Investigation of social olfaction in a Neuroligin 3 Knockout mouse model

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

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Declaration

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Abstract

Mouse models contribute a lot to our understanding of human illness by allowing assessment of the effect of both genes and environment which can be used to answer important questions. Autism spectrum disorder (ASD) is a developmental disorder that does not have one single cause but can be due to a number of different causes. This ranges from single gene de novo mutations to an accumulation of mutations and with the addition of environmental effects ASD is a challenging disorder to understand.

One of the genes associated with ASD is the *NLGN3,* an X-linked gene which encodes the protein Neuroligin 3. Neuroligin 3 forms a cell adhesion molecule found in the synapses of neurons and its main function is maintaining the stability of synapses. Neuroligin 3 knockout (*Nlgn3*$^{y/-}$) mice have been studied for behavioural modifications and it was identified that *Nlgn3*$^{y/-}$ mice have a deficit in social memory. One of the main symptoms of ASD is deficits in social communication so this phenotype is worth exploring. As social odour production and detection is an important factor in social communication in mice we decided to pursue the social memory deficit of *Nlgn3*$^{y/-}$ mice in this context.

I identified reduced interest for social cues and altered discrimination behaviour in *Nlgn3*$^{y/-}$ mice. Also an environmental effect where the genetics of the mice in a home environment also affect reactions to social cues. Both Neuroligin 3 knockout and housing affected cFos signal in discrete brain regions in response to a particular scent cue known as major urinary protein 20 (MUP20), particularly in the dentate gyrus. Neuroligin-3 was identified in the Vomeronasal organ (VNO), which is an olfactory tissue, however the role that this protein plays in VNO function has yet to be identified.

These findings suggests that NLGN3 is a gene of importance to the social behaviour of mice and could contribute to social memory phenotypes identified in *Nlgn3*$^{y/-}$ mice.

Abbreviations

AOB: Accessory olfactory bulb

ASD: Autism spectrum disorder

CNVs: Copy number variations

DZ: Dizygotic twins

FPR: Formyl peptide receptors

GFAP: Glial fibrillary acidic protein

GFP: Green fluorescent protein

HEX: Hexadecanal

HMW: High molecular weight

IP: Immunoprecipitation

LMW: Low molecular weight

MGH: Mixed genotype housed

MOB: Main olfactory bulb

MOE: Main olfactory epithelium

MUPs: Major urinary proteins

MZ: Monozygotic

OECs: Olfactory ensheathing cells

OMP: Olfactory Marker protein

SGH: Single genotype housed

SNVs: Single nucleotide variations

V1R: Vomeronasal receptor type 1

V2R: Vomeronasal receptor type 2

VNO: Vomeronasal organ

Contents

List of figures

Chapter 1

General introduction

1.1 Social smell in mice

1.1.1 Structure of the mouse social olfactory system

The olfactory system of mice is comprised of two pathways, the main olfactory pathway and the accessory olfactory pathway. Anatomically the two olfactory pathways have similarities in their structure, they both have sensory epithelium containing olfactory sensory neurons positioned in the nasal cavity which extend axons to the central nervous system.

The main olfactory pathway sensory epithelium is known as the main olfactory epithelium (MOE) and is situated at the back of the nasal cavity resting against the bone (Figure 1.1a). The MOE has a convoluted structure made up largely of supportive cells which hold the bi-polar structured olfactory sensory neurons in place so they extend receptor expressing cilia into the nasal cavity. Odour molecules from the external environment can be inhaled and stick to the surface of the MOE where they can bind with receptors on the cilia. The olfactory glomeruli are organised in a manner that reflects the topography of the MOE so each region of the MOE is mapped to a specific region of the main olfactory bulb (MOB). The glomeruli are also grouped by the receptor content of particular olfactory neurons.

The accessory olfactory pathway sensory epithelium is known as the vomeronasal organ (VNO) and is situated between the anterior palatine foramen bones in the nasal septum. The VNO is a tube shaped structure made up of neuronal tissue on one side with cartilage supporting it and forming a lumen in the centre (Figure 1.1b). The olfactory sensory neurons cell bodies form layers which extend microvilli inward into the lumen. Unlike the MOE the VNO does not extend microvilli into the nasal cavity but instead relies on the transport of molecules in mucus flow through the cartilage lumen by active sniffing. The axons of the VNO also form bundles which reflect the receptors present in the microvilli and form

topographically organised glomeruli in the accessory olfactory bulb (AOB), which is situated at the back of the MOB, which map the apical and basal layers of the VNO (Figure 1.1b).

