, F = 4.0, df = 1*, $Nlgn3^{y/+}$ mice from MGH n = 12, $Nlgn3^{y/-}$ mice from MGH n = 10, $Nlgn3^{y/+}$ from SGH n = 9, $Nlgn3^{y/-}$ from SGH n = 14, $Nlgn3^{+/+}$ mice from MGH n = 14, $Nlgn3^{+/+}$ mice from SGH n = 12, $Nlgn3^{-/-}$ mice from MGH n = 15, $Nlgn3^{-/-}$ mice from SGH n = 20). As there is a main effect of sex, a targeted analysis of the male and female juvenile mice was conducted separately.

3.3.3.1. The impact of the social dominance environment and Nlgn3 re-expression on the social behaviours of juvenile $Nlgn3^{y/+}$ and $Nlgn3^{y/-}$ mice

It was observed that there is an impact of social dominance environment on the social behaviours of male mice, however, further analysis is needed to determine if there is an impact of re-expression in *Pvalb*-expressing cells on the behaviours of $Nlgn3^{y/+}Pvalb^{cre/+}$ and $Nlgn3^{y/-}Pvalb^{cre/+}$ mice. It was found that juvenile $Nlgn3^{y/+}$ mice and $Nlgn3^{y/-}$ mice from MGH show decreased social interaction compared to $Nlgn3^{y/+}$ mice and $Nlgn3^{y/-}$ mice from SGH, and $Nlgn3^{y/+}Pvalb^{cre/+}$ and $Nlgn3^{y/-}Pvalb^{cre/+}$ mice (**figure 3.7.B,** two-way ANOVA, social dominance environment: *P = 0.0002, F=9.5, df= 2,* Bonferroni post-hoc analysis, MGH and SGH: *P = 0.0008*, MGH and *Pvalb: P = 0.0009*, $Nlgn3^{y/+}$ mice from MGH n = 12, $Nlgn3^{y/-}$ mice from MGH n = 10, $Nlgn3^{y/+}$ from SGH n = 9, $Nlgn3^{y/-}$ from SGH n = 14, $Nlgn3^{y/+}Pvalb^{cre/+}$ n = 11, and $Nlgn3^{y/-}Pvalb^{cre/+}$ n = 15). Therefore, it appears that MGH impacts the social behaviour of juvenile $Nlgn3^{y/+}$ and $Nlgn3^{y/-}$ mice, and that re-expression of *Nlgn3* in $Nlgn3^{y/-}Pvalb^{cre/+}$ mice

restores their own and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates' levels of social interaction, to levels comparable with *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from SGH. This suggests that re-expression of *Nlgn3* in *Pvalb*-expressing cells influences the social behaviours of juvenile mice.

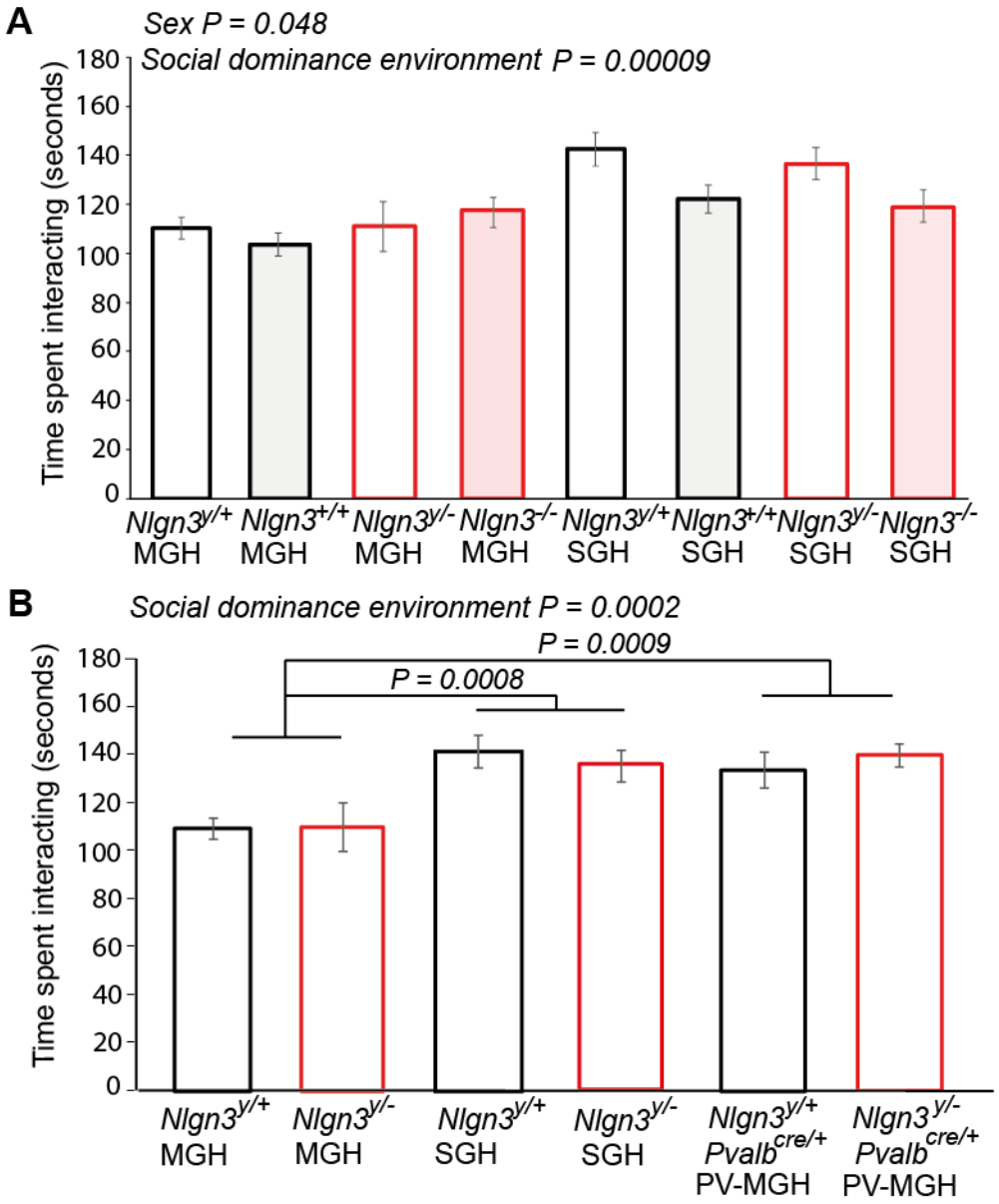


Figure 3.7. The impact of social dominance environment on the social interaction of juvenile male and female wild-type and knockout mice, and the impact of social dominance environment and re-expression on juvenile male *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice. *A*, the average time (seconds) and standard error of the mean (SE) spent interacting with an unfamiliar adult female in oestrous of *Nlgn3*$^{y/+}$ mice from MGH, 110.0(SE:4.4), *Nlgn3*$^{y/-}$ mice from MGH, 111.0(SE:10.3), *Nlgn3*$^{y/+}$ mice from SGH, 142.6(SE:6.8), *Nlgn3*$^{y/-}$ mice from SGH, 136.6(SE:6.6), *Nlgn3*$^{+/+}$ mice from MGH 103.6(SE:5.2), *Nlgn3*$^{+/+}$ mice from SGH 122.2(SE:5.7), *Nlgn3*$^{-/-}$ mice from MGH 116.6(SE:6.2), and *Nlgn3*$^{-/-}$ mice from SGH 119.4(SE:5.9). Two-way ANOVA, social dominance environment: *P = 0.000086,* Sex *P = 0.048.* ***B*,** the average time (seconds) and standard error of the mean (SE) spent interacting with an unfamiliar adult female in oestrous of *Nlgn3*$^{y/+}$ mice from MGH, 110.0(SE:4.4), *Nlgn3*$^{y/-}$ mice from MGH, 111.0(SE:10.3), *Nlgn3*$^{y/+}$ mice from SGH, 142.6(SE:6.8), *Nlgn3*$^{y/-}$ mice from SGH, 136.6(SE:6.6), *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$, 134.8(SE:7.5), and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$, 140.9(SE:4.8). A two-way ANOVA revealed a significant effect of social dominance environment (*P = 0.002*), and the results of the Bonferroni post-hoc analysis are shown on the figure.

3.3.3.2. The influence of the social dominance environment and re-expression on the social interaction of juvenile Nlgn3$^{+/+}$ mice, Nlgn3$^{+/-}$, and Nlgn3$^{-/-}$ mice.

In this section, the social interactions of juvenile *Nlgn3*$^{+/+}$ mice, *Nlgn3*$^{+/-}$ mice, and *Nlgn3*$^{-/-}$ mice from different social dominance environment conditions and of *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice were assessed. Previously it was shown that there is an effect of social dominance environment on *Nlgn3*$^{+/+}$ mice and *Nlgn3*$^{-/-}$ mice, but the phenotype of *Nlgn3*$^{+/-}$ mice compared to their littermates and the impact of social dominance environment on *Nlgn3*$^{+/-}$ mice were not explored. To that end, the social interaction of *Nlgn3*$^{+/+}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-WT) littermates was compared with *Nlgn3*$^{-/-}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates. It was observed that there is no significant difference in the social interaction with an unfamiliar female of juvenile *Nlgn3*$^{-/-}$ mice from MGH, their *Nlgn3*$^{+/-}$ (H-KO) littermates, *Nlgn3*$^{+/+}$ mice from MGH, and their *Nlgn3*$^{+/-}$ (H-WT) littermates, (**figure 3.8.A,** one-way ANOVA, *Nlgn3*$^{-/-}$ mice from MGH n = 15, *Nlgn3*$^{+/-}$ (H-KO) mice n = 8, *Nlgn3*$^{+/+}$ mice from MGH n = 14, *Nlgn3*$^{+/-}$ (H-WT) mice n = 8). Therefore, juvenile *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice do not have social behaviour changes relative to their littermates, and *Nlgn3*$^{+/-}$ mice do not have altered social behaviour dependent upon their social dominance environment condition.

Nlgn3$^{+/+}$ mice from MGH were compared with *Nlgn3*$^{+/+}$ mice from SGH, revealing that *Nlgn3*$^{+/+}$ mice from MGH have significantly lower social interactions than *Nlgn3*$^{+/+}$ mice from SGH (**figure 3.8.B**, independent samples t - test, *P = 0.027, df =24, Nlgn3*$^{+/+}$ mice from MGH n = 14, *Nlgn3*$^{+/+}$ mice from SGH n = 12). These data show that the social dominance environment does influence the social behaviours of juvenile *Nlgn3*$^{+/+}$ mice, confirming the impact of social dominance environment shown in **figure 3.7.A**.

Finally, comparison of juvenile *Nlgn3*$^{-/-}$ mice from MGH, *Nlgn3*$^{-/-}$ mice from SGH, and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ mice revealed no impact of social dominance environment or re-expression of *Nlgn3* in *Pvalb*-expressing cells on the time spent socially interacting with an unfamiliar female in oestrous (**figure 3.8.C,** one-way ANOVA, *Nlgn3*$^{-/-}$ mice from MGH n = 15, *Nlgn3*$^{-/-}$ mice from SGH n = 20, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice n = 10). These results suggest that neither the social dominance environment nor re-expression of *Nlgn3* within *Pvalb*-expressing cells influences the social behaviours of juvenile *Nlgn3*$^{-/-}$ mice. Of note is that the results shown in **figure 3.7.A** don't show an interaction of sex, social dominance environment and genotype, with more mice it is possible that this would be observed.

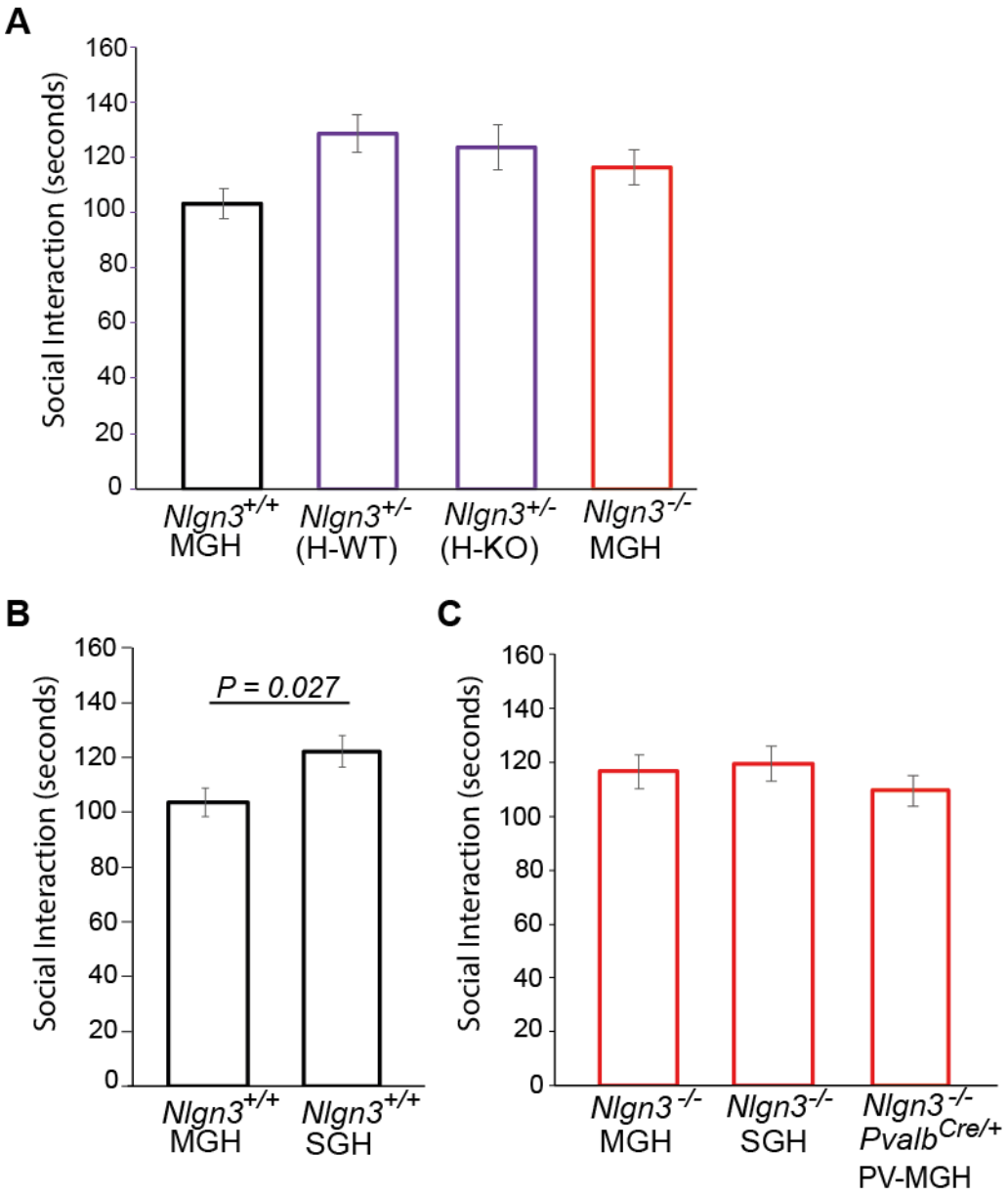


Figure 3.8. The impact of the social dominance environment on the time spent by juvenile *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice interacting with an unfamiliar mouse. ***A***, average time(seconds) and standard error of the mean (SE) spent interacting with an unfamiliar female of *Nlgn3*$^{+/+}$ mice from MGH, 103.6(SE:5.4), *Nlgn3*$^{+/-}$ (H-WT) mice, 128.9(SE:6.7), *Nlgn3*$^{+/-}$(H-KO) mice, 123.9(SE:8.0), *Nlgn3*$^{-/-}$ mice from MGH 116.6(SE:6.2). A one-way ANOVA revealed no significant difference between the groups. ***B***, average time (seconds) and standard error of the mean (SE) spent interacting with an unfamiliar female of *Nlgn3*$^{+/+}$ mice from MGH, 103.6(SE:5.2) *Nlgn3*$^{+/+}$ mice from SGH, 122.2(SE:5.7). An independent samples t-test revealed a significant difference between the groups (*P = 0.027*). **C**, average time (seconds) and standard error of the mean (SE) spent interacting with an unfamiliar female of *Nlgn3*$^{-/-}$ mice from MGH, 116.6(SE:5.9), *Nlgn3*$^{-/-}$ mice from SGH, 119.4(SE:5.9), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 109.0(SE:7.9). A one-way ANOVA revealed no significant difference between the groups.

3.4. Discussion

A summary of the statistically significant influence of MGH compared to SGH on the social behaviours of male and female mice as adults and juveniles (**table 3.3**).

Table 3.3. A summary of the statistically significant impact of mixed genotype housing on the social behaviours of *Nlgn3$^{y/+}$, Nlgn3$^{y/-}$, Nlgn3$^{-/-}$,* and *Nlgn3$^{+/+}$* mice.

	Adult Male in MGH	***Adult Female in MGH***	***Juvenile Male in MGH***	***Juvenile Female in MGH***
Dominance against unfamiliar males	***Nlgn3$^{y/+}$*** ↓ ***Nlgn3$^{y/-}$*** ↓	*N/A*	*N/A*	*N/A*
Urinary testosterone	***Nlgn3$^{y/+}$*** ↓ *No change:* ***Nlgn3$^{y/-}$***	*N/A*	*N/A*	*N/A*
Vocalisation	No change	*N/A*	*N/A*	*N/A*
Social interaction	*N/A*	*No change:* ***Nlgn3$^{+/+}$*** ***Nlgn3$^{-/-}$*** ↓	***Nlgn3$^{y/+}$*** ↓ ***Nlgn3$^{y/-}$*** ↓	***Nlgn3$^{+/+}$*** ↓ *No change:* ***Nlgn3$^{-/-}$***

3.4.1. Adult male social behaviours

3.4.1.1. Dominance behaviours of adult male mice are impacted by the social dominance environment and genotype

The data presented in this chapter support the initial hypothesis that *Nlgn3$^{y/-}$* mice are submissive to their *Nlgn3$^{y/+}$* littermates, showing that a phenotype relating to social hierarchy does exist in these mice. Furthermore, this social submission within the hierarchy is not seen in the *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, revealing that re-expression of Neuroligin-3 within *Pvalb*-expressing cells is sufficient to ensure that *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice are not submissive towards their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates.

This observation was expanded upon by assessing the relative dominance of the mice in the tube test compared to unfamiliar mice. *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice from MGH show a decreased number of victories compared to *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice from SGH and to *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice. This suggests that *Nlgn3$^{y/-}$* mice disrupt the cage hierarchy leading to submissive behaviour of the group when tested against mice that are not peers. Interestingly, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice have tube test victories that are similar to those from SGH conditions, suggesting that re-expression of Neuroligin-3 in *Pvalb*-expressing cells is not only restoring the within cage hierarchy but also relative dominance levels against unfamiliar mice. Of note is that while *Nlgn3$^{y/+}$* mice from MGH show increased dominance versus their *Nlgn3$^{y/-}$* littermates, they are submissive to unfamiliar wild-type mice. These data suggest that dominance levels are relative to the social dominance environment, and are not innate, meaning that just because a mouse is dominant in one condition, this does not translate to dominance in the other condition.

3.4.1.2. Testosterone, the social dominance environment, and social competition

Levels of testosterone are associated with behaviours relating to social dominance, including aggressive behaviours against unfamiliar wild-type male mice, and counter marking of territory (Juntti et al 2010). In rhesus monkeys, higher levels of testosterone have been associated with displays of dominance (Higley et al 1996) and in humans levels of testosterone have been associated with non-violent dominance in teenage boys (Rowe et al 2004). Supporting this notion is that the *Nlgn3$^{y/-}$* mice and *Nlgn3$^{y/+}$* mice from MGH show decreased levels of testosterone and decreased wins against unfamiliar mice. However, *Nlgn3$^{y/-}$* mice from SGH and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice show decreased testosterone levels

compared to *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice, despite having comparable numbers of wins tube test wins as *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice. These data suggest that testosterone levels do not necessarily correlate with number of wins against unfamiliar male mice, therefore are not a direct readout of the dominance status of the mice. Another important point to consider is that testosterone levels have also been shown to be influenced by perception of challenge. Levels of testosterone increase in non-human primates and in humans in anticipation of a challenge (Mazur et al 1992, Wobber et al 2010), and testosterone has been shown to increase in humans following a victory (Mazur et al 1980, Mazur et al 1992). In the context of the within cage hierarchy, given that *Nlgn3*$^{y/-}$ mice from MGH are submissive to their *Nlgn3*$^{y/+}$ littermates, a lack of challenge may lead to decreased overall testosterone of all the mice. This could be a potential mechanism by which the *Nlgn3*$^{y/-}$ mice from MGH influence the testosterone levels of their *Nlgn3*$^{y/+}$ littermates. In support of this notion is that testosterone levels of mice have been shown to be influenced by the social dominance environment of the mice. In one study, group housed male mice which were undergoing morphine withdrawal were assessed. Two groups were assessed, either all mice of the group received saline injections, or a group comprised of morphine withdrawal mice housed with saline treated non-morphine withdrawal mice. It was observed that morphine withdrawal mice, and their saline treated littermates, showed decreased levels of testosterone relative to saline treated only mice (Hofford et al 2011). This could be perceived as due to a change in the behaviour of the morphine withdrawal mice leading to an altered social dominance environment that then impacts the testosterone levels of the saline-treated mice, potentially reflecting a similar phenomenon that is seen in the MGH mice.

3.4.1.3. Divergence of courtship USV behaviour and other markers of dominance

Testosterone levels and position within the social hierarchy have been shown to influence levels of courtship USV in mice (Nunez et al 1978, Wang et al 2011). Given that both testosterone levels and position within the social hierarchy were changed dependent upon genotype and social dominance environment conditions, it seemed plausible that USV may also change. However, this was not observed. In the work of Wang et al (2011) it was shown that a more dominant mouse emits more courtship USV, but in contrast to this is that the data presented in this chapter show that position in the hierarchy and relative levels of dominance do not necessarily influence levels of courtship USV. *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice, which

have restored within cage tube test dominance, do not show an increase in courtship USV, indicating that these behaviours do not change in relation to each other. Additionally, both *Nlgn3*$^{y/-}$ mice from SGH and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice have restored relative dominance levels against unfamiliar wild-types, but again do not show an increase in courtship USV. However, in the case of the *Nlgn3*$^{y/-}$ mice from SGH and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice this could be due to a lack of *Nlgn3* expression in neurones that mediate USV causing this deficit. Another point is that *Nlgn3*$^{y/+}$ mice from MGH and SGH, and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice show similar levels of USV despite having different relative dominance levels as assessed by unfamiliar male wild-type mice, again indicating that dominance does not necessarily increase levels of courtship USV. However, it is possible that dominance relative to unfamiliar wild-type mice may not share the same neurobiology as within cage hierarchy. Further to this, is that courtship USV and tube test dominance has been shown to be controlled by activity of the medial prefrontal cortex (mPFC) and influencing mPFC activity can increase or decrease both behaviours (Wang et al 2011). However, other brain regions, such as the hypothalamic ventral premammillary nucleus have also been associated with hierarchy behaviours (Stagkourakis et al 2018), and others may remain to be discovered. It is therefore possible there may also be a divergence of relative dominance, within-cage hierarchy, and levels of courtship USV on a neurological level, leading to the influence of social dominance environment on the dominance as measured by the tube test, but not by courtship USV.

Furthermore, it was shown by Wang et al (2011) that a linear correlation of tube test and USV can be observed in wild-type mice, and while this was observed in the *Nlgn3*$^{y/+}$ mice from SGH, this is not seen in the MGH cages. Therefore, it appears that *Nlgn3*$^{y/-}$ mice from MGH disrupt the formation of their hierarchy in such a way that the dominant in the tube test is no longer the dominant in levels of USV. Similar observations have been made in work in which mixed genotype cages containing wild-type mice and *Cdkn1c*^{BACx1} mice, which have altered dominance behaviours, no longer show a correlation of tube test victories and water access, while a correlation between the two tasks is observed in the control group (McNamara et al 2017, McNamara et al 2018). Therefore, disrupted cage hierarchy may be commonly seen in group housed mice of mixed dominance levels.

3.4.2. Adult female social behaviour

3.4.2.1. Adult female mice, like male mice, influence and are influenced by the social dominance environment

The results of the female behaviour demonstrate that, like in the males, MGH alters the behaviours of the mice within that environment. In adulthood, both *Nlgn3*$^{-/-}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates have decreased social interaction compared to *Nlgn3*$^{+/+}$ mice and their *Nlgn3*$^{+/-}$ (H-WT) littermates. The decreased social behaviour of *Nlgn3*$^{+/-}$ mice must be due to their *Nlgn3*$^{-/-}$ littermates, as *Nlgn3*$^{+/-}$ (H-WT) and *Nlgn3*$^{+/-}$ (H-KO) have identical genotypes of male littermates as juveniles and dams, with the only difference being that they have either *Nlgn3*$^{-/-}$ or *Nlgn3*$^{+/+}$ littermates. Additionally, in adulthood, the social behaviours of *Nlgn3*$^{-/-}$ mice, like *Nlgn3*$^{y/-}$ mice, seems to be restored by SGH social dominance environment, and by re-expression of *Nlgn3* within *Pvalb*-expressing cells.

3.4.2.2. A trend for no changes in maternal and sexual behaviour of female mice

The work in this chapter aimed to assess if there may be an impact of *Nlgn3* deletion on the maternal behaviours. Given that different genotypes of breeding females are used during this work, it is important to ensure there are no gross differences in the pup retrieval behaviour of the mice. While the results of the pup retrieval assay suggest that there are no differences between *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice, it is by no means exhaustive, and other behavioural assays assessing maternal care could be used, such as scoring the maternal behaviours of the female mice, and assessing criteria such as time spent in the nest and the time spent grooming the pups. If taken on face value, however, these results indicate that the contribution of the maternal behaviours to the phenotypes of the litter should not largely differ between the groups. Furthermore, the assessment of *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates provides a control for the maternal behaviours, as the dams of both the *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates and of *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from MGH mice are always *Nlgn3*$^{+/-}$. Additionally, the use of *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ mice provides a control for prenatal events, as the parvalbumin promotor is activated at around p10-p14 (Del Rio et al 1992).

Finally, the results of the assessment of courtship USV towards female mice demonstrated that there is no difference in the levels of USV towards the female mice. This could indicate that there is possibly no difference in the pheromone status of the *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice

compared to *Nlgn3*$^{+/+}$ mice; it could also indicate that there is no difference in the sexual receptivity. However, this is not a confirmed protocol, and it could be that it is not a sensitive enough measure to detect these changes if present.

3.4.3. Influence of the social dominance environment on juvenile male and female mice

It has been demonstrated that fighting behaviours of juvenile mice from p15-p30 only make up a small amount of the interactions of the mice, while in adult male mice a much larger amount of time is spent in agonistic interactions, with a large increase in aggressive behaviours at p35 and onwards (Pellis et al 1999, Terranova et al 1998). This shows that the social dominance environment between males is different at adulthood compared to as juveniles. When looking at the behaviours of the juvenile male mice, it was observed that there was an impact of social dominance environment, with *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from MGH showing decreased social interaction with an unfamiliar female, compared to *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from SGH and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ littermates. However, like in adults, male juvenile mice also show an impact of MGH that is restored by SGH and re-expression, indicating that juvenile male mice are also sensitive to their social dominance environment despite the different types of social interactions of their peers. It is not known at what age the hierarchy begins to manifest, but it is possible that some degree of hierarchy without associated aggressive behaviours has begun to develop in the juvenile males. In order to determine if this is true, long term scoring of home cage behaviours of the mice over development into adulthood is needed for further characterisation of the behaviours of the mice.

In the juvenile female mice, no difference is observed between *Nlgn3*$^{-/-}$ from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates and *Nlgn3*$^{+/+}$ from MGH mice and their *Nlgn3*$^{+/-}$ (H-WT) littermates, nor is there any difference observed between *Nlgn3*$^{-/-}$ mice from MGH and *Nlgn3*$^{-/-}$ mice from SGH and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice. However, *Nlgn3*$^{+/+}$ mice from MGH were found to have decreased social interaction compared to *Nlgn3*$^{+/+}$ mice from SGH. Interestingly, *Nlgn3*$^{+/+}$ mice from MGH show decreased social interactions as juveniles, a stage in which they are housed with *Nlgn3*$^{y/-}$ mice, however, as adults in same sex housing, they do not show decreased social interaction. This indicates that *Nlgn3*$^{y/-}$ mice could be influencing the behaviours of *Nlgn3*$^{+/+}$ mice from MGH as juveniles, and this may be reversed following weaning. However, to truly understand if opposite sex *Nlgn3* knockout mice influence each other this would have to be explored further. The degree in which opposite sex littermates

influence each other's behaviours is not known. Some evidence suggests that opposite sex littermates can influence the behaviours of their opposite sex littermates (Crews et al 2009), however, this study assessed the behaviour of mice with estrogen receptor alpha knockout, that leads to the females adopting male behaviours, therefore this change may be due to interactions with the opposite sex that would not normally exist.

3.4.4. Concluding remarks

As hypothesised, adult *Nlgn3*$^{y/-}$ mice are socially submissive to their *Nlgn3*$^{y/+}$ littermates, while re-expression of *Nlgn3* within *Pvalb*-expressing cells restores this. Following this, it was observed that adult male MGH mice showed decreased dominance and decreased testosterone levels compared to mice from SGH and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates. Furthermore, juvenile male mice from MGH show decreased social interaction. A similar trend was observed in the female mice; adult *Nlgn3*$^{-/-}$ mice and their *Nlgn3*$^{+/-}$ (H-KO) littermates show decreased social interactions compared to *Nlgn3*$^{+/+}$ mice and their *Nlgn3*$^{+/-}$ (H-WT) littermates, demonstrating that adult *Nlgn3*$^{-/-}$ mice influence the social behaviours of their littermates. Additionally, MGH leads to decreased social behaviours of adult *Nlgn3*$^{-/-}$ mice, and SGH or re-expression of *Nlgn3* in *Pvalb*-expressing cells in *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice restores this. These data demonstrate that the presence of *Nlgn3* knockout mice leads to measurable changes in the social behaviour of the group. Given that social interactions between peers shapes behaviours such as anxiety, it is therefore of importance to assess how these are impacted by the different genotype social dominance environment conditions.

Chapter 4: The effect of the social dominance environment and *Nlgn3* re-expression on exploratory and anxiety-related behaviours of *Nlgn3* knockout mice and their wild-type littermates

4.1. Introduction

The results from chapter three show that mixed genotype housing (MGH) impacts the social behaviours of male and female mice. In mice and rats, the social dominance environment has been shown to impact behaviours such as locomotion (Schiavone et al 2009, George et al 2010, Ashby et al 2010, Schiavone et al 2012, Liu et al 2016), levels of anxiety (Romeo et al 2003, Chourbaji et al 2005, Branchi and Alleva 2006, Branchi, D'andrea, Sietzema, et al 2006, Millstein and Holmes 2007, George et al 2010), and learning and memory (Rice et al 2008, Ibi et al 2008, Benner et al 2014). Furthermore, the results from chapter three show that *Nlgn3*$^{y/-}$ mice have altered dominance-related behaviours. The position within the social hierarchy of mice has been shown to differentially influence the behaviours and physiology of mice, including anxiety and corticosterone levels (Ely et al 1978, Doulames et al 2015, Larrieu et al 2017, Horii et al 2017). It is therefore of interest to assess how MGH and single genotype housing (SGH) impacts the locomotive and anxiety-related behavioural phenotypes of the mice, and if re-expression of *Nlgn3* in *Pvalb*-expressing cells influences the individual and group behaviours. These questions will be addressed in this chapter.

4.2. Aims and objectives

- To understand if MGH influences the locomotive and anxiety-related phenotypes of male and female wild-type and *Nlgn3* knockout mice.
- To understand if this is ameliorated at the individual and group level by re-expressing *Nlgn3* within *Pvalb*-expressing cells.

4.3. Results

4.3.1. The influence of the social dominance environment and Nlgn3 re-expression on the locomotive and anxiety-related behaviours of adult male Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

This section of the chapter aims to determine how the social dominance environment influences the locomotive and anxiety-related behaviours of adult male mice. The social dominance environment conditions are the same as those outlined in **table 3.1** of chapter three.

4.3.1.1. The influence of the social dominance environment and Nlgn3 re-expression on open field arena exploration of adult Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

The activity of *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates within the open field arena was assessed to determine if there is an effect of social dominance environment on locomotive behaviour. It was observed that *Nlgn3$^{y/+}$* mice from SGH have lower distance travelled than *Nlgn3$^{y/+}$* mice from MGH and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice. *Nlgn3$^{y/-}$* mice were found to be hyperactive regardless of social dominance environment or re-expression (**figure 4.1**, two-way ANOVA, Genotype: *P = 2.4e-10, F = 52.1, df = 1,* social dominance environment: *P = 0.035, F = 3.5, df = 2.* Genotype x social dominance environment: *P = 0.0002, F = 9.5, df = 2.* Bonferroni post-hoc analysis, MGH and SGH*: P = 0.001,* SGH and *Pvalb: P = 0.016.* Bonferroni adjusted pairwise analysis, *Nlgn3$^{y/+}$* mice from MGH and *Nlgn3$^{y/-}$* mice from MGH: *P = 0.00002*, *Nlgn3$^{y/+}$* mice from SGH and *Nlgn3$^{y/-}$* mice from SGH: *P = 1.6e-09, Nlgn3$^{y/+}$* mice from MGH and *Nlgn3$^{y/+}$* mice from SGH: *P = 0.0003, Nlgn3$^{y/+}$* mice from SGH and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice: *P = 0.00009*, *Nlgn3$^{y/+}$* mice from MGH n = 21, *Nlgn3$^{y/-}$* mice from MGH n = 14, *Nlgn3$^{y/+}$* mice from SGH n = 16, *Nlgn3$^{y/-}$* mice from SGH n = 9, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 14, and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice n = 14).

Consistent with previous reports, these data demonstrate that *Nlgn3$^{y/-}$* mice are hyperactive in the open field arena (Radyushkin et al 2009, Rothwell et al 2014). Furthermore, these data suggest that MGH does not influence the activity of the *Nlgn3$^{y/-}$* mice within the open field arena, while MGH does influence the behaviours of *Nlgn3$^{y/+}$* mice. Interestingly, while *Nlgn3$^{y/-}$* mice from SGH are hyperactive compared to *Nlgn3$^{y/+}$* mice from SGH, and *Nlgn3$^{y/-}$* mice from MGH are hyperactive compared to *Nlgn3$^{y/+}$* mice from MGH, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice are not hyperactive compared to their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates. This suggests that *Nlgn3*

expression within *Pvalb*-expressing cells may be influencing the hyperactivity of the mice. However, as no difference between the activity of *Nlgn3*$^{y/-}$ mice from SGH or MGH conditions and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice was observed, it is not possible to conclude that *Nlgn3* re-expression within *Pvalb*-expressing cells does mediate open field activity.

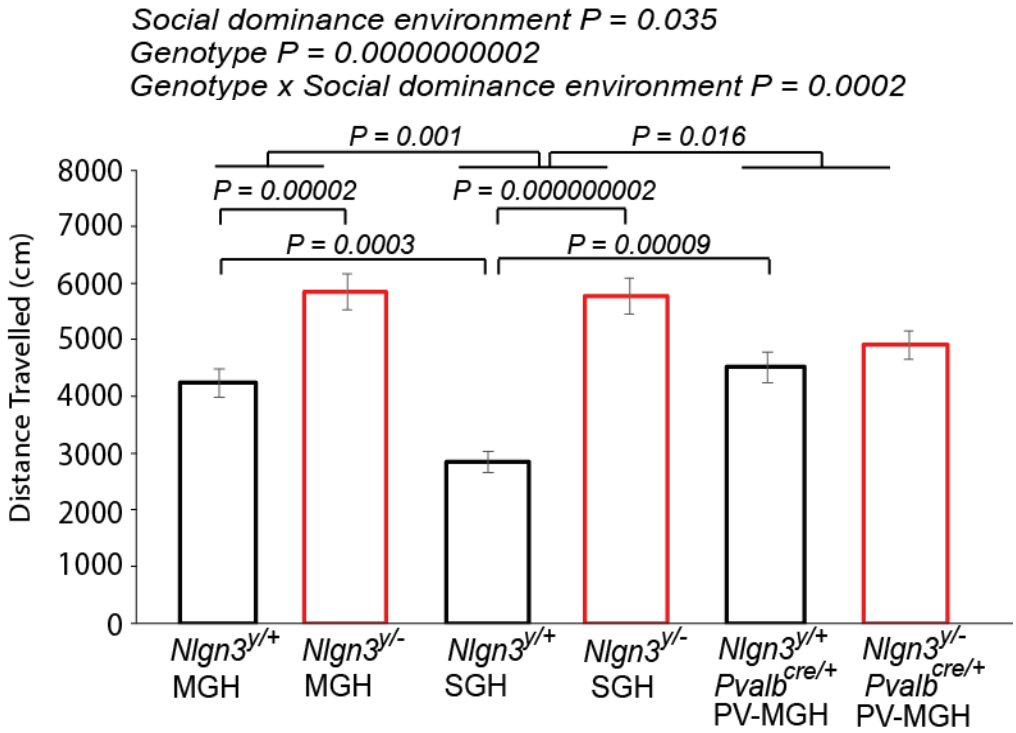


Figure 4.1. The impact of the social dominance environment and re-expression on the open field activity of adult *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice. The average distance travelled (cm) and the standard error of the mean (SE) of adult *Nlgn3$^{y/+}$* mice from MGH, 4242.2(SE:251.7), *Nlgn3$^{y/-}$* mice from MGH, 5845.0(SE:321.5), *Nlgn3$^{y/+}$* mice from SGH, 2848.8(SE:186.3), *Nlgn3$^{y/-}$* mice from SGH, 5765.2(SE:316.1), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 4515.5(SE:277.7), and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$*, 4907.3(SE:258.8). A two-way ANOVA revealed a significant impact of genotype (*P = 0.0000000002*), housing (*P = 0.035*), and an interaction of genotype and social dominance environment (*P = 0.0002*). The results of the Bonferroni post-hoc analysis and Bonferroni adjusted post-hoc analysis and pairwise analysis are shown on the figure.

4.3.1.2. The influence of the social dominance environment and Nlgn3 re-expression on thigmotaxis within the open field arena of adult Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

The distribution of activity within the open field arena can be used to assess anxiety of mice. The thigmotaxis of *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates within the open field arena was assessed. It was observed that *Nlgn3$^{y/-}$* mice have decreased thigmotaxis as shown by their increased time in the centre and increased ratio of distance travelled in the centre compared to *Nlgn3$^{y/+}$* mice. Furthermore, it was observed that social dominance environment does influence the thigmotaxis of the mice within the open field arena. *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH have decreased thigmotaxis, as shown by an increased ratio of distance travelled in the centre and an increased time in the centre when compared to *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and an increased time in the centre when compared to *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates (ratio distance travelled in the centre/ total distance travelled**, figure 4.2.A,** two-way ANOVA, social dominance environment: *P = 0.007, F = 5.3, df = 2,* Genotype*: P = 0.001, F = 12.4, df = 1*, Bonferroni post-hoc analysis: MGH and SGH *P = 0.001*. Time in the centre, **figure 4.2.B,** two-way ANOVA, social dominance environment: *P = 0.002, F = 6.9, df = 2*, Genotype: *P = 0.012, F = 6.5, df = 1*, Bonferroni post-hoc analysis, MGH and SGH: *P = 0.002*, MGH and *Pvalb: P = 0.011*, *Nlgn3$^{y/+}$* mice from MGH n = 21. *Nlgn3$^{y/-}$* mice from MGH n = 14, *Nlgn3$^{y/+}$* mice from SGH n = 16, *Nlgn3$^{y/-}$* mice from SGH n = 9, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 14, and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice n = 14).