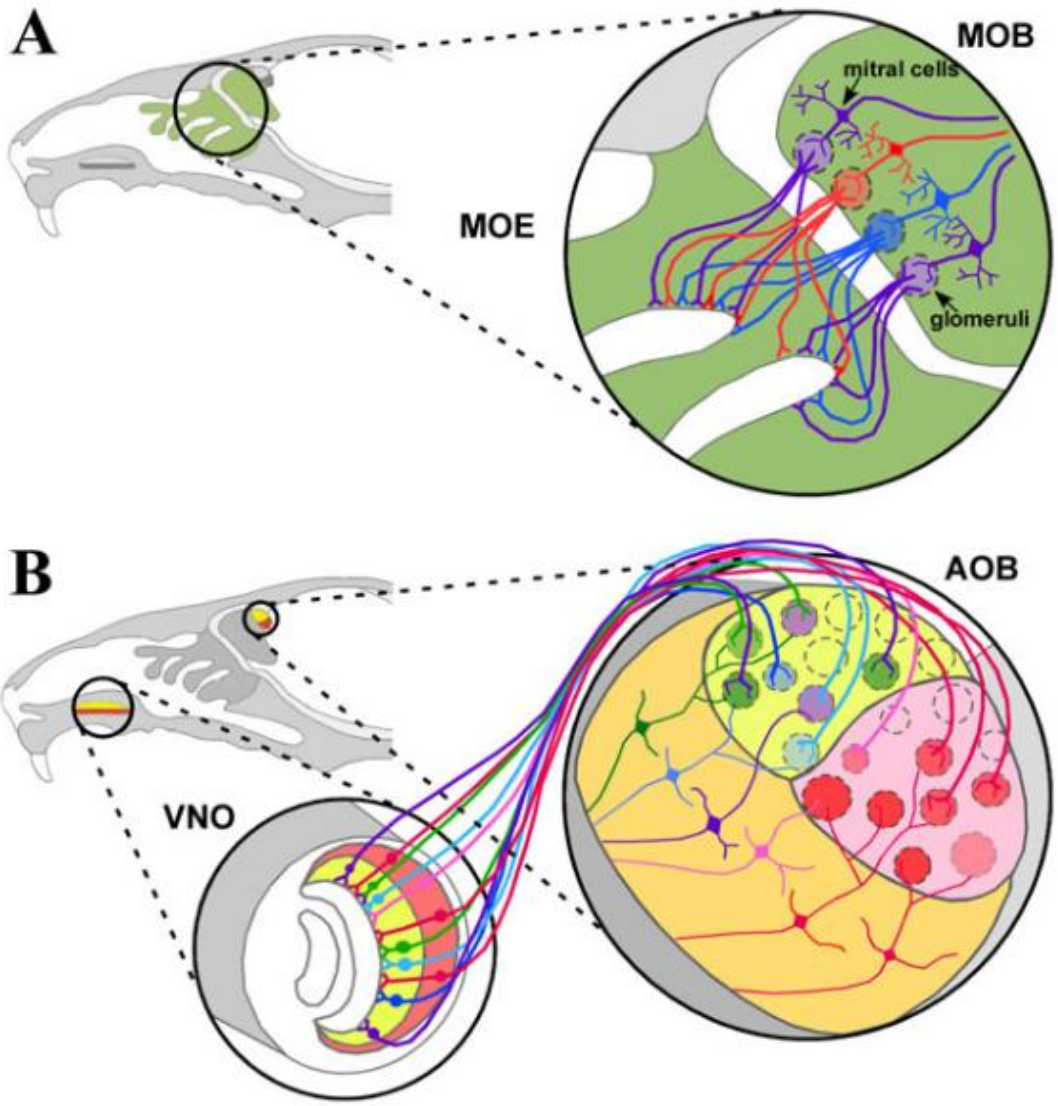


Figure 1.1: Anatomy of the mouse olfactory system. a) Olfactory sensory neurons in the MOE synapse with mitral cells in the MOB in glomeruli. b) Two layers of the VNO olfactory sensory neurons synapse on two separate regions of the AOB (Figure 3 from Dulac & Wagner 2006)

1.1.2 Structure of the Vomeronasal organ

The role of the mouse VNO is to detect conspecific and interspecies scent cues for recognition which influences mouse behaviours, this requires an abundance of different molecules.

The curved structure of the VNO is formed of two distinct layers of olfactory sensory neurons which express two main families of membrane receptors, vomeronasal receptor type 1 (V1R) and vomeronasal receptor type 2 (V2R) (Dulac & Axel, 1995, Matsunami & Buck, 1997, Ryba & Tirindelli, 1997, Herrada & Dulac, 1997). Broadly the apical layer of the VNO is made up of V1R containing vomeronasal sensory neurons (VSNs; the sensory olfactory neurone of the VNO) and the basal layer is made up of V2R containing VSNs with supportive cells dispersed throughout (Figure 1.2). Both V1Rs and V2Rs are G protein-coupled receptors, V1Rs couple with Gαi2 and V2Rs couple with Gαo.