These data show that *Nlgn3$^{y/-}$* mice have decreased thigmotaxis and that both *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH have decreased thigmotaxis. This shows that social dominance environment does influences thigmotaxis, possibly indicating an effect on anxiety. Furthermore, the *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* littermates showed similar behaviour to *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice from SGH conditions, particularly for the time in the centre, indicating that re-expression of *Nlgn3* within *Pvalb*-expressing cells restores the thigmotaxis behaviours of these mice.

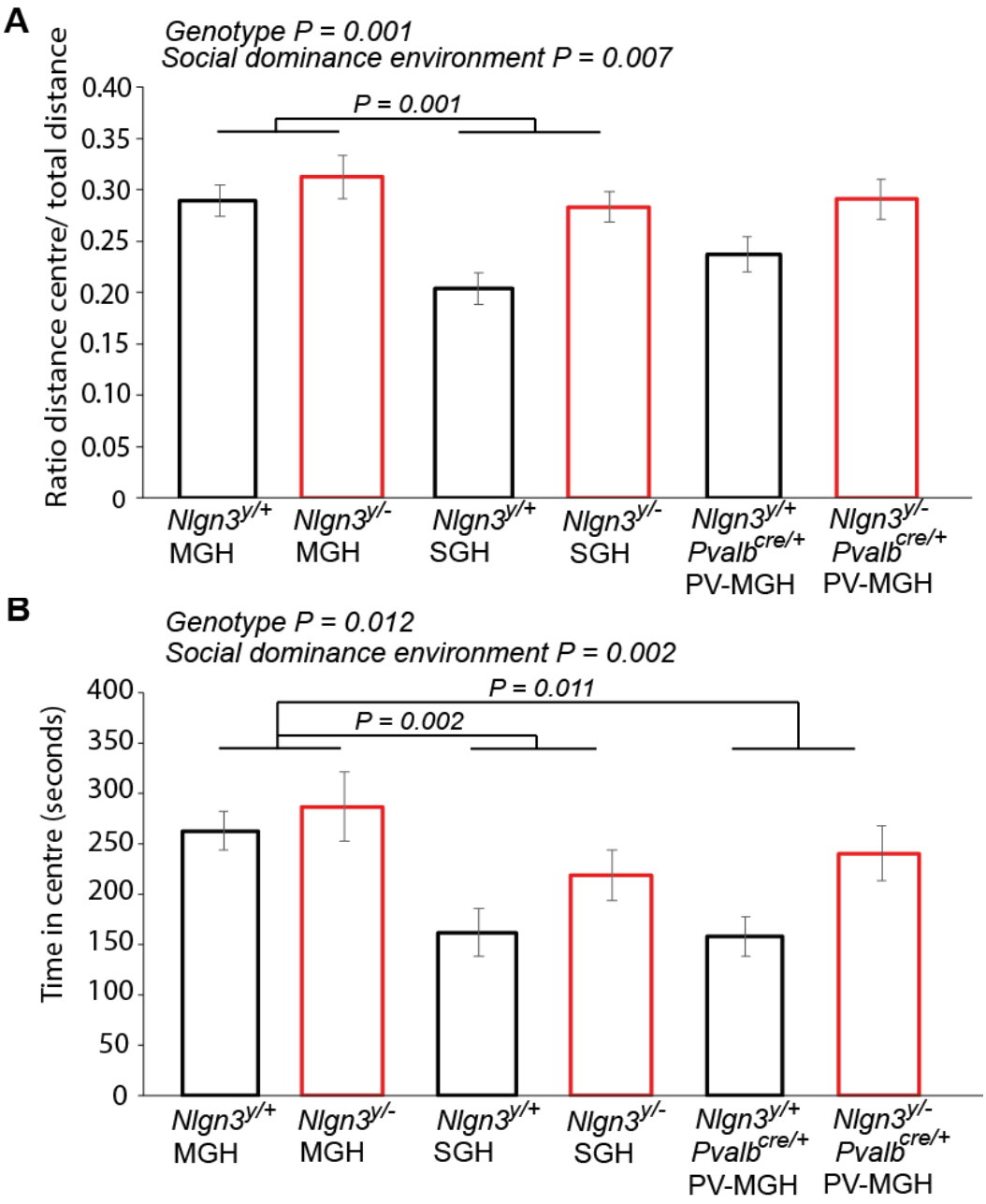


Figure 4.2. The impact of the social dominance environment and re-expression on the thigmotaxis of adult *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice in the open field arena. *A*, the average ratio in the centre/ total distance and standard error of the mean (SE) travelled in the open field arena of adult *Nlgn3$^{y/+}$* mice from MGH, 0.28(SE:0.015), *Nlgn3$^{y/-}$* mice from MGH, 0.31(SE:0.021), *Nlgn3$^{y/+}$* mice from SGH, 0.20(SE:0.015), *Nlgn3$^{y/-}$* mice from SGH, 0.28(SE:0.014), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 0.23(SE:0.017), and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$*, 0.29(SE:0.019). A two-way ANOVA revealed a significant impact of genotype (*P = 0.001*), housing (*P = 0.007*). The results of the Bonferroni post-hoc analysis are shown on the figure. ***B***, the average time in the centre (seconds) and the standard error of the mean (SE) of adult *Nlgn3$^{y/+}$* mice from MGH, 263.1(SE:19.5), *Nlgn3$^{y/-}$* mice from MGH, 287.3(SE:34.4), *Nlgn3$^{y/+}$* mice from SGH, 162.2(SE:23.8), *Nlgn3$^{y/-}$* mice from SGH, 218.7(SE:25.1), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 158.5(SE:19.5), and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$*, 240.8(SE:27.5). A two-way ANOVA revealed a significant impact of social dominance environment (*P = 0.002*) and genotype (*P = 0.012*). The results of the Bonferroni post-hoc are shown on the figure.

4.3.1.3. The influence of social dominance environment and Nlgn3 re-expression on elevated plus maze exploration of adult Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

The anxiety levels of the mice can be explored further by using the elevated plus maze. The time spent in the open arms of the elevated plus maze of *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice was assessed, revealing both an impact of genotype and social dominance environment on the time in the open arms of the elevated plus maze (**figure 4.3**, two-way ANOVA, social dominance environment: *P = 0.048, F = 3.2, df = 2.* Genotype: *P = 0.036, F = 4.6, df = 1*, *Nlgn3$^{y/+}$* mice from MGH n = 20, *Nlgn3$^{y/-}$* mice from MGH n = 14, *Nlgn3$^{y/+}$* mice from SGH n = 12, *Nlgn3$^{y/-}$* from SGH n = 13, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 12, and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* n = 12). These data show that both genotype and social dominance environment led to alterations of the time spent in the open arms of the elevated plus maze. However, the post-hoc analysis of the social dominance environment conditions revealed no significant interactions, so this was not explored further.

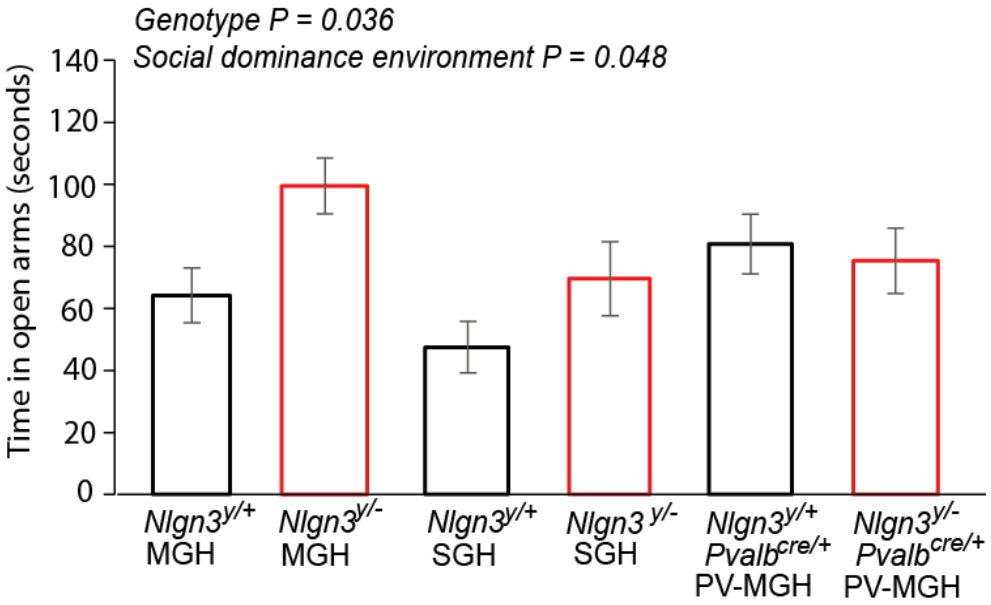


Figure 4.3. The impact of the social dominance environment and re-expression on the time spent by adult *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice in the open arms of the elevated plus maze. The average time spent in the open arms (seconds) and the standard error of the mean (SE) for *Nlgn3$^{y/+}$* mice from MGH, 64.2(SE:8.8), *Nlgn3$^{y/-}$* mice from MGH, 99.5(SE:9.0), *Nlgn3$^{y/+}$* mice from SGH, 47.5(SE:8.3), *Nlgn3$^{y/-}$* mice from SGH, 69.6(SE:11.9), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 80.7(SE:9.6), and for *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 75.4(SE:10.2). A two-way ANOVA revealed a significant effect of social dominance environment (*P = 0.048*) and of genotype (*P = 0.036*).

4.3.2. The impact of the social dominance environment and re-expression on the exploratory and anxiety-related behaviours of adult female Nlgn3$^{+/+}$, Nlgn3$^{-/-}$, and Nlgn3$^{+/-}$ mice

This section of the chapter aims to determine how the social dominance environment impacts anxiety-related behaviours of adult female mice. The social dominance environment conditions were the same as those outlined in **table 3.2** of chapter three. An initial analysis was conducted on *Nlgn3$^{+/+}$* mice from MGH, their *Nlgn3$^{+/-}$* (H-WT) littermates, *Nlgn3$^{-/-}$* mice from MGH, and their *Nlgn3$^{+/-}$* (H-KO) littermates. Following this a targeted approach was utilised, in which the *Nlgn3$^{+/+}$* mice from MGH and *Nlgn3$^{+/+}$* mice from SGH were compared, followed by a comparison of the *Nlgn3$^{-/-}$* mice from MGH, with *Nlgn3$^{-/-}$* mice from SGH and *Nlgn3$^{-/-}$Pvalb$^{cre/+}$* mice.

4.3.2.1. The influence of the social dominance environment and Nlgn3 re-expression on the open field locomotive behaviour of adult Nlgn3$^{+/+}$ mice, Nlgn3$^{+/-}$ mice, and Nlgn3$^{-/-}$ mice

To investigate if locomotive behaviours are altered in the female mice with *Nlgn3* knockout, the open field activity of *Nlgn3$^{-/-}$* mice from MGH, their *Nlgn3$^{+/-}$* (H-KO) littermates, *Nlgn3$^{+/+}$* mice from MGH, and their *Nlgn3$^{+/-}$* (H-WT) littermates was assessed, revealing that *Nlgn3$^{-/-}$* mice from MGH are hyperactive (**figure 4.4.A**, Kruskal-Wallis test, *P = 0.000,* Dunn's pairwise analysis, *Nlgn3$^{-/-}$* mice from MGH and *Nlgn3$^{+/+}$* mice from MGH: *P = 0.023*, *Nlgn3$^{-/-}$* mice from MGH and *Nlgn3$^{+/-}$* (H-WT) mice: *P = 0.001*, *Nlgn3$^{-/-}$* mice from MGH and *Nlgn3$^{+/-}$* (H-KO) mice: *P = 0.001*, *Nlgn3$^{+/+}$* mice from MGH n = 13, *Nlgn3$^{+/-}$* (H-WT) mice n = 13, *Nlgn3$^{-/-}$* mice from MGH n = 16, *Nlgn3$^{+/-}$* (H-KO) mice n = 12). These data demonstrate that adult *Nlgn3$^{-/-}$* mice from MGH conditions are hyperactive when compared to *Nlgn3$^{+/+}$* mice and *Nlgn3$^{+/-}$* mice, reflecting the hyperactivity seen in *Nlgn3$^{y/-}$* mice. Furthermore, adult *Nlgn3$^{+/-}$* mice do not have altered activity dependent upon their social dominance environment.

To assess if the social dominance environment influences the locomotive behaviour of *Nlgn3$^{+/+}$* mice, the open field activity of the *Nlgn3$^{+/+}$* mice from MGH was compared with the activity of *Nlgn3$^{+/+}$* mice from SGH. Analysis of the distance travelled within the open field arena revealed that *Nlgn3$^{+/+}$* mice from SGH show significantly lower distance travelled in the open field compared to *Nlgn3$^{+/+}$* mice from MGH (**figure 4.4.B,** Mann-Whitney U test, *P = 0.0004*, *Nlgn3$^{+/+}$* mice from MGH n = 13, *Nlgn3$^{+/+}$* mice from SGH n = 8). This shows that the social dominance environment is a factor in influencing the locomotive activity of adult

Nlgn3$^{+/+}$ mice and is consistent with the observation of decreased open field locomotion of adult *Nlgn3*$^{y/+}$ mice from SGH. This could be due to the presence of the *Nlgn3*$^{+/-}$ mice, but the early life social dominance environment could also be a contributing factor.

To assess if social dominance environment and re-expression impacts the locomotive behaviours of *Nlgn3*$^{-/-}$ mice, the open field activity of the *Nlgn3*$^{-/-}$ mice from MGH was compared to that of *Nlgn3*$^{-/-}$ mice from SGH and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice. This revealed no significant difference between the groups (**figure 4.4.C**, one-way ANOVA, *Nlgn3*$^{-/-}$ mice from MGH n = 16, *Nlgn3*$^{-/-}$ mice from SGH n = 13, *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice n = 10). These data suggest that the social dominance environment does not influence the open field activity levels of the *Nlgn3*$^{-/-}$ mice, reflecting what is seen in adult *Nlgn3*$^{y/-}$ mice. Furthermore, re-expression within *Pvalb*-expressing cells does not significantly influence the activity of *Nlgn3*$^{-/-}$ mice, however, a trend for a decrease is observed, reflecting the non-significant decrease seen in *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice.

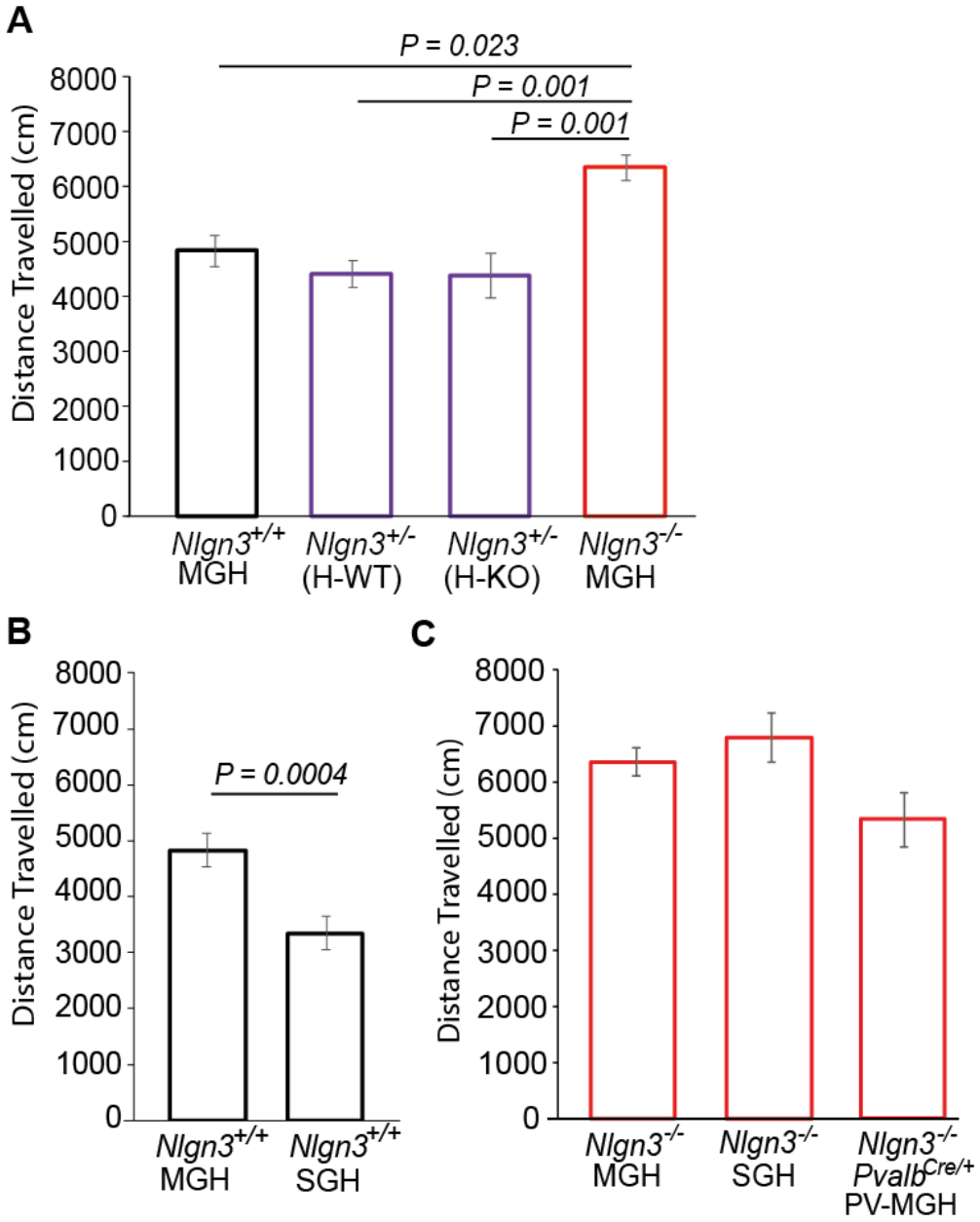


Figure 4.4. The impact of the social dominance environment on the activity within the open field arena of adult *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice. ***A***, average distance travelled (cm) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 4831.0 (SE:292.6), *Nlgn3*$^{+/-}$ (H-WT) mice, 4418.07 (SE:209.1), *Nlgn3*$^{+/-}$ (H-KO) mice, 4387.9 (SE:401.1), *Nlgn3*$^{-/-}$ mice from MGH, 6348.3 (SE:232.6). A Kruskal-Wallis test revealed a significant difference between the groups (*P = 0.000*). ***B***, average distance travelled (cm) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 4831.5(SE:292.6), *Nlgn3*$^{+/+}$ mice from SGH, 3342.2(SE:298.5). A Mann-Whitney U test revealed a significant difference between the groups (*P = 0.0004*). ***C***, Average distance travelled (cm) and standard error of the mean (SE) of *Nlgn3*$^{-/-}$ mice from MGH, 6348.3 (SE:232.1), *Nlgn3*$^{-/-}$ mice from SGH, 6791.1 (SE:437.3), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 5751.9 (SE:484.2). A one-way ANOVA revealed no significant difference between the groups.

4.3.2.2. The influence of the social environment and Nlgn3 re-expression on the levels of thigmotaxis in the open field arena of adult Nlgn3$^{+/+}$ mice, Nlgn3$^{+/-}$ mice, and Nlgn3$^{-/-}$ mice

To explore the anxiety levels of the mice, the levels of thigmotaxis from the open field area were determined. The thigmotaxis of the *Nlgn3$^{+/+}$* mice from MGH, their *Nlgn3$^{+/-}$* (H-WT) littermates, *Nlgn3$^{-/-}$* mice from MGH and their *Nlgn3$^{+/-}$* (H-KO) littermates were compared. *Nlgn3$^{-/-}$* mice from MGH were found to have an increased ratio of the distance travelled in the centre of the open field arena/ distance, and a non-significant increased time in the centre (ratio of the distance travelled in the centre of the open field arena/ distance, **figure 4.5.A,** one-way ANOVA, *P = 0.0001, F = 8.6, df= 3,* Tukey's post-hoc analysis, *Nlgn3$^{-/-}$* mice from MGH and *Nlgn3$^{+/+}$* mice from MGH: *P = 0.001*, *Nlgn3$^{-/-}$* mice from MGH and *Nlgn3$^{+/-}$* (H-WT) mice: *P = 0.001*, *Nlgn3$^{-/-}$* mice from MGH and *Nlgn3$^{+/-}$* (H-KO) mice: *P = 0.004.* Time in centre, **figure 4.5.B,** Kruskal-Wallis test, *Nlgn3$^{+/+}$* mice from MGH n = 13, *Nlgn3$^{+/-}$* (H-WT) mice n = 13, *Nlgn3$^{-/-}$* mice from MGH n = 16, *Nlgn3$^{+/-}$* (H-KO) mice n = 12). These results show that *Nlgn3$^{-/-}$* mice from MGH have an increased ratio of distance travelled in the centre and a trend for increased time in the centre, possibly indicating decreased anxiety. This also shows that adult *Nlgn3$^{+/-}$* mice do not have altered thigmotaxis dependent upon social dominance environment.

To determine if the social dominance environment is affecting the anxiety of *Nlgn3$^{+/+}$* mice, the thigmotaxis behaviour of the *Nlgn3$^{+/+}$* mice from MGH was compared to that of *Nlgn3$^{+/+}$* mice from SGH. It was observed that there is a significantly increased time in the centre of *Nlgn3$^{+/+}$* mice from MGH, and a non-significantly increased ratio of the distance travelled in the centre of the open field arena/ distance of *Nlgn3$^{+/+}$* mice from MGH (ratio of the distance travelled in the centre of the open field arena/ distance, **figure 4.5.C,** independent samples t-test, time in the centre, **figure 4.5.D,** independent samples t-test *P = 0.019, df = 19, Nlgn3$^{+/+}$* mice from MGH n = 13, *Nlgn3$^{+/+}$* mice from SGH n = 8). These data show that the social environment is a factor in mediating the thigmotaxic behaviour of *Nlgn3$^{+/+}$* mice, possibly indicating decreased anxiety in adult *Nlgn3$^{+/+}$* mice from MGH.

To determine if the social dominance environment impacts the thigmotaxis behaviours of *Nlgn3$^{-/-}$* mice, the *Nlgn3$^{-/-}$* mice from MGH were compared to *Nlgn3$^{-/-}$* mice from SGH and to *Nlgn3$^{-/-}$Pvalb$^{cre/+}$* mice. This comparison revealed no significant effect of social dominance environment or re-expression on the levels of thigmotaxis of the mice (ratio distance travelled in the centre/ total distance, **figure 4.5.E**, one-way ANOVA. Time in centre, **figure**

4.5.F, one-way ANOVA, *Nlgn3*$^{-/-}$ mice from MGH n = 16, *Nlgn3*$^{-/-}$ mice from SGH n = 13, *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ n = 10). These data indicate that there is no effect of social dominance environment or re-expression of *Nlgn3* in *Pvalb*-expressing cells on the thigmotaxic behaviour of adult *Nlgn3*$^{-/-}$ mice.

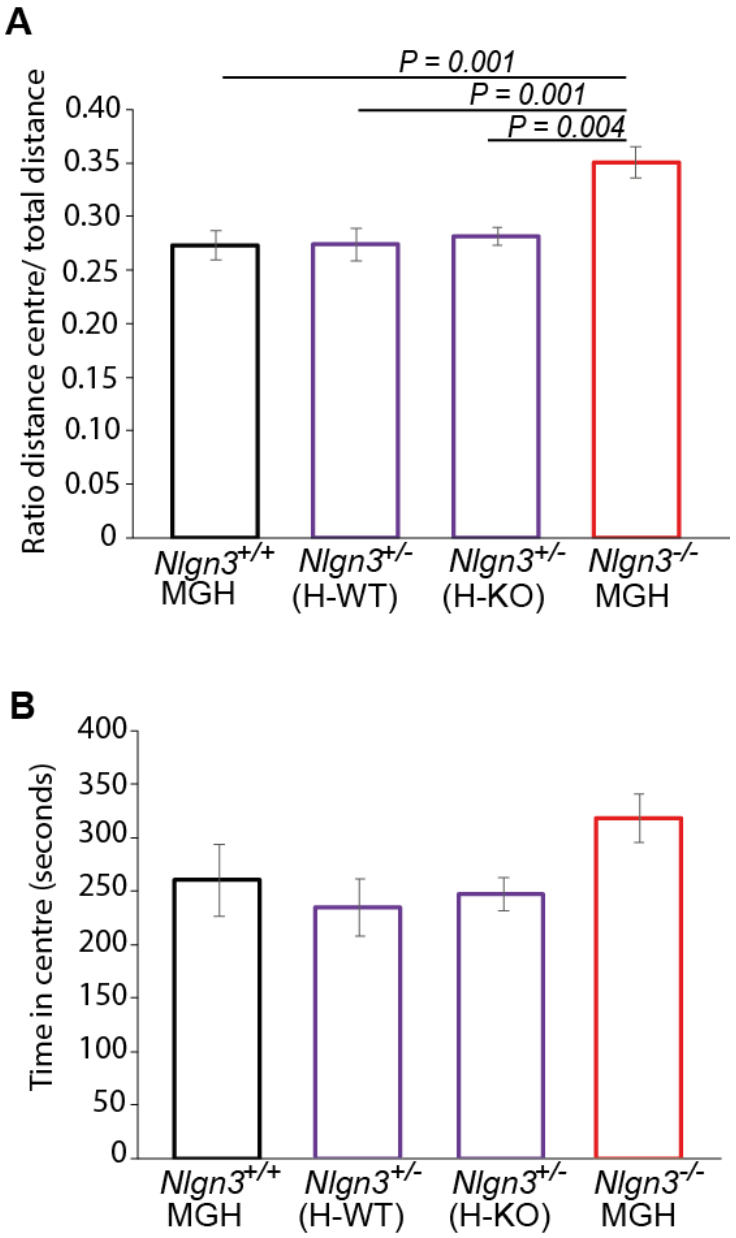


Figure 4.5. The impact of the social dominance environment on the thigmotaxis in the open field arena of adult *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice. *A*, average ratio distance in the centre/ total distance and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 0.27(SE:0.013), *Nlgn3*$^{+/-}$ (H-WT) mice, 0.27(SE:0.015), *Nlgn3*$^{+/-}$ (H-KO) mice, 0.28(SE:0.009), and *Nlgn3*$^{-/-}$ mice from MGH, 0.35(SE:0.015). A one-way ANOVA revealed a significant difference between the groups (*P = 0.0001*), and the results of the post-hoc analysis are shown on the figure. ***B*,** average time in the centre (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 260.2 (SE:33.1), *Nlgn3*$^{+/-}$ (H-WT) mice, 235.2(SE:26.8), *Nlgn3*$^{+/-}$ (H-KO) mice, 247.7(SE:14.9), and *Nlgn3*$^{-/-}$ mice from MGH, 317.9(SE:25.3). A Kruskal-Wallis test reveals no significant difference between the groups.

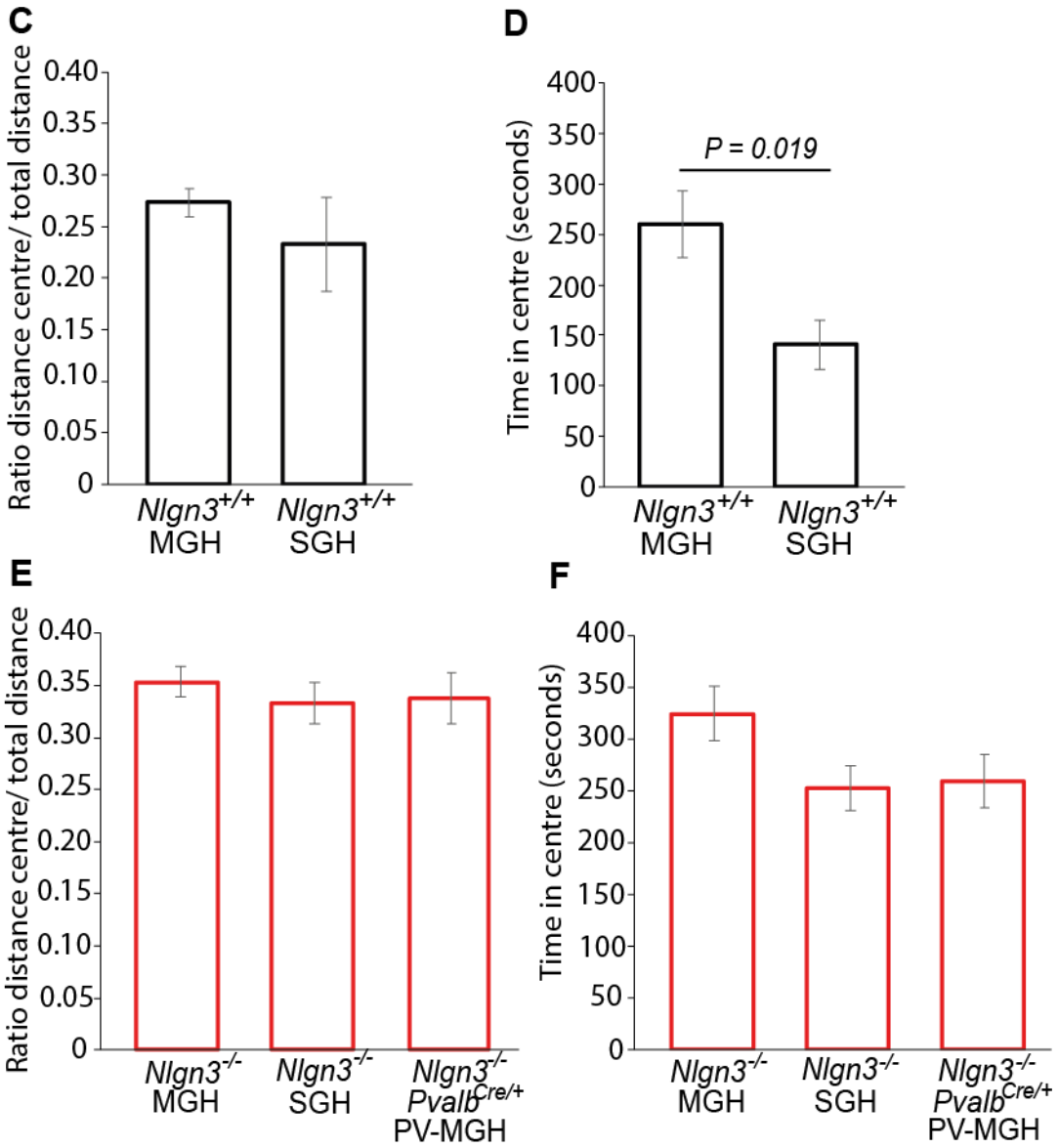


Figure 4.5 continued. ***C***, average ratio distance in the centre/ total distance and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 0.27(SE:0.013), and *Nlgn3*$^{+/+}$ mice from SGH, 0.23(SE:0.05). An independent samples t-test revealed no significant difference between the groups. ***D***, average time in the centre (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 260.2(SE:33.1), and *Nlgn3*$^{+/+}$ mice from SGH, 140.7(SE:24.4). An independent samples t-test revealed a significant difference between the groups (*P = 0.019*). ***E*,** average ratio distance in the centre/ total distance and standard error of the mean (SE) of *Nlgn3*$^{-/-}$ mice from MGH, 0.35(SE:0.015), *Nlgn3*$^{-/-}$ mice from SGH, 0.33(SE:0.02), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 0.34(SE:0.024). A one-way ANOVA revealed no significant difference between the groups. ***F*,** average time in the centre (seconds) and standard error of the mean (SE) of *Nlgn3*$^{-/-}$ mice from MGH, 317.9(SE:25.3), *Nlgn3*$^{-/-}$ mice from SGH, 252.8(SE:22.01), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 259(SE:25.8). A one-way ANOVA revealed no significant difference between the groups.

4.3.2.3. The influence of the social dominance environment and Nlgn3 re-expression on the exploration of the elevated plus maze of adult Nlgn3$^{+/+}$ mice, Nlgn3$^{+/-}$ mice, and Nlgn3$^{-/-}$ mice

The exploration of the elevated plus maze of *Nlgn3*$^{+/+}$ mice from MGH, their *Nlgn3*$^{+/-}$ (H-WT) littermates, *Nlgn3*$^{-/-}$ mice from MGH, and their *Nlgn3*$^{+/-}$ (H-KO) littermates was compared, revealing that there is no significant difference between the groups (**figure 4.6.A**, one-way ANOVA, *Nlgn3*$^{+/+}$ mice from MGH n = 13, *Nlgn3*$^{+/-}$ (H-WT) mice n = 11, *Nlgn3*$^{-/-}$ mice from MGH n = 19, *Nlgn3*$^{+/-}$ (H-KO) mice n = 13). This shows that neither adult *Nlgn3*$^{-/-}$ mice or *Nlgn3*$^{+/-}$ mice have altered elevated plus maze exploration relative to their littermates. Additionally, adult *Nlgn3*$^{+/-}$ mice do not have altered elevated plus maze exploration dependent upon if they are housed with *Nlgn3*$^{+/+}$ mice or *Nlgn3*$^{-/-}$ mice.

To determine if the social dominance environment of adult *Nlgn3*$^{+/+}$ mice impacts their elevated plus maze exploration, the *Nlgn3*$^{+/+}$ mice from MGH were compared to *Nlgn3*$^{+/+}$ mice from SGH. It was observed that *Nlgn3*$^{+/+}$ mice from MGH spent significantly more time in the open arms of the elevated plus maze compared to *Nlgn3*$^{+/+}$ mice from SGH (**figure 4.6.B**, independent samples t-test, *P = 0.0006, df = 19, Nlgn3*$^{+/+}$ mice from MGH n = 13, *Nlgn3*$^{+/+}$ mice from SGH n = 8). This shows that social dominance environment impacts the open arm exploration of *Nlgn3*$^{+/+}$ mice, possibly indicating that adult *Nlgn3*$^{+/+}$ mice from MGH have decreased anxiety compared to adult *Nlgn3*$^{+/+}$ mice from SGH.

To explore how the social dominance environment and re-expression of *Nlgn3* in *Pvalb*-expressing cells mediates the exploration of the elevated plus maze behaviour of adult *Nlgn3*$^{-/-}$ mice, the *Nlgn3*$^{-/-}$ mice from MGH were compared with *Nlgn3*$^{-/-}$ mice from SGH, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice. It was observed that *Nlgn3*$^{-/-}$ mice from MGH have increased time in the open arms compared to *Nlgn3*$^{-/-}$ mice from SGH and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice (**figure 4.6.C**, one-way ANOVA, *P = 0.004, F = 6.2, df = 2,* Tukey's post-hoc analysis, *Nlgn3*$^{-/-}$ mice from MGH and *Nlgn3*$^{-/-}$ mice from SGH: *P = 0.008*, *Nlgn3*$^{-/-}$ mice from MGH and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice: *P = 0.032, Nlgn3*$^{-/-}$ mice in MGH n =19, *Nlgn3*$^{-/-}$ mice from SGH n = 13, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice n =9). These data show that the social dominance environment impacts the elevated plus maze exploration of *Nlgn3*$^{-/-}$ mice and indicates that the anxiety of adult female *Nlgn3*$^{-/-}$ mice is influenced by the social dominance environment and re-expression of *Nlgn3* in *Pvalb*-expressing cells.

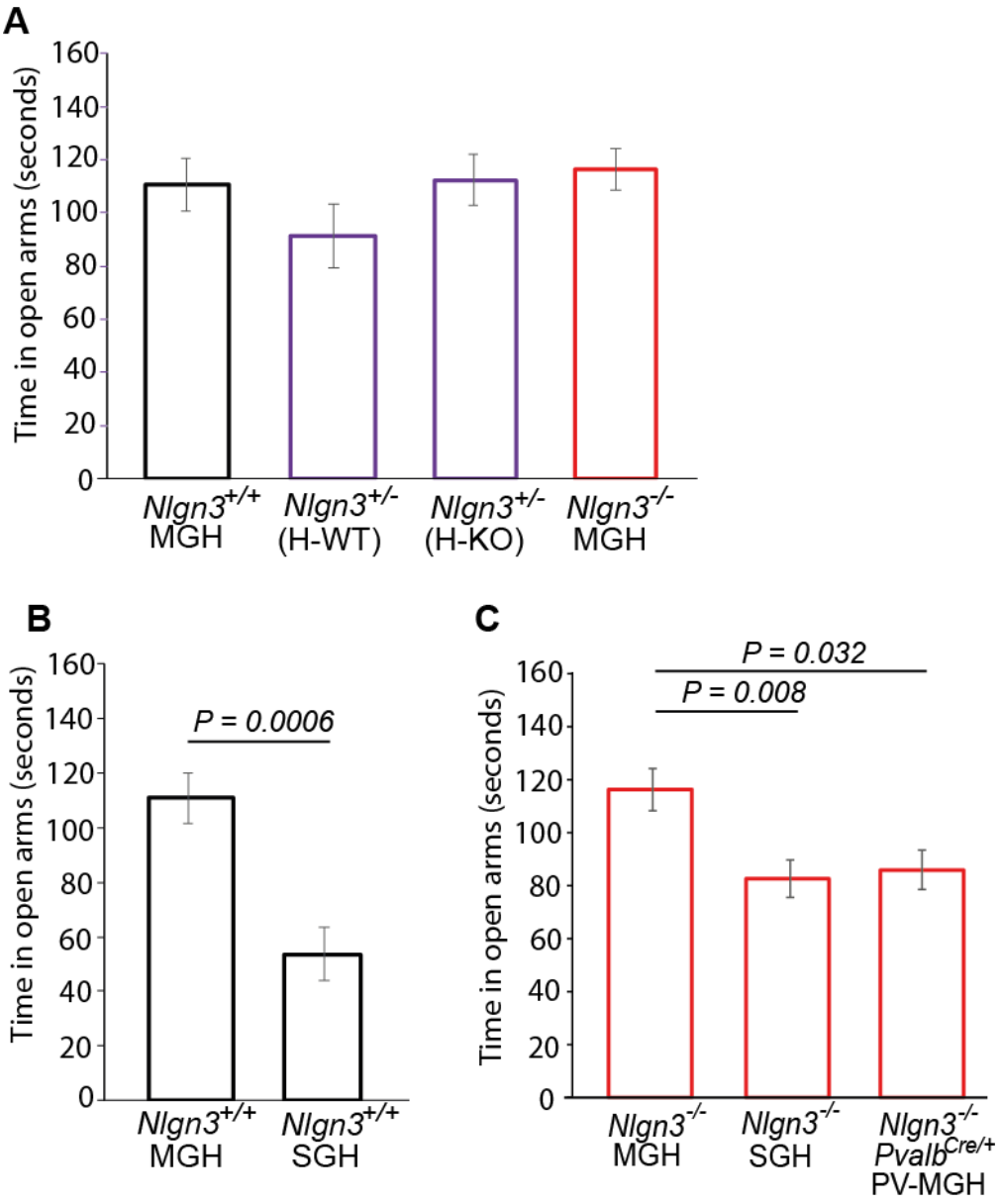


Figure 4.6. The impact of the social dominance environment on the time spent in the open arms of the elevated plus maze of adult *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice. *A*, average time spent in the open arms (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 110.8(SE:9.9), *Nlgn3*$^{+/-}$ (H-WT) mice, 91.3(SE:12.1), *Nlgn3*$^{+/-}$ (H-KO) mice 112.4(SE: 9.4), *Nlgn3*$^{-/-}$ mice from MGH, 116.3(SE:7.9). A one-way ANOVA revealed no significant difference between the groups. ***B***, Average time spent in the open arms (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 110.8(SE:9.9), *Nlgn3*$^{+/+}$ mice from SGH, 53.7(SE:9.6). An independent sample test revealed a significant difference between the groups (*P = 0.0006*). ***C***, Average time spent in the open arms (seconds) and standard error of the mean (SE) of *Nlgn3*$^{-/-}$ mice from MGH, 116.3(SE:7.9), *Nlgn3*$^{-/-}$ mice from SGH, 82.7(SE:7.0), *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 85.9(SE:7.4). A one-way ANOVA revealed a significant difference between the groups (*P = 0.004*). The results of the Tukey's post-hoc analysis are shown in the figure.

4.3.3. The impact of the social dominance environment and Nlgn3 re-expression on the exploratory and anxiety-related behaviours of juvenile male and female mice

In this section, the exploratory behaviours of male and female juvenile mice were assessed. As before, the juvenile male and female mice were not yet weaned into their separate sex housing conditions. The social dominance environment conditions are the same as those outlined in **table 3.1** and **table 3.2**. As shown in chapter 3, the social interaction behaviour is different between the sexes, indicating a sexual dimorphism at juvenile stages; because of this, the sexes were split in the following analysis.

4.3.3.1. The influence of the social dominance environment and Nlgn3 re-expression on the open field arena exploration of juvenile Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

To assess how knockout of *Nlgn3* and the social dominance environment influences the locomotive behaviours of juvenile mice, recordings in the open field arena were taken. Two recordings of open field activity were taken on sequential days to assess if initial reactivity and habituation to the box is impacted by genotype, social dominance environment, and *Nlgn3* re-expression. The distance travelled of *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* littermates was compared, revealing that *Nlgn3$^{y/-}$* mice are hyperactive, and that the behaviour of MGH mice on day two is different to that of mice from SGH and the PV-MGH mice (**figure 4.7**, two-way ANOVA, Day: *P = 1.0e-12, F = 73.4, df = 1.* social dominance environment: *P = 0.0003, F =8.9, df = 2*. Genotype: *P = 0.00002, F = 20.4, df = 1*. Day x social dominance environment: *P = 0.007, F = 5.1, df = 2,* Bonferroni post-hoc analysis, MGH and SGH: *P = 0.049*, MGH and *Pvalb: P = 0.0006*. Bonferroni adjusted pair-wise comparisons, day two MGH and day two *Pvalb*: *P = 9.01e-7*, day two MGH and day two SGH: *P = 0.0003*, MGH day one to day two: *P = 0.011*, SGH day one to day two: *P = 2.57e-08*, PV-MGH day one to day two: *P = 2.36e-08*, *Nlgn3$^{y/+}$* mice from MGH n = 24, *Nlgn3$^{y/-}$* mice from MGH n = 17, *Nlgn3$^{y/+}$* mice from SGH n = 19, *Nlgn3$^{y/-}$* mice from SGH n = 21, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 15, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice n = 13).

These results show that, like in adulthood, juvenile *Nlgn3$^{y/-}$* mice are also hyperactive, and this hyperactivity is not influenced by the social dominance environment condition or re-expression of *Nlgn3* in *Pvalb*-expressing cells. Furthermore, these results show that *Nlgn3$^{y/-}$* mice and *Nlgn3$^{y/+}$* mice from MGH have increased activity within the open field arena on day

two compared to mice from SGH and PV-MGH mice, indicating that social dominance environment and re-expression of *Nlgn3* influences habituation within the open field arena.

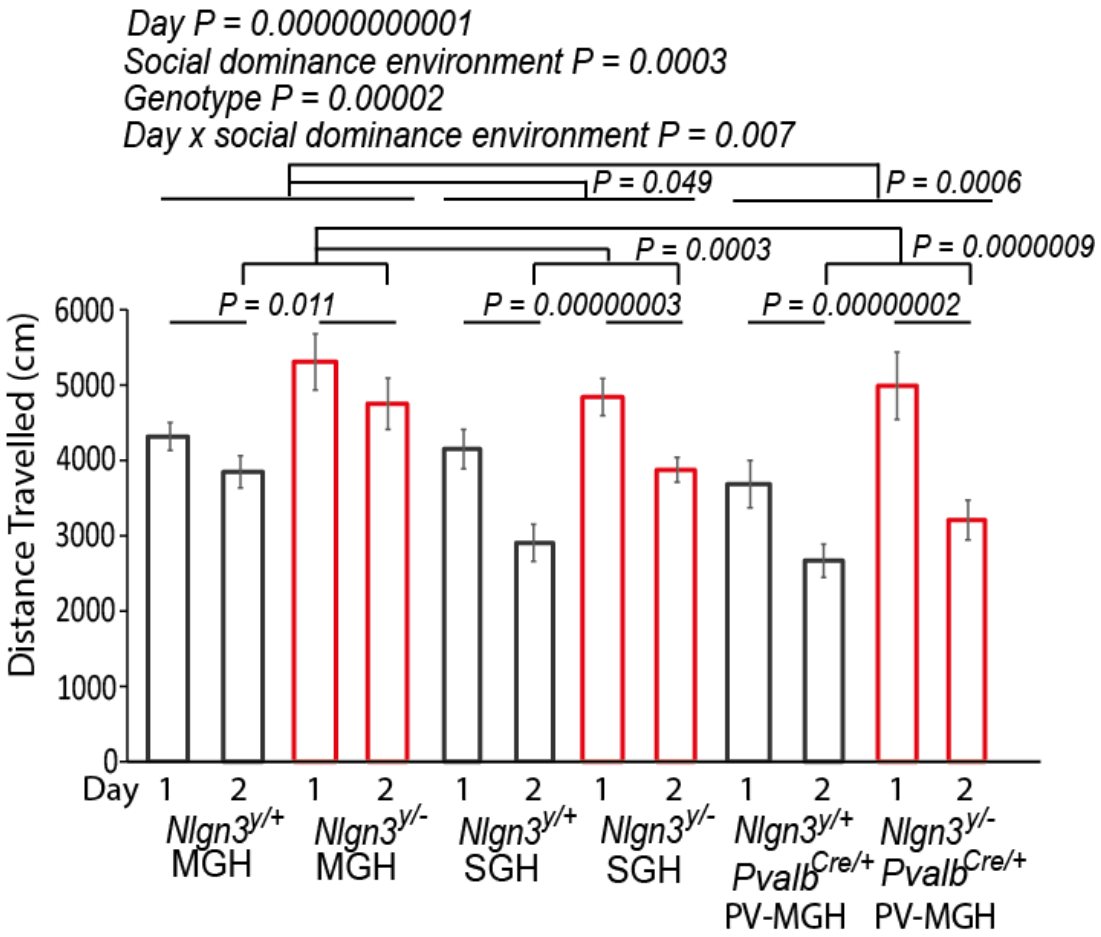


Figure 4.7. The impact of the social dominance environment and re-expression on the activity within the open field arena of juvenile *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice. Average distance travelled (cm) and standard error of the mean (SE) on day one of *Nlgn3$^{y/+}$* mice from MGH, 4316.4(SE:182.6), *Nlgn3$^{y/-}$* mice from MGH, 5307.0(SE:378.0), *Nlgn3$^{y/+}$* mice from SGH, 4150.8(SE:260.0), *Nlgn3$^{y/-}$* mice from SGH, 4839.9(SE:248.1), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 3683.3(SE:314.9), *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 4990.3(SE:445.3), and on day two, *Nlgn3$^{y/+}$* mice from MGH, 3847.1(SE:212.4), *Nlgn3$^{y/-}$* mice from MGH, 4751.6(SE:341.6), *Nlgn3$^{y/+}$* mice from SGH, 2904.7(SE:245.9), *Nlgn3$^{y/-}$* mice from SGH, 3874.5(SE:166), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$*, 2667.4(SE:218.2), *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 3206.9(SE:261.4). The result of the two-way ANOVA found significant impact of day (*P = 0.00000000001)*, genotype (*P = 0.00002)*, social dominance environment (*P = 0.0003*), and day x social dominance environment (*P = 0.007)*, and the results of the Bonferroni post-hoc analysis and Bonferroni adjusted pair-wise analysis are shown on the figure.

4.3.3.2. The influence of the social dominance environment and Nlgn3 re-expression on the levels of thigmotaxis in the open field arena of juvenile Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

To assess if genotype or social dominance environment influences the anxiety of the juvenile male mice within the open field arena, the open field thigmotaxis of the *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates was compared. It was observed that *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* have decreased time in the centre and increased ratio of distance in the centre/ total distance. From day one to day two, it was observed that mice from MGH show no decrease in the ratio of distance travelled in the centre (ratio distance travelled in the centre/ total distance, **figure 4.8.A**, two-way ANOVA, Day: *P = 1.65e-09, F = 43.5, df = 1.* Genotype: *P = 0.004, F = 8.8, df= 2*. social dominance environment: *P = 0.00001, F = 12.9, df=2*. Day x social dominance environment: *P = 0.001, F = 6.9, df = 2*. Bonferroni post-hoc: MGH and PV-MGH *P = 5.2e-06*, SGH and PV-MGH *P = 0.0004,* Bonferroni pairwise comparison, day two MGH and *Pvalb: P = 1.32e-08*, day two SGH and *Pvalb: P = 4.44e-06*, PV-MGH day one to day two: *P = 1.21e-08*, SGH day one and day two: *P = 0.007*. Time in centre, **figure 4.8.B**, two-way ANOVA, Day: *P = 0.0004, F = 16.4, df = 1.* Genotype *P = 0.0002, F =15.18, df =1*. social dominance environment: *P = 0.002, F = 6.8, df = 2*, Bonferroni post-hoc analysis: MGH and PV-MGH *P = 0.001*, SGH and PV-MGH *P = 0.005*, *Nlgn3$^{y/+}$* mice from MGH n = 24, *Nlgn3$^{y/-}$* mice from MGH n = 17, *Nlgn3$^{y/+}$* mice from SGH n = 19, *Nlgn3$^{y/-}$* mice from SGH n = 21, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 15, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice n = 13).

Interestingly, a significant impact of genotype was observed for both the ratio of distance travelled and the time in the centre, with *Nlgn3$^{y/-}$* mice showing decreased thigmotaxis, consistent with what is observed in adult *Nlgn3$^{y/-}$* mice. This appears to be more pronounced on day two, but no interaction of social dominance environment, day and genotype was observed so this was not explored further. A main effect of social dominance environment and day was observed for the ratio of distance in the centre/ total distance, and investigation into this revealed that both the mice from SGH and the *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates show a significant decrease from day one to day two, while mice from MGH do not. However, *Nlgn3$^{y/-}$* mice from SGH do not appear to have this decrease. As there was no interaction of day, social dominance environment, and genotype, this was not investigated further. Additionally, these data indicate that the time in the centre and the ratio of the distance in the centre/ total distance is influenced by re-expression of *Nlgn3* in

Pvalb-expressing cells, possibly indicating that re-expression of *Nlgn3* within parvalbumin containing neurones could be having an additional effect on thigmotaxic behaviour.

A

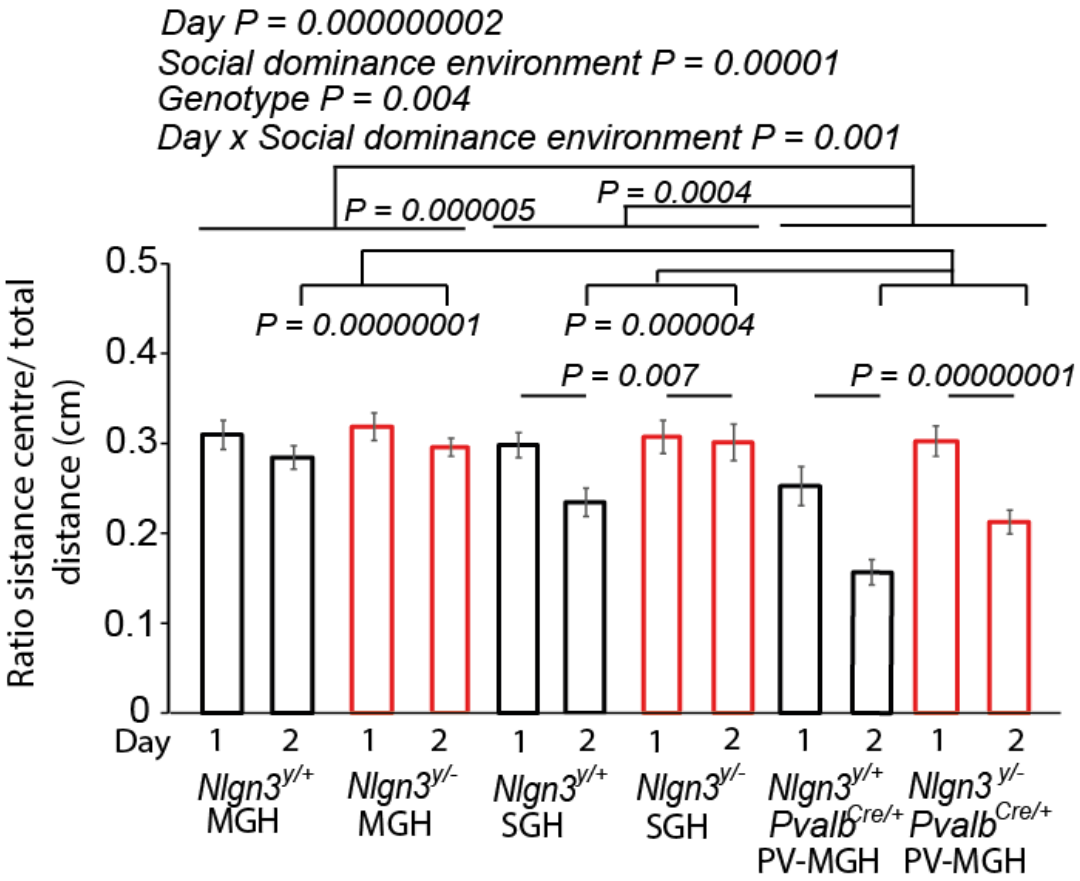


Figure 4.8. The impact of social dominance environment and re-expression on thigmotaxis of juvenile *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice within the open field arena. *A*, average ratio of distance travelled in the centre/ total distance and standard error of the mean (SE) on day one of *Nlgn3$^{y/+}$* mice from MGH, 0.31 (SE:0.016), *Nlgn3$^{y/-}$* mice from MGH, 0.31(SE:0.015), *Nlgn3$^{y/+}$* mice from SGH, 0.30(SE:0.014), *Nlgn3$^{y/-}$* mice from SGH, 0.31(SE:0.018), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 0.25(SE:0.022), *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 0.30(SE:0.017), and on day two of *Nlgn3$^{y/+}$* mice from MGH, 0.28(SE:0.013), *Nlgn3$^{y/-}$* mice from MGH, 0.30(SE:0.010), *Nlgn3$^{y/+}$* mice from SGH, 0.23(SE:0.016), *Nlgn3$^{y/-}$* mice from SGH, 0.30 (SE:0.020), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 0.15(SE:0.014), *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 0.21(SE:0.013). The result of the two-way ANOVA found significant impact of day (*P = 1.65e-09*), genotype (*P = 0.004*), social dominance environment (*P = 0.00001*), and an interaction of day and social dominance environment (*P = 0.001*). The results of the Bonferroni post-hoc analysis and pairwise comparisons are shown on the figure.

B

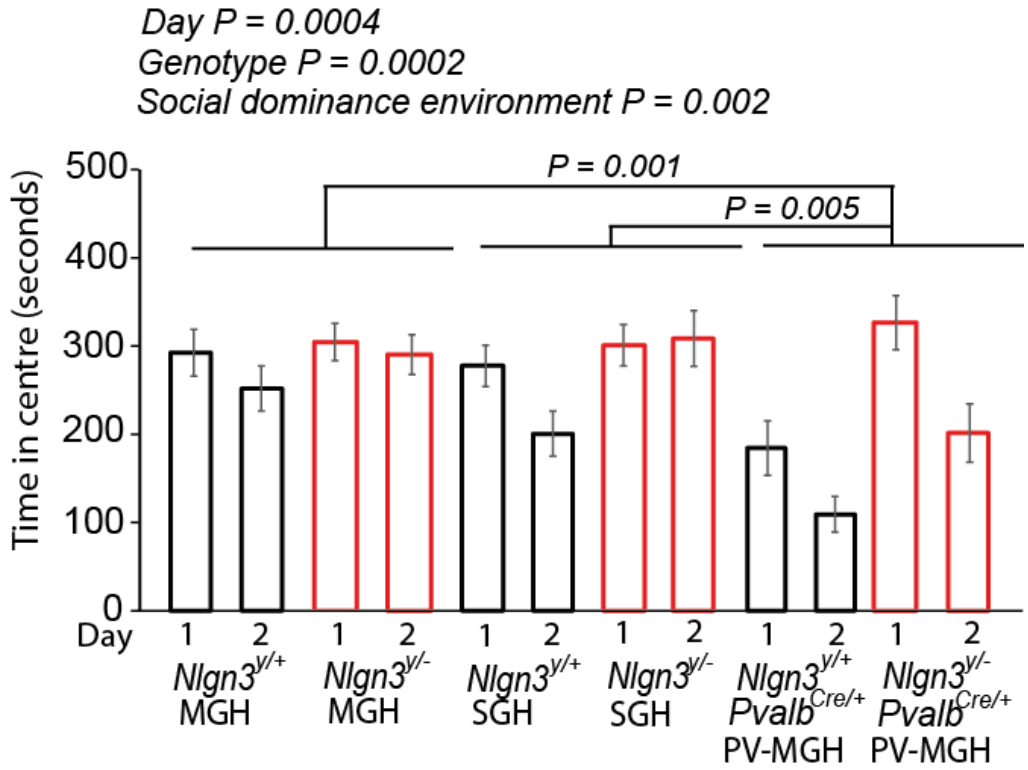


Figure 4.8, continued. *B*, average time in the centre (seconds) and the standard error of the mean (SE) on day one of *Nlgn3*$^{y/+}$ mice from MGH, 292.5(SE:26.5), *Nlgn3*$^{y/-}$ mice from MGH, 304.5(SE:20.9), *Nlgn3*$^{y/+}$ mice from SGH, 277.8(SE:23.5), *Nlgn3*$^{y/-}$ mice from SGH, 300.9(SE:23.3), *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice, 183.4(SE:30.9), *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice, 326.7(SE:30.7), and on day two of *Nlgn3*$^{y/+}$ mice from MGH, 251.8 (SE:25.6), *Nlgn3*$^{y/-}$ mice from MGH, 290.4(SE:22.5), *Nlgn3*$^{y/+}$ mice from SGH, 200.6(SE:25.6), *Nlgn3*$^{y/-}$ mice from SGH, 308.6(SE:31.4), *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice, 109.2(SE:20.4), *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice, 201.5(SE:33.0). The result of the two-way ANOVA found significant impact of day (*P = 0.0004*), genotype (*P = 0.0002*), and social dominance environment (*P = 0.002*), and the results of the Bonferroni post-hoc analysis are shown on the figure.

4.3.3.3. The influence of the social dominance environment and Nlgn3 re-expression on the exploration of the elevated plus maze of juvenile Nlgn3$^{y/+}$ and Nlgn3$^{y/-}$ mice

The anxiety levels of the juvenile mice were further assessed using the elevated plus maze. The time in the open arms of *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from MGH, *Nlgn3$^{y/+}$* mice and *Nlgn3$^{y/-}$* mice from SGH, and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* and their *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* littermates were compared to assess the effect of social dominance environment and genotype. It was found that *Nlgn3$^{y/-}$* mice have increased time in the open arms, and that mice from SGH show an overall decreased time in the open arms (**figure 4.9,** two-way ANOVA, Genotype: *P = 0.022, F = 5.3, df = 1,* Social dominance environment: *P = 0.001, F = 7.6, df = 2*, Bonferroni post-hoc analysis: SGH and MGH *P = 0.004*, SGH and PV-MGH *P = 0.029*, *Nlgn3$^{y/+}$* mice from MGH n = 18, *Nlgn3$^{y/-}$* mice from MGH n = 9, *Nlgn3$^{y/+}$* mice from SGH n = 19, *Nlgn3$^{y/-}$* mice from SGH n = 22, *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice n = 11, *Nlgn3$^{y/-}$Pvalb$^{cre/+}$*mice n = 15). These data show that juvenile *Nlgn3$^{y/-}$* mice, like adult *Nlgn3$^{y/-}$* mice, have increased time spent in the open arms, possibly reflecting decreased anxiety. There appears to be an effect of SGH, but not re-expression in *Pvalb*-expressing cells, on the time spent in the open arms on the mice, possibly indicating an altered anxiety of the mice.

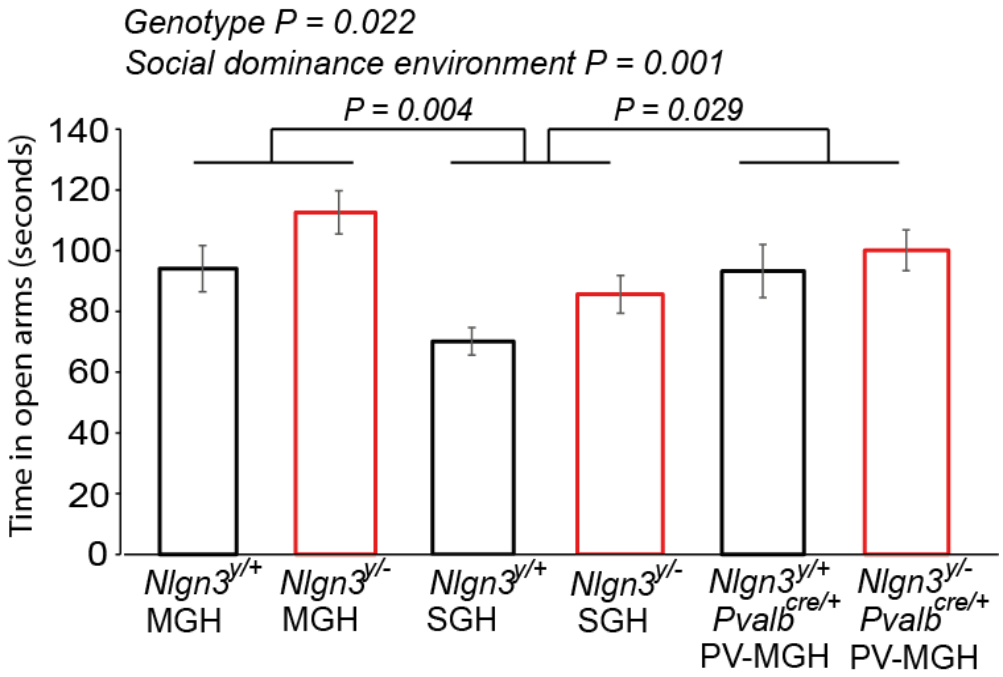


Figure 4.9. The impact of the social dominance environment and re-expression on elevated plus maze exploration of juvenile *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* mice. Average time in the open arms (seconds), and standard error of the mean (SE) of *Nlgn3$^{y/+}$* mice from MGH, 94.1(SE:7.60), *Nlgn3$^{y/-}$* mice from MGH, 112.6(SE:7.11), *Nlgn3$^{y/+}$* mice from SGH, 70.1(SE:4.52), *Nlgn3$^{y/-}$* from SGH, 85.7(SE:6.20), *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice, 93.3(SE:8.78), *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice, 100.1(SE:6.74). A two-way ANOVA revealed a main effect of genotype (*P = 0.022*) and a main effect of social dominance environment (*P = 0.001*), the results of the Bonferroni post-hoc analysis are shown on the figure.

4.3.3.5. The influence of the social dominance environment and re-expression of Nlgn3 on the open field activity of juvenile Nlgn3$^{+/+}$, Nlgn3$^{+/-}$, and Nlgn3$^{-/-}$ mice

The activity within the open field arena on two sequential days of juvenile *Nlgn3$^{+/+}$* mice from MGH, *Nlgn3$^{+/-}$* (H-WT) mice, *Nlgn3$^{+/-}$* (H-KO) mice, and *Nlgn3$^{-/-}$* mice from MGH was assessed, revealing that *Nlgn3$^{-/-}$* mice from MGH are hyperactive (**figure 4.10.A**, repeated measures ANOVA, Day: *P = 0.009, F = 7.2, df = 1.* Group: *P = 0.00004, F = 9.2, df = 3*, Bonferroni post-hoc analysis, *Nlgn3$^{+/+}$* mice from MGH and *Nlgn3$^{-/-}$* mice from MGH: *P = 0.00008*, *Nlgn3$^{+/-}$* (H-WT) mice and *Nlgn3$^{-/-}$* mice from MGH: *P = 0.009*, *Nlgn3$^{+/-}$* mice (H-KO) and *Nlgn3$^{-/-}$* mice from MGH: *P = 0.007*, *Nlgn3$^{+/+}$* mice from MGH n = 13, *Nlgn3$^{+/-}$* (H-WT) mice n = 13, *Nlgn3$^{+/-}$* (H-KO) mice n = 14, *Nlgn3$^{-/-}$* mice from MGH n = 24). These data show that *Nlgn3$^{-/-}$* mice from MGH have significantly increased locomotion in the open field arena compared to their *Nlgn3$^{+/-}$* (H-KO) littermates, and *Nlgn3$^{+/+}$* mice and their *Nlgn3$^{+/-}$* (H-WT) littermates. This demonstrates that like adult *Nlgn3$^{-/-}$* mice, and adult and juvenile *Nlgn3$^{y/-}$* mice, juvenile *Nlgn3$^{-/-}$* mice are hyperactive. Additionally, *Nlgn3$^{+/-}$* mice do not show altered activity dependent upon social environment, reflecting what is seen in adult *Nlgn3$^{+/-}$* mice. Overall there was an impact of day, but no interaction of day and group, however, *Nlgn3$^{+/+}$* mice do appear to have a smaller decrease in activity from day one to day two compared to the other groups, which was not explored further due to no interaction of day and group.

To assess how the social dominance environment impacts the open field activity of *Nlgn3$^{+/+}$* mice over two days, *Nlgn3$^{+/+}$* mice from MGH were compared to *Nlgn3$^{+/+}$* mice from SGH. This revealed that the activity of *Nlgn3$^{+/+}$* mice on both days was impacted by social dominance environment. Relative to *Nlgn3$^{+/+}$* mice from MGH, *Nlgn3$^{+/+}$* mice from SGH show an increased distance travelled on day one, and a decreased distance travelled on day two (**figure 4.10.B,** repeated measures ANOVA, Day: *P = 0.0006, F = 15.6, df = 1,* Day x environment: *P = 0.0003, F = 18.2, df = 1*, Bonferroni corrected pair-wise analysis, *Nlgn3$^{+/+}$* mice from SGH, day one to day two: *P = 8.4e-06*, Day one *Nlgn3$^{+/+}$* mice from SGH and *Nlgn3$^{+/+}$* mice from MGH: *P = 0.002*, Day two *Nlgn3$^{+/+}$* mice from SGH and *Nlgn3$^{+/+}$* mice from MGH: *P = 0.017, Nlgn3$^{+/+}$* mice from MGH n = 13, *Nlgn3$^{+/+}$* mice from SGH n = 12). These data demonstrate that the social dominance environment does influence the activity levels of *Nlgn3$^{+/+}$* mice in the open field arena over the two-day exposure, reflecting what is seen in *Nlgn3$^{y/+}$* juvenile mice. Interestingly, as day one activity is also elevated in *Nlgn3$^{+/+}$* mice from

SGH compared to *Nlgn3*$^{+/+}$ mice from MGH, it appears that initial reactivity to the open field arena, as well as habituation, is impacted by social dominance environment.

To assess if the social dominance environment or re-expression of *Nlgn3* in *Pvalb*-expressing cells influences the activity of juvenile *Nlgn3*$^{-/-}$ mice, the activity in the open field arena on day one, and day two, of *Nlgn3*$^{-/-}$ mice from MGH, *Nlgn3*$^{-/-}$ mice from SGH, and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ was assessed. It was revealed that there is a significant impact of day and group (**figure 4.10.C,** repeated measures ANOVA, Day: *P = 0.00009, F = 18.2, df = 1.* Group: *P = 0.035, F = 3.6, df = 2,* Bonferroni post-hoc, *Nlgn3*$^{-/-}$ MGH and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice: *P = 0.031,* *Nlgn3*$^{-/-}$ mice from MGH n = 24, *Nlgn3*$^{-/-}$ mice from SGH n = 20, *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ n = 10). This demonstrates that re-expression of *Nlgn3* in *Pvalb*-expressing cells leads to an overall decrease in activity, however, day one to day two activity is not impacted by social dominance environment or re-expression.

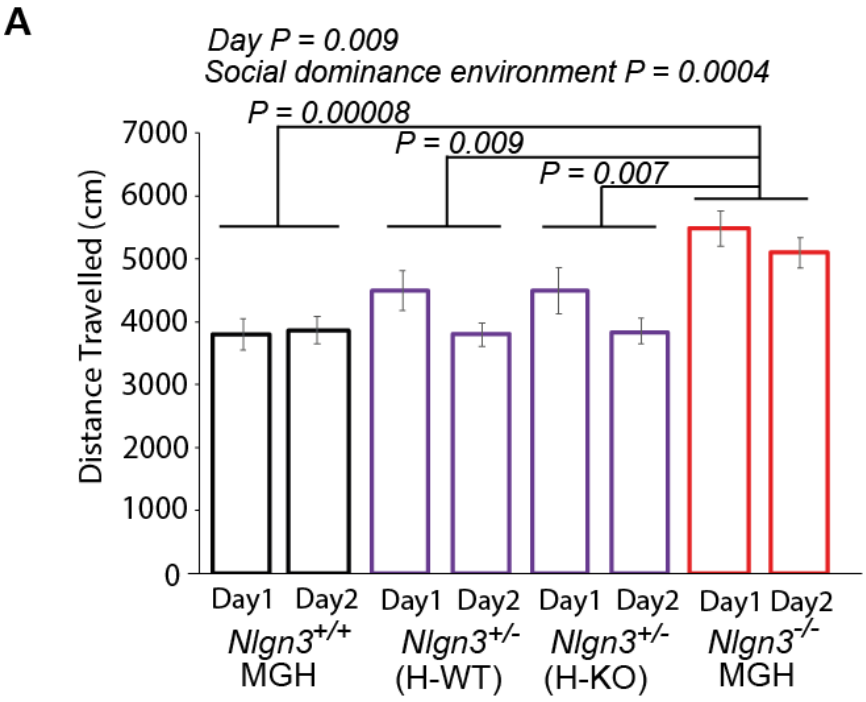
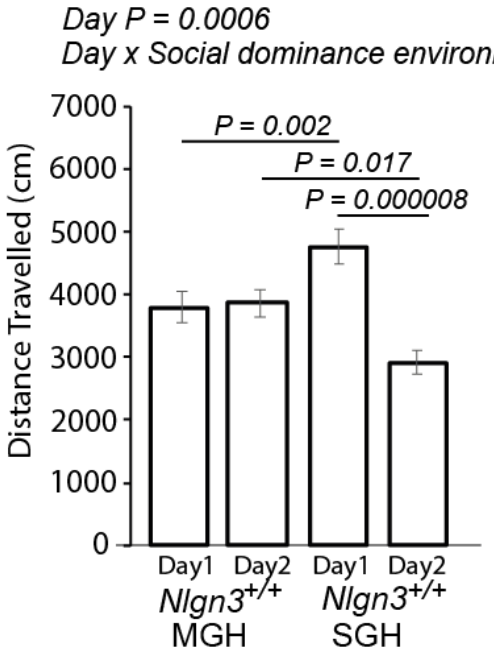


Figure 4.10. The impact of the social dominance environment on activity in the open field arena of juvenile *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice. ***A***, the average distance travelled (cm) and standard error of the mean (SE) on day one of *Nlgn3*$^{+/+}$ mice from MGH, 3795.6(SE:248.5), *Nlgn3*$^{+/-}$ (H-WT) mice, 4625.7(SE:315.7), *Nlgn3*$^{+/-}$ (H-KO) mice, 4625.3(SE:366.0), and *Nlgn3*$^{-/-}$ mice from MGH, 5480(SE:282.5), and on day 2, *Nlgn3*$^{+/+}$ mice from MGH, 3866.0(SE:214.5), *Nlgn3*$^{+/-}$ (H-WT) mice, 3876.8(SE:184.7), *Nlgn3*$^{+/-}$ (H-KO) mice, 3885.4(SE:203.4), *Nlgn3*$^{-/-}$ mice from MGH, 5049.0(SE:245.2). A repeated measures ANOVA found a significant impact of day (*P = 0.009*) and of group (*P = 0.0004*), the results of the Bonferroni post-hoc analysis are shown on the figure.

B



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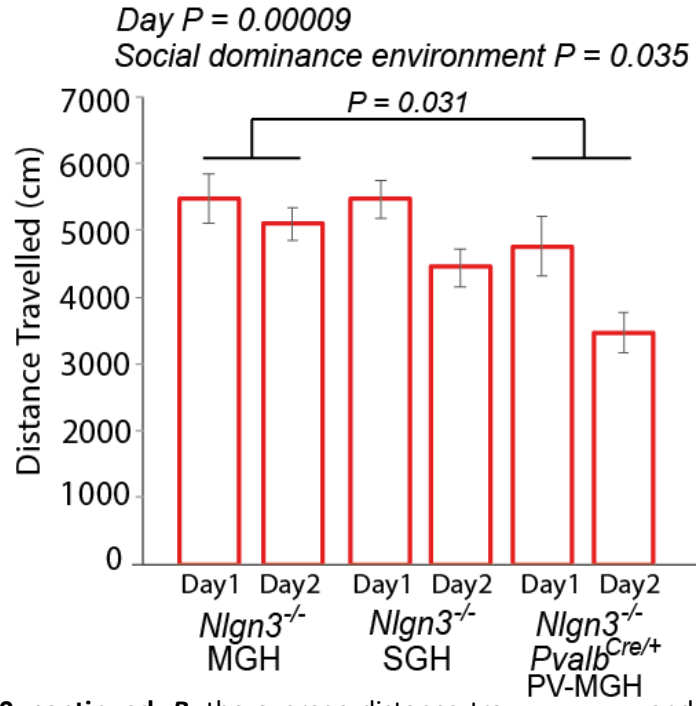


Figure 4.10, continued. ***B***, the average distance travelled (cm) and standard error of the mean (SE) on day one of *Nlgn3*$^{+/+}$ mice from MGH, 3795.6(SE:248.5), and *Nlgn3*$^{+/+}$ mice from SGH, 4752.8(SE:277.3) and on day 2, *Nlgn3*$^{+/+}$ mice from MGH, 3866.0(SE:214.5) and *Nlgn3*$^{+/+}$ mice from SGH, 2884.3(SE:170.1). A significant impact of day (*P = 0.0006*), and of day and genotype (*P = 0.0003*). The results of the Bonferroni adjusted pairwise comparisons are shown on the figure. ***C***, Average distance travelled (cm) and standard error of the mean (SE) on day one *Nlgn3*$^{-/-}$ mice from MGH, 5480(SE:282.5), *Nlgn3*$^{-/-}$ mice from SGH, 5473.5(SE:375.4), *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 4751.6(SE:282.2), and on day 2, *Nlgn3*$^{-/-}$ mice from MGH, 5049.0(SE:245.2), *Nlgn3*$^{-/-}$ mice from SGH, 4459.8(SE:282.6), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 3473.2(SE:302.3). A repeated measures ANOVA revealed a significant effect of day (*P = 0.00009*), and of group (*P = 0.035*); the results of the Bonferroni post-hoc analysis are shown on the figure.

4.3.3.6. The influence of the social dominance environment and Nlgn3 re-expression on the levels of thigmotaxis within the open field arena of juvenile Nlgn3$^{+/+}$, Nlgn3$^{+/-}$, and Nlgn3$^{-/-}$ mice

Differences in open field exploration and habituation could be explained by anxiety within the open field arena. The levels of thigmotaxis of *Nlgn3*$^{+/+}$ mice from MGH, *Nlgn3*$^{+/-}$ (H-WT) mice, *Nlgn3*$^{+/-}$ (H-KO) mice, and *Nlgn3*$^{-/-}$ mice from MGH were assessed. The ratio of the distance travelled in the centre/ total distance travelled and the time in the centre was analysed, revealing no significant impact of day, group, or interaction of group and day (ratio of the distance travelled in the centre/ total distance, **figure 4.11.A**, repeated measures ANOVA. Time in the centre, **figure 4.11.B**, repeated measures ANOVA, *Nlgn3*$^{+/+}$ mice from MGH n = 13, *Nlgn3*$^{+/-}$ (H-WT) mice n = 13, *Nlgn3*$^{+/-}$ (H-KO) mice n = 14, and *Nlgn3*$^{-/-}$ mice from MGH n = 24). These data demonstrate that there is no difference in thigmotaxis of *Nlgn3*$^{-/-}$ mice from MGH, their *Nlgn3*$^{+/-}$ (H-KO) littermates, and *Nlgn3*$^{+/+}$ mice from MGH, and their *Nlgn3*$^{+/-}$ (H-WT) littermates.

To assess how the social dominance environment impacts the thigmotaxis of juvenile *Nlgn3*$^{+/+}$ mice, the thigmotaxis behaviour of *Nlgn3*$^{+/+}$ mice from MGH was compared to that of *Nlgn3*$^{+/+}$ mice from SGH. *Nlgn3*$^{+/+}$ mice from SGH but not *Nlgn3*$^{+/+}$ mice from MGH showed a significant decrease in their ratio of distance travelled in the centre/ total distance travelled from day one to day two, and a similar, but not significant, trend is seen in the time in the centre (ratio of distance travelled in the centre/ total distance, **figure 4.11.C***,* repeated measures ANOVA, day x social dominance environment: *P = 0.009, F = 7.9, df = 1,* Bonferroni corrected pair-wise analysis: day one to day two of *Nlgn3*$^{+/+}$ mice from SGH: *P = 0.008*. Time in the centre, **figure 4.11.D,** repeated measures ANOVA, *Nlgn3*$^{+/+}$ mice from MGH n = 13, *Nlgn3*$^{+/+}$ mice from SGH n = 12). These data show that the social dominance environment influences the thigmotaxis behaviour of *Nlgn3*$^{+/+}$ mice on day one to day two, possibly indicating an influence of social dominance environment on the anxiety of the mice over the course of exposure to the open field arena.

To assess if the social dominance environment or the re-expression of *Nlgn3* in *Pvalb*-expressing cells of *Nlgn3*$^{-/-}$ mice influences the thigmotaxis behaviour within the open field arena, the thigmotaxis behaviour of *Nlgn3*$^{-/-}$ mice from MGH, *Nlgn3*$^{-/-}$ mice from SGH, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice within the open field arena were assessed. This revealed no impact of social dominance environment or re-expression on thigmotaxis of *Nlgn3*$^{-/-}$ mice (ratio of the

distance travelled in the centre/ total distance, **figure 4.11.E**, repeated measures ANOVA, day*: P = 0.012, F = 6.8, df =1*, Time in centre, **figure 4.11.F**, repeated measures ANOVA, *Nlgn3*$^{-/-}$ mice from MGH n = 24, *Nlgn3*$^{-/-}$ mice from SGH n = 20, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ n = 10). These results indicate that the thigmotaxis of juvenile *Nlgn3*$^{-/-}$ mice is not impacted by the social dominance environment condition and that *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, unlike juvenile *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ mice, have no significant change in their thigmotaxis behaviour.