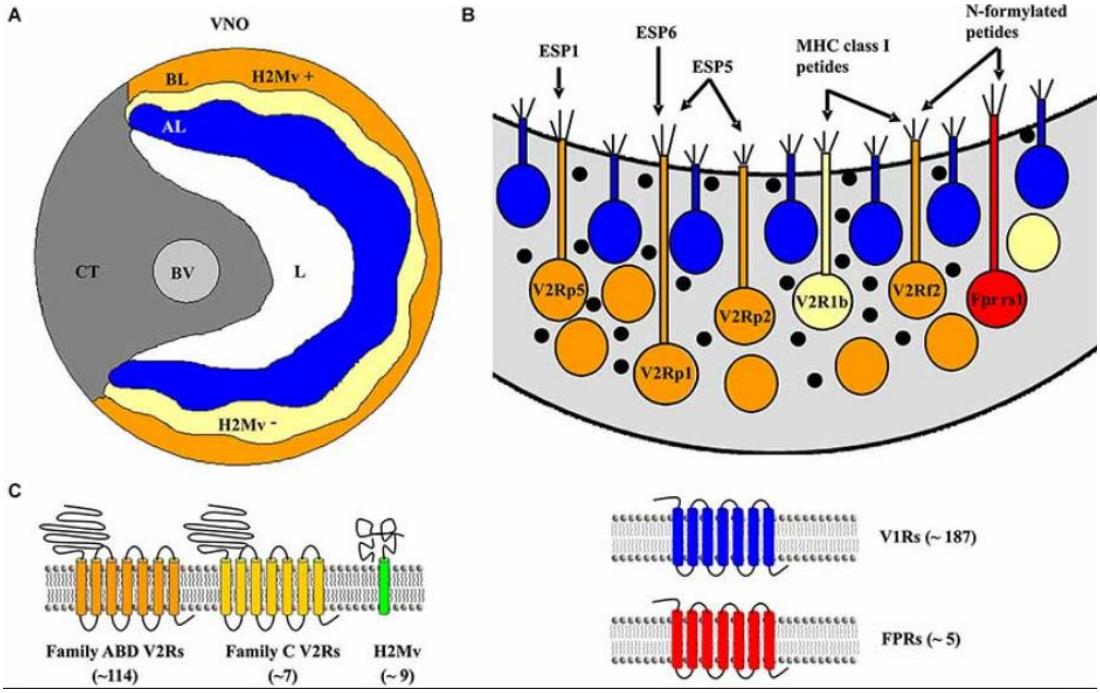


Figure 1.2: Receptor types in the VNO. A) The VNO olfactory sensory neurons are situated on one side of the lumen and can be divided into layers based on the receptors present in the cells. The apical layer (blue; AL) contains mainly V1Rs and the basal layer (yellow and orange; BL) contains mainly ABD and C V2Rs and FPRs. B) Molecules bind to some V2Rs individually and others can bind to two V2Rs. C) Schematic of basal layer receptors. (Figure 1 from Pérez-Gómez et al. 2014)

V1R-positive VSNs have been found to be reactive to the filtered urine containing small organic molecules (Holekamp, Turaga and Holy, 2008) and some sulphated steroids including members of the androgen, estrogen, pregnanolone and glucocorticoid families (Turaga and Holy, 2012) and have been implicated in detection of and behavioural response of mice to the female urine (Chamero, Leinders-Zufall and Zufall, 2012).

A number of families of V2R VSNs have been identified (A-E) and basal VSNs can express a single type of these receptors or a combination (Silvotti *et al.*, 2011). V2R-positive VSNs detect large peptide or protein families such as MUPs (Chamero *et al.*, 2007), though they seem to be more finely tuned to specific cues than V1R-positive VSNs (Isogai *et al.*, 2011), and have been associated with a number of behaviours in mice such as inbreeding avoidance, male countermarking and female sexual interest (Hurst et al., 2001; Sherborne et al., 2007; Roberts et al., 2010; Kaur et al., 2014)

As well as these more abundant receptors there are others which are less abundant. A subset of the VSNs contain formyl peptide receptors (FPR), one of which is co-expressed with Gα0 and the others that are co-expressed with Gαi2 (Chamero, Leinders-Zufall and Zufall, 2012) that are expressed in the basal layer of the VNO. These receptors are thought to be involved in the detection of pathogens (Challet *et al.*, 2009). The VSNs also contain calcium activated chloride channels, such as Trpc2, that are involved in the amplification of signal induced by molecules binding to dendritic receptors (Liman, Corey and Dulac, 1999) and is a target of genetic knockout to generate anosmic mice. Neurones in the VNO vary in their ligand binding specificity from single ligand binding to 'broadly tuned' neurons which can detect a number of molecules, however major urinary proteins (MUPs) are detected directly by Vmn2r pheromone receptors (V2Rs) located in the basilar part of the VNO which tend to be more finely tuned.