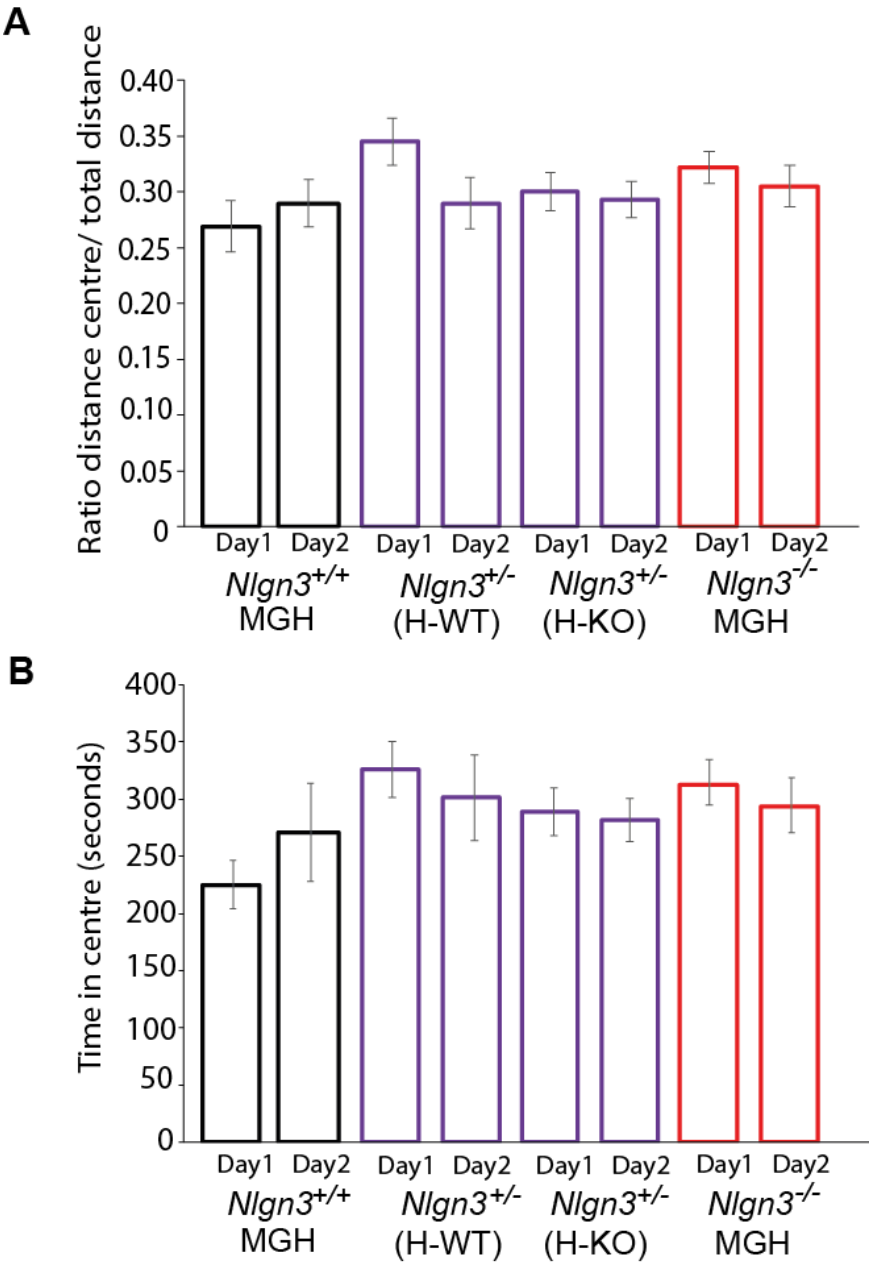


Figure 4.11. The impact of the social dominance environment on thigmotaxis of juvenile *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice in the open field arena. ***A***, the average ratio distance in the centre/ total distance, and the standard error of the mean (SE) on day one of *Nlgn3*$^{+/+}$ mice from MGH, 0.27(SE:0.019), *Nlgn3*$^{+/-}$ (H-WT) mice, 0.35(SE:0.021), *Nlgn3*$^{+/-}$ (H-KO) mice, 0.30(SE:0.017), and *Nlgn3*$^{-/-}$ mice from MGH, 0.32(SE:0.001). On day two, *Nlgn3*$^{+/+}$ mice from MGH, 0.28(SE:0.021), *Nlgn3*$^{+/-}$ (H-WT), 0.29(SE:0.022), *Nlgn3*$^{+/-}$ (H-KO), 0.29(SE:0.016), and *Nlgn3*$^{-/-}$ mice from MGH, 0.30(0.018). A repeated measures ANOVA revealed no significant interactions. ***B***, the average time in the centre (seconds), and the standard error of the mean (SE), on day one of *Nlgn3*$^{+/+}$ mice from MGH, 225.1(SE:21.2), *Nlgn3*$^{+/-}$ (H-WT) mice, 325.9(SE:24.6), *Nlgn3*$^{+/-}$ (H-KO) mice, 289.0(SE:20.9), and *Nlgn3*$^{-/-}$ mice from MGH, 312.7(SE:19.9). On day two, *Nlgn3*$^{+/+}$ mice from MGH, 271.4(SE:42.8), *Nlgn3*$^{+/-}$ (H-WT) mice, 301.4(SE:37.5), *Nlgn3*$^{+/-}$ (H-KO) mice, 281.8(SE:18.8), and *Nlgn3*$^{-/-}$ mice from MGH, 293.8(24.2). A repeated measures ANOVA revealed no significant interactions.

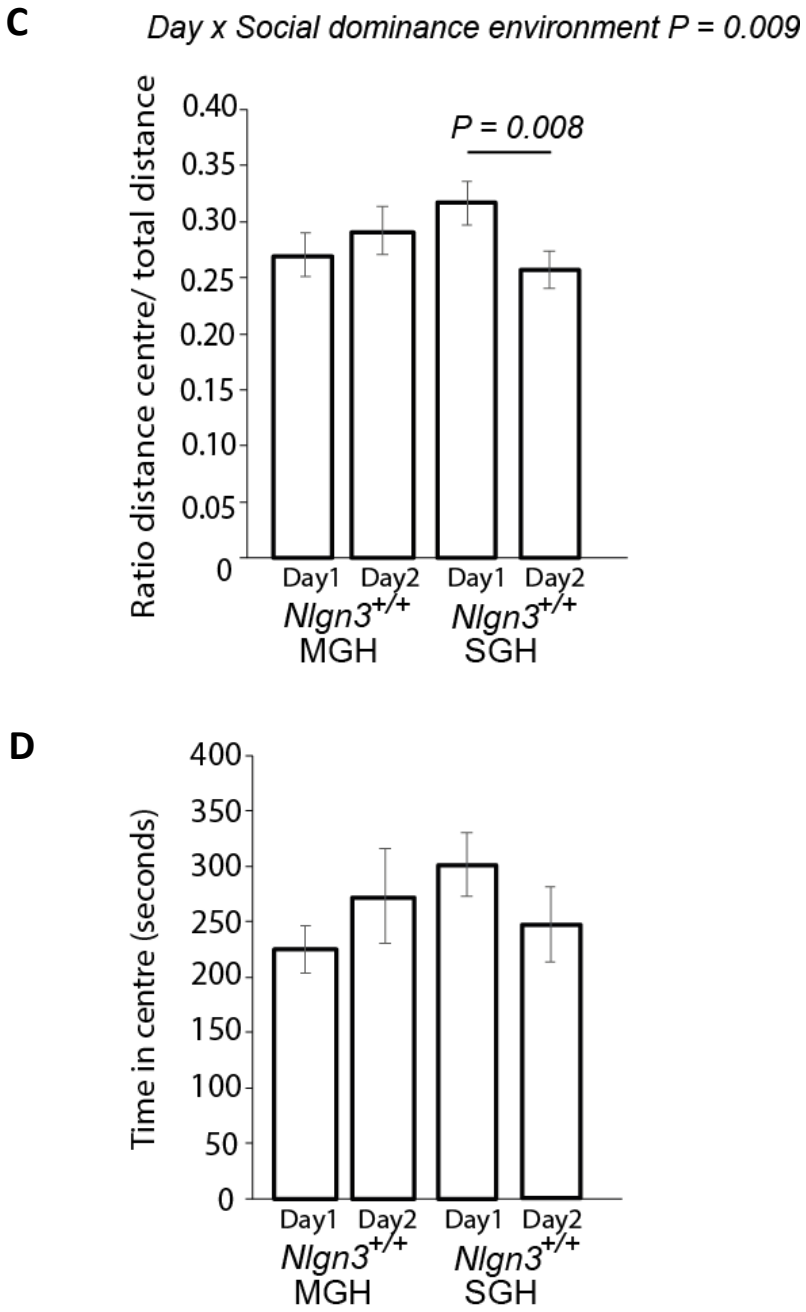


Figure 4.11, continued. ***C***, average ratio distance centre/ total distance travelled and standard error of the mean (SE) on day one, of *Nlgn3*$^{+/+}$ mice from MGH, 0.26(SE:0.019), and *Nlgn3*$^{+/+}$ mice from SGH, 0.32(SE:0.019). On day two, for *Nlgn3*$^{+/+}$ mice from MGH, 0.28(SE:0.021), and for *Nlgn3*$^{+/+}$ mice from SGH, 0.26(SE:0.017). A repeated measures ANOVA revealed a significant interaction of day and social dominance environment (*P = 0.009*). The result of the Bonferroni adjusted pair-wise analysis are shown on the figure. ***D,*** average time in the centre (seconds) and standard error of the mean (SE) on day one, of *Nlgn3*$^{+/+}$ mice from MGH, 225.1(SE:21.3), and *Nlgn3*$^{+/+}$ mice from SGH, 301.0(SE:28.5). On day two, for *Nlgn3*$^{+/+}$ mice from MGH, 271.5(SE:42.8), and for *Nlgn3*$^{+/+}$ mice from SGH, 246.1(SE:34.1). A repeated measures ANOVA revealed no significant interactions.

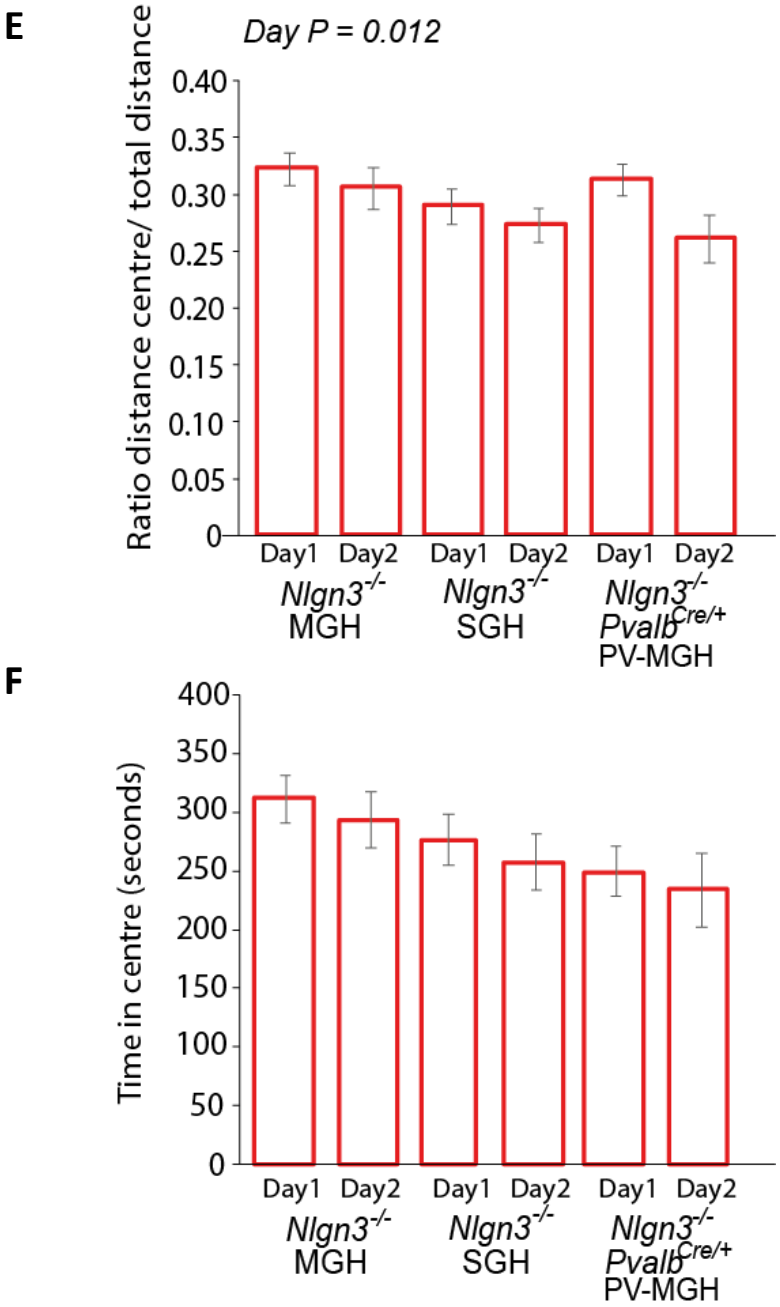


Figure 4.11, continued. ***E***, the average ratio of distance in the centre/ total distance travelled and standard error of the mean (SE) on day one of *Nlgn3*$^{-/-}$ mice from MGH, 0.32(SE:0.014), *Nlgn3*$^{-/-}$ mice from SGH, 0.28(SE:0.015), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 0.31(SE:0.014). On day two, for *Nlgn3*$^{-/-}$ mice from MGH, 0.31(SE:0.018), *Nlgn3*$^{-/-}$ mice from SGH, 0.27(SE:0.014), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$, 0.26(SE:0.021). A repeated measures ANOVA found a significant interaction of day (*P = 0.012*). ***F*,** the average time in the centre (seconds) and standard error of the mean (SE) on day one of *Nlgn3*$^{-/-}$ mice from MGH, 312.7(SE:19.9), *Nlgn3*$^{-/-}$ mice from SGH, 275.9(SE:21.5), and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 248.9(SE:21.3). On day two, for *Nlgn3*$^{-/-}$ mice from MGH, 293.8(SE:24.2), *Nlgn3*$^{-/-}$ mice from SGH, 257.3(SE:24.0), and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ mice, 235.2(SE:31.8). A repeated measures ANOVA found no significant interactions.

4.3.3.7. The influence of the social dominance environment and Nlgn3 re-expression on the elevated plus maze exploration of Nlgn3$^{+/+}$, Nlgn3$^{+/-}$ and Nlgn3$^{-/-}$ mice

The elevated plus maze exploration of juvenile female mice was assessed. The time in the open arms of *Nlgn3*$^{-/-}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates were compared with *Nlgn3*$^{+/+}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-WT) littermates, revealing that there is no significant difference between the groups (**figure 4.12.A**, Kruskal-Wallis test, *Nlgn3*$^{-/-}$ mice from MGH n = 13, *Nlgn3*$^{+/-}$ (H-KO) mice n = 7, *Nlgn3*$^{+/+}$ mice from MGH n = 10, *Nlgn3*$^{+/-}$ (H-WT) mice n = 9). This shows that juvenile *Nlgn3*$^{-/-}$ mice and *Nlgn3*$^{+/-}$ mice do not have significant differences in their levels of elevated plus maze exploration compared to their *Nlgn3*$^{+/-}$ littermates, and juvenile *Nlgn3*$^{+/-}$ mice do not show altered elevated plus maze exploration dependent on whether their social environment has *Nlgn3*$^{-/-}$ mice or *Nlgn3*$^{+/+}$ mice present.

To determine if the social dominance environment of *Nlgn3*$^{+/+}$ mice influences their elevated plus maze exploration, the time spent in the open arms of the elevated plus maze of *Nlgn3*$^{+/+}$ mice from MGH was compared to *Nlgn3*$^{+/+}$ mice from SGH, revealing no significant difference between the groups (**figure 4.12.B**, independent samples t-test, *Nlgn3*$^{+/+}$ mice from MGH n = 10, *Nlgn3*$^{+/+}$ mice from SGH n = 11).

The elevated plus maze exploration of *Nlgn3*$^{-/-}$ mice from MGH, *Nlgn3*$^{-/-}$ mice from SGH conditions, and *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice were assessed to determine if the social environment and the re-expression of *Nlgn3* in *Pvalb*-expressing cells of *Nlgn3*$^{-/-}$ mice influences this behaviour. No statistically significant difference between the groups was revealed (**figure 4.12.C,** Kruskal-Wallis, *Nlgn3*$^{-/-}$ mice from SGH n = 19, *Nlgn3*$^{-/-}$ mice from MGH conditions n = 13, *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice n = 10).

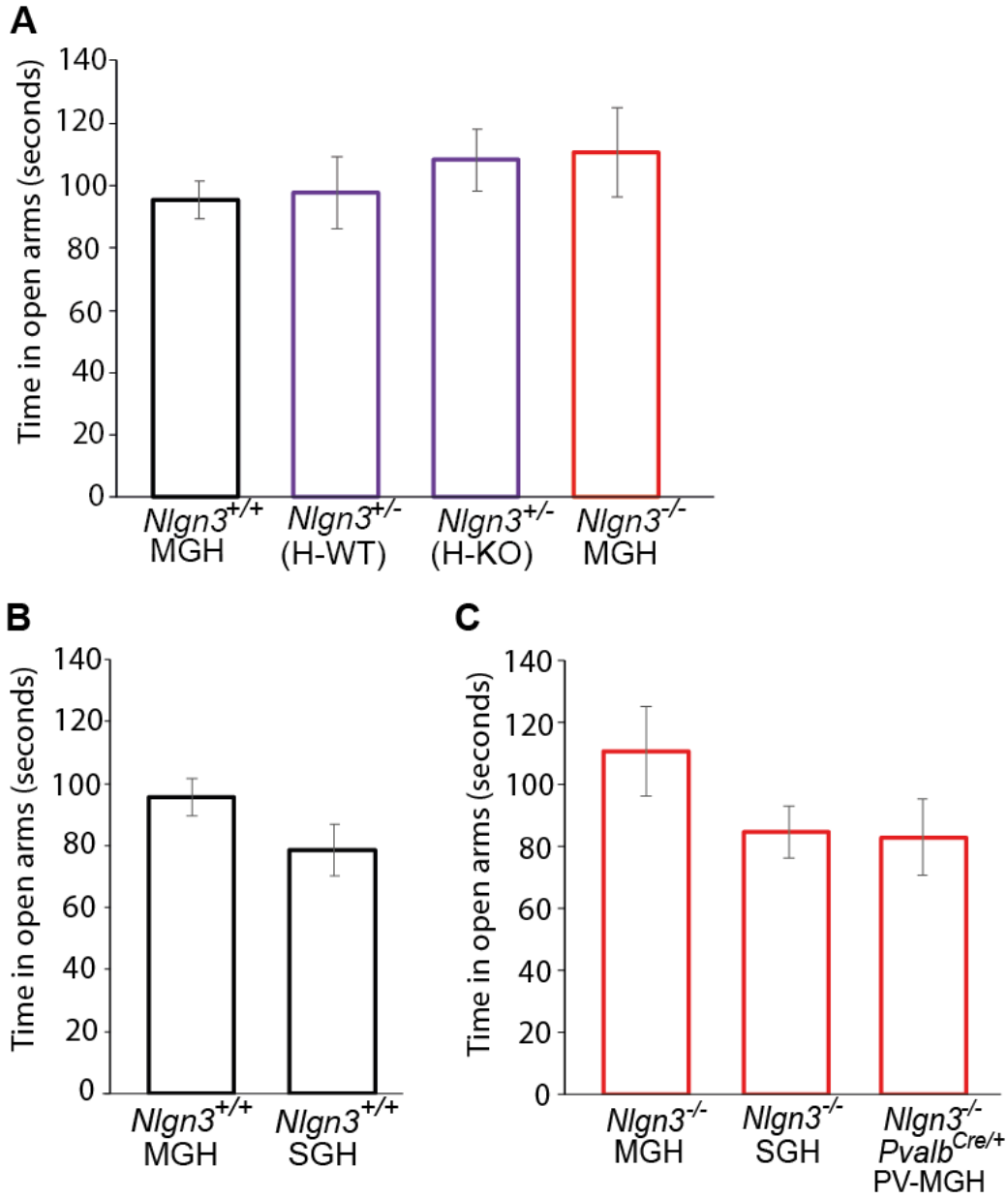


Figure 4.12. The impact of the social dominance environment on the time spent by juvenile *Nlgn3*$^{+/+}$, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ mice in the open arms of the elevated plus maze. *A*, average time spent in the open arms (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 97.9 (SE:11.5), *Nlgn3*$^{+/-}$ (H-WT) mice, 97.8(SE:11.5), *Nlgn3*$^{+/-}$ (H-KO) mice, 108.1(SE:9.9), and *Nlgn3*$^{-/-}$ mice from MGH, 110.8(SE:14.3). A Kruskal-Wallis test revealed no significant difference between the groups ***B,*** average time spent in the open arms (seconds) and standard error of the mean (SE) of *Nlgn3*$^{+/+}$ mice from MGH, 97.9(SE:11.5), and *Nlgn3*$^{+/+}$ mice from SGH, 78.4(SE:8.2). An independent samples t-test revealed no significant difference between the groups ***C,*** average time spent in the open arms (seconds) and standard error of the mean (SE) of *Nlgn3*$^{-/-}$ mice from MGH, 110.8(SE:14.3), *Nlgn3*$^{-/-}$ mice from SGH, 84.8(SE:8.5), *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, 82.9, (SE:12.4). A Kruskal-Wallis test revealed no significant difference between the groups.

4.4. Discussion

A summary of the influence of MGH compared to SGH on the activity and anxiety-related behaviours of male and female mice as adults and juveniles (**table 4.1**).

Table 4.1. A summary of the statistically significant impact of MGH on the locomotive and anxiety-related behaviours of *Nlgn3$^{y/+}$, Nlgn3$^{y/-}$, Nlgn3$^{-/-}$,* and *Nlgn3$^{+/+}$* mice.

	Adult Male in MGH	***Adult Female in MGH***	***Juvenile Male in MGH***	***Juvenile Female in MGH***
Activity	***Nlgn3$^{y/+}$*** ↑ ***Nlgn3$^{y/-}$*** *No change*	***Nlgn3$^{+/+}$*** ↑ ***Nlgn3$^{-/-}$*** *No change*	***Nlgn3$^{y/+}$*** *Day two* ↑ ***Nlgn3$^{y/-}$*** *Day two* ↑	***Nlgn3$^{+/+}$*** *Day one* ↓ *Day two* ↑ ***Nlgn3$^{-/-}$*** *No change*
Anxiety (thigmotaxis)	***Nlgn3$^{y/+}$*** ↓ ***Nlgn3$^{y/-}$*** ↓	***Nlgn3$^{+/+}$*** ↓ ***Nlgn3$^{-/-}$*** *No change*	***Nlgn3$^{y/+}$*** Change from day one to day two ↓ ***Nlgn3$^{y/-}$*** Change from day one to day two ↓	***Nlgn3$^{+/+}$*** Change from day one to day two ↓ ***Nlgn3$^{-/-}$*** *No change*
Anxiety (elevated plus maze)	*No change*	***Nlgn3$^{+/+}$*** ↓ ***Nlgn3$^{-/-}$*** ↓	***Nlgn3$^{y/+}$*** ↓ ***Nlgn3$^{y/-}$*** ↓	***Nlgn3$^{+/+}$*** *No change* ***Nlgn3$^{-/-}$*** *No change*

4.4.1. Levels of activity and anxiety are influenced by the social dominance environment and Nlgn3 re-expression

In adult male and female mice, it is observed that *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{+/+}$ mice show activity that is impacted by their social dominance environment conditions, while adult male and female *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{-/-}$ mice show hyperactivity that is not influenced by the social dominance environment conditions. The hyperactivity seen in the *Nlgn3*$^{y/-}$ mice is consistent with previous reports (Radyushkin et al 2009, Rothwell et al 2014). *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice do not show increased activity compared to their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates, and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice do not show a significant decrease compared to *Nlgn3*$^{y/-}$ mice from MGH and SGH. Like *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice, *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice also show a non-significant trend for decreased activity. Altogether, these results indicate that there is not a sexual dimorphism in the control of the influence of social dominance environment, genotype, and re-expression the locomotive behaviours of adult mice. Furthermore, these data indicate that there is no significant influence of *Nlgn3* re-expression within *Pvalb*-expressing cells on locomotive behaviours of mice. Previously it has been demonstrated that mice with selective deletion of *Nlgn3* within parvalbumin containing neurones have an overall decrease in open field locomotion compared to wild-type mice (Rothwell et al 2014). Together, this would indicate that *Nlgn3* deletion or re-expression within parvalbumin containing cells does not simply induce and reduce hyperactivity, and the relationship between this would have to be explored futher.

The characterisation of the activity over two days of the juvenile mice allowed the assessment of the initial reaction to the novel environment, and the assessment of how following habituation the behaviour changes. It was found that juvenile *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{-/-}$ mice were consistently hyperactive. However, on day two it was observed that there is an effect of social dominance environment, and re-expression of *Nlgn3*, with *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice from MGH showing increased activity compared to mice from SGH and *Pvalb*$^{cre/+}$ mice. Unlike the *Nlgn3*$^{y/-}$ mice, social dominance environment did not impact the activity of juvenile *Nlgn3*$^{-/-}$ mice, while re-expression led to an overall decrease in the activity of *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, indicating some sexually dimorphic effect of social dominance environment and re-expression in juvenile *Nlgn3*$^{-/-}$ mice. Interestingly, *Nlgn3*$^{+/+}$ mice from MGH, like *Nlgn3*$^{y/+}$ mice from MGH, show an elevated activity on day two compared to SGH

conditions, indicating that for wild-type mice the social dominance environment doesn't influence the habituation within the open field arena in a sexually dimorphic manner.

Adult and juvenile *Nlgn3*$^{y/-}$ mice were found to spend more time in the open arms of the elevated plus maze, and have decreased thigmotaxis, indicating a decreased anxiety. A similar result of decreased open field thigmotaxis was observed in adult *Nlgn3*$^{-/-}$ female mice. Furthermore, like *Nlgn3*$^{y/-}$ mice from MGH, *Nlgn3*$^{-/-}$ from MGH show increased time in the open arms of the elevated plus maze that is influenced by social dominance environment and re-expression. Of note is that it has previously been shown that there is no significant difference in time spent in the open arms of adult male *Nlgn3*$^{y/-}$ mice compared to their *Nlgn3*$^{y/+}$ littermates (Radyushkin et al 2009). Similarly, it was previously reported that adult male *Nlgn3*$^{y/-}$ mice do not show a decrease in their thigmotaxis compared to their littermates (Radyushkin et al 2009, Rothwell et al 2014). This could possibly be explained by the protocol which was used. In the work presented in this thesis, by adulthood, the mice had already been exposed to the open field multiple times during development. Furthermore, handling of mice can impact behavioural outcomes, including exploration of the elevated plus maze (Hurst and West 2010, Ghosal et al 2015, Gouveia and Hurst 2017, Clarkson et al 2018). All mice used in this work were habituated to handling from an early age, and were not held by the tail, in order to reduce baseline stress levels for experimental work. Therefore, a lower baseline of stress may allow the differences between the genotypes to be observed.

Finally, both *Nlgn3*$^{+/+}$ and *Nlgn3*$^{y/+}$ mice when in mixed genotype housing show either significantly, or a trend towards, decreased open field thigmotaxis and increased open arms exploration. All together, these results from the open field arena and elevated plus maze show that mixed genotype housing leads to an apparent decrease in anxiety in both male and female wild-type and knockout mice. Taken on face value, this would indicate a decreased anxiety. However, this could also be explained by an increased exploration behaviour. To explore if this really is a stress response, additional measures such as corticosterone levels and body temperature could be utilised (Veening et al 2004).

Of note is that the result of the open field locomotion of the adult male mice differ to those that are seen in Kalbassi et al (2017) where it was observed that *Nlgn3*$^{y/-}$ mice have activity that is impacted by the social dominance environment. Two main differences could explain the difference observed. Firstly, all *Nlgn3*$^{y/-}$ mice from SGH presented in this thesis come from *Nlgn3*$^{-/-}$ dams, whereas in Kalbassi et al (2017) only *Nlgn3*$^{+/-}$ dams were used to generate SGH

breedings. As all *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice are generated from *Nlgn3*$^{+/-}$ mice, and they show behavioural differences to *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH, whilst sharing many behavioural phenotypes with *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH it seems less likely, but not impossible, that the maternal genotype could be accounting for this difference. Furthermore, *Nlgn3*$^{+/-}$ mice have a lack of overt phenotype, making it seem unlikely that they are influencing the behaviours of their littermates. Another important difference is that for this thesis, all mice have been tested at juvenile and adult stages, whereas mice in Kalbassi et al (2017) were only tested when adults. As is shown in the juvenile open field locomotive behaviour, previous exposure appears to influence the subsequent locomotive activity within the open field arena, and therefore exposures during development are a factor to consider.

4.4.2. Concluding remarks

The data presented in this chapter demonstrate that the presence of *Nlgn3* knockout mice within the social dominance environment shapes the activity and anxiety-related behaviours of both male and female mice, both in adulthood and as juveniles. The behavioural changes from *Nlgn3* knockout and the social dominance environment conditions were typically consistent between male and female mice, with only a few examples of sexually dimorphic response. Furthermore, re-expression of *Nlgn3* within *Pvalb*-expressing cells seems to restore many of these behaviours. In females, this was only explored on an individual level in the *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, and in males it was shown to impact both the *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates. As shown in the literature discussed in this chapter, and in the general introduction, changes in the social dominance environment of mice can influence a wide range of phenotypes related to anxiety. Given that it was observed that the social behaviours of *Nlgn3* knockout mice are disrupted, it seems likely that these aberrant social behaviours of the knockout mice led to an abnormal home-cage social dominance environment that ultimately led to the shift in the behaviours of the mice within the open field arena and elevated plus maze. Altogether, these results show that the mixed genotype housing leads to a shift of the mice on the behavioural level. Expanding from this, it is important to address how mixed genotype housing impacts other physiological parameters of the mice.

Chapter 5: The impact of the social dominance environment and genotype on the transcriptomes of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice

5.1. Introduction

From chapters three and four, it was observed that mixed genotype housing (MGH) of *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice influences numerous behaviours, including dominance-related behaviours and exploratory and anxiety-related behaviours. As discussed previously, manipulations of the social environment have been shown to alter the physiology of the rodents. In both mice and rats, it has been demonstrated that social defeat, social isolation, and the position within the social hierarchy leads to differential expression of mRNAs and proteins and alters dopamine signalling within the striatum (Tidey et al 1996, Berton et al 2006, Krishnan et al 2007, Anstrom et al 2009, Schiavone et al 2009, Cao et al 2010, Nesher et al 2015, Kudryavtseva et al 2017). Experiments in mice and rats have also shown that the hippocampus is impacted by manipulations of the early life social environment, social instability, and the position within the social hierarchy, leading to altered expression of mRNAs and proteins, as well as altered cellular properties (Roceri et al 2004, Branchi, D'andrea, Sietzema, et al 2006, Sterlemann et al 2009, Leasure et al 2009, Harte et al 2009, Benner et al 2014, So et al 2015, Nesher et al 2015, Horii et al 2017). As the striatum and hippocampus are sensitive to the social environment, these studies indicate that the striatum and hippocampus are two regions of interest in the exploration of the impact of mixed genotype housing on the behaviours of the mice.

An additional reason to explore the striatum and the hippocampus is that *Nlgn3* expression within the striatum and hippocampus have been shown to mediate some of the behavioural phenotypes of *Nlgn3* knockout mice. *Nlgn3* expression in the ventral tegmental area (VTA) and the nucleus accumbens (NAc) influences social behaviours and motor learning, respectively (Rothwell et al 2014, Bariselli et al 2018), and *Nlgn3* knockout within the hippocampus has been shown to lead to changes in hippocampal dependent learning (Polepalli et al 2017). This indicates that these regions are important in the regulation of the phenotypes seen in the *Nlgn3*$^{y/-}$ mice.

To investigate how the striatum and the hippocampus are impacted by MGH and single genotype housing (SGH), the transcriptome was investigated using RNA sequencing (RNAseq).

5.2. Aims and objectives

- To determine if the transcriptome of the striatum and the hippocampus is impacted by MGH or SGH conditions in adult *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice.
- To confirm targets of interest using qPCR.

5.3. Results

5.3.1. Impact of MGH and SGH on the transcriptome

RNAseq was performed separately on both the hippocampus and striatum of adult male *Nlgn3*$^{y/+}$ mice from MGH, *Nlgn3*$^{y/-}$ mice from MGH, *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH. The social dominance environment conditions are the same as those outlined in **table 3.1**. A principal component analysis was conducted on the data from the RNAseq. Two principal components that explain the variance between the groups were identified. For the striatum, principal component 1 (PC1) accounts for 55.6% of the variance, and principal component 2 (PC2) accounts for 20.7% of the variance (**figure 5.1.A,** *Nlgn3*$^{y/+}$ mice from MGH n = 3, *Nlgn3*$^{y/-}$ mice from MGH n = 3, *Nlgn3*$^{y/+}$ mice from SGH n = 3, *Nlgn3*$^{y/-}$ mice from SGH n = 3). For the hippocampus, PC1 accounts for 42.0% of the variance, and PC2 accounts for 29.9% of the variance (**figure 5.1.B,** *Nlgn3*$^{y/+}$ mice from MGH n = 3, *Nlgn3*$^{y/-}$ mice from MGH n = 3, *Nlgn3*$^{y/+}$ mice from SGH n = 3, *Nlgn3*$^{y/-}$ mice from SGH n = 3).

For PC1 of both the striatum and the hippocampus, it was observed that there is a significant difference between *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH, and there is no significant difference between *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH, possibly indicating a convergence of the striatal transcriptome of mice from MGH (Striatum: **figure 5.1.C,** Kruskal-Wallis test *P = 0.025,* Dunn's pairwise comparison, *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH: *P = 0.013*, *Nlgn3*$^{y/+}$ mice from MGH n = 3, *Nlgn3*$^{y/-}$ mice from MGH n = 3, *Nlgn3*$^{y/+}$ mice from SGH n = 3, *Nlgn3*$^{y/-}$ mice from SGH n = 3. Hippocampus: **figure 5.1.D,** Kruskal-Wallis *P = 0.022,* Dunn's pairwise analysis, *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$

mice from SGH: *P = 0.028*, *Nlgn3$^{y/+}$* mice from MGH n = 3, *Nlgn3$^{y/-}$* mice from MGH n = 3, *Nlgn3$^{y/+}$* mice from SGH n = 3, *Nlgn3$^{y/-}$* mice from SGH n = 3).

For the PC2 of the striatum, it was observed that there is no significant difference between the groups (**figure 5.1.E,** Kruskal-Wallis test, *Nlgn3$^{y/+}$* mice from MGH n = 3, *Nlgn3$^{y/-}$* mice from MGH n = 3, *Nlgn3$^{y/+}$* mice from SGH n = 3, *Nlgn3$^{y/-}$* mice from SGH n = 3). For the PC2 of the hippocampus it was observed that there is a significant difference between *Nlgn3$^{y/+}$* mice from MGH and *Nlgn3$^{y/-}$* mice from SGH (**figure 5.1.F**, Kruskal-Wallis test *P = 0.033*, Dunn's pairwise analysis *Nlgn3$^{y/+}$* mice from MGH and *Nlgn3$^{y/-}$* mice from SGH *P = 0.019*, *Nlgn3$^{y/+}$* mice from MGH n = 3, *Nlgn3$^{y/-}$* mice from MGH n = 3, *Nlgn3$^{y/+}$* mice from SGH n = 3, *Nlgn3$^{y/-}$* mice from SGH n = 3).

Together, these data indicate that the largest differences are between *Nlgn3$^{y/+}$* mice from SGH and *Nlgn3$^{y/-}$* mice from SGH, with few differences between *Nlgn3$^{y/+}$* mice from MGH and *Nlgn3$^{y/-}$* mice from MGH, suggesting a convergence of the expression of mRNA levels in MGH, particulary for PC1. For PC2 for the striatum and the hippocampus, a slight trend is observed for divergence of *Nlgn3$^{y/+}$* mice from MGH, indicating that MGH of *Nlgn3$^{y/+}$* mice is impacting the transcriptome of the mice in a manner different to the divergence seen along PC1.

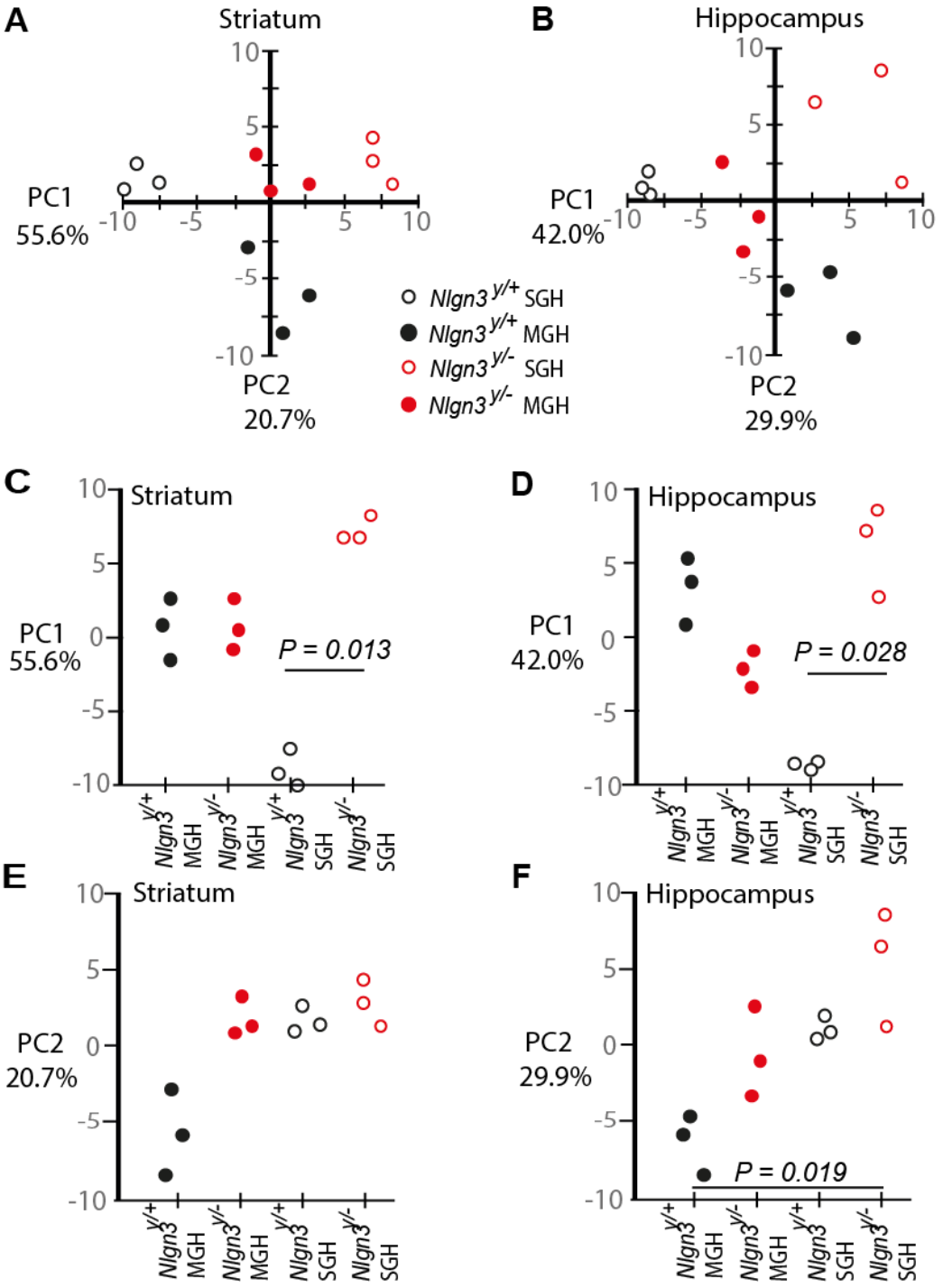


Figure 5.1. Principal component analysis of the striatum and hippocampus of mice from MGH and SGH conditions. ***A***, PC1 and PC2 of the striatum. ***B***, PC1 and PC2 of the hippocampus. ***C***, Kruskal-Wallis analysis of PC1 for the striatum revealed a significant difference between the groups (*P = 0.025*), results of the Dunns pairwise analysis are shown on the figure. ***D***, Kruskal-Wallis analysis of PC1 for the hippocampus revealed a significant difference between the groups (*P = 0.022*), results of the Dunns pairwise analysis are shown on the figure. ***E***, Kruskal-Wallis analysis of PC2 for the striatum revealed no significant difference between the groups. ***F***, Kruskal-Wallis analysis of PC2 for the hippocampus revealed a significant difference between the groups (*P = 0.033*), results of the Dunns pairwise analysis are shown on the figure.

The principal component analysis looks at dimensions of variance, however, from the analysis of the RNAseq data, it is also possible to compare the expression of the different mRNAs between groups. From this data, volcano plots were generated comparing the $\log_2$ (fold change) and the $-\log_{10}$(p-value) of the mRNAs. Of note is that for any comparisons between *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice, the point denoting *Nlgn3* mRNA is removed due to the high $\log_2$ (fold change) and high-$\log_{10}$ (p-value), leading to a scale change in the figures, making the figures unreadable.

For the striatum, *Nlgn3*$^{y/+}$ mice from SGH when compared to *Nlgn3*$^{y/+}$ mice from MGH have 15 mRNAs that are significantly different in their expression, indicating that social dominance environment does influence striatal expression of mRNAs in *Nlgn3*$^{y/+}$ mice (**figure 5.2.A**, 15 significantly different mRNAs, *Nlgn3*$^{y/+}$ mice from SGH n = 3, *Nlgn3*$^{y/+}$ mice from MGH n = 3). Comparison of *Nlgn3*$^{y/-}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from SGH revealed that social dominance environment has lesser influence on the number of striatal mRNAs that are significantly different in *Nlgn3*$^{y/-}$ mice (**figure 5.2.B,** 0 significantly different mRNAs, *Nlgn3*$^{y/-}$ mice from SGH n = 3, *Nlgn3*$^{y/-}$ mice from MGH n = 3). Furthermore, *Nlgn3*$^{y/+}$ mice from SGH compared to *Nlgn3*$^{y/-}$ mice from SGH have 14 mRNAs that are significantly different in their expression, a result that is consistent with the result of the principal component analysis (**figure 5.2.C,** 14 significantly different mRNAs, *Nlgn3*$^{y/+}$ mice from SGH n = 3, *Nlgn3*$^{y/-}$ mice from SGH n = 3). Also consistent with the results of the principal component analysis is that *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH have very little variance in the expression of their mRNAs, indicating a convergence of the transcriptome (**figure 5.2.D,** 1 significant different mRNA, *Nlgn3*$^{y/+}$ mice from MGH n = 3, *Nlgn3*$^{y/-}$ mice from MGH n = 3).

For the hippocampus, *Nlgn3*$^{y/+}$ mice from SGH compared to *Nlgn3*$^{y/+}$ mice from MGH have 30 mRNAs that are differentially expressed, indicating that social dominance environment is influencing mRNA expression within the hippocampus of *Nlgn3*$^{y/+}$ mice (**figure 5.2.E,** 30 significantly different mRNAs, *Nlgn3*$^{y/+}$ mice from SGH n = 3, *Nlgn3*$^{y/+}$ mice from MGH n = 3). Unlike in the striatum, in the hippocampus there are more significantly differentially expressed mRNAs of *Nlgn3*$^{y/-}$ mice from SGH compared to *Nlgn3*$^{y/-}$ mice from MGH, with 13 mRNAs being significantly different (**figure 5.2.F,** 13 significantly different mRNAs, *Nlgn3*$^{y/-}$ mice from SGH n = 3, *Nlgn3*$^{y/-}$ mice from MGH n = 3). Consistent with the results of the principal component analysis is that in the hippocampus, *Nlgn3*$^{y/+}$ mice from SGH when compared to *Nlgn3*$^{y/-}$ mice from SGH show many significantly regulated mRNAs (**Figure 5.2.G,**

27 significant different mRNAs, *Nlgn3*$^{y/+}$ mice from SGH n = 3, *Nlgn3*$^{y/-}$ mice from SGH n = 3). Finally, the *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH show 3 significantly different differentially regulated mRNAs in the hippocampus (**figure 5.2.H,** 3 significantly different mRNAs**,** *Nlgn3*$^{y/+}$ mice from MGH n = 3, *Nlgn3*$^{y/-}$ mice from MGH n = 3). This suggests that like the striatum, the hippocampus shows little divergence between *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH.

These data demonstrate that on a transcriptional level, *Nlgn3*$^{y/+}$ mice from MGH diverge less from *Nlgn3*$^{y/-}$ mice from MGH, than *Nlgn3*$^{y/+}$ mice from SGH diverge from *Nlgn3*$^{y/-}$ mice from SGH. This is seen both in the principal component analysis and on the level of the individual mRNAs. Per group, there is only an n of 3; with an increased number of mice it would be of interest to see if more targets become significant.

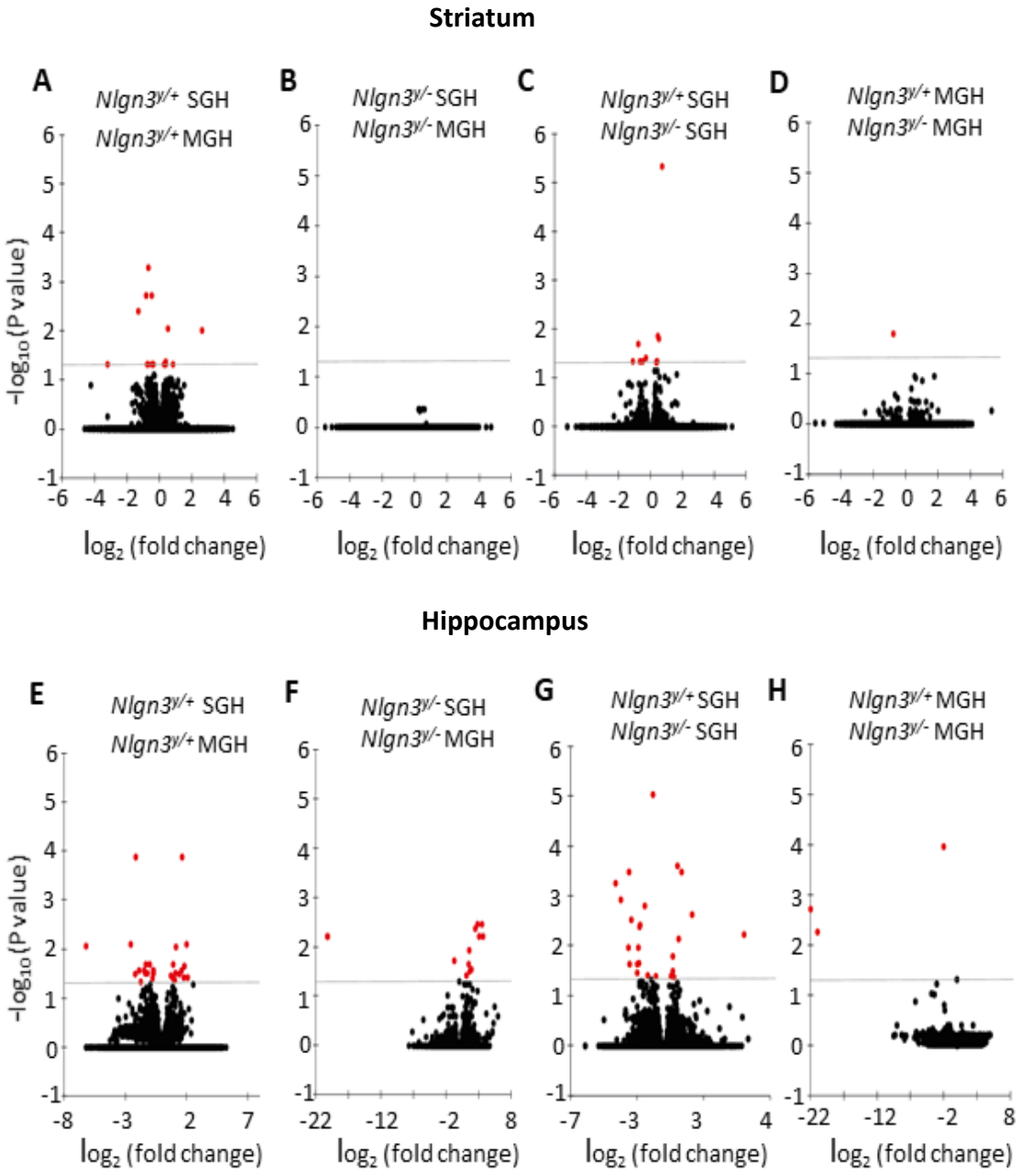


Figure 5.2. Comparison of the expression of different mRNAs from the RNAseq. Targets in red are significantly different, the line at 1.3 indicates –log10(0.05), and all points above this are significant. ***A,*** comparison of striatal expression of mRNAs from *Nlgn3*$^{y/+}$ mice from MGH and SGH. ***B,*** comparison of striatal expression of mRNAs from *Nlgn3*$^{y/-}$ mice from MGH and SGH. **C,** comparison of striatal expression of mRNAs from *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH. ***D,*** comparison of striatal of mRNAs from from *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH. ***E,*** comparison of hippocampal expression of mRNAs from *Nlgn3*$^{y/+}$ mice from MGH and SGH. ***F,*** comparison of hippocampal expression of mRNAs from *Nlgn3*$^{y/-}$ mice from MGH and SGH. **G,** comparison of hippocampal expression of mRNAs from *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH. ***H,*** comparison of striatal expression of mRNAs from *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH.

5.3.2. Quantitative real-time polymerase chain reaction (qPCR) analysis of C3 and C4 mRNA levels

From the results of the RNAseq, it was observed that two of the mRNAs that showed altered expression between *Nlgn3$^{y/-}$* mice from SGH and *Nlgn3$^{y/+}$* mice from SGH are complement component 4 (C4) and compliment component 3 (C3). From the RNAseq, C4 and C3 were shown to be increased in the striatum of *Nlgn3$^{y/+}$* mice from SGH relative to *Nlgn3$^{y/-}$* mice from SGH. Furthermore, a nonsignificant trend for increased striatal levels of both C4 and C3 were found for *Nlgn3$^{y/+}$* mice from SGH compared to *Nlgn3$^{y/+}$* mice from MGH. In addition to the samples used in the RNAseq, additional samples from independent breedings were also included in the analysis to verify and further explore this observation from the RNAseq. In the figures, the samples used for the initial RNAseq are represented as circles while the new samples are represented as squares.

qPCR analysis of C4 expression, normalised to 18S ribosomal RNA (18S), within the striatum and hippocampus revealed that there are tissue dependent changes, with striatal, but not hippocampal, changes in C4 expression. An effect of social dominance environment and genotype was observed in the striatum, with mice from SGH and *Nlgn3$^{y/+}$* mice showing higher levels of C4 (**figure 5.3.A**, two-way ANOVA, social dominance environment: *P = 0.007, F = 8.7*, *df = 1*, Genotype: *P = 0.010, F = 7.9, df = 1*, *Nlgn3$^{y/+}$* mice from MGH n = 7, *Nlgn3$^{y/+}$* mice from SGH n = 7, *Nlgn3$^{y/-}$* from MGH n = 7, *Nlgn3$^{y/-}$* mice from SGH n = 6). Analysis of the C4 expression within the hippocampus led to no significant effect of social dominance environment or genotype, and no interaction of social dominance environment and genotype (**figure 5.3.B**, two-way ANOVA, *Nlgn3$^{y/+}$* mice from MGH n = 6, *Nlgn3$^{y/+}$* mice from SGH n = 7, *Nlgn3$^{y/-}$* from MGH n = 6, *Nlgn3$^{y/-}$* mice from SGH n = 6). These data show that changes in C4 levels are tissue specific. For the striatum, with increased mice it may be possible that an interaction of social dominance environment and genotype could appear, as *Nlgn3$^{y/+}$* mice from SGH appear to have the highest levels of C4, consistent with the increased striatal levels of C4 seen in the RNAseq.

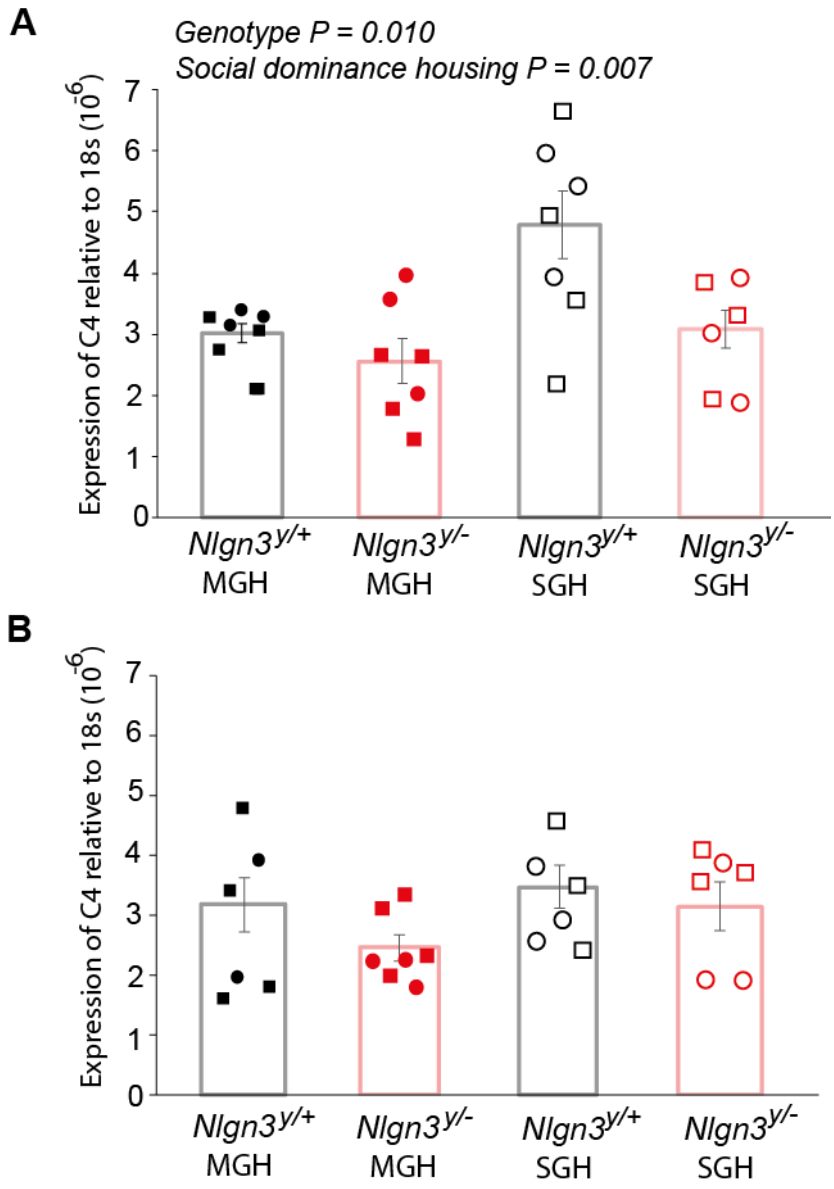


Figure 5.3. qPCR determination of C4 mRNA levels of *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from MGH and SGH conditions, circles represent samples used in the RNAseq, squares represent additional samples included for the qPCR. Δct C4 relative to Δct 18S. *A*, Average C4/18S levels and standard error of the mean (SE) in the striatum of *Nlgn3*$^{y/+}$ mice from MGH, 3.02e-06(SE:1.53e-07), *Nlgn3*$^{y/-}$ mice from MGH, 2.56e-06(SE:3.71e-07), *Nlgn3*$^{y/+}$ mice from SGH, 4.78e-06(5.62e-07), *Nlgn3*$^{y/-}$ mice from SGH, 3.08e-06(SE:3.40e-07). A two-way ANOVA revealed a significant impact of social dominance environment (*P = 0.007*) and genotype (*P = 0.010*). ***B*,** Average C4/18S levels and standard error of the mean (SE) in the hippocampus, *Nlgn3*$^{y/+}$ mice from MGH, 3.17e-06(SE:4.46e-07), *Nlgn3*$^{y/-}$ mice from MGH, 2.46e-06(SE:2.24e-07), *Nlgn3*$^{y/+}$ mice from SGH, 3.47e-06(SE:3.53e-07), *Nlgn3*$^{y/-}$ mice from SGH, 3.15e-06(SE:4.02e-07). A two-way ANOVA revealed no significant effects.

Next, using qPCR analysis, levels of C3, relative to 18S, within the striatum and hippocampus were assessed. Analysis of levels of C3 within the striatum revealed a significant effect of genotype (**figure 5.4.A**, two-way ANOVA, Genotype: *P = 0.043, F = 4.7, df = 1*, *Nlgn3*$^{y/+}$ mice from MGH n = 6, *Nlgn3*$^{y/+}$ mice from SGH n = 5, *Nlgn3*$^{y/-}$ from MGH n = 6, *Nlgn3*$^{y/-}$ mice from SGH n = 6). The results from the qPCR reflect the difference detected between *Nlgn3*$^{y/+}$ mice from SGH and *Nlgn3*$^{y/-}$ mice from SGH by the analysis of the RNAseq data. However, there is no interaction of genotype and social dominance environment. The qPCR results also show a difference between *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{y/-}$ mice from MGH, which is not seen in the RNAseq dataset. Within the hippocampus the qPCR results revealed no significant differences in the levels of C3 (**figure 5.4.A**, two-way ANOVA, *Nlgn3*$^{y/+}$ mice from MGH n = 6, *Nlgn3*$^{y/+}$ mice from SGH n = 6, *Nlgn3*$^{y/-}$ from MGH n = 7, *Nlgn3*$^{y/-}$ mice from SGH n = 6). This corresponds with the lack of difference in C3 levels seen in the RNAseq data set.

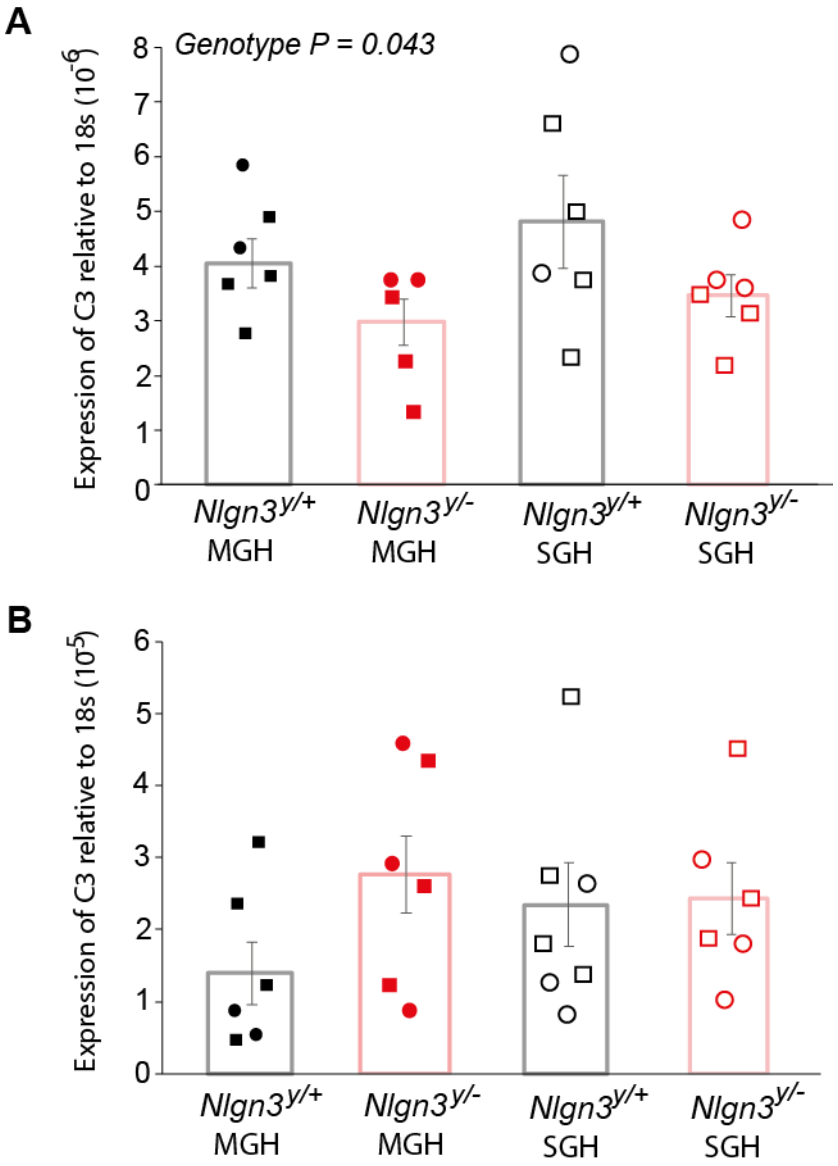


Figure 5.4. qPCR determination of C3 mRNA levels of *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from MGH and SGH conditions, circles represent samples used in the RNAseq, squares represent additional samples included for the qPCR. Δct C3 relative to Δct 18S. *A*, average C3/18S levels and standard error of the mean (SE) in the striatum, *Nlgn3*$^{y/+}$ mice from MGH, 4.05e-06(SE:4.54e-07), *Nlgn3*$^{y/-}$ mice from MGH, 2.97e-06(SE:4.13e-07), *Nlgn3*$^{y/+}$ mice from SGH, 4.81e-06(8.56e-07), *Nlgn3*$^{y/-}$ mice from SGH, 3.46e-06(SE:3.89e-07). A two-way ANOVA revealed a significant impact of genotype (*P = 0.043*). ***B*,** average C3/18S levels and standard error of the mean (SE) in the hippocampus, *Nlgn3*$^{y/+}$ mice from MGH, 1.39e-05(SE:4.34e-06), *Nlgn3*$^{y/-}$ mice from MGH, 2.77e-05(SE:5.33e-06), *Nlgn3*$^{y/+}$ mice from SGH, 2.36e-05(SE:5.84e-06), *Nlgn3*$^{y/-}$ mice from SGH, 2.43e-05(SE:4.93e-06). A two-way ANOVA revealed no significant effects.

5.4. Discussion

5.4.1. RNA sequencing

The principal component analysis revealed that there is a significant impact of social dominance environment on the transcription of mRNAs within the hippocampus and the striatum. This shows that there is a significant divergence not only on the behavioural level but also on the transcriptional level. *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from MGH show largely similar profiles of mRNA expression, consistent with the convergence of some of their behaviours. In contrast to this, a larger divergence of *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from SGH was observed.

Interestingly, *Nlgn3*$^{y/-}$ mice from SGH and MGH show smaller divergence in the mRNAs than *Nlgn3*$^{y/+}$ mice from SGH and MGH. This could be seen as being consistent with more adult *Nlgn3*$^{y/+}$ mouse behaviours being influenced by social dominance environment than *Nlgn3*$^{y/-}$ mouse behaviours. Indeed, adult *Nlgn3*$^{y/+}$ mice but not *Nlgn3*$^{y/-}$ mice show altered activity in the open field arena dependent upon social dominance environment, and *Nlgn3*$^{y/+}$ mice show a larger increase in testosterone levels dependent upon social dominance environment conditions (chapters 3 and 4). Furthermore, there are many other behaviours that could be changed but were not assessed in the work in this thesis. Another point to consider is that it is possible that in different brain regions there would be larger variance in the levels of the mRNAs to account for this change. For example, dominance related behaviours change in *Nlgn3*$^{y/-}$ mice from SGH compared to *Nlgn3*$^{y/-}$ mice from MGH (chapter 3), and the mPFC has been shown to be important in these behaviours (Wang et al 2011). Given that both *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice show altered dominance behaviours dependent upon social dominance environment conditions, it is possible that a similar degree of change between the group's dependent upon social dominance environment condition would be observed in the mPFC. Furthermore, the transcription and activity within the mPFC has been shown to be influenced by the social environment and the position within the social hierarchy (Caldji et al 2000, Covington et al 2005, Wang et al 2011) making it an attractive region to explore further. It would be of interest to explore in which other regions have altered transcription dependent upon the environment. Identification of the genes that are differentially regulated dependent upon the social dominance environment would give insight into which genes are sensitive to the social environment and could be an interesting target in the exploration of the understanding of social behaviours.

In future, more information could be yielded by utilising a method such as gene enrichment analysis. This would allow the identification of genes relating to specific phenotypes that are over or under enriched dependent upon genotype and social dominance environment condition. This could help to better understand the relationship between the mRNAs that are up or down regulated, and the change in behaviours seen in the groups. This in combination with the sequencing of other brain regions of interest could yield a greater understanding of the mechanisms and neuronal networks implicated in the altered behaviour in response to the MGH. Regarding the qPCR confirmation of targets of interest, the samples taken from the RNAseq were analysed with samples taken from an independent experimental cohort. In future, a qPCR analysis of the samples from the RNAseq that does not include any new samples could be conducted, to assess how comparable the results of the RNAseq are to the results from the qPCR. After this, the question of the potential impact of cohort could then be addressed.

The RNAseq and qPCR identified increased levels of compliment component 3 (*C3*) mRNA and compliment component 4 (*C4*) mRNA within the striatum of *Nlgn3*$^{y/+}$ mice from SGH compared to *Nlgn3*$^{y/-}$ mice from SGH. The results from the RNAseq demonstrated a trend for increased *C3* and *C4* within the striatum of *Nlgn3*$^{y/+}$ mice from SGH compared to *Nlgn3*$^{y/+}$ mice from MGH, which was confirmed for C4 using qPCR. *C3* and *C4* make up part of the compliment system that are important for immune response. Variants of C4 have been shown to have a strong risk associated with schizophrenia (Sekar et al 2016). Additionally, decreased serum levels of *C4* have been seen in those with schizophrenia and decreased levels of *C4* in the cingulate cortex have been observed in a rat model of schizophrenia (Tao et al 2017, Duchatel et al 2018). Furthermore, C3, like C4, makes up part of the compliment system, and, like *C4*, has also been associated with schizophrenia (Tao et al 2017). Additionally, the RNAseq revealed that within the hippocampus, *Hspb1* mRNA, encoding heat shock protein beta-1, was observed to be decreased in *Nlgn3*$^{y/+}$ mice from SGH compared to *Nlgn3*$^{y/+}$ mice from MGH. *Hspb1* has been shown to be dysregulated in brain tissue of those with ASD (Ginsberg et al 2012), however, the directionality was unconfirmed in the paper. Nonetheless, *Hspb1* has also been shown to be increased in the brain tissue of those with schizophrenia (Arion et al 2007). Schizophrenia has been associated with social behavioural abnormalities, as well as broader behavioural changes. *Hspb1* would therefore be an interesting target to explore in relation to the change in the behaviour of the *Nlgn3*$^{y/+}$ mice from the different social dominance environment conditions.

Given that schizophrenia impacts social behaviours, these results indicate that *Hsbp1*, *C3* and *C4* levels could have some role in the social behaviour differences seen in the mice; however, this would need much more exploration. Therefore, *C3* and *C4* could be interesting targets to explore further to better understand their relationship with the social dominance environment and social behaviour changes. Additionally, models of schizophrenia or ASD that influence activity or levels of *Hsbp1*, *C3*, or *C4*, may also have social dominance environment conditions compromised by MGH. It would be worth exploring if these targets are impacted by other paradigms in which the social environment impacts behaviour, as they could potentially serve as a marker when assessing the presence of an effect of MGH in experimental groups.

5.4.2. Conclusions

The results presented in this chapter demonstrate that social dominance environment and genotype leads to a measurable impact on the transcriptome of the mice. The results of the principal component analysis revealed that *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from SGH show larger degrees of divergence, while *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from MGH appear to converge more. Furthermore, the results of the RNAseq revealed genes that are influenced by genotype and social dominance environment that have been related to schizophrenia in humans. Additionally, these genes appear to be differentially impacted in different tissues. Future work could expand upon these initial observations by looking at additional brain regions, and to seek to better understand the relationship between social dominance environment and the regulation of these genes.

Chapter 6: General discussion

6.1. Summary of results

The results in presented in this thesis demonstrate that the presence of mice with *Nlgn3* knockout leads to an influence on the behaviours of their littermates and that their littermates influence the *Nlgn3* knockout mice. Initially, adult *Nlgn3*$^{y/-}$ mice were found to be socially submissive to their *Nlgn3*$^{y/+}$ littermates, and this social submission was reversed in *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice. Next, *Nlgn3*$^{y/-}$ mice and their *Nlgn3*$^{y/+}$ littermates in mixed genotype housed (MGH) conditions were found to show an overall decrease in their dominance behaviours when compared to *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from single genotype housing (SGH) and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice and their *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ littermates. Reflecting this is that *Nlgn3*$^{y/-}$ mice and their *Nlgn3*$^{y/+}$ littermates in MGH have decreased urinary testosterone compared to *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from SGH and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice. However, while levels of courtship ultrasonic vocalisation (USV) were decreased in *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice compared to *Nlgn3*$^{y/+}$ mice, these were not affected by tube test dominance. Additionally, the transcriptome of the striatum and hippocampus of adult *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from MGH and SGH conditions was compared, revealing that there appears to be a degree of convergence of *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from MGH, and a divergence of the *Nlgn3*$^{y/+}$ mice and *Nlgn3*$^{y/-}$ mice from SGH. Additionally, region specific changes in mRNA levels of ASD-related genes were identified, that could be of interest to explore further. These data demonstrate that social environment impacts not only the behaviours of mice but also impacts the physiology of the mice.

Social behaviour changes were also found in adult female and juvenile male and female mice. Adult female *Nlgn3*$^{-/-}$ mice but not *Nlgn3*$^{+/-}$ mice were found to phenocopy the males. *Nlgn3*$^{-/-}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates were found to have decreased sociability, and like in the males, the social phenotype was restored in *Nlgn3*$^{-/-}$ mice by either re-expression of *Nlgn3* in *Pvalb*-expressing cells in *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, or by being in SGH conditions. Juvenile male *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice show decreased sociability when in MGH conditions, which is restored by SGH or re-expression in *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice. For the juvenile females, only *Nlgn3*$^{+/+}$ mice show decreased sociability when in MGH conditions.

As well as social behaviour changes, MGH was shown to impact anxiety-related behaviours of adult and juvenile and male and female mice. *Nlgn3*$^{y/-}$ and *Nlgn3*$^{-/-}$ mice, regardless of

social dominance environment showed increased activity and reduced anxiety. Additionally, habituation within the open field of *Nlgn3*$^{y/-}$ mice was decreased in MGH and restored by SGH and re-expression. Adult and juvenile *Nlgn3*$^{y/+}$ mice from MGH and *Nlgn3*$^{+/+}$ mice from MGH showed increased activity, reduced juvenile habituation, and reduced anxiety-related behaviour compared to mice from SGH or *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ mice, demonstrating that the social dominance environment impacts group behaviours of both male and female wild-type mice. Re-expression of *Nlgn3* in *Pvalb*-expressing cells of *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice and *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ mice led to altered anxiety-related and habituation behaviours. For the *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ mice, these changes were also seen in their *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates. This indicates that the re-expression influences both individual and group behaviours.

To conclude, the results in this thesis demonstrate that the presence of mice with *Nlgn3* knockout influences the behaviours and physiology of their littermates in both male and female, and adult and juvenile mice. Furthermore, the wild-type littermates also influence the *Nlgn3* knockout mice. Finally, re-expression of *Nlgn3* in *Pvalb*-expressing cells restores some of these phenotypes in the knockout mice, and their wild-type littermates.

6.2. The results presented in this thesis expand on the knowledge of the impact of Nlgn3 knockout in mice

Previous studies have demonstrated that adult *Nlgn3*$^{y/-}$ mice show phenotypes relating to autism spectrum disorder (ASD). These include decreased sociability in a three-chamber test and decreased social conditioned place preference (Radyushkin et al 2009, Bariselli et al 2018), decreased courtship USV (Radyushkin et al 2009, Fischer and Hammerschmidt 2011), hyperactivity (Radyushkin et al 2009, Rothwell et al 2014) and altered motor learning and fear extinction (Baudouin et al 2012, Rothwell et al 2014, Polepalli et al 2017). The results in this thesis expand upon these findings by revealing that *Nlgn3*$^{y/-}$ mice are socially submissive to their *Nlgn3*$^{y/+}$ littermates, and have altered interest in female mice. Furthermore, previous studies did not reveal any change in anxiety measures as assessed by the elevated plus maze and thigmotaxis behaviours (Radyushkin et al 2009, Rothwell et al 2014), however, the work presented in this thesis did, likely due to altered handling methods leading to decreased overall baseline anxiety allowing the differences to be observed. Additionally, juvenile *Nlgn3*$^{y/-}$ mice had not been characterised at all, therefore the finding that *Nlgn3*$^{y/-}$ mice are hyperactive, have decreased sociability, and altered anxiety behaviours are all novel. Further to this is that *Nlgn3*$^{+/-}$ mice had only been phenotyped in one task (Dere et al 2018) and *Nlgn3*$^{-/-}$ mice had not been phenotyped at all. Therefore, the characterisation of the

behavioural phenotypes of *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ mice both as juveniles and as adults are all novel findings. Importantly, the work presented in this thesis demonstrates a novel finding that the social dominance environment of *Nlgn3* knockout mice and their different genotype littermates is a major factor that influences the behaviour and physiology of the mice, and that many of these behaviours can be reversed by SGH. Importantly, the effect of social dominance environment was seen in male and female, and adult and juvenile mice. Furthermore, the work in this thesis characterised the effect of selective *Nlgn3* re-expression in *Pvalb*-expressing cells, revealing that individual phenotypes are restored that also led to a change in the behaviours of their littermates.

In summary, the work presented in this thesis identifies new behavioural and physiological phenotypes through further a characterisation that also includes juveniles and females, as well revealing an effect of MGH. Finally, the effect of re-expression of *Nlgn3* in parvalbumin containing neurones on individual as well as group behaviours was characterised.

6.3. The re-expression of Nlgn3 in Pvalb-expressing cells

Re-expression of *Nlgn3* within *Pvalb*-expressing cells restores social behaviour deficit whenever present, as seen by the restored social behaviour of juvenile *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and of adult *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice. This shows that Neuroligin-3 in *Pvalb*-expressing cells is important in the control of the social behaviours, consistent with the knowledge that *Pvalb*-expressing cells mediate social behaviour (see **section 1.3**). Additionally, in adult male mice, re-expression of Neuroligin-3 restored the hierarchy and dominance behaviours of the mice. Social hierarchy has so far been linked to specific neuronal circuitry, and manipulations of the medial prefrontal cortex (mPFC) and hypothalamic ventral premammillary nucleus have been shown to lead to long lasting changes to the position within the social hierarchy (Wang et al 2011, Zhou et al 2017, Stagkourakis et al 2018). Excitation and inhibition within the mPFC influences the position within the social hierarchy, and manipulations of this can either increase or decrease the position within that social hierarchy as assessed by the tube test, and numbers of courtship USV (Wang et al 2011). It is possible that re-expression of *Nlgn3* within *Pvalb*-expressing cells could shift this balance of excitation and inhibition within the mPFC, allowing the social dominance behaviours of the mice to be restored. However, while the work of Wang et al (2011) demonstrated that manipulations of excitation and inhibition of the mPFC led to increased USV as well as increased tube test rank, only increased tube test rank, but not increased courtship USV, was observed when *Nlgn3* was re-expressed,

indicating a divergence of the hierarchy behaviours at this level. It is therefore possible that this increase in dominance is mediated by different brain regions, which do not influence courtship USV, or that USV while being mediated by the mPFC, needs functional Neuroligin-3 activity in the mPFC or in other neuronal populations and brain regions.

Furthermore, re-expression of *Nlgn3* within *Pvalb*-expressing cells of *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice led to restored social behaviour of their juvenile *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates, and restored dominance behaviours in their adult *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ littermates. This influenced other phenotypes, including adult testosterone levels and thigmotaxis behaviours, and juvenile habituation behaviours and thigmotaxis behaviours. This indicates that *Nlgn3* expression in *Pvalb*-expressing cells mediates behaviours that influence the group social environment, altogether showing that *Nlgn3* expression in *Pvalb*-expressing cells influences both individual and group behaviours of mice.

6.4. Translation of the results to other models and ASD in humans

6.4.1. Application to other animal models

Another question that needs addressing is whether the observations are valid in other models with a phenotype relating to social dominance. Given that many models of ASD have been identified as having phenotypes relating to social dominance (Cheh et al 2006, Jamain et al 2008, Spencer et al 2005, Yang et al 2015, Wöhr et al 2011), it is of interest to determine how widespread this effect could be. Indeed, as discussed in **section 1.2.7,** other studies looking at animal models with altered dominance behaviours also observe a significant impact on the wild-type behaviours of the littermates (Saxena et al 2018, McNamara et al 2018), and other studies looking at mouse models of ASD or with social deficit also report an impact of a shared social environment (Crews et al 2009, Yang et al 2011, Lipina et al 2013, Yang et al 2015). This demonstrates that this effect is not specific to Neuroligin-3 deficient mice. The fragile X mental retardation 1 (*Fmr1*) knockout rats were shown have altered dominance levels dependent upon their housing conditions (Saxena et al 2018) and, interestingly, in *Fmr1* knockout mice, levels of Neuroligin-3 have been shown to be upregulated (Chmielewska et al 2018). The *Fmr1* knockout rats were shown to be submissive compared to their wild-type littermates (Saxena et al 2018) and, similarly, in a study looking at the tube test behaviours of mice, *Fmr1* knockout mice have decreased tube test victories compared to their wild-type littermates (Spencer et al 2005). However, if decreased Neuroligin-3 is associated with submissive behaviour, then increased Neuroligin-3 may be

associated with dominant behaviour. While increased dominance of *Fmr1* knockout mice has been observed in one study (de Esch et al 2015), this is in direct contrast to submissive phenotypes of *Fmr1* knockout animals in the aforementioned works (Spencer et al 2005, Saxena et al 2018). Much more work is needed to determine if Neuroligin-3 may be mediating the dominance phenotype of these other models, and then to determine the relationship of this.

Furthermore, as discussed previously, *Nlgn3* knockout mice show pathology that is shared with other models of ASD, including aberrant synaptic properties and mGluR dysfunction (**section 1.1.2**), and phenotypes that are mediated by *Pvalb*-expressing cells (**section 1.1.3**, **section 1.1.4**, **chapter 3**, and **chapter 4**). It is possible that other models that share these core pathological features may share a phenotype in which they influence the behaviours of their littermates. Additionally, shared biological pathways may also indicate other factors that could lead to similar phenotypes as is seen in *Nlgn3* knockout mice. Neuroligin-3 has been shown to interact with neurexins, and *FMRP* has been shown to control levels of *Nlgn3* mRNA and levels of Neuroligin-3 protein (Ichtchenko et al 1996, Chmielewska et al 2018). As Neuroligin-3 is known to interact with ASD-related proteins, it is possible that they share similar modes of pathophysiology.