1.1.3 Major urinary proteins

Many of the signalling molecules that affect social behaviours among mice are volatile and non-volatile molecules excreted in the urine, though social cues can also be found in other bodily fluids. Volatile molecules can become airborne and so may be inhaled from a distance, attracting mice to the urine mark (Humphries et al. 1999). Non-volatile molecules tend to be larger and unable to become airborne from urine so require direct contact to affect behaviour (Roberts et al. 2012).

Major urinary proteins (MUPs) are non-volatile molecules that are thought to be important in the social signalling properties of mouse urine. These proteins are synthesised in the liver and excreted in the urine (Watson *et al.*, 2011). Mice have complex urinary olfactory scent cues with many MUPs, some of which are specific to males (Phelan et al. 2014; Asaba et al. 2014).

A number of MUPs have been identified to play an important role in initiating some mouse behaviours and in some cases have been identified as the direct cause. Male mice attack duration was found to be maintained in males that were exposed to a combination of rMUPs alone compared to male high molecular weight (HMW) urine fraction which contains MUPs and volatile urine components. Also aggression promoting MUPs stimulate V2R positive VSNs (Chamero *et al.*, 2007) though only a subset of male MUPs cause aggressive behaviour such as MUP3 and MUP20 where others such as MUP7 do not (Kaur *et al.*, 2014). Also genetic knockout of the Gαo protein, which is commonly found in the V2R positive VSNs, caused a reduction in male-male aggression (Chamero *et al.*, 2011).

MUP20 (otherwise known as Darcin; Roberts et al. 2010) is only produced by male mice and maintains its tertiary structure in high urea concentrations and effectively binds to pheromone ligands (the volatile component of the urine) allowing it to effectively slow the release of volatiles (Hurst et al. 1998, Phelan et al. 2014). This keeps the urine cue effective as a marker of territory or to attract a mate for longer and so is considered a beneficial MUP for male mice to produce. MUP20 has also been found to induce behavioural effects such as increasing

aggression in males, affecting sexual selection in females and to impact cognition (Roberts et al. 2010; Hoffman et al. 2015; Phelan et al. 2014; Roberts et al. 2012).

In the absence of direct interactions between mice males will still preform assertive behaviours such as countermarking when they were exposed to the urine of a stranger male and though they will not perform this behaviour in the presence of their own urine, the addition of MUPs can be enough to induce it (Kaur *et al.*, 2014). Taken together this suggests that MUPs are important cues in male-male mouse social behaviour and that mice can detect identity based on MUPs present in urine.

MUPs in the male urine do not only affect the behaviour of male mice but also the behaviour of female mice. This was also found to be dependent not just on MUP concentration but the specific MUPs presented (Roberts *et al.*, 2010). MUP20 in particular is an attractive MUP to female mice causing increased interest and inducing a lasting memory of scent cues (Roberts et al. 2010; Roberts et al. 2012; Hoffman et al. 2015).

Female urine also affects behaviours of other mice. Exposure to urine of females in oestrus causes increased marking micturition in dominant male mice (Hou *et al.*, 2016), decreases avoidance of predictor scent cues and attenuated reduced testosterone and increased corticosterone levels caused by exposure to predator scent (Kavaliers *et al.*, 2016).

1.1.4 Sex difference in the VNO

A number of sex specific differences in behavioural responses to social scent cues have been identified in mice despite no apparent difference in activity of sensory neurons (Dean, Mazzatenta and Menini, 2004). Some anatomical features in the number and distribution of V2R VSNs in the rat VNO (Herrada and Dulac, 1997) based on sex in the rat and a number of behavioural differences have been identified in mice (Figure 1.3). The male scent cue ESP1, secreted from the tear duct, binds with V2Rp5 and while this initiates lordosis behaviour in

female mice (Haga *et al.*, 2010) it enhances aggressive behaviours in male mice (Hattori *et al.*, 2016).