Furthermore, in addition to the previously known interactions, preliminary work conducted in addition to the main finding of this thesis has identified novel interactions of Neuroligin-3 and other ASD-related proteins within the synaptosome (**figure 6.1,** and **figure A1.2** for additional characterisation of the synaptosome, and validations of the MeCP2 antibody). The synaptosome extraction is used to extract functional presynaptic and postsynaptic terminals that contain other elements of the synapse including vesicles and mitochondria (Hebb and Whittaker 1958, Gray and Whittaker et al 1962, Nagy et al, 1976, Lu et al 1998, Phillips et al 2001, Grady et al 2002,). Within the synaptosomes, Neuroligin-3 was shown to interact with cytoplasmic FMRP interacting protein 1 (CYFIP1), Wiskott-Aldrich syndrome protein family verprolin homologous protein 1 (WAVE1), and non-catalytic region of tyrosine kinase adaptor protein associated protein 1 (NCKAP1). Together CYFIP1, WAVE1, and NCKAP1 form part of the WAVE regulatory complex (WRC), which functions to regulate the actin cytoskeleton, and dysregulation of the WRC has been associated with ASD (reviewed in: Campellone et al 2010, Waltes et al 2014, Wang et al 2015, Wang et al 2016). Furthermore, Neuroligin-3 was shown to interact with methyl-CpG-binding protein 2 (MeCP2), which is associated with Rett

syndrome (Amir et al 1999). These interactions of Neuroligin-3 with other ASD-related proteins indicates that they could share common pathways of pathophysiology, or that levels of Neuroligin-3 could be dysregulated in models involving these interacting proteins. This could indicate models with dysregulation of any of these associated genes could recapitulate some of the phenotypes seen in *Nlgn3* knockout mice, including the impact of MGH.

It is of importance to continue to explore the impact of MGH in other models, as rethinking how the social environment influences the behaviours and physiology of experimental animals could be an important step in the optimisation of research of disorders effecting behaviour. Given the difficulty in developing effective therapeutic interventions for ASD, as well as other neurodevelopmental, neuropsychiatric and mood disorders, taking into account any potential impact of social dominance environment on the phenotypes of the mice could improve the efficiency and output of this area of research by ensuring that the experimental groups are optimal.

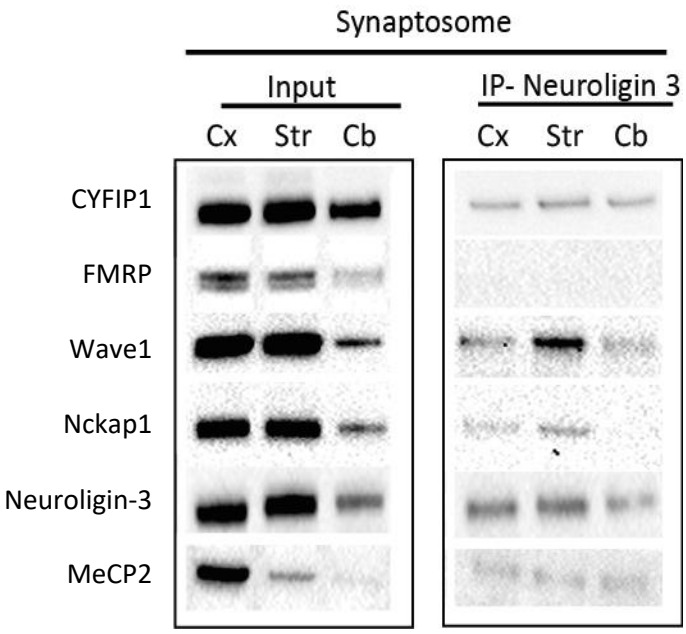


Figure 6.1. **Interaction of Neuroligin-3 with ASD-related proteins.** Coimmunoprecipitation of Neuroligin-3 with ASD-related proteins in synaptosome preparation of the cortex (Cx), striatum (Str) and cerebellum (Cb) of wild-type mice.

6.4.2. Application to the human condition

Siblings of those with ASD not only have increased risk of ASD, but also show abnormal social behaviours, anxiety, and emotional reactivity (Yirmiya et al 2006, Toth et al 2007, Shivers et al 2018). Given the shared genetics, it is likely that these represent endophenotypes. However, some evidence suggests that social support can alleviate to some degree these behaviours in the siblings of those with ASD (Hastings 2003), indicating that the social environment could influence some of these behaviours. Furthermore, children with ASD who have older siblings without ASD show better outcomes than those with ASD that do not have older siblings without ASD (Ben-Itzchak, Zukerman, and Zachor 2016, Ben-Itzchak, Nachshon, and Zachor 2018). These studies indicate that the social environment of children with ASD and their siblings can influence their behavioural outcomes. Whilst the social environment of lab mice is far removed from that of humans, it appears that the impact of MGH of *Nlgn3* knockout in mice may be recapitulating what could be seen in the human condition. Through the study of MGH, it may be possible to further identify brain regions that mediate this influence of social environment, which could possibly have some utility in understanding the effect seen in humans.

6.5. Phenotypes of female Nlgn3 knockout mice and wild-type mice

6.5.1. Minimal influence of sex on the impact of social environment and re-expression of Nlgn3

Several factors could lead to sexual dimorphism in behaviours of female mice. Female mouse models of ASD do not necessarily phenocopy the male mice (see **section 1.1.6)**, and in some cases, the social environment has been shown to impact male and female mice and rats differently (see **section 1.2.3)** possibly due to different manifestations of social hierarchy or social inequality (see **section 1.2.4).** Finally, there is some evidence to suggest that there may be a sexual dimorphism in the control of behaviours by *Pvalb*-expressing cells (see **sections 1.3.2** and **1.3.3**).

Firstly, adult *Nlgn3*$^{-/-}$ mice from MGH show hyperactivity, decreased social interest, and decreased thigmotaxis, reflecting what is seen in the *Nlgn3*$^{y/-}$ mice from MGH. Adult *Nlgn3*$^{-/-}$ mice, unlike *Nlgn3*$^{y/-}$ mice, do not show increased open arm exploration of the elevated plus maze relative to their littermates. Furthermore, juvenile *Nlgn3*$^{-/-}$ mice do not show a social deficit, perhaps indicating that these phenotypes emerge later in development for *Nlgn3*$^{-/-}$

mice. Nonetheless, despite a small amount of divergence, these data show that *Nlgn3*$^{y/-}$ mice largely phenocopy *Nlgn3*$^{y/-}$ mice.

Secondly, the influence of the social dominance environment of the female mice largely did not differ in its impact on behaviours compared to that in males. Adult *Nlgn3*$^{-/-}$ mice were shown to influence the social behaviours of their *Nlgn3*$^{+/-}$ (H-KO) littermates, reflecting the observation that *Nlgn3*$^{y/-}$ mice from MGH influence the social behaviours of their *Nlgn3*$^{y/+}$ littermates. Unlike the juvenile *Nlgn3*$^{y/-}$ mice, juvenile *Nlgn3*$^{-/-}$ mice do not show an impact of the MGH, as they do not have altered behaviours relative to *Nlgn3*$^{-/-}$ mice from SGH, and juvenile *Nlgn3*$^{+/-}$ mice do not show a difference in their social behaviour dependent upon whether they are housed with *Nlgn3*$^{+/+}$ mice or *Nlgn3*$^{-/-}$ mice. These effects may be due to juvenile *Nlgn3*$^{-/-}$ mice not showing a social phenotype. However, *Nlgn3*$^{+/+}$ mice, like *Nlgn3*$^{y/+}$ mice, show altered behaviours dependent upon being in MGH conditions or SGH conditions, indicating that sex of the wild-types also does not lead to dimorphism of response to the MGH. Nonetheless, the female mice do not always show the same influence of environment on the same behavioural measures, indicating that some degree a dimorphism exists. In the males, the influence of the social dominance environment could be attributed to a disruption of the within cage hierarchy. As discussed in the literature review (**section 1.2.4**), the females may have a social hierarchy, despite having decreased home cage aggression between peers compared to the levels of home cage aggression reported in males. In this thesis the dominance behaviours of female mice were not explored due to preliminary experiments utilising the tube test being unsuccessful, due to a lack of completion of the task by the females. In future experiments, dominance and hierarchy of the female cages could be determined by using a method such as that by Schuhr et at (1987), in which home cage interactions that are associated with dominance in females could be correlated with hormone levels. This would yield more information on if whether *Nlgn3*$^{-/-}$ mice have abnormal behaviours associated with female dominance.

Finally, re-expression of *Nlgn3* within *Pvalb*-expressing cells restores the social behaviours of adult *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice, reflecting the restored dominance of *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice and the restored social behaviours of juvenile *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice. Some small differences can be seen in the impact of re-expression on the exploratory behaviours and anxiety-related behaviours of *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice, and often this is due to a lack of a

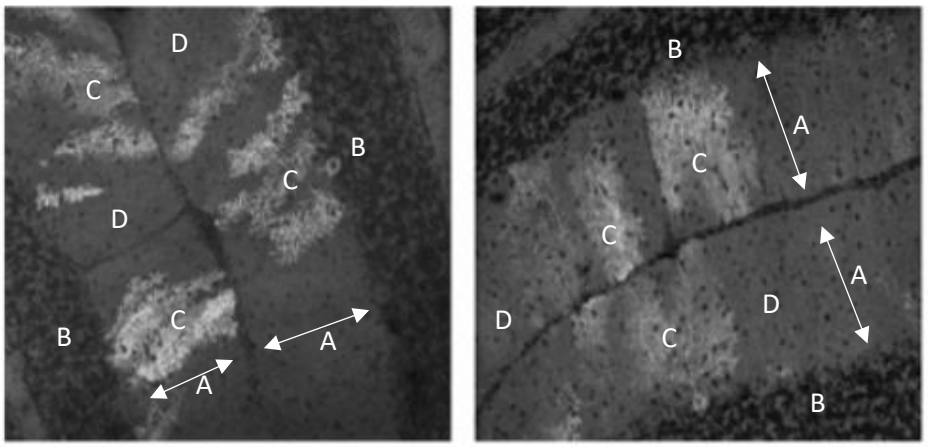
significance despite a trend, however with some exceptions, the data mostly shows the same directionality as in the males.

Altogether, these data indicate that sex does not largely influence the phenotypes of the mice. *Nlgn3*$^{-/-}$ mice show behavioural phenotypes that frequently mirror *Nlgn3*$^{y/-}$ mice, the impact of social dominance environment is seen on all genotypes of female mice, and re-expression of *Nlgn3* restores many phenotypes in *Nlgn3*$^{-/-}$*Pvalb*$^{cre/+}$ mice. However, some small differences are seen in the emergence of the phenotypes of *Nlgn3*$^{-/-}$ mice, and while the social dominance environment and re-expression influences the behaviours of the female mice, there are some sex differences dependent upon the task.

6.5.2. X-inactivation of Neuroligin-3 and the phenotypes of Nlgn3$^{+/-}$ *mice*

When an organism has two X chromosomes, one of them is subjected to X-inactivation, whereby the gene expression of one of the chromosomes is silenced, with only few genes escaping the X-inactivation (Carrel and Willard 2005). As one X chromosome has genes expressed, while the other X chromosome does not, this leads to mosaicism of the genetic expression. *Nlgn3* is an X-linked gene, which has been shown to be subjected to X-inactivation (**figure 6.2**). Due to the X-inactivated nature of *Nlgn3*, in *Nlgn3*$^{+/-}$ mice there is a mosaicism of expression of Neuroligin-3 in the different neuronal populations, with some neurones expressing Neuroligin-3, and others not expressing Neuroligin-3. Mosaicism of expression of Neuroligin-3 could lead to neuronal competition, a phenomenon in which homogeneous populations of wild-type or knockout neurones show no phenotype, while mixed populations do. Neuronal competition has been demonstrated in neuronal populations either homogeneous for Neuroligin-1 being present, homogeneous for Neuroligin-1 deficit, or in mixed populations of neurones both expressing Neuroligin-1 and not expressing Neuroligin-1. Only the mixed populations of neurones either expressing or not expressing Neuroligin-1 showed synaptic phenotypes (Kwon et al 2012). Therefore, it is possible that in *Nlgn3*$^{+/-}$ mice, the mixed population of neurones that are expressing Neuroligin-3 with neurones that do not express Neuroligin-3 could lead to a phenotype that is distinguishable from *Nlgn3*$^{+/+}$ mice and *Nlgn3*$^{-/-}$ mice. However, *Nlgn3*$^{+/-}$ (H-WT) mice did not show a behavioural divergence from their *Nlgn3*$^{+/+}$ littermates. This would indicate that despite the *Nlgn3*$^{+/-}$ mice having only half the Neuroligin-3 expression, there may be synaptic compensation from the synapses expressing Neuroligin-3 that prevents an observable phenotype.

A



A: Molecular layer

B: Purkinje layer

C: Purkinje cell dendrites expressing Neuroligin-3

D: Purkinje cell dendrites not expressing Neuroligin-3

B

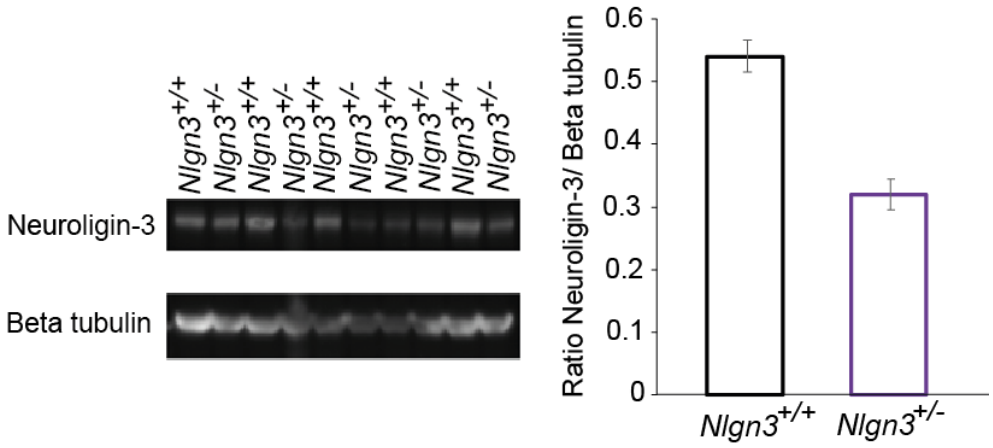


Figure 6.2. X-inactivation of *Nlgn3*. *A,* *Nlgn3*$^{+/-}$ mice expressing *Pcp2-tta*, which encodes a tetracycline-controlled transactivator (tta) expressed in purkinje cells only. As the STOP-TetO contruct includes a tetracycline operator (TetO), this leads to over expression of Neuroligin-3, as seen in the figure. In *Nlgn3*$^{+/-}$ mice, a pattern of mosiac expression of Neuroligin-3 is seen, consistent with *Nlgn3* being subjected to X-inactivation. This experiment was conducted by Dr Baudouin. ***B,*** western blot of cerebellum lystate taken from *Nlgn3*$^{+/+}$ mice and *Nlgn3*$^{+/-}$ mice. Relative to *Nlgn3*$^{+/+}$ mice, *Nlgn3*$^{+/-}$ mice show approximatly a 50% reduction in Neuroligin-3 levels, consistent with *Nlgn3* being subjected to X-inactivation.

6.6. The social dominance environment shapes behaviour

6.6.1. The impact of MGH is consistent with the results of a manipulated social environment

MGH was shown to impact a variety of behaviours, and some of these are consistent with previous reports of environmental manipulations on the behaviours of mice. Early life maternal deprivation has been shown to lead to decreased social dominance in adulthood, while early life social enrichment has been shown to lead to increased dominance, more rapid hierarchy formation, and increased behaviours related to dominance in adulthood (Pietropaolo et al 2004, Branchi, D'Andrea, Fiore et al 2006, D'Andrea et al 2007, Benner et al 2014). This is consistent with *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from MGH showing decreased dominance, indicating that hierarchy is malleable and a product of social experiences. Furthermore, social isolation in adult male mice leads to increased social interest (Naert et al 2011) and early life social manipulations in female mice and rats leads to increased direct social interaction as adults (Bondar et al 2018, Farrell et al 2016) and decreased aggression during lactation (Veenema et al 2007), demonstrating that the social environment can shape the social behaviours of mice. Adult *Nlgn3*$^{-/-}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates show reduced social interaction, and further exploration would be needed to understand what is leading to the reduced social interaction of the *Nlgn3*$^{-/-}$ mice from MGH and their *Nlgn3*$^{+/-}$ (H-KO) littermates.

From the results presented in chapter three, it was observed that juvenile *Nlgn3*$^{y/+}$ mice, *Nlgn3*$^{y/-}$ mice, and *Nlgn3*$^{+/+}$ mice from MGH show altered habituation within the open field over two days. This increased activity of the juvenile mice from MGH within the open field arena on day two could be representative of altered processing of the spatial environment, leading to increased exploratory behaviours. Learning and memory have been shown to be impacted by levels of maternal care and social isolation (Rice et al 2008, Ibi et al 2008, Benner et al 2014), indicating that the social environment could be influencing the ability of the mice to retain spatial information about the open field arena. The hippocampus is important in the encoding of spatial information, and mice from MGH have altered transcriptomes of the hippocampus, indicating that hippocampal dependent processing of spatial information could be impacted by MGH.

Furthermore, manipulations of maternal care, communal nesting, and social environment have been shown to lead to changes in the locomotive activity of mice and rats (Palanza et al 2001, Schiavone et al 2009, George et al 2010, Ashby et al 2010, Naert et al 2011, Schiavone

et al 2012), indicating that aberrant locomotion could be commonly seen when the social environment could be a variable influencing behaviour. Indeed, in one of these studies, anxiety levels were also found to be altered, with the early life separated mice showing increased locomotion as well as increased time in the centre of the open arena (George et al 2010). Consistent with this is that elevated plus maze exploration has been shown to be impacted by the social environment (Romeo et al 2003, Weiss et al 2004, Schmidt et al 2007, Sterlemann et al 2008, Schmidt et al 2010, Larrieu et al 2017, Bondar et al 2018). A similar trend is also observed in the data presented in this thesis; the social environment impacts the anxiety-related behaviours, with both MGH and knockout of *Nlgn3* being associated with decreased thigmotaxis and increased open arm exploration of the elevated plus maze. These data suggest that the altered locomotive activity and anxiety-related phenotypes are consistent with previous observations of the impact of the social environment.

Furthermore, the results from the RNAseq are consistent with the social environment having a measurable effect on the expression of mRNAs within the brain (Schmidt et al 2007 Sterlemann et al 2008, So et al 2015, Nesher et al 2015, Horii et al 2017). These results further demonstrate that MGH, like the position within the social hierarchy and the manifestation of the social environment, can impact mRNA expression within the brain.

6.6.2. Mechanisms of transfer of social behaviours

The results presented in this thesis demonstrate that MGH leads to changes in behaviours of the mice relative to SGH mice and mice with *Nlgn3* re-expression in *Pvalb*-expressing cells. This might be explained by aberrant home cage social interactions. As discussed extensively in **section 1.2**, the deprivation, enrichment, or instability of the social environment, as well as position within the social hierarchy, leads to altered behaviours and physiology, demonstrating the interaction between peers is key in shaping these factors. Given that MGH impacts the social behaviours of male and female wild-type and *Nlgn3* knockout mice, it is likely that abnormal home cage interactions could be leading to the altered behaviours and physiology reported in this work, this may be especially true as the male, and possibly female, mice have hierarchies that are disrupted by the presence of *Nlgn3* knockout mice. This is consistent with the reports of other animal models of ASD, and models with social or dominance-related deficits in their behaviours impacting their littermates (see **section 1.2.7**). However, without scoring the home cage interactions, it is not possible to say that the home

cage interactions of mice from MGH conditions are actually different to those from SGH conditions.

Alternative explanations may also explain in this shift in group behaviour. The gut microbiome has been shown to shape behaviour and physiology of both mice and humans. Individual species of bacteria have been shown to exert different behavioural outcomes in mice, for example some species of bacteria have been shown to lead to altered stress response and altered levels of brain derived neurotrophic factor and N-methyl-D-aspartate receptor subunit composition, while germ-free mice have altered postsynaptic density-95, synaptophysin, and monoamine levels (Sudo et al 2004, Heijtz et al 2011). Additionally, the gut biome has been shown to mediate behaviours relevant to the changes reported in this thesis; germfree mice have alterations in their open field activity and anxiety as measured by the elevated plus maze (Heijtz et al 2011). As the mice reported in this thesis spend their lives housed with each other, and the ingestion of faecal matter is common in mice, it is possible that there may be a transfer of this bacteria that may lead to alterations of the behaviours of all mice within the social environment. Such an effect has been observed in experiments in which BALB/c mice and NIH Swiss mice, which have distinct microbiomes, had an exchange of gut microbiome. Initially, the two strains were raised in germ free conditions before the gut biome of the other strain was introduced into them. Following this, the mice then showed the behavioural profile of the strain of mice for which the bacteria came from (Bercik et al 2011). This notion of potential alterations of gut biome in the *Nlgn3* mice is further supported by evidence from people with ASD. People with ASD have alterations in the populations their gut biome (Parracho et al 2005, Krajmalnik-Brown et al 2015). Interestingly this change in gut biome has also been found in a maternal valproic acid exposure as a model of ASD, and in a *Shank3* knockout model of ASD (de Theije et al 2014, Tabouy et al 2018). Analysis of the gut biome could be of interest, to observe if there is a potential influence of the gut microbiome of the behaviours of the mice. This could be followed by the rearing in germ-free conditions, to determine if the microbiome is indeed a vector for the social behaviours.

Similarly, olfactory cues play a large part in the communication of social information in mice. Mice have been shown to release alarm pheromones and anxiogenic pheromones, which leads to behavioural changes such as aversion, decreased locomotion, enhanced or impaired fear acquisition and extinction of fear, and altered perception of pain (Rottman and

Snowdown 1972, Lapin 1990, Bredy and Barad 2009). Interestingly, the social environment has been shown to impact the olfactory receptors in the vomeronasal organ, main olfactory epithelium, and olfactory bulb of both male and female mice. Mice that were housed in same-sex or combined-sex housing showed significantly altered receptor composition due to exposure or lack of exposure to pheromones related to the opposite sex (Linden et al 2018) therefore olfactory reception could be altered. Additionally, in drosophila melanogaster, it has been shown that the social environment can influence pheromone production (Kent et al 2008, Krupp et al 2008). It is therefore possible that if *Nlgn3* knockout mice are emitting different pheromones this could alter the olfactory response of their littermates leading to behavioural consequence, or that the pheromones being emitted by the group are changed as a consequence of MGH. This is supported by *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from MGH having different interest in social odours compared to *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from SGH and *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ and *Nlgn3*$^{y/-}$*Pvalb*$^{cre/+}$ mice (Kalbassi et al 2017). It is therefore possible that altered olfactory cues could be having a role in the influence of behaviours of the mice from MGH.

It would be worthwhile to determine if abnormal home cage interactions, or another cause, are leading to the shift in behaviours of the mice in the MGH conditions. Understanding the cause would give a better understanding of how the group social behaviour of mice manifests, for example, if the home cage social interactions are abnormal, this could be a useful tool in the assessment of social phenotypes of mouse models of ASD. If another cause, such a bacteria, was identified, this could provide invaluable information in understanding the link between the gut microbiome and social behaviours.

6.6.3. Nlgn3 knockout mice could influence long-term group fitness

Indirect genetic effects are a way of assessing how individual genotypes within a population influence the phenotypes of other within that population. In this thesis, it was observed that mice lacking *Nlgn3* impact the phenotypes of their littermates. This led to altered dominance, social, locomotive, and anxiety-related behaviours, and, additionally, both hormonal status and the transcriptomes of mice in MGH were shown to be altered. Therefore, behaviourally and physiologically, mice in MGH may have altered group fitness.

The consequence of altered group fitness could be investigated in transgenerational and complex social housing experiments. In the original ‘mouse utopia’ experiments, it was observed that there was an increase in the number of autistic-like mice, that may have

disrupted the population of mice leading to collapse of the social structures via rejection of normal social behaviours (social interaction, territory defence, courtship, maternal behaviours), leading to the demise of the population (Calhoun 1973). It has been speculated that the demise of mouse utopia may have been mediated via deleterious indirect genetic effects from the autistic-like mice (Woodley of Menie et al 2017).

In complex social environments, mice split into groups and claim their own territories, and levels of territorial protection and patrol behaviours naturally vary in the mice and are influenced by hierarchy behaviours (Crowcroft and Rowe 1963). Utilising a transgenerational and complex social housing experiment that includes either wild-type mice, or wild-type mice with *Nlgn3* knockout mice, the long-term impact of *Nlgn3* knockout mice on the group could be better assessed. Given the decrease in competitive behaviours of *Nlgn3*$^{y/-}$ mice and their *Nlgn3*$^{y/+}$ littermates, it would be of interest to explore if MGH groups would fail to split up into smaller groups, and, if they didn't, it would be of interest to explore how the mice would adapt to these larger, less competitive groups. However, it is also possible that instead of compromising the entire groups' fitness, there would be selection pressure against the mice with *Nlgn3* deletion, leading to the removal of the number of mice with *Nlgn3* knockout and restoring the group fitness.

6.7. Concluding remarks

The results presented in this thesis demonstrate that male and female mice lacking Neuroligin-3 influence the social and anxiety-related behaviours of their different genotype littermates and that this effect is bi-directional as the different genotype littermates influence the social and anxiety-related behaviours of Neuroligin-3 knockout mice. *Nlgn3* re-expression within *Pvalb*-expressing cells was shown to mediate the effect of *Nlgn3* on both an individual and group level. These results are of importance as this gives a better understanding of the role of Neuroligin-3, and of Neuroligin-3 expression in *Pvalb*-expressing cells, in the control of individual and group social behaviours, and further highlights the social environment as an important variable for studies of animal models of ASD. Additionally, the data from the RNA sequencing revealed that the transcriptomes reflect the influence of MGH, as well as identifying brain regions and targets that could be of interest in exploring this effect further. Altogether, these results expand the literature that animals with social phenotypes can shift the behaviours and physiology of their different genotype littermates. This is of importance as greater understanding of the social environment could optimise

preclinical models, improving research into animal models of neurodevelopmental, neuropsychiatric and mood disorders.

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Appendix

1.0. Cre doesn't influence the behaviour of adult female wild-type mice

Preliminary experiments demonstrated that the presence of the *Pvalb$^{cre/+}$* construct had no effect on the behaviours of wild-type mice. This work was conducted on adult female *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* mice and their *Nlgn3$^{+/+}$* littermates. The litters were generated from *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* females crossed with *Nlgn3$^{y/+}$* mice. When the direct social interaction of the mice with an unfamiliar female was assessed, no difference was observed in the social behaviour of *Nlgn3$^{+/+}$* mice compared to *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* mice (Figure A1.1A, *Nlgn3$^{+/+}$* mice n = 9, *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* mice n = 10, independent samples t-test *P = 0.904*). No difference was observed on the open field activity of *Nlgn3$^{+/+}$* mice compared to *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* mice (Figure A1.1B, *Nlgn3$^{+/+}$* mice n = 9, *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* mice n = 10, independent samples t-test *P = 0.573*). No difference was observed on the open field thigmotaxis of *Nlgn3$^{+/+}$* mice compared to *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* mice (ratio distance in the centre / total distance travelled, figure A1.1C, independent samples t-test *P = 0.545*, time in the centre, figure A1.1D, independent samples t-test *P = 0.757*, *Nlgn3$^{+/+}$* mice n = 9, *Nlgn3$^{+/+}$Pvalb$^{cre/+}$* mice n = 10). This indicates that the presence of the *Pvalb$^{cre/+}$* construct itself does not influence behaviour.

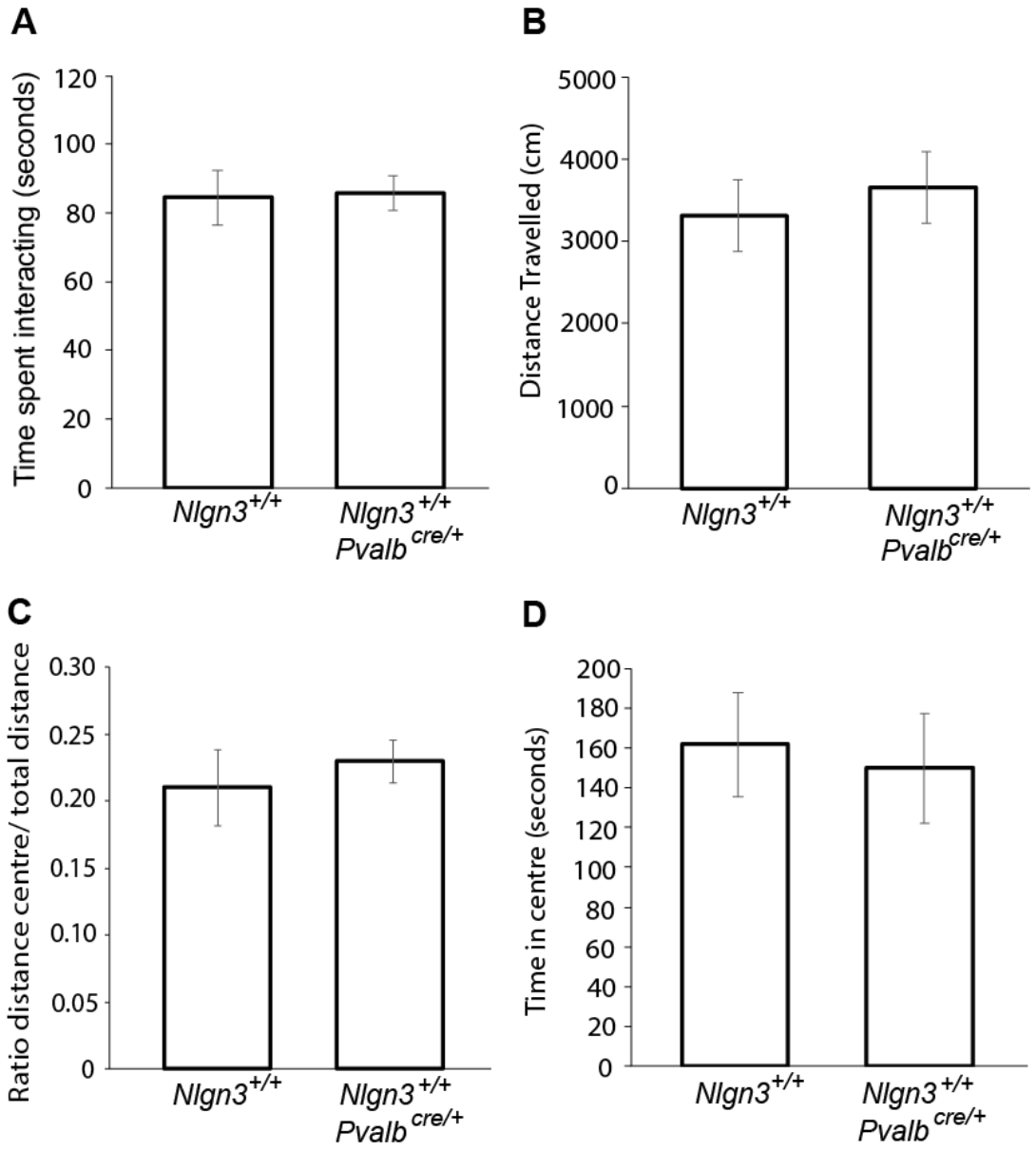

Figure A1.1, Cre has no effect on the behaviour on adult wild-type female mice. ***A*****,** Average time and standard error (SE) in interaction (seconds) of *Nlgn3*$^{+/+}$ mice, 84.4 (SE:8.1), *Nlgn3*$^{+/+}$*Pvalb*$^{cre/+}$ mice, 85.7(SE:4.9). ***B*****,** Average distance travelled and standard error (SE) (cm) of *Nlgn3*$^{+/+}$ mice, 3302.2 (SE:434.8), *Nlgn3*$^{+/+}$*Pvalb*$^{cre/+}$ mice, 3656.8 (SE:435.3. ***C*****,** Average ratio distance in the centre / total distance travelled and standard error (SE) of *Nlgn3*$^{+/+}$ mice, 0.209 (SE:0.028), *Nlgn3*$^{+/+}$*Pvalb*$^{cre/+}$ mice, 0.229 (SE:0.015). ***D*****,** Average ratio distance in the centre / total distance travelled and standard error (SE) of *Nlgn3*$^{+/+}$ mice, 162.0 (SE:26.3), *Nlgn3*$^{+/+}$*Pvalb*$^{cre/+}$ mice, 150.0 (SE:27.6).

2.0 Details of reagents for materials and methods

2.1. Reagents used for genotyping

TBE buffer

Tris Base 54 grams

Boric acid 27.5 grams

EDTA (pH 8.0) 20ml 0.5 M

pH to 8.3

2% agarose gel

Agarose 1.4 grams

TBE 70 ml

Ethidium bromide 1.4 µl

1% agarose gel

Agarose 0.7 grams

TBE 70 ml

Ethidium bromide 1.4 µl

2.2. Reagents used for protein extraction and western blot

20x MES running buffer

MES 97.60 grams

Tris Base 60.60 grams

SDS 10.0 grams

EDTA 3.0 grams

Water to 500 ml

10x NuPage buffer

Bicine 40.8 grams

Bis-Tris 52.3 grams

EDTA 3 grams

NuPage buffer working buffer

10x NuPage buffer 100 ml

Isopropanol 142 ml

Water 758 ml

10x TBS

Tris Base 30 grams

NaCl 80 grams

KCl 2 grams

pH to 7.5 with HCl

Water to 1 litre

TBS-T

100 ml 10x TBS

100 µl tween

900 ml water

Blocking agent

5 grams milk

TBS-T to 100 ml

3.0. Western blots used for quantification of Neuroligin-3 expression in Nlgn3$^{y/-}$Pvalb$^{cre/+}$ and Nlgn3$^{y/+}$Pvalb$^{cre/+}$ mice

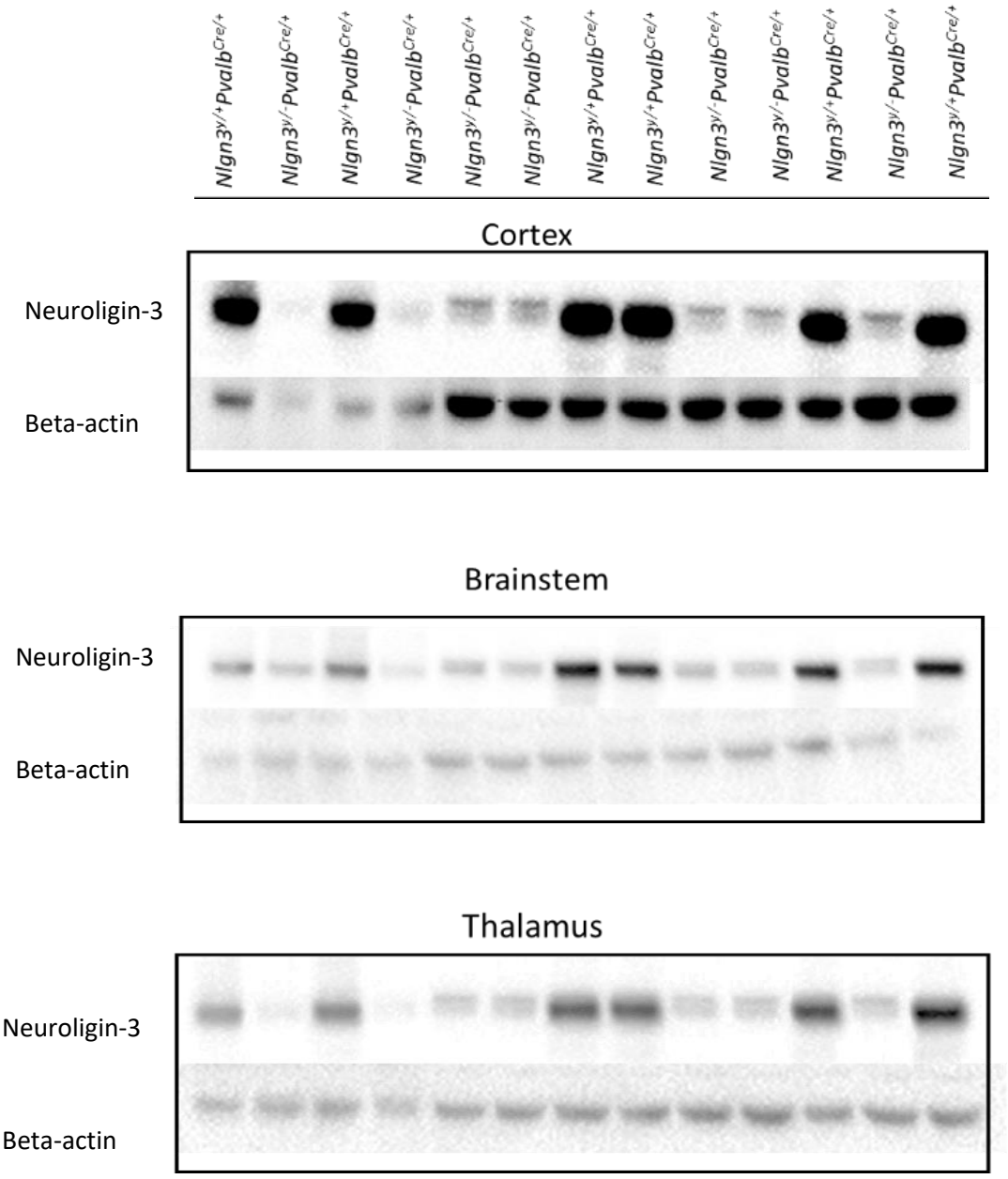

Figure A3.1. Western blots used for quantification of Neuroligin-3 levels in *Nlgn3$^{y/-}$Pvalb$^{cre/+}$* mice and *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* mice.

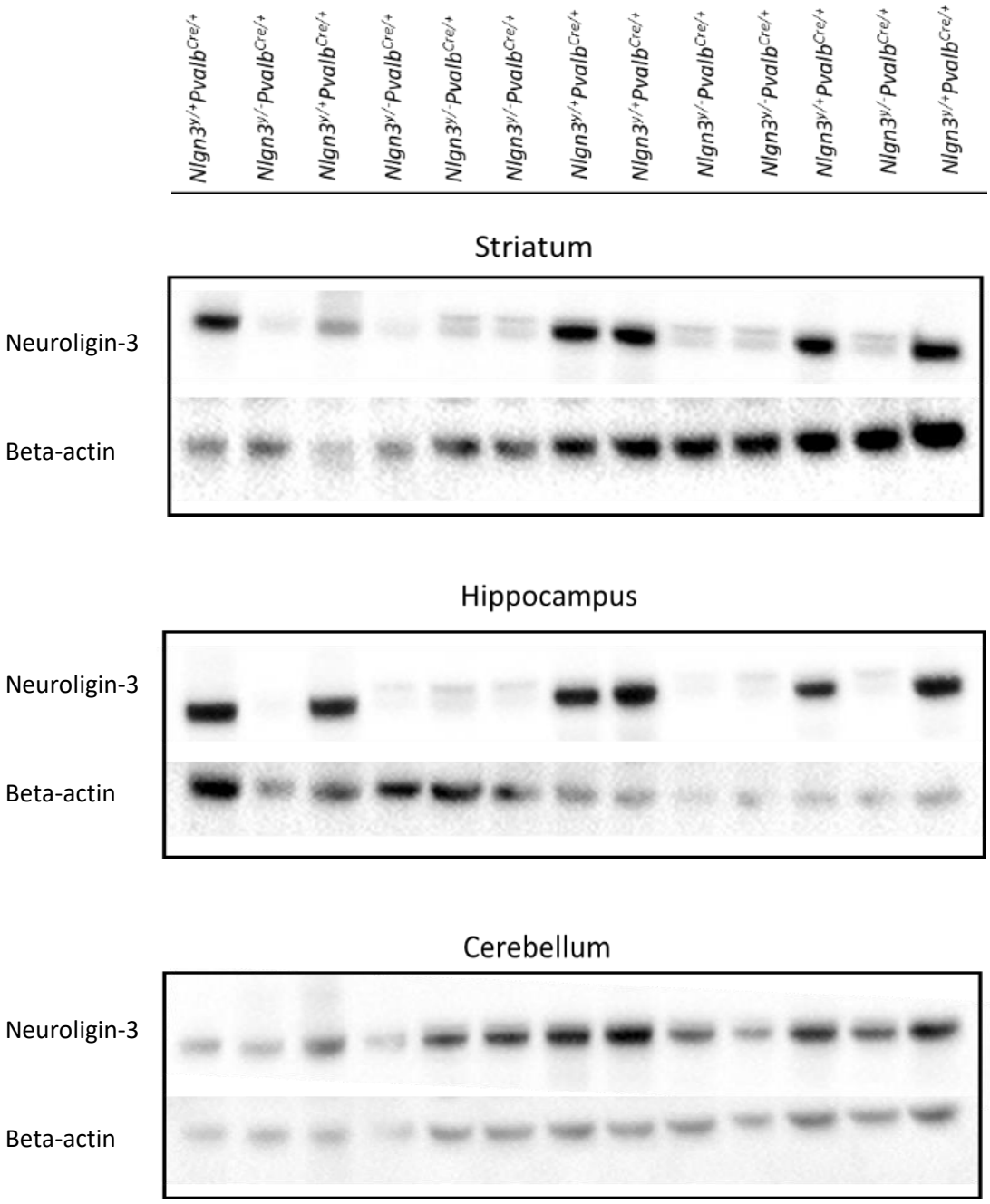

Figure A3.1, continued.