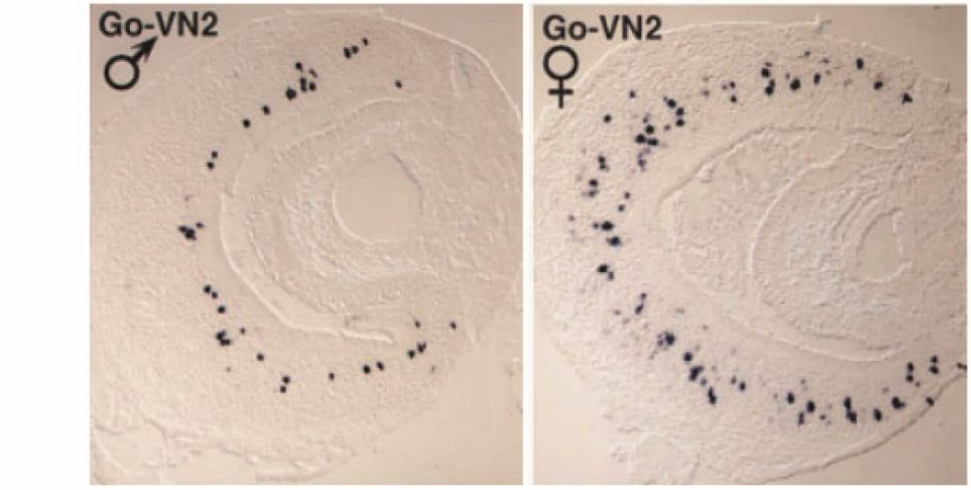


Figure 1.3:The distribution of G_o VSNs in the male and female VNO identified using in situ hybridisation (From Figure 5 from Herrada & Dulac 1997).

Also some responses to urinary cues are not only sex specific but also vary depending on particular circumstances. Male-male aggression in mice is induced by male scent cues (Chamero *et al.*, 2007) via V2R(Gαo) VSNs and though many male cues usually are involved in sexual selection in females (Roberts *et al.*, 2010; Ishii *et al.*, 2016) they cause aggression in lactating females (Chamero *et al.*, 2011).

It is therefore important, in the study of VNO function, not to assume that findings in the male mouse will apply to the female mouse and to treat them as separate experimental groups.

1.1.5 Variation in MUP profiles and signals of identity in mice

MUPs have been associated with a number of behavioural outcomes, including territorial marking and mate selection. These behaviours require that scent cues provide information about the scent cue maker and it has been identified that male mice secrete a few of the

selection of possible MUPs. This is described as a MUP profile and it has been found to transmit information to other mice about the individual identity of the scent cue maker (Roberts *et al.*, 2018).

This requires a level of variation in MUP output of individuals. There are at least 21 protein encoding genes in the gene cluster on chromosome 4 associated with MUPs (Bishop *et al.*, 1982) and though there is greater variation in the wild mouse population than in in-bred strains differences in individuals from these groups were small (Beynon and Hurst, 2004; Beynon *et al.*, 2014). Also differences in behaviour, such as territorial behaviours which rely on identification with urine marks (Hurst and Beynon, 2004), emerge between in-bred mice from the same strain (Freund *et al.*, 2013). This suggests that the differences between individuals of inbred strains is small but detectable by mice allowing them to identify individuals among other inbred mice.

Expression of MUPs is controlled by genetic variation which limits the production of the RNA of particular MUPs in the liver and also a number of sex and pituitary hormones such as testosterone (Mucignat-Caretta *et al.*, 2014), growth hormone and thyroxine (Knopf, Gallagher and Held, 1983).

Individual differences can even be seen in closely related males showing similar MUP profiles. In these mice the specific MUPs they produce due to genetics are often the same but the relative intensity of MUPs differs between individuals (Roberts *et al.*, 2018).

Environmental conditions such as social isolation have been found to affect MUP production in male mice which is thought to be associated with fluctuation in testosterone levels (Nowell, 1972; Mucignat-Caretta *et al.*, 2014). As MUPs have a significant effect on the behaviour of mice it is important to consider the potential of the environment to influence MUP production, after all MUPs themselves are become an environmental factor when urine marks are produced. It is possible to imagine in this manner that can be an interaction between MUPs in the environment and MUP production. This could mean that males with different MUP

profiles could influence each other's behaviour and MUP production. It has already been identified that testosterone levels can be reduced by stress such as changes in environment, altered housing density and social experience and change over time with the age of the mouse (Chichinadze and Chichinadze, 2008) which could in turn affect MUP production (Nowell, 1972; Mucignat-Caretta *et al.*, 2014). This means the social housing and social interactions of mice can have a significant long term impact on mouse behaviour and to be considered in the study of mouse social behaviour.

1.2 Mouse models for Autism Spectrum Disorder (ASD)

1.2.1 Convergence in mouse models for ASD?

Mouse models are commonly used in the study of social and developmental disorders, often to better understand the contribution of specific genes and even to measure the effectiveness of treatments. It is therefore important to have a good understanding of the social behaviours of experimental mice so that they can better inform our understanding of human conditions.

Autism spectrum disorder (ASD) is a developmental disorder characterised mainly by deficit in social communication and stereotyped behaviours (Tuchman, Rapin and Shinnar, 1991). Some of the more common symptoms include delayed speech and poor communicative ability, rigid thinking and repetitiveness in behaviour and intellectual disability. However there is a lot of behavioural and genetic variation in patients due to the many varied genetic presentations of ASD, this adds to the complexity of searching for therapeutic targets to treat ASD (Neale *et al.*, 2012; Roak *et al.*, 2012; De Rubeis *et al.*, 2014; Chang *et al.*, 2015). For example some patients show sensitivity to sensory cues, aggression and/or hyperactivity but others do not.

Monogenetic forms of ASD are often specific syndromes with distinct clinical symptoms such as Fragile X, Angelman, and Down's syndrome which are sometimes co-morbid with ASD (Rutter *et al.*, 1994; Miles *et al.*, 2005). These are often *de novo* mutations which cause severe

symptoms and shorten life expectancy which greatly reduces the possibility of inheritance. Polygenetic ASD, where there is no association with specific clinical disorders, is based on a combination of an accumulation of mutations in risk genes that may be *de novo* or inherited and risk factors from the environment. Diagnosis focuses more on specific behavioural characteristics that are associated with ASD. Some physical biomarkers such as microcephaly are predictive of poor outcomes in ASD (Miles *et al.*, 2005) and though post-mortem observations have identified more refined morphological features they were quite varied among the ASD patients (Ebrahimi-Fakhari and Sahin, 2015). In many ways the diagnosis of ASD depends on the clinical characteristics presented and less on biomarkers (Geschwind 2011) and as a result some characteristics are less explored than others in a therapeutic setting. As Geschwind (2011) argues, there is not even fully a consensus on whether ASD is considered "a unitary disorder versus a spectrum of dysfunction", taken together it is important to consider that there is a lot of variation between the presentations of ASD and there is a need for more flexible and nuanced therapeutic approaches to treatment.

When considering different presentations of ASD genetics are a key factor. Studies of monozygotic twins (MZ) and dizygotic twins (DZ) show that heritability of ASD is high (88% MZ and 31% DZ; Rosenberg et al. (2009)) though the interaction between genetics and environmental factors shows that the combination of these factors often necessary for ASD presentation (Hallmayer *et al.*, 2015). Many genetic mutations have been associated with ASD but not all mutations represent an equal risk for developing ASD. While risk genes can be inherited there are also a number of *de novo* mutations with high penetrance which result in ASD.

In monogenic forms of ASD single gene mutations have been associated with ASD symptoms which in some cases is part of a syndrome with a number of other characteristic symptoms. Examples of this include mutations of *FMR1* which results in Fragile X syndrome (Mclennan *et al.*, 2011) or *MECP2* which results in Rett syndrome (Bienvenu *et al.*, 2000). Syndromes such as these tend to be severe in symptom presentation with both physical and behavioural

symptoms in patients. For example Rett syndrome causes a number of physical disabilities including reduced mobility and fine movement as well as social behavioural symptoms such as social withdrawal and reduced eye contact.

Other genes with a high penetrance have been identified that are not always associated with a particular syndrome such as *NLGN3* and *NLGN4* (Jamain *et al.*, 2003) and share a common feature with a number of other high penetrance genes such as *SHANK3* (Durand et al. 2006) as that they are all synaptic genes. Analysis of *de novo* single nucleotide variations (SNVs) and copy number variations (CNVs) that identified in ASD patients shows that many of the genetic risk factors in ASD affect a few biological functions; postsynaptic density, chromatin modification/remodelling, channel activity and neuronal signalling/cytoskeleton (Figure 1.4, Chang et al. 2015).

The analysis of biological mechanisms that cause ASD can link many of the genetic factors together and give targets for research and intervention. As Cheng et al. (2015) identified, a number of genes for proteins found in the postsynaptic density are associated with ASD and so disruption of the normal functioning of the postsynaptic density could be a biological mechanism connecting a number of different genetic presentations of ASD. By addressing the biological mechanisms that affect particular behaviours I would have a greater capacity to understand the individual presentations of ASD. One of the best methods to address this is with genetic models, in this way I could analyse the symptoms that are present in animals due to specific mutations. A more thorough symptom to genetics understanding is key if ASD patients are to be assessed more individually.