4.0. Characterisation of the synaptosome and the MeCP2 antibody

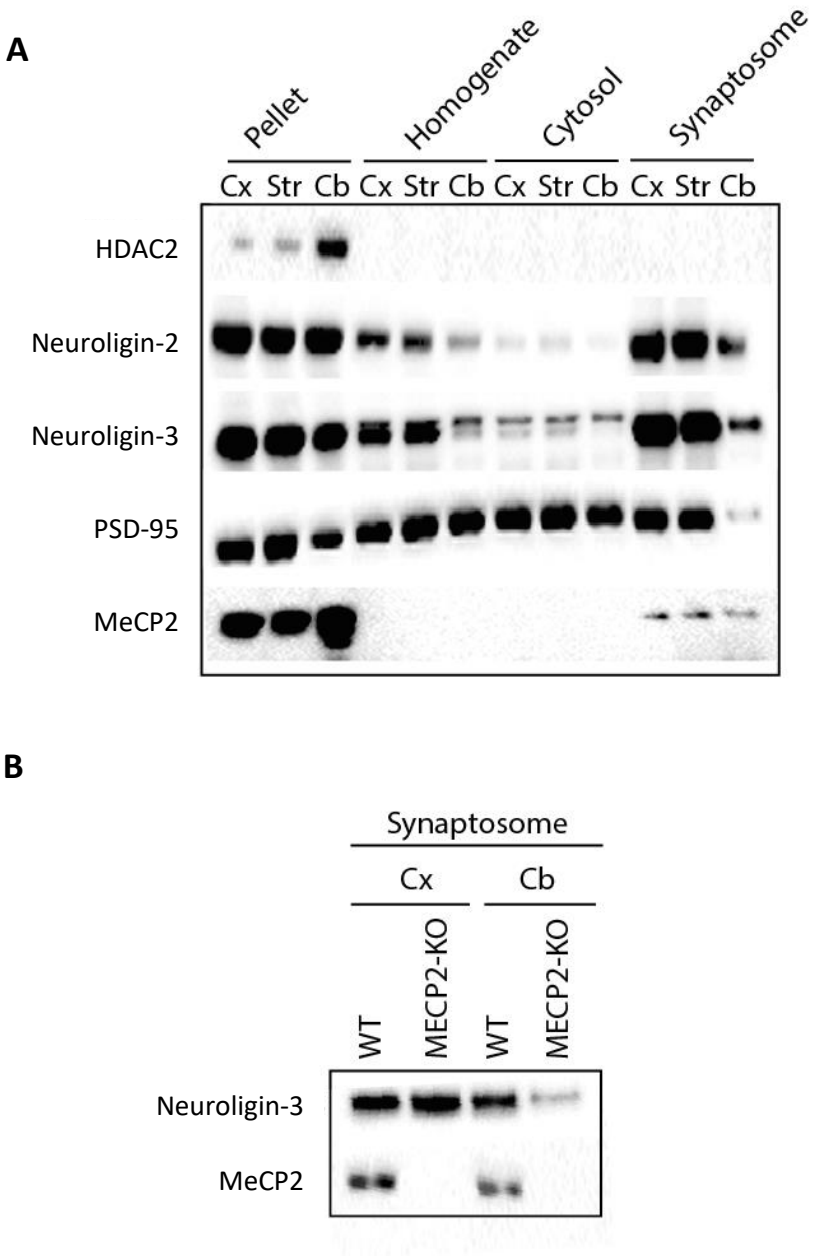

Figure A4.1. Validation of synaptosome prep and MeCP2 antibody. A, Validation of the synaptosome preperation. The synaptosome does not contain nuclear markers (HDAC), it contains markers for inhibitory synapses (Neuroligin-2), excitatory synapses (PSD-95). It also contains neuroligin 3 and MeCP2. Cx indicates cortex, Str indicates striatum, Cb indicates cerebellum. **B**, validation of the MeCP2 antibody on the synapses taken from wither wild-type mice or MECP2 knockout mice. Cx indicates cortex, Str indicates striatum, Cb indicates cerebellum.

5.0. Additional information pertaining to the experiments

- Table A5.1 Detailed information on the mice used in the experiments.
- Table A5.2. Detailed accounts of the statistical outputs.

Table A5.1 Detailed information on the mice used in the experiments.

Table A5.1 Detailed information on the mice used in the experiments

Figure	Experimental group	Number of different cages of mice	Number of different breeding females used	Past experiments
3.1.A Within cage tube test MGH		N = 15	n = 10	N = 15, adult open field activity and courtship USV vocalisation N = 3, Juvenile open field activity and social behaviour
3.1.D Within cage tube test PV-MGH		N = 5	n = 4	N = 5, Juvenile open field activity, social behaviour N = 5, adult open field and courtship USV
3.2.A, tube test against unfamiliar mice	MGH	N = 7	n = 5	N = 5, Juvenile open field activity, social behaviour, elevated plus maze N = 7, Adult open field activity, courtship USV
	SGH*Nlgn3*$^{y/+}$	N = 7	n = 6	N = 3, Juvenile open field activity, social behaviour N = 7, Adult open field activity, courtship USV
	SGH *Nlgn3*$^{y/-}$	N = 5	n = 3	N = 3, Juvenile open field activity, social behaviour, elevated plus maze N = 5, adult open field, courtship USV, elevated plus maze
	Pvalb	N = 5	n = 4	N = 5, Juvenile open field activity, social behaviour N = 5, adult open field and courtship USV
3.2.B, testosterone levels	MGH	N = 5	n = 4	N = 5, Juvenile open field activity, social behaviour, elevated plus maze N = 5, Adult open field activity, courtship USV, tube test

Table A5.1 Detailed information on the mice used in the experiments

	SGH*Nlgn3*$^{y/+}$	N = 7	n = 6	N = 4, Juvenile open field activity, social behaviour, elevated plus maze N = 7, Adult open field activity, courtship USV, tube test
	SGH *Nlgn3*$^{y/-}$	N = 5	n = 5	N = 5, Juvenile open field activity, social behaviour, elevated plus maze N = 5, adult open field, courtship USV,
	Pvalb	N = 6	n = 4	N = 6, Juvenile open field activity, social behaviour N = 6, adult open field and courtship USV
3.3.A, Number of USV 3.3.B, Duration of USV 3.3.C, time in interaction with female	MGH	N = 7	n = 5	N = 5, Juvenile open field activity, social behaviour, elevated plus maze N = 7, Adult open field activity, courtship USV
	SGH*Nlgn3*$^{y/+}$	N = 4	n = 3	N = 4, Juvenile open field activity, social behaviour, elevated plus maze N = 4, Adult open field activity, elevated plus maze.
	SGH *Nlgn3*$^{y/-}$	N = 3	n = 3	N = 3, Juvenile open field activity, social behaviour, elevated plus maze N = 3, Adult open field, courtship USV.
	Pvalb	N = 5	n = 4	N = 5, Juvenile open field activity, social behaviour N = 5, Adult open field, and courtship USV.
3.3.D, correlation of USV and tube test	MGH	N = 6	n = 3	N = 6 Juvenile open field activity, social behaviour N = 6 Adult open field activity, courtship USV, tube test
	SGH*Nlgn3*$^{y/+}$	N = 7	n = 5	N = 7, Juvenile open field activity, social behaviour N = 7, Adult open field, courtship USV, tube test.

Table A5.1 Detailed information on the mice used in the experiments

3.4.A, Social interaction of adult female mice	*Nlgn3*$^{+/+}$ MGH *Nlgn3*$^{+/-}$ (H-WT)	N = 7	n = 6	N = 7, juvenile open field activity, social behaviour, and elevated plus maze N = 7, adult open field activity, elevated plus maze.
	Nlgn3$^{-/-}$MGH *Nlgn3*$^{+/-}$ (H-KO)	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze N = 4, adult open field activity, elevated plus maze.
3.4.B, Social interaction of adult *Nlgn3*$^{+/+}$ mice	*Nlgn3*$^{+/+}$ MGH	N = 7	n = 6	N = 7, juvenile open field activity, social behaviour, and elevated plus maze N = 7, adult open field activity, elevated plus maze.
	Nlgn3$^{+/+}$ SGH	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze N = 4, adult open field activity, elevated plus maze.
3.4.C, social interaction of adult *Nlgn3*$^{-/-}$ mice	*Nlgn3*$^{-/-}$MGH	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze N = 4, adult open field activity, elevated plus maze.
	Nlgn3$^{-/-}$ SGH	N = 3	n = 3	N = 3, juvenile open field activity, social behaviour, and elevated plus maze N = 3, adult open field activity, elevated plus maze.
	Nlgn3$^{-/-}$ *Pvalb*$^{cre/+}$	N = 6	n = 2	N = 6, juvenile open field activity, social behaviour, and elevated plus maze N = 6, adult open field activity, elevated plus maze.
3.5.A, total duration calling to female mice 3.5.B, number of calls to female mice	*Nlgn3*$^{+/+}$	N = 5	n = 5	N = 5, juvenile open field activity, social behaviour, and elevated plus maze N = 5, adult open field activity, elevated plus maze.
	Nlgn3$^{+/-}$	N = 6	n = 6	N = 6, juvenile open field activity, social behaviour, and elevated plus maze N = 6, adult open field activity, elevated plus maze.
	Nlgn3$^{-/-}$	N = 3	n = 3	N = 3, juvenile open field activity, social behaviour, and elevated plus maze

Table A5.1 Detailed information on the mice used in the experiments

				N = 3, adult open field activity, elevated plus maze.
3.5.C, pup retrieval of female mice	*Nlgn3*$^{+/+}$	N = 7	n = 7	N = 2, juvenile open field activity, social behaviour, and elevated plus maze N = 2, adult open field activity, elevated plus maze.
	Nlgn3$^{+/-}$	N = 9	n = 8	N = 2, juvenile open field activity, social behaviour, and elevated plus maze N = 2, adult open field activity, elevated plus maze.
	Nlgn3$^{-/-}$	N = 7	n = 6	N = 4, juvenile open field activity, social behaviour, and elevated plus maze N = 4, adult open field activity, elevated plus maze.
3.6.A. Juvenile male and female social interaction	MGH*Nlgn3*$^{y/+}$	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	MGH*Nlgn3*$^{+/+}$	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	MGH*Nlgn3*$^{y/-}$	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	MGH*Nlgn3*$^{-/-}$	N = 4	n = 3	N = 4, open field activity, elevated plus maze
	SGH*Nlgn3*$^{y/+}$	N = 4	n = 3	N = 4, open field activity, elevated plus maze
	SGH*Nlgn3*$^{y/-}$	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	SGH*Nlgn3*$^{+/+}$	N = 4	n = 3	N = 4, open field activity, elevated plus maze
	SGH*Nlgn3*$^{-/-}$	N = 5	n = 4	N = 5, open field activity, elevated plus maze
3.6B Juvenile male social interaction	MGH	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	SGH*Nlgn3*$^{y/+}$	N = 4	n = 3	N = 4, open field activity, elevated plus maze
	SGH *Nlgn3*$^{y/-}$	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	Pvalb	N = 6	n = 4	N = 6, open field activity, elevated plus maze
3.7A, social interaction of juvenile female mice	*Nlgn3*$^{+/+}$ MGH *Nlgn3*$^{+/-}$ (H-WT)	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	Nlgn3$^{-/-}$MGH *Nlgn3*$^{+/-}$ (H-KO)	N = 4	n = 3	N = 4, open field activity, elevated plus maze

Table A5.1 Detailed information on the mice used in the experiments

3.7B, social interaction of juvenile *Nlgn3*$^{+/+}$ mice	*Nlgn3*$^{+/+}$ MGH	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	Nlgn3$^{+/+}$ SGH	N = 4	n = 3	N = 4, open field activity, elevated plus maze
3.7C, social interaction of juvenile *Nlgn3*$^{-/-}$ mice	*Nlgn3*$^{-/-}$MGH	N = 4	n = 3	N = 4, open field activity, elevated plus maze
	Nlgn3$^{-/-}$ SGH	N = 5	n = 4	N = 5, open field activity, elevated plus maze
	Nlgn3$^{-/-}$ *Pvalb*$^{cre/+}$	N = 6	n = 2	N = 6, juvenile open field activity, social behaviour, and elevated plus maze
4.1. Adult male activity 4.2.A. Adult male ratio distance in the centre 4.2.B. Adult male time in the centre	MGH	N = 8	n = 7	N = 8, juvenile open field activity, elevated plus maze, social behaviour
	SGH*Nlgn3*$^{y/+}$	N = 4	n = 3	N = 4, juvenile open field activity, elevated plus maze, social behaviour
	SGH *Nlgn3*$^{y/-}$	N = 3	n = 2	N = 3, juvenile open field activity, elevated plus maze, social behaviour
	Pvalb	N = 6	n = 4	N = 6, Juvenile open field activity, social behaviour
4.3. Adult male elevated plus maze	MGH	N = 8	n = 7	N = 8, juvenile open field activity, elevated plus maze, social behaviour, adult open field arena
	SGH*Nlgn3*$^{y/+}$	N = 4	n = 3	N = 4, juvenile open field activity, elevated plus maze, social behaviour, adult open field arena
	SGH *Nlgn3*$^{y/-}$	N = 3	n = 2	N = 3, juvenile open field activity, elevated plus maze, social behaviour
	Pvalb	N = 6	n = 2	N = 6, juvenile open field activity, social behaviour, and elevated plus maze

Table A5.1 Detailed information on the mice used in the experiments

4.4.A. Adult *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH activity 4.5.A-B. Adult *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH ratio distance centre and time in centre	*Nlgn3*$^{+/+}$ MGH *Nlgn3*$^{+/-}$ (H-WT)	N = 7	n = 6	N = 7, juvenile open field activity, social behaviour, and elevated plus maze
	Nlgn3$^{-/-}$MGH *Nlgn3*$^{+/-}$ (H-KO)	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze
4.4.B. Adult female activity *Nlgn3*$^{+/+}$ 4.5.C-D. Adult *Nlgn3*$^{+/+}$ ratio distance centre and the time in the centre	*Nlgn3*$^{+/+}$ MGH	N = 7	n = 6	N = 7, juvenile open field activity, social behaviour, and elevated plus maze
	Nlgn3$^{+/+}$ SGH	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze
4.4.C. Adult female *Nlgn3*$^{-/-}$ activity 4.5.E-F. Adult *Nlgn3*$^{-/-}$ ratio distance centre and time in centre	*Nlgn3*$^{-/-}$MGH	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze
	Nlgn3$^{-/-}$ SGH	N = 4	n = 4	N = 4, juvenile open field activity, social behaviour, and elevated plus maze
	Nlgn3$^{-/-}$ *Pvalb*$^{cre/+}$	N = 6	n = 2	N = 6, juvenile open field activity, social behaviour, and elevated plus maze
4.6.A. Adult *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH	*Nlgn3*$^{+/+}$ MGH *Nlgn3*$^{+/-}$ (H-WT)	N = 7	n = 6	N = 7, juvenile open field activity, social behaviour, and elevated plus maze N = 7, adult open field activity

Table A5.1 Detailed information on the mice used in the experiments

elevated plus maze				
	Nlgn3$^{-/-}$MGH *Nlgn3*$^{+/-}$ (H-KO)	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze N = 4, adult open field activity
4.6.B. Adult *Nlgn3*$^{+/+}$ elevated plus maze	*Nlgn3*$^{+/+}$ MGH	N = 7	n = 6	N = 7, juvenile open field activity, social behaviour, and elevated plus maze N = 7, adult open field activity
	Nlgn3$^{+/+}$ SGH	N = 3	n = 3	N = 3, juvenile open field activity, social behaviour, and elevated plus maze N = 3, adult open field activity
4.6.C. Adult female *Nlgn3*$^{-/-}$ elevated plus maze	*Nlgn3*$^{-/-}$MGH	N = 4	n = 3	N = 4, juvenile open field activity, social behaviour, and elevated plus maze N = 4, adult open field activity
	Nlgn3$^{-/-}$ SGH	N = 4	n = 4	N = 4, juvenile open field activity, social behaviour, and elevated plus maze N = 4, adult open field activity
	Nlgn3$^{-/-}$ *Pvalb*$^{cre/+}$	N = 6	n = 2	N = 6, juvenile open field activity, social behaviour, and elevated plus maze N = 6, adult open field activity
4.7. Juvenile male activity 4.8.A-B. Juvenile male ratio distance in the centre and time in the centre	MGH	N = 8	n = 7	Handling only
	SGH*Nlgn3*$^{y/+}$	N = 4	n = 3	Handling only
	SGH *Nlgn3*$^{y/-}$	N = 6	n = 5	Handling only
	Pvalb	N = 6	n = 2	Handling only
	MGH	N = 8	n = 7	N = 8, juvenile open field arena

Table A5.1 Detailed information on the mice used in the experiments

4.9. Juvenile male elevated plus maze	SGH*Nlgn3*$^{y/+}$	N = 4	n = 3	N = 4, juvenile open field arena
	SGH *Nlgn3*$^{y/-}$	N = 6	n = 5	N = 6, juvenile open field arena
	Pvalb	N = 6	n = 2	N = 6, juvenile open field arena
4.10.A. Juvenile *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH female activity 4.11.A-B. Juvenile *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH ratio distance centre and time in centre	*Nlgn3*$^{+/+}$ MGH *Nlgn3*$^{+/-}$ (H-WT)	N = 8	n = 8	Handling only
	Nlgn3$^{-/-}$MGH *Nlgn3*$^{+/-}$ (H-KO)	N = 7	n = 4	Handling only
4.10.B. Juvenile *Nlgn3*$^{+/+}$ activity 4.11.C-D Juvenile *Nlgn3*$^{+/+}$ MGH and SGH ratio distance centre and time in centre	*Nlgn3*$^{+/+}$ MGH	N = 8	n = 8	Handling only
	Nlgn3$^{+/+}$ SGH	N = 4	n = 3	Handling only
4.10.C. Juvenile *Nlgn3*$^{-/-}$ activity 4.11.E-F. Juvenile *Nlgn3*$^{-/-}$ MGH and SGH, and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ ratio distance centre	*Nlgn3*$^{-/-}$MGH	N = 7	n = 4	Handling only
	Nlgn3$^{-/-}$ SGH	N = 4	n = 4	Handling only

Table A5.1 Detailed information on the mice used in the experiments

and time in centre	*Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$	N = 6	n = 2	Handling only
4.12.A. Juvenile *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH elevated plus maze	*Nlgn3*$^{+/+}$ MGH *Nlgn3*$^{+/-}$ (H-WT)	N = 8	n = 8	N = 8, juvenile open field activity
	Nlgn3$^{-/-}$MGH *Nlgn3*$^{+/-}$ (H-KO)	N = 7	n = 4	N = 7, juvenile open field activity
4.12.B Juvenile *Nlgn3*$^{+/+}$ MGH and SGH elevated plus maze	*Nlgn3*$^{+/+}$ MGH	N = 8	n = 8	N = 8, juvenile open field activity
	Nlgn3$^{+/+}$ SGH	N = 4	n = 3	N = 4, juvenile open field activity
4.12.C. Juvenile *Nlgn3*$^{-/-}$ MGH and SGH, and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ elevated plus maze	*Nlgn3*$^{-/-}$MGH	N = 7	n = 4	N = 7, juvenile open field activity
	Nlgn3$^{-/-}$ SGH	N = 4	n = 4	N = 4, juvenile open field activity
	Nlgn3$^{-/-}$ *Pvalb*$^{cre/+}$	N = 6	n = 2	N = 6, juvenile open field activity
5.1.A - F PC1 and PC2 Striatum and hippocampus	MGH	N = 2	n = 2	Handling only
	SGH*Nlgn3*$^{y/+}$	N = 1	n = 1	Handling only
	SGH *Nlgn3*$^{y/-}$	N = 1	n = 1	Handling only
5.3.A-B C4 Hippocampus and striatum	MGH	N = 4	n = 4	Handling only
	SGH*Nlgn3*$^{y/+}$	N = 2	n = 2	Handling only
	SGH *Nlgn3*$^{y/-}$	N = 2	n = 2	Handling only
5.4.A-B C3 Striatum and hippocampus	MGH	N = 4	n = 4	Handling only
	SGH*Nlgn3*$^{y/+}$	N = 2	n = 2	Handling only
	SGH *Nlgn3*$^{y/-}$	N = 2	n = 2	Handling only
A1.1A *Nlgn3*$^{+/+}$ and		N = 3	n = 2	N = 3 open field activity

Table A5.1 Detailed information on the mice used in the experiments

Nlgn3$^{+/+}$*Pvalb*$^{cre/+}$ social interaction				
A1.1B-C *Nlgn3*$^{+/+}$ and *Nlgn3*$^{+/+}$*Pvalb*$^{cre/+}$ thigmotaxis		N = 3	n = 2	N = 3 open field activity and social interaction

Table A5.2. Detailed accounts of the statistical outputs.

Table A5.2. Detailed accounts of the statistical outputs

Figure	Shapiro-Wilk test of normality	Test for Equality of variances	Type of test	Sample size
3.1.A Within cage tube test MGH	*Nlgn3$^{y/-}$* mice *P = 0.00015* *Nlgn3$^{y/+}$* mice *P = 0.001*	Levenes test *P = 0.716*	Mann-Whitney U *P = 0.025* *Test statistic = 104.5*	*Nlgn3$^{y/-}$* mice n = 19 *Nlgn3$^{y/+}$* mice n = 19
3.1.D Within cage tube test PV-MGH	*Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P = 0.195* *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* mice *P = 0.408*	Levenes test *P = 0.732*	Independent samples t-test *P = 0.732* *df = 15*	*Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 8 *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* mice n = 9
3.2.A, tube test against unfamiliar mice	*Nlgn3$^{y/+}$* SGH *P* =0.008 *Nlgn3$^{y/-}$*SGH *P =0.011* *Nlgn3$^{y/+}$* MGH *P* = 0.02 *Nlgn3$^{y/-}$* MGH *P* =0.004 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* *P* =0.194 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* =0.025	Levenes test *P = 0.123* *F = 1.811*	Independent samples Kruskal-Wallis *P = 0.027* Two-way ANOVA **Social dominance environment:** *P = 0.003, F $_{(2,74)}$ = 6.423, df = 2, Power = 0.892* **Genotype:** *P = 0.809, F $_{(1,74)}$ = 0.020, df = 1, Power = 0.053* **Social dominance environment x genotype:** *P = 0.527, F $_{(1,74)}$ = 0.145, df = 2, Power = 0.172* Followed by Bonferroni adjusted post-hoc analysis and confirmed on a transformed dataset.	*Nlgn3$^{y/+}$* SGH n = 15 *Nlgn3$^{y/-}$*SGH n = 12 *Nlgn3$^{y/+}$*MGH n = 16 *Nlgn3$^{y/-}$*MGH n =13 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 9 *Nlgn3$^{y/-}$ pvalb$^{cre/+}$* n =8
3.2.B, testosterone levels	*Nlgn3$^{y/+}$* SGH *P* =0.868 *Nlgn3$^{y/-}$*SGH *P* =0.230 *Nlgn3$^{y/+}$* MGH *P* = 0.006 *Nlgn3$^{y/-}$* MGH *P* = 0.015	Levenes test *P = 0.073* *F = 2.125*	Independent samples Kruskal-Wallis *P* = 0.002 Two-way ANOVA **Social dominance environment:** *P = 0.00039, F $_{(2,54)}$ = 9.07, df = 2, Power = 0.968* **Genotype:**	*Nlgn3$^{y/+}$* SGH n = 15 *Nlgn3$^{y/-}$*SGH n = 12 *Nlgn3$^{y/+}$* MGH n = 10 *Nlgn3$^{y/-}$* MGH n = 8

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{y/+}$ Pvalb$^{cre/+}$ *P* =0.297 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* =0.745		*P = 0.045, F $_{(1,54)}$ = 3.290, df = 2, Power = 0.601* **Social dominance environment x genotype:** *P = 0.026, F $_{(2,54)}$ = 3.901, df = 2, Power = 0.680* Followed by Bonferroni adjusted post-hoc analysis and Bonferroni adjust pair-wise analysis and confirmed on a transformed dataset	*Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* n = 9 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 6
3.3.A, Number of USV	*Nlgn3$^{y/+}$*MGH *P* = 0.188 *Nlgn3$^{y/-}$*MGH *P* = 0.034 *Nlgn3$^{y/+}$* SGH *P* = 0.251 *Nlgn3$^{y/-}$*SGH *P* = 0.008 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* *P* = 0.961 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.00	Levenes test *P = 0.333* *F = 1.171*	Independent samples Kruskal-Wallis *P = 0.041* Two-way ANOVA **Social dominance environment:** *P = 0.756, F $_{(2,68)}$ = 0.281, df = 2, Power = 0.093* **Genotype:** *P = 0.00009, F $_{(1,68)}$ = 10.61, df = 2, Power = 0.987* **Social dominance environment x genotype:** *P = 0.664, F $_{(2,68)}$ = 0.412, df = 2, Power = 0.114* Followed by Bonferroni adjusted post-hoc analysis and confirmed on a transformed dataset	*Nlgn3$^{y/+}$*MGH n = 15 *Nlgn3$^{y/-}$*MGH n = 11 *Nlgn3$^{y/+}$* SGH n = 15 *Nlgn3$^{y/-}$*SGH n = 12 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 11 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 10
3.3.B, Duration of USV	*Nlgn3$^{y/+}$ MGH* *P* = 0.716 *Nlgn3$^{y/-}$ MGH* *P* = 0.024 *Nlgn3$^{y/+}$ SGH* *P* = 0.269 *Nlgn3$^{y/-}$ SGH* *P* = 0.007 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* *P* = 0.830	Levenes test *P = 0.498,* *F = 0.881*	Independent samples Kruskal-Wallis *P = 0.003* Two-way ANOVA **Social dominance environment:** *P = 0.937, F $_{(1,68)}$ = 0.065, df = 1, Power = 0.059* **Genotype:** *P = 0.000081, F $_{(2,68)}$ = 17.58, df =2, Power = 0.985*	*Nlgn3$^{y/+}$ MGH* n = 15 *Nlgn3$^{y/-}$ MGH* n = 11 *Nlgn3$^{y/+}$ SGH* n = 15 *Nlgn3$^{y/-}$ SGH* n = 12 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 11 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 10

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{y/-}$ Pvalb$^{cre/+}$ *P* = 0.000		**Social dominance environment x genotype:** *P = 0.951, F $_{(2,68)}$ = 0.051, df = 2, Power = 0.057* Followed by Bonferroni adjusted post-hoc analysis and confirmed on a transformed dataset	
3.4.A, time in interaction with female	*Nlgn3$^{y/+}$ MGH* *P* = 0.118 *Nlgn3$^{y/-}$ MGH* *P* = 0.743 *Nlgn3$^{y/+}$ SGH* *P* = 0.017 *Nlgn3$^{y/-}$ SGH* *P* = 0.900 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* *P* = 0.697 *Nlgn3$^{y/-}$ pvalb$^{cre/+}$* *P* = 0.23	Levenes test *P = 0.003, F = 3.94*	Independent samples Kruskal-Wallis *P = 0.013* Two-way ANOVA **Social dominance environment:** *P = 0.44, F $_{(2,66)}$ = 0.84, df = 2, Power = 0.188* **Genotype:** *P = 0.000000012, F $_{(1,66)}$ = 42.290, df = 1, Power = 1.00* **Social dominance environment x genotype:** *P = 0.291, F $_{(2,66)}$ = 1.259, df = 2, Power = 0.264* Followed by Bonferroni adjusted post-hoc analysis and confirmed on a transformed dataset	*Nlgn3$^{y/+}$ MGH* n = 15 *Nlgn3$^{y/-}$ MGH* n = 11 *Nlgn3$^{y/+}$ SGH* n = 13 *Nlgn3$^{y/-}$ SGH* n = 12 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 11 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 10
3.4.B, correlation of USV and tube test			*Nlgn3$^{y/+}$* SGH Pearson Correlation r^2 = 0.84 *Nlgn3$^{y/+}$* and *Nlgn3$^{y/-}$* MGH Pearson Correlation r^2 = 0.43	*Nlgn3$^{y/+}$* SGH N = 7 MGH N = 6
3.5.A, Social interaction of adult female mice	*Nlgn3$^{+/+}$ MGH* *P* =0120 *Nlgn3$^{+/-}$* (*H-WT)* *P* =0.150 *Nlgn3$^{+/-}$* (H-KO) *P* =0.634 *Nlgn3$^{-/-}$* MGH *P* =0.166	Levenes test *P = 0.051 F = 2.879*	One-way ANOVA: *P = 0.0004 F $_{(3,40)}$ = 7.87, df = 3 Power = 0.98* Followed by Tukey's post-hoc analysis	*Nlgn3$^{+/+}$ MGH* n = 10 *Nlgn3$^{+/-}$* (*H-WT)* n =10 *Nlgn3$^{+/-}$* (H-KO) n =7 *Nlgn3$^{-/-}$ MGH* n = 12
3.5.B, Social interaction	*Nlgn3$^{+/+}$* MGH *P* = 0.278	Levenes test *P = 0.930*	Independent samples t-test, two tailed.	*Nlgn3$^{+/+}$* MGH n =10

Table A5.2. Detailed accounts of the statistical outputs

of adult *Nlgn3*$^{+/+}$ mice	*Nlgn3*$^{+/+}$ SGH *P* = 0.210	*F = 0.008*	*P = 0.59, df = 19*	*Nlgn3*$^{+/+}$ SGH n = 11
3.5.C, social interaction of adult *Nlgn3*$^{-/-}$ mice	*Nlgn3*$^{-/-}$ MGH *P* =0.166 *Nlgn3*$^{-/-}$ SGH *P* =0.465 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.860	Levenes test *P = 0.657* *F = 4.62*	One-way ANOVA: *P = 0.00001, F* $_{(2,30)}$ *= 17.22, df = 2, Power = 0.999* Followed by Tukey's post-hoc analysis	*Nlgn3*$^{-/-}$ MGH n = 12 *Nlgn3*$^{-/-}$ SGH n = 11 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n = 10
3.6.A, total duration calling to female mice	*Nlgn3*$^{+/+}$ *P* =0.584 *Nlgn3*$^{+/-}$ *P* = 0.096 *Nlgn3*$^{-/-}$ *P* = 0.118	Levenes test *P = 0.151* *F = 2.063*	One-way ANOVA *P = 0.15, F* $_{(2,22)}$ *= 2.20* *df = 2, Power = 0.374*	*Nlgn3*$^{+/+}$ n = 8 *Nlgn3*$^{+/-}$ n = 9 *Nlgn3*$^{-/-}$ n = 8
3.6.B, number of calls to female mice	*Nlgn3*$^{+/+}$ *P* = 0.728 *Nlgn3*$^{+/-}$ *P* = 0.049 *Nlgn3*$^{-/-}$ *P* = 0.196	Levenes test *P = 0.793* *F = 2.35*	Kruskal-Wallis test: *P = 0.54*	*Nlgn3*$^{+/+}$ n = 8 *Nlgn3*$^{+/-}$ n = 9 *Nlgn3*$^{-/-}$ n =8
3.6.C, pup retrieval of female mice	**First contact:** *Nlgn3*$^{+/+}$ *P* = 0.097 *Nlgn3*$^{+/-}$ *P* = 0.540 *Nlgn3*$^{-/-}$ *P* = 0.001 **First pup**: *Nlgn3*$^{+/+}$ *P* = 0.406 *Nlgn3*$^{+/-}$ *P* = 0.434 *Nlgn3*$^{-/-}$ *P* = 0.724 **Second pup:** *Nlgn3*$^{+/+}$ *P* = 0.457 *Nlgn3*$^{+/-}$ *P* = 0.547 *Nlgn3*$^{-/-}$ *P* = 0.066 **Third pup:**	Levenes test First contact: *P = 0.131* *F = 2.191* First pup: *P = 0.481* *F = 0.752* second pup: *P = 0.069* *F = 2.951* Third pup: *P = 0.171* *F = 1.885*	Kruskal-Wallis test **First contact:** *P = 0.022* (Followed by Dunn's adjusted pairwise analysis) **First pup:** *P = 0.43* **Second pup:** *P = 0.48* **Third pup:** *P = 0.23*	*Nlgn3*$^{+/+}$ n = 13 *Nlgn3*$^{+/-}$ n = 9 *Nlgn3*$^{-/-}$ n = 9

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{+/+}$ *P* = 0.200 *Nlgn3*$^{+/-}$ *P* = 0.160 *Nlgn3*$^{-/-}$ *P* = 0.004			
3.7.A. Juvenile male and female social interaction	*Nlgn3*$^{y/+}$ MGH *P* = 0.891 *Nlgn3*$^{y/-}$ MGH *P* = 0.759 *Nlgn3*$^{y/+}$ SGH *P* = 0.813 *Nlgn3*$^{y/-}$ SGH *P* = 0.77 *Nlgn3*$^{+/+}$ MGH MGH: *P* = 0.734 *Nlgn3*$^{+/+}$ SGH *P* = 0.206 *Nlgn3*$^{-/-}$ MGH *P* = 0.266 *Nlgn3*$^{-/-}$ SGH *P* = 0.714	Levenes test *P = 0.123* *F = 1.680*	Two-way ANOVA **Social dominance environment***: P = 0.000087, F* $_{(1,55)}$ *= 16.77, df = 1, power = 0.98* **Genotype** *P = 0.247, F* $_{(1,55)}$ *= 0.64 df = 1, power = 0.06* **Sex** *P = 0.048, F* $_{(1,55)}$ *= 4.03, df = 1, power = 0.51* **Social dominance environment x genotype** *P = 0.247, F* $_{(1,55)}$ *= 1.355 df = 1, power = 0.211* **Social dominance environment x sex** *P = 0.062, F* $_{(1,55)}$ *= 3.56 df = 1, power = 0.463* **Genotype x sex** *P = 0.424, F* $_{(1,55)}$ *= 0.646 df = 1, power = 0.13* **Social dominance environment x genotype x sex** *P = 0.84, F* $_{(1,55)}$ *= 0.24 df = 1, power = 0.076*	*Nlgn3*$^{y/+}$ MGH n = 12 *Nlgn3*$^{y/-}$ MGH n = 10 *Nlgn3*$^{y/+}$ SGH n = 9 *Nlgn3*$^{y/-}$ SGH n = 14 *Nlgn3*$^{+/+}$ MGH n = 14 *Nlgn3*$^{+/+}$ SGH n = 12 *Nlgn3*$^{-/-}$ MGH n = 15 *Nlgn3*$^{-/-}$ SGH n = 20
3.7.B Juvenile male social interaction	*Nlgn3*$^{y/+}$ MGH *P* = 0.891 *Nlgn3*$^{y/-}$ MGH *P* = 0.759 *Nlgn3*$^{y/+}$ SGH *P* = 0.813 *Nlgn3*$^{y/-}$ SGH *P* = 0.77 *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ *P* = 0.890	Levenes test *P = 0.168* *F = 1.618*	Two-way ANOVA **Social dominance environment:** *P = 0.00024, F* $_{(2,63)}$ *= 9.51, df = 2, Power = 0.98* **Genotype:** *P =0.92, F* $_{(1,63)}$ *= 0.010, df = 1, Power = 0.05* **Social dominance environment x genotype:**	*Nlgn3*$^{y/+}$ MGH n = 12 *Nlgn3*$^{y/-}$ MGH n = 10 *Nlgn3*$^{y/+}$ SGH n = 9 *Nlgn3*$^{y/-}$ SGH n = 14 *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ n = 11 *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ n = 15

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{y/-}$ *Pvalb$^{cre/+}$* *P* = 0.709		*P = 0.65, F $_{(2,63)}$ = 0.43, df = 2, Power = 0.12* Followed by Bonferroni adjusted post-hoc analysis	
3.8.A, social interaction of juvenile female mice	*Nlgn3$^{+/+}$* MGH *P* = 0.734 *Nlgn3$^{+/-}$* (H-WT) *P* = 0.909 *Nlgn3$^{+/-}$* (H-KO) *P* = 0.356 *Nlgn3$^{-/-}$* MGH *P* = 0.266	Levenes test *P = 0.660* *F = 0.536*	One-way ANOVA: *P = 0.052,* *F $_{(3,41)}$ = 2.79, df = 3, Power = 0.63*	*Nlgn3$^{+/+}$* n = 14 *Nlgn3$^{+/-}$* (H-WT) n = 8 *Nlgn3$^{+/-}$* (H-KO) n = 8 *Nlgn3$^{-/-}$* MGH n = 15
3.8.B, social interaction of juvenile *Nlgn3$^{+/+}$* mice	*Nlgn3$^{+/+}$* MGH MGH: *P* = 0.734 *Nlgn3$^{+/+}$* SGH *P* = 0.206	Levenes test *P = 0.660* *F = 0.536*	Independent samples test *P = 0.027, df = 24*	*Nlgn3$^{+/+}$* MGH n = 14 *Nlgn3$^{+/+}$* SGH n = 12
3.8C, social interaction of juvenile *Nlgn3$^{-/-}$* mice	*Nlgn3$^{-/-}$* MGH *P* = 0.266 *Nlgn3$^{-/-}$* SGH *P* = 0.714 *Nlgn3$^{-/-}$* *Pvalb$^{cre/+}$* *P* = 0.244	Levenes test *P = 0.146* *F = 2.018*	One-way ANOVA *P= 0.607, F $_{(2,42)}$ = 0.51, df = 2, Power = 0.13*	*Nlgn3$^{-/-}$* MGH n = 15 *Nlgn3$^{-/-}$* SGH n = 20 *Nlgn3$^{-/-}$* *Pvalb$^{cre/+}$* n =10
4.1. Adult male activity	*Nlgn3$^{y/+}$* MGH *P = 0.431* *Nlgn3$^{y/-}$* MGH *P = 0.396* *Nlgn3$^{y/+}$* SGH *P = 0.514* *Nlgn3$^{y/-}$* SGH *P* = 0.630 *Nlgn3$^{y/+}$* *Pvalb$^{cre/+}$* *P* =0.723 *Nlgn3$^{y/-}$* *Pvalb$^{cre/+}$* *P* = 0.894	*Levenes test* *P = 0.349* *F = 1.133*	Two-way ANOVA **Social dominance environment** *P = 0.035, F $_{(2,82)}$ = 3.49* *df = 2, Power = 0.64* **Genotype** *P = 2.41e -10, F $_{(1,82)}$ = 52.10* *df = 1, Power = 0.99* **Social dominance environment x Genotype** *P = 0.0002, F $_{(2,82)}$ = 9.48* *df = 2, Power = 0.98* Followed by Bonferroni post-hoc analysis and Bonferroni adjusted pair-wise analysis.	*Nlgn3$^{y/+}$* MGH n = 21 *Nlgn3$^{y/-}$* MGH n = 14 *Nlgn3$^{y/+}$* SGH n = 16 *Nlgn3$^{y/-}$* SGH n = 9 *Nlgn3$^{y/+}$* *Pvalb$^{cre/+}$* n = 14 *Nlgn3$^{y/-}$* *Pvalb$^{cre/+}$* n = 14
4.2.A. Adult male ratio	*Nlgn3$^{y/+}$* MGH *P* = 0.881	*Levenes test* *P = 0.395*	Two-way ANOVA	*Nlgn3$^{y/+}$* MGH n = 21