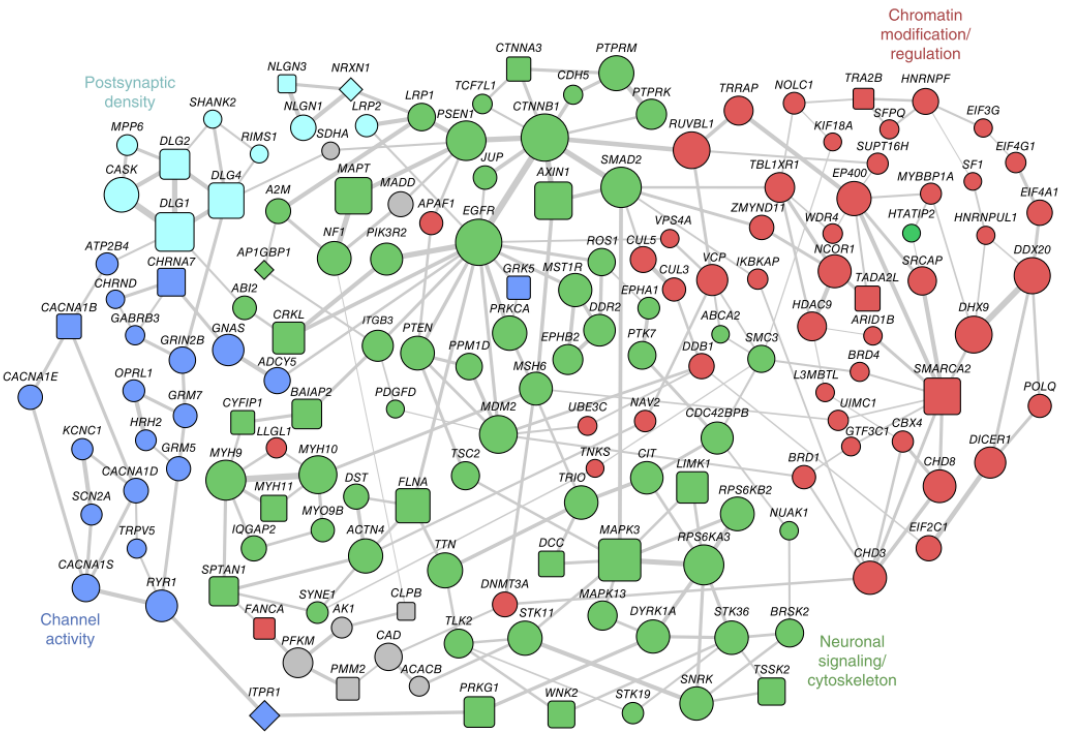


Figure:1.4: NETBAG+ and DAVID were used to generate a list of 159 genes (131 affected by de novo SNVs and 31 by de novo CNVs) with biological functions for hierarchical analysis to identify clusters of genes by biological function (Figure 1 from Chang et al. 2015)

1.1.2. The case study of Neuroligin-3

NLGN3 is a protein coding gene that has been identified as a high penetrance gene in ASD (Jamain *et al.*, 2003). Neuroligin-3 is the protein produced by *NLGN3* and is most typically recognised in its biological role as a cell adhesion molecule that binds to presynaptic proteins. In the post synaptic density Neuroligin 3 binds to directly to PSD95 in the post synaptic density and then to GKAP, SHANK and Homer proteins (Figure 1.5 Feng & Zhang 2009). Neuroligin-3 then binds Neurexin proteins across the synaptic cleft, this is thought to stabilize the synapse. Mouse models of Neuroligin 3 knockout have shown that the function of the synapse is altered (Rawson *et al.*, 2006; Tabuchi *et al.*, 2007; Baudouin *et al.*, 2012) and symptoms of ASD can be identified (Radyushkin *et al.*, 2009; Fuccillo *et al.*, 2014).

However a number of other biological functions have been found for Neuroligin 3 in addition to synaptic adhesion. Transfection of Neuroligin 3 into a human neuroblastoma cell line (SH-SY5Y) identified a role for this protein in regulation of cytosolic calcium (Shen, Huo and Zhao, 2015). Also Neuroligin 3 has been identified as having a unique role in the morphogenesis of astrocytes in young mice (Stogsdill *et al.*, 2017). Using postnatal astrocyte labelling by electroporation (PALE) astrocyte morphogenesis in brain tissue was measured and it was identified that, between postnatal age 7 days and postnatal age 21 days, knockdown of Neuroligin-3 severely affected astrocyte growth (Stogsdill et al. 2017).

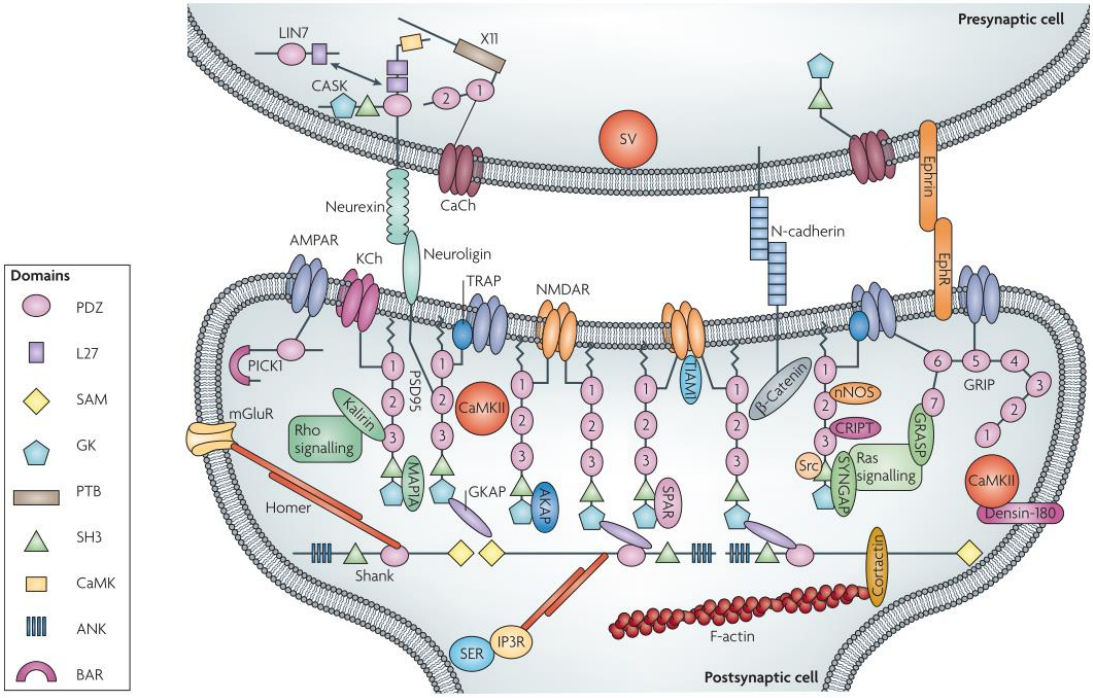


Figure 1.5: Structure of the postsynaptic density; Neuroligin-3 can be seen in the postsynaptic density extracellularly with an intracellular tail which binds to PSD95 (Figure 1 from Feng & Zhang 2009)

A number of different mutations in Neuroligin-3 have been identified in ASD patients, which are rare mutations but are thought to contribute to ASD phenotypes none the less (Jamain et al., 2003, Steinberg *et al.*, 2012; Xu, Xiong and Zhang, 2014). Mouse models exploring the contribution of Neuroligin-3 in ASD have identified a number of phenotypes of interest. Radyushkin et al. (2009) completed a battery of behavioural tests on a Neuroligin 3 knockout mouse. In particular they identified that male-male interactions between mice of the same genotype reveals no differences between *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice, likewise no differences in the interest for a novel male in the three chamber test was found. However there was a distinctive phenotype in that the social memory of the *Nlgn3*$^{y/-}$ mice where *Nlgn3*$^{y/-}$ mice had no preference for a novel male mouse over a familiar mouse suggesting that they had not formed a memory for the mouse that they previously encountered. This has also been identified in Neuroligin-3 R451C mutant mice who show no preference for novel mice and may have difficulty identifying social cues so this is not limited to the knockout model (Tabuchi *et al.*, 2007). When considered with the affect that scent cues have been identified to have on behaviours such as territory maintenance, which require memory of social cues, and also the finding that *Nlgn3*$^{y/-}$ and *Nlgn3*$^{+/-}$ *mice* have reduced magnitude of interest for scent cues of mice of the opposite sex (Dere *et al.*, 2018) it is possible that there is an issue of scent cue processing in the Neuroligin 3 knockout mouse. What the exact nature of this modification is remains to be investigated.

The *Nlgn3*$^{y/-}$ mice studied show very similar home cage behaviours and appearance to their *Nlgn3*$^{y/+}$ counterparts however pre-weaning the *Nlgn3*$^{y/-}$ mice were visibly smaller in size making them very sensitive to low temperature. To prevent this affecting survival rates in our colonies we put home cages into a heating cabinet for a few days post weaning. The *Nlgn3*$^{y/-}$ mice caught up in body size to *Nlgn3*$^{y/+}$ mice after weaning. Aside from this *Nlgn3*$^{y/-}$ mice did not show physical symptoms such as mobility issues or altered survival that would affect their ability to perform behavioural tasks.

1.3 Social olfaction in ASD

1.3.1 Anatomy of olfactory system in humans

Detection of scent cues depends on the transport of molecules to the olfactory sensory neurons in the olfactory epithelium of the nasal cavity (Figure 1.6). Air drawn in through the nose into the nasal cavity carries odorants from the environment which stick to the mucus film covering the inside of the nasal cavity, which can be aided by olfactory binding proteins (Briand *et al.*, 2002). The olfactory sensory neurons have a bipolar structure with receptors in the extending into the nasal cavity and axons forming collections of bundles which pass through the cribiform plate and synapse on olfactory bulb glomeruli in groups reflecting an organisation of the olfactory sensory neurons depending on receptor type.

Doubts surrounding the importance of olfaction in human behaviour could be inhibiting beneficial research into an element of human social communication. McGann (2017) links the undervaluation of human olfaction to Paul Broca's 19th Century analysis of the structure and function of the olfactory bulb relative to the frontal lobe. From anatomical and behavioural analysis available at the time, Broca asserted that size and function of brain regions were inherently linked. McGann (2017) considered Broca's "reductionist views" as more politically than scientifically driven and suggests that rather than consider absolute size of the olfactory bulb in different animals it is more important to consider the number of neurons, the complexity of connections and influence of olfactory