Table A5.2. Detailed accounts of the statistical outputs

distance in the centre	*Nlgn3$^{y/-}$* MGH *P* = 0.530 *Nlgn3$^{y/+}$* SGH *P* = 0.261 *Nlgn3$^{y/-}$* SGH *P* = 0.692 *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* *P* =0.664 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.137	*F = 1.049*	**Social dominance environment** *P = 0.007, F $_{(1,82)}$ = 5.32 df =1, Power = 0.83* **Genotype** *P = 0.001, F $_{(2,82)}$ = 12.39 df = 2, Power = 0.94* **Genotype x Social dominance environment** *P = 0.295, F $_{(2,82)}$ = 1.24 df = 2, Power = 0.26* Followed by Bonferroni post-hoc analysis	*Nlgn3$^{y/-}$* MGH n = 14 *Nlgn3$^{y/+}$* SGH n = 16 *Nlgn3$^{y/-}$* SGH n = 9 *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* n = 14 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 14
4.2.B. Adult male time in the centre	*Nlgn3$^{y/+}$* MGH *P* = 0.358 *Nlgn3$^{y/-}$* MGH *P* = 0.377 *Nlgn3$^{y/+}$* SGH *P* = 0.359 *Nlgn3$^{y/-}$* SGH *P* = 0.119 *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* *P* =0.835 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.919	*Levenes test* *P = 0.203* *F = 1.486*	Two-way ANOVA **Social dominance environment** *P = 0.002, F $_{(1,82)}$ = 6.95 df = 1, Power = 0.92* **Genotype** *P = 0.012, F $_{(2,82)}$ = 6.54 df = 2, Power = 0.72* **Genotype x Social dominance environment** *P = 0.500, F $_{(2,82)}$ = 0.699 df = 2, Power = 0.164* Followed by Bonferroni post-hoc analysis	*Nlgn3$^{y/+}$* MGH n = 21 *Nlgn3$^{y/-}$* MGH n = 14 *Nlgn3$^{y/+}$* SGH n = 16 *Nlgn3$^{y/-}$* SGH n = 9 *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* n = 14 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 14
4.3. Adult male elevated plus maze	*Nlgn3$^{y/+}$* MGH *P* = 0.720 *Nlgn3$^{y/-}$* MGH *P* = 0.942 *Nlgn3$^{y/+}$* SGH *P* = 0.272 *Nlgn3$^{y/-}$* SGH *P* = 0.904 *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* *P* = 0.898 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.485	Levenes test *P = 0.781* *F = 0.493*	Two-way ANOVA **Genotype** *P = 0.036, F $_{(1,77)}$ = 4.56, df = 1, Power = 0.590* **Social dominance environment** *P = 0.048, F $_{(2,77)}$ = 3.16 df =2, Power = 0.55* **Genotype x Social dominance environment** *P = 0.119, F $_{(2,77)}$ =2.19 df =2, Power = 0.44* Followed by Bonferroni post-hoc analysis	*Nlgn3$^{y/+}$* MGH n = 20 *Nlgn3$^{y/-}$* MGH n = 14 *Nlgn3$^{y/+}$* SGH n = 12 *Nlgn3$^{y/-}$* SGH n = 13 *Nlgn3$^{y/+}$ Pvalb$^{cre/+}$* n = 12 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 12
4.4.A. Adult *Nlgn3$^{+/+}$* MGH, *Nlgn3$^{+/-}$* ,	*Nlgn3$^{+/+}$* MGH *P = 0.015* *Nlgn3$^{+/-}$* (H-WT) *P = 0.475*	Levenes test *P = 0.367* *F = 1.078*	Kruskal Wallis test *P = 0.000*	*Nlgn3$^{+/+}$* MGH n =13 *Nlgn3$^{+/-}$* (H-WT) n =13

Table A5.2. Detailed accounts of the statistical outputs

and *Nlgn3*$^{-/-}$ MGH activity	*Nlgn3*$^{+/-}$ (H-KO) *P = 0.751* *Nlgn3*$^{-/-}$ MGH *P = 0.294*		Followed by a Dunn's pairwise analysis	*Nlgn3*$^{+/-}$ (H-KO) n = 12 *Nlgn3*$^{-/-}$ MGH n =16
4.4.B. Adult female activity *Nlgn3*$^{+/+}$	*Nlgn3*$^{+/+}$ MGH *P* = 0.015 *Nlgn3*$^{+/+}$ SGH *P* = 0.465	Levenes test *P = 0.188* *F = 1.754*	Mann-Whitney U *P = 0.0004* *Test statistic = 7.00*	*Nlgn3*$^{+/+}$ MGH n =13 *Nlgn3*$^{+/+}$ SGH n = 8
4.4.C. Adult female *Nlgn3*$^{-/-}$ activity	*Nlgn3*$^{-/-}$ MGH *P = 0.294* *Nlgn3*$^{-/-}$ SGH *P = 0.843* *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P = 0.762*	Levenes test *P = 0.187* *F = 1.755*	One-way ANOVA *P = 0.19, F* $_{(2,36)}$ *= 1.72* *df = 2, Power = 0.34*	*Nlgn3*$^{-/-}$ MGH n = 16 *Nlgn3*$^{-/-}$ SGH n = 13 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n = 10
4.5.A. Adult *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH ratio distance centre	*Nlgn3*$^{+/+}$ MGH *P* = 0.139 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.700 *Nlgn3*$^{+/-}$ (H-KO) *P* = 0.552 *Nlgn3*$^{-/-}$ MGH *P* = 0.653	Levenes test *P = 0.250* *F = 1.412*	One-way ANOVA *P = 0.0001, F* $_{(3,50)}$ *= 8.56* *df =3, Power = 0.99* Followed by Tukey's post-hoc analysis	*Nlgn3*$^{+/+}$ MGH n =13 *Nlgn3*$^{+/-}$ (H-WT) n =13 *Nlgn3*$^{+/-}$ (H-KO) n=12 *Nlgn3*$^{-/-}$ MGH n =16
4.5.B. Adult *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$ and *Nlgn3*$^{-/-}$ MGH time in centre	*Nlgn3*$^{+/+}$ MGH *P* = 0.044 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.354 *Nlgn3*$^{+/-}$ (H-KO) *P* = 0.371 *Nlgn3*$^{-/-}$ MGH *P* = 0.784	Levenes test *P = 0.219* *F = 1.525*	Kruskal-Wallis test *P = 0.088*	*Nlgn3*$^{+/+}$ MGH n =13 *Nlgn3*$^{+/-}$ (H-WT) n =13 *Nlgn3*$^{+/-}$ (H-KO) n =12 *Nlgn3*$^{-/-}$ MGH n =16
4.5.C. Adult *Nlgn3*$^{+/+}$ ratio distance centre	*Nlgn3*$^{+/+}$ MGH *P* = 0.102 *Nlgn3*$^{+/+}$ SGH *P* = 0.210	Levenes test *P = 0.106* *F = 2.883*	Independent samples t-test *P = 0.31, df = 19*	*Nlgn3*$^{+/+}$ MGH n =13 *Nlgn3*$^{+/+}$ SGH n = 8
4.5.D. Adult *Nlgn3*$^{+/+}$ time in centre	*Nlgn3*$^{+/+}$ MGH *P* =0.044 *Nlgn3*$^{+/+}$ SGH *P* = 0.763	Levenes test *P = 0.281* *F = 1.231*	Independent samples t-test *P = 0.019, df = 19*	*Nlgn3*$^{+/+}$ MGH n =13 *Nlgn3*$^{+/+}$ SGH n = 8
4.5.E. Adult *Nlgn3*$^{-/-}$ ratio distance centre	*Nlgn3*$^{-/-}$ MGH *P* = 0.784 *Nlgn3*$^{-/-}$ SGH *P* = 0.965	Levenes test *P = 0.103* *F = 2.422*	One-way ANOVA *P = 0.083, F* $_{(2,36)}$ *= 2.67* *df = 2, Power = 0.49*	*Nlgn3*$^{-/-}$ MGH n = 16 *Nlgn3*$^{-/-}$ SGH n = 13 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n =10

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0336			
4.5.F. Adult *Nlgn3*$^{-/-}$ time in centre	*Nlgn3*$^{-/-}$ MGH *P* = 0.757 *Nlgn3*$^{-/-}$ SGH *P* = 0.678 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.641	Levenes test *P = 0.707* *F = 0.351*	One-way ANOVA *P = 0.713, F* $_{(2,36)}$ *= 0.34* *df = 2, Power = 0.10*	*Nlgn3*$^{-/-}$ MGH n = 16 *Nlgn3*$^{-/-}$ SGH n = 13 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n =10
4.6.A. Adult *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH elevated plus maze	*Nlgn3*$^{+/+}$ MGH *P* = 0.234 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.423 *Nlgn3*$^{+/-}$ (H-KO) *P* = 0.079 *Nlgn3*$^{-/-}$ MGH *P* = 0.185	Levenes test *P =0.965* *F = 0.05*	One-way ANOVA *P = 0.30, F* $_{(3,52)}$ *= 1.24* *df = 3, Power = 0.314*	*Nlgn3*$^{+/+}$ MGH n =13 *Nlgn3*$^{+/-}$ (H-WT) n =11 *Nlgn3*$^{+/-}$ (H-KO) n =13 *Nlgn3*$^{-/-}$ MGH n =19
4.6.B. Adult *Nlgn3*$^{+/+}$ elevated plus maze	*Nlgn3*$^{+/+}$ MGH *P* = 0.234 *Nlgn3*$^{+/+}$ SGH *P* = 0.193	Levenes test *P = 0.268* *F = 1.300*	Independent samples t-test *P = 0.0006, df = 19*	*Nlgn3*$^{+/+}$ MGH n =13 *Nlgn3*$^{+/+}$ SGH n = 8
4.6.C. Adult female *Nlgn3*$^{-/-}$ elevated plus maze	*Nlgn3*$^{-/-}$ MGH *P* = 0.185 *Nlgn3*$^{-/-}$ SGH *P* = 0.595 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.487	Levenes test *P = 0.295* *F = 1.26*	One-way ANOVA *P = 0.004, F* $_{(2,39)}$ *= 6.245* *df = 2, Power = 0.870*	*Nlgn3*$^{-/-}$ MGH n = 19 *Nlgn3*$^{-/-}$ SGH n = 13 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n = 10
4.7. Juvenile male activity	***Day one:*** *Nlgn3*$^{y/-}$ MGH *P* = 0.653 *Nlgn3*$^{y/+}$ MGH *P* = 0.072 *Nlgn3*$^{y/-}$ SGH *P* = 0.149 *Nlgn3*$^{y/+}$ SGH *P* = 0.186 *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ *P* = 0.081 *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ *P* = 0.990 ***Day two:*** *Nlgn3*$^{y/-}$ MGH *P* = 0.708	Levenes test Day one *P = 0.248* *F = 1.354* Day two: *P = 0.191* *F = 1.518*	Repeated measures two-way ANOVA **Day** *P = 1.11e-13, F* $_{(1,103)}$ *= 73.38* *Df = 1, Power = 0.99* **Day x Social dominance environment** *P = 0.007, F* $_{(2,103)}$ *= 5.14* *Df = 2, Power = 0.81* **Day x Genotype** *P = 0.465, F* $_{(1,103)}$ *= 0.54* *Df = 1, Power = 0.112* **Day x Social dominance environment x genotype** *P = 0.288, F* $_{(2,103)}$ *= 1.26* *Df = 2, Power = 0.27*	*Nlgn3*$^{y/-}$ MGH n = 17 *Nlgn3*$^{y/+}$ MGH n = 24 *Nlgn3*$^{y/-}$ SGH n = 21 *Nlgn3*$^{y/+}$ SGH n = 19 *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ n = 13 *Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ n = 15

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{y/+}$ MGH *P* = 0.008 *Nlgn3$^{y/-}$* SGH *P* = 0.890 *Nlgn3$^{y/+}$* SGH *P* = 0.001 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.623 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$ P* = 0.489		**Genotype:** *P = 0.000016, F $_{(1,103)}$ = 20.393* *df = 1, Power = 0.994* **Social dominance environment:** *P = 0.0003, F $_{(2,103)}$ = 8.78* *df = 2, Power = 0.97* **Social dominance environment x Genotype** *P = 0.981, F $_{(2,103)}$ = 0.019* *df = 2, Power = 0.053* Followed by Bonferroni post-hoc analysis and Bonferroni adjusted pair-wise analysis	
4.8.A. Juvenile male ratio distance in the centre	***Day one:*** *Nlgn3$^{y/+}$*MGH *P* = 0.711 *Nlgn3$^{y/-}$* MGH *P* = 0.641 *Nlgn3$^{y/+}$* SGH *P* = 0.598 *Nlgn3$^{y/-}$* SGH *P* = 0.927 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* *P* = 0.060 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.004 ***Day two:*** *Nlgn3$^{y/+}$* MGH *P* = 0.767 *Nlgn3$^{y/-}$* MGH *P* = 0.028 *Nlgn3$^{y/+}$* SGH *P* = 0.516 *Nlgn3$^{y/-}$* SGH *P* = 0.180 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* *P* = 0.694	Levenes test Day one: *P = 0.607* *F = 0.723* Day two: *P = 0.336* *F = 1.155*	Repeated measures two-way ANOVA **Day** *P = 1.65e -09, F $_{(1,103)}$ = 43.5* *df = 1, Power = 0.999* **Day x Social dominance environment** *P = 0.001, F $_{(2,103)}$ = 6.94* *df = 2, Power = 0.92* **Day x Genotype** *P = 0.154, F $_{(1,103)}$ = 2.07* *df = 1, Power = 0.29* **Day x Social dominance environment x genotype** *P = 0.234, F $_{(2,103)}$ = 1.47* *df = 2, Power = 0.31* **Genotype:** *P = 0.004, F $_{(1,103)}$ = 8.80* *df = 1, Power = 0.84* **Social dominance environment:** *P = 0.00001, F $_{(2,103)}$ = 12.89* *df =2, Power = 0.99* **Social dominance environment x Genotype** *P = 0.30, F $_{(2,103)}$ = 1.22*	*Nlgn3$^{y/+}$* MGH n = 17 *Nlgn3$^{y/-}$* MGH n = 24 *Nlgn3$^{y/+}$* SGH n = 21 *Nlgn3$^{y/-}$* SGH n = 19 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 13 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 15

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{y/-}$ Pvalb$^{cre/+}$ *P* = 0.665		*df = 2, Power = 0.26* Followed by Bonferroni post-hoc analysis and Bonferroni adjusted pair-wise analysis	
4.8.B. Juvenile male time in the centre	***Day one:*** *Nlgn3$^{y/+}$* MGH *P* = 0.417 *Nlgn3$^{y/-}$* MGH *P* = 0.310 *Nlgn3$^{y/+}$* SGH *P* = 0.121 *Nlgn3$^{y/-}$* SGH *P* = 0.724 *Nlgn3^{y+-} Pvalb$^{cre/+}$* *P* = 0.906 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.313 ***Day two:*** *Nlgn3$^{y/+}$* MGH *P* = 0.011 *Nlgn3$^{y/-}$* MGH *P* = 0.537 *Nlgn3$^{y/+}$* SGH *P* = 0.155 *Nlgn3$^{y/-}$* SGH *P* = 0.573 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* *P* = 0.087 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* *P* = 0.004	Levenes test Day one: *P =0.631* *F = 0.691* Day two: *P = 0.653* *F = 0.662*	Repeated measures two-way ANOVA **Day** *P = 0.0004, F $_{(1,103)}$ = 16.46* *df = 1, Power = 0.98* **Day x genotype** *P = 0.444, F $_{(1,103)}$ = 0.59* *df = 1, Power = 0.12* **Day x Social dominance environment** *P = 0.074, F $_{(2,103)}$ = 2.676* *df = 2, Power = 0.520* **Day x genotype x Social dominance environment** *P = 0.140, F $_{(2,103)}$ = 2.01* *df = 2, Power = 0.41* **Social dominance environment** *P = 0.002, F $_{(2,103)}$ = 6.820* *df = 2, Power = 0.913* **Genotype:** *P = 0.0002, F (1,103) = 15.18* *df = 1, Power = 0.97* **Social dominance environment x Genotype** *P = 0.129, F $_{(2,103)}$ = 2.09* *df = 2, Power = 0.42* Followed by Bonferroni post-hoc analysis	*Nlgn3$^{y/+}$* MGH n = 17 *Nlgn3$^{y/-}$* MGH n = 24 *Nlgn3$^{y/+}$* SGH n = 21 *Nlgn3$^{y/-}$* SGH n = 19 *Nlgn3$^{y/+}$Pvalb$^{cre/+}$* n = 13 *Nlgn3$^{y/-}$ Pvalb$^{cre/+}$* n = 15
4.9. Juvenile male elevated plus maze	*Nlgn3$^{y/+}$* MGH *P* = 0.481 *Nlgn3$^{y/-}$* MGH *P* = 0.126 *Nlgn3$^{y/+}$* SGH *P* = 0.788 *Nlgn3$^{y/-}$* SGH *P* = 0.463	Levenes test *P = 0.253* *F = 1.344*	Two-way ANOVA **Genotype** *P = 0.022, F $_{(1,95)}$ = 5.38* *df = 1, Power = 0.63* **Social dominance environment** *P = 0.001, F $_{(2,95)}$ = 7.63* *df = 2, Power = 0.94*	*Nlgn3$^{y/+}$* MGH n = 18 *Nlgn3$^{y/-}$* MGH n = 11 *Nlgn3$^{y/+}$* SGH n = 19 *Nlgn3$^{y/-}$* SGH n = 22

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3^{y+-} *Pvalb*$^{cre/+}$ *P* = 0.751 *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ *P* = 0.642		**Genotype x Social dominance environment** *P = 0.724, F* $_{(2,95)}$ *= 0.32, df = 2, Power = 0.10*	*Nlgn3*$^{y/+}$*Pvalb*$^{cre/+}$ n = 11 *Nlgn3*$^{y/-}$ *Pvalb*$^{cre/+}$ n = 15
4.10.A. Juvenile *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH female activity	*Day one:* *Nlgn3*$^{+/+}$ MGH *P* = 0.589 *Nlgn3*$^{+/+}$ (H-WT) *P* = 0.954 *Nlgn3*$^{+/-}$ (H-KO) *P* = 0.486 *Nlgn3*$^{-/-}$ MGH *P* = 0.317 Day two: *Nlgn3*$^{+/+}$ MGH *P* = 0.658 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.519 *Nlgn3*$^{+/-}$ (H-KO) *P* = 0.538 *Nlgn3*$^{-/-}$ MGH *P* = 0.016	Levenes test Day one *P = 0.396 F = 1.007* Day two *P = 0.237 F = 1.451*	Repeated measures ANOVA **Day** *P = 0.009, F* $_{(1,60)}$ *= 7.19 df =1, Power = 0.75* **Group** *P = 0.00004, F* $_{(3,60)}$ *= 9.2 df = 3, Power = 0.99* **Day x group** *P = 0.358, F* $_{(3,60)}$ *= 1.10, df = 3, Power = 0.28* Followed by Bonferroni post-hoc analysis and confirmed on transformed data set	*Nlgn3*$^{+/+}$ MGH n = 13 *Nlgn3*$^{+/-}$ (H-WT) n = 13 *Nlgn3*$^{+/-}$ (H-KO) n = 14 *Nlgn3*$^{-/-}$ MGH n = 24
4.10.B. Juvenile *Nlgn3*$^{+/+}$ activity	***Day one:*** *Nlgn3*$^{+/+}$ MGH *P* = 0.589 *Nlgn3*$^{+/+}$ MGH *P* = 0.059 ***Day two****:* *Nlgn3*$^{+/+}$ MGH *P* = 0.658 *Nlgn3*$^{+/+}$ MGH *P* = 0.417	Levenes test Day one *P = 0.658 F = 0.201* Day two *P = 0.952 F = 0.004*	Repeated measures ANOVA **Day** *P = 0.0006, F* $_{(1,23)}$ *= 15.64 df = 1, Power = 0.96* **Group** *P = 0.959, F* $_{(1,23)}$ *= 0.003 df = 1, Power = 0.05* **Day x group** *P = 0.00029, F* $_{(1,23)}$ *= 18.2 df = 1, Power = 0.98* Followed by Bonferroni adjusted pairwise comparisons	*Nlgn3*$^{+/+}$ MGH n = 13 *Nlgn3*$^{+/+}$ MGH n = 12
4.10.C. Juvenile *Nlgn3*$^{-/-}$ activity	*Day one:* *Nlgn3*$^{-/-}$ SGH *P* = 0.679 *Nlgn3*$^{-/-}$ MGH *P* = 0.317	Day one: *P = 0.714 F = 0.339* Day one:	Repeated measures ANOVA **Day** *P = 0.000087, F* $_{(1,51)}$ *= 18.17*	*Nlgn3*$^{-/-}$ SGH n = 20 *Nlgn3*$^{-/-}$ MGH n = 24

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{-/-}$ Pvalb$^{cre/+}$ *P* = 0.277 *Day two:* *Nlgn3*$^{-/-}$ SGH *P* = 0.431 *Nlgn3*$^{-/-}$ MGH *P* = 0.016 *Nlgn3*$^{-/-}$ Pvalb$^{cre/+}$ *P* = 0.669	*P = 0.251* *F = 1.420*	*df = 1, Power = 0.99* **Group** *P = 0.035, F* $_{(2,51)}$ *= 3.58* *df = 2, Power = 0.64* **Day x group** *P = 0.236, F* $_{(2,51)}$ *= 1.49* *df = 2, Power = 0.30*	*Nlgn3*$^{-/-}$ Pvalb$^{cre/+}$ n = 10
4.11.A. Juvenile *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH ratio distance centre	**Day one** *Nlgn3*$^{+/+}$ MGH *P* = 0.031 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.931 *Nlng3*$^{+/-}$ (H-KO) *P* = 0.190 *Nlgn3*$^{-/-}$ MGH *P* = 0.546 **Day two** *Nlgn3*$^{+/+}$ MGH *P* = 0.570 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.310 *Nlng3*$^{+/-}$ (H-KO) *P* = 0.135 *Nlgn3*$^{-/-}$ MGH *P* = 0.120	Levenes test Day one *P = 0.970* *F = 0.081* Day two *P = 0.402* *F = 0.993*	Repeated measures ANOVA **Day** *P = 0.225, F* $_{(1,60)}$ *= 1.50* *df =1, Power = 0.23* **Day x Group** *P = 0.218, F* $_{(3,60)}$ *= 1.523* *df = 3, Power = 0.38* **Group** *P = 0.285, F* $_{(3,60)}$ *= 1.29* *df = 3, Power = 0.32*	*Nlgn3*$^{+/+}$ MGH n = 13 *Nlgn3*$^{+/-}$ (H-WT) n = 13 *Nlng3*$^{+/-}$ (H-KO) n = 14 *Nlgn3*$^{-/-}$ MGH n = 24
4.11.B. Juvenile *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH time in centre	**Day one** *Nlgn3*$^{+/+}$ MGH *P* = 0.966 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.864 *Nlng3*$^{+/-}$ (H-KO) *P* = 0.173 *Nlgn3*$^{-/-}$ MGH *P* = 0.990 **Day two** *Nlgn3*$^{+/+}$ MGH *P* = 0.147 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.076	Levenes test Day one *P = 0.601* *F = 0.626* Day two *P = 0.074* *F = 2.427*	Repeated measures ANOVA **Day** *P = 0.944, F* $_{(1,60)}$ *= 0.01* *df = 1, Power = 0.05* **Day x group** *P = 0.389, F* $_{(3,60)}$ *= 1.02* *df = 3, Power = 0.26* **Group** *P = 0.207, F* $_{(3,60)}$ *= 1.57* *df = 3, Power = 0.39*	*Nlgn3*$^{+/+}$ MGH n = 13 *Nlgn3*$^{+/-}$ (H-WT) n = 13 *Nlng3*$^{+/-}$ (H-KO) n = 14 *Nlgn3*$^{-/-}$ MGH n = 24

Table A5.2. Detailed accounts of the statistical outputs

	Nlng3$^{+/-}$ (H-KO) *P* = 0.178 *Nlgn3*$^{-/-}$ MGH *P* = 0.660			
4.11.C Juvenile *Nlgn3*$^{+/+}$ MGH and SGH ratio distance centre	*Day one* *Nlgn3*$^{+/+}$ MGH *P* = 0.031 *Nlgn3*$^{+/+}$ SGH *P* = 0.448 *Day two* *Nlgn3*$^{+/+}$ MGH *P* = 0.570 *Nlgn3*$^{+/+}$ SGH *P* = 0.835	Levenes test Day one *P = 0.222* *F = 1.577* Day two *P = 0.939* *F = 0.006*	Repeated measures ANOVA **Day** *P = 0.188, F* $_{(1,23)}$ *= 1.84* *df = 1, Power = 0.25* **Day x group** *P = 0.009, F* $_{(1,23)}$ *= 7.94* *df = 1, Power = 0.77* **Group** *P = 0.757, F* $_{(1,23)}$ *= 0.10* *df = 1, Power = 0.06* Confirmed on log transformed data and followed by Bonferroni adjusted pairwise comparisons	*Nlgn3*$^{+/+}$ MGH n = 13 *Nlgn3*$^{+/+}$ SGH n = 12
4.11.D. Juvenile *Nlgn3*$^{+/+}$ MGH and SGH time in centre	*Day one* *Nlgn3*$^{+/+}$ MGH *P* = 0.966 *Nlgn3*$^{+/+}$ SGH *P* = 0.341 *Day two* *Nlgn3*$^{+/+}$ MGH *P* = 0.147 *Nlgn3*$^{+/+}$ SGH *P* = 0.138	Levenes test Day one *P = 0.480* *F = 0.517* Day two *P = 0.166* *F = 2.048*	Repeated measures ANOVA **Day** *P = 0.855, F* $_{(1,23)}$ *= 0.04* *df = 1, Power = 0.05* **Day x group** *P = 0.052, F* $_{(1,23)}$ *= 4.21* *df = 1, Power = 0.50* **Group** *P = 0.521, F* $_{(1,23)}$ *= 0.43* *df = 1, Power = 0.09*	*Nlgn3*$^{+/+}$ MGH n = 13 *Nlgn3*$^{+/+}$ SGH n = 12
4.11.E. Juvenile *Nlgn3*$^{-/-}$ MGH and SGH, and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ ratio distance centre	***Day one*** *Nlgn3*$^{-/-}$ MGH *P* = 0.990 *Nlgn3*$^{-/-}$ SGH *P* = 0.022 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.253 **Day two** *Nlgn3*$^{-/-}$MGH *P* = 0.660 *Nlgn3*$^{-/-}$ SGH *P* = 0.387	Day one Levenes test *P = 0.441* *F = 0.832* Day two *P = 0.704* *F = 0.354*	Repeated measures ANOVA **Day** *P = 0.239, F* $_{(1,51)}$ *= 1.42* *df = 1, Power = 0.22* **Day x group** *P = 0.990, F* $_{(2,51)}$ *= 0.10* *df = 2, Power = 0.05* **Group** *P = 0.139, F* $_{(2,51)}$ *= 2.05* *df = 2, Power = 0.40*	*Nlgn3*$^{-/-}$ SGH n = 20 *Nlgn3*$^{-/-}$ MGH n = 24 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n = 10

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.802			
4.11.F. Juvenile *Nlgn3*$^{-/-}$ MGH and SGH, and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ time in centre	***Day one*** *Nlgn3*$^{-/-}$ MGH *P* = 0.546 *Nlgn3*$^{-/-}$ SGH *P* = 0.357 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.060 **Day two** *Nlgn3*$^{-/-}$ MGH *P* = 0.120 *Nlgn3*$^{-/-}$ SGH *P* = 0.058 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.026	Levenes test Day one *P = 0.233* *F = 1.498* Day two *P = 0.457* *F = 0.795*	Repeated measures ANOVA **Day** *P = 0.012, F* $_{(1,51)}$ *= 6.84* *df = 1, Power = 0.73* **Day x group** *P = 0.406, F* $_{(2,51)}$ *= 0.92* *df = 2, Power 0.20* **Group** *P = 0.181, F* $_{(2,51)}$ *= 1.77* *df = 2, Power = 0.35*	*Nlgn3*$^{-/-}$ MGH n = 24 *Nlgn3*$^{-/-}$ SGH n = 20 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n = 10
4.12.A. Juvenile *Nlgn3*$^{+/+}$ MGH, *Nlgn3*$^{+/-}$, and *Nlgn3*$^{-/-}$ MGH elevated plus maze	*Nlgn3*$^{+/+}$ MGH *P* = 0.175 *Nlgn3*$^{+/-}$ (H-WT) *P* = 0.396 *Nlgn3*$^{+/-}$ (H-KO) *P* = 0.233 *Nlgn3*$^{-/-}$ MGH *P* = 0.031	Levenes test *P = 0.013* *F = 1.498*	Kruskal-Wallis test *P = 0.286* (confirmed on transformed data)	*Nlgn3*$^{+/+}$ MGH n = 10 *Nlgn3*$^{+/-}$ (H-WT) n = 13 *Nlgn3*$^{+/-}$ (H-KO) n = 7 *Nlgn3*$^{-/-}$ MGH n = 13
4.12.B Juvenile *Nlgn3*$^{+/+}$ MGH and SGH elevated plus maze	*Nlgn3*$^{+/+}$ MGH *P* = 0.175 *Nlgn3*$^{+/+}$ SGH *P* = 0.999	Levenes test *P = 0.488* *F = 0.500*	Independent samples t-test *P = 0.122* *df = 20*	*Nlgn3*$^{+/+}$ MGH n = 10 *Nlgn3*$^{+/+}$ SGH n = 11
4.12.C. Juvenile *Nlgn3*$^{-/-}$ MGH and SGH, and *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ elevated plus maze	*Nlgn3*$^{-/-}$ MGH *P = 0.031* *Nlgn3*$^{-/-}$ SGH *P = 0.712* *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ *P* = 0.878	Levenes test *P = 0.128* *F = 1.261*	Kruskal-Wallis test *P = 0.128*	*Nlgn3*$^{-/-}$ MGH n = 13 *Nlgn3*$^{-/-}$ SGH n = 19 *Nlgn3*$^{-/-}$ *Pvalb*$^{cre/+}$ n = 10

Table A5.2. Detailed accounts of the statistical outputs

5.1.C. PC1 Striatum	*Nlgn3$^{y/+}$* MGH *P = 0.488* *Nlgn3$^{y/-}$* MGH *P = 0.482* *Nlgn3$^{//+}$* SGH *P = 0.930* *Nlgn3$^{y/-}$* SGH *P = 0.066*	Levenes test *P = 0.563* *F = 0.730*	Kruskal-Wallis test *P = 0.025* (Followed by Dunn's adjusted pair-wise comparisons)	*Nlgn3$^{y/+}$* MGH n = 3 *Nlgn3$^{y/-}$* MGH n = 3 *Nlgn3$^{//+}$* SGH n = 3 *Nlgn3$^{y/-}$* SGH n = 3
5.1.D. PC1 Hippocampus	*Nlgn3$^{y/+}$* MGH *P = 0.543* *Nlgn3$^{y/-}$* MGH *P = 0.652* *Nlgn3$^{//+}$* SGH *P = 0.594* *Nlgn3$^{y/-}$* SGH *P = 0.266*	Levenes test P = 0.047 F = 4.171	Kruskal-Wallis test *P = 0.022* (Followed by Dunn's adjusted pair-wise comparisons)	*Nlgn3$^{y/+}$* MGH n = 3 *Nlgn3$^{y/-}$* MGH n = 3 *Nlgn3$^{//+}$* SGH n = 3 *Nlgn3$^{y/-}$* SGH n = 3
5.1.E. PC2 Striatum	*Nlgn3$^{y/+}$* MGH *P = 0.522* *Nlgn3$^{y/-}$* MGH *P = 0.314* *Nlgn3$^{//+}$* SGH *P = 0.802* *Nlgn3$^{y/-}$* SGH *P = 0.882*	Levenes test P = 0.318 F = 1.375	Kruskal-Wallis test *P = 0.055*	*Nlgn3$^{y/+}$* MGH n = 3 *Nlgn3$^{y/-}$* MGH n = 3 *Nlgn3$^{//+}$* SGH n = 3 *Nlgn3$^{y/-}$* SGH n = 3
5.1F. PC2 Hippocampus	*Nlgn3$^{y/+}$* MGH *P = 0.389* *Nlgn3$^{y/-}$* MGH *P = 0.564* *Nlgn3$^{y/+}$* SGH *P = 0.804* *Nlgn3$^{y/-}$* SGH *P = 0.475*	Levenes test *P = 0.216* *F = 1.850*	Kruskal-Wallis test *P = 0.033* (Followed by Dunn's adjusted pair-wise comparisons)	*Nlgn3$^{y/+}$* MGH n = 3 *Nlgn3$^{y/-}$* MGH n = 3 *Nlgn3$^{y/+}$* SGH n = 3 *Nlgn3$^{y/-}$* SGH n = 3
5.3.A. C4 Striatum	*Nlgn3$^{y/+}$* MGH *P = 0.030* *Nlgn3$^{y/-}$* MGH *P = 0.655* *Nlgn3$^{//+}$* SGH *P = 0.824* *Nlgn3$^{y/-}$* SGH *P = 0.398*	Levenes test *P = 0.117* *F = 2.189*	Two-way ANOVA **Genotype** *P = 0.010, F $_{(1,23)}$ = 7.96, df = 1, power = 0.77* **Social dominance environment** *P = 0.007, F $_{(1,23)}$ = 8.77, df = 1,* **Genotype x Social dominance environment** *P = 0.120, F $_{(1,23)}$ = 2.61, df = 1, power = 0.34*	*Nlgn3$^{y/+}$* MGH n = 7 *Nlgn3$^{y/-}$* MGH n = 7 *Nlgn3$^{y/+}$* SGH n = 7 *Nlgn3$^{y/-}$* SGH n = 6

Table A5.2. Detailed accounts of the statistical outputs

5.3.B C4 Hippocampus	*Nlgn3$^{y/+}$* MGH *P = 0.967* *Nlgn3$^{y/-}$* MGH *P = 0.410* *Nlgn3$^{y/+}$* SGH *P = 0.126* *Nlgn3$^{y/-}$* SGH *P = 0.082*	Levenes test *P = 0.449* *F = 0.917*	Two-way ANOVA **Genotype**: *P = 0.158, F $_{(1,22)}$ = 2.14, df = 1, power = 0.29* **Social dominance environment** *P = 0.182, F $_{(1,22)}$ = 1.90, df = 1, P = 0.26* **Genotype x Social dominance environment** *P = 0.589, F $_{(1,22)}$ = 0.30, df = 1, power = 0.08*	*Nlgn3$^{y/+}$* MGH n = 7 *Nlgn3$^{y/-}$* MGH n = 7 *Nlgn3$^{y/+}$* SGH n = 7 *Nlgn3$^{y/-}$* SGH n = 6
5.4.A. C3 Striatum	*Nlgn3$^{y/+}$* MGH *P = 0.857* *Nlgn3$^{y/-}$* MGH *P = 0.873* *Nlgn3$^{y/+}$* SGH *P = 0.151* *Nlgn3$^{y/-}$* SGH *P = 0.816*	Levenes test *P = 0.122* *F = 2.182*	Two-way ANOVA **Genotype** *P = 0.043, F $_{(1,20)}$ = 4.65, df = 1, power = 0.54* **Social dominance environment** *P = 0.282, F $_{(1,20)}$ = 1.22, df = 1, P = 0.18* **Genotype x Social dominance environment** *P = 0.815, F $_{(1,20)}$ = 0.06, df = 1, power = 0.06*	*Nlgn3$^{y/+}$* MGH n = 6 *Nlgn3$^{y/-}$* MGH n = 7 *Nlgn3$^{y/+}$* SGH n = 7 *Nlgn3$^{y/-}$* SGH n = 6
5.4.B. C3 Hippocampus	*Nlgn3$^{y/+}$* MGH *P = 0.190* *Nlgn3$^{y/-}$* MGH *P = 0.192* *Nlgn3$^{y/+}$* SGH *P = 0.652* *Nlgn3$^{y/-}$* SGH *P = 0.657*	Levenes test *P = 0.897* *F = 0.197*	Two-way ANOVA **Genotype** *P = 0.175, F $_{(1,22)}$ = 1.96, df = 1, power = 0.27* **Social dominance environment** *P = 0.562, F $_{(1,22)}$ = 0.35, df = 1* **Genotype x Social dominance environment** *P = 0.233, F $_{(1,22)}$ = 1.50, df = 1, power = 0.22*	*Nlgn3$^{y/+}$* MGH n = 6 *Nlgn3$^{y/-}$* MGH n = 7 *Nlgn3$^{y/+}$* SGH n = 7 *Nlgn3$^{y/-}$* SGH n = 6
A1.1A	*Nlgn3$^{+/+}$* *P = 0.109* *Nlgn3$^{+/+}$Pvalb^{cre} $^{/+}$ P = 0.856*	Levenes test *P = 0.061* *F = 4.016*	Independent samples t-test *P = 0.573*	*Nlgn3$^{+/+}$* *n = 9,* *Nlgn3$^{+/+}$Pvalb$^{cre/}$ $^{+}$* n = 10
A1.1B	*Nlgn3$^{+/+}$* *P = 0.032* *Nlgn3$^{+/+}$Pvalb^{cre} $^{/+}$ P = 0.852*	Levenes test *P = 0.831* *F = 0.047*	Independent samples t-test *P = 0.573* Mann-Whitney U test *P =* 0.905	*Nlgn3$^{+/+}$* *n = 9,* *Nlgn3$^{+/+}$Pvalb$^{cre/}$ $^{+}$* n = 10
A1.1C	*Nlgn3$^{+/+}$* *P = 0.451*	Levenes test *P = 0.135*	Independent samples t-test *P = 0.545*	*Nlgn3$^{+/+}$*

Table A5.2. Detailed accounts of the statistical outputs

	Nlgn3$^{+/+}$Pvalb^{cre} $^{/+}$ P = 0.744	*F = 2.458*		*n = 9, Nlgn3$^{+/+}$Pvalb$^{cre/}$ $^{+}$* n = 10
A1.1D	*Nlgn3$^{+/+}$ P = 0.889 Nlgn3$^{+/+}$Pvalb^{cre} $^{/+}$ P = 0.286*	Levenes test *P = 0.929 F = 0.008*	Independent samples t-test *P = 0.757*	*Nlgn3$^{+/+}$ n = 9, Nlgn3$^{+/+}$Pvalb$^{cre/}$ $^{+}$* n = 10

6.0. Published work

Some of the findings presented in this thesis have been published in:

"Kalbassi, S., Bachmann, S.O., Cross, E., Roberton, V.H. and Baudouin, S.J., 2017. Male and female mice lacking neuroligin-3 modify the behavior of their wild-type littermates. eNeuro, 4(4), pp.ENEURO-0145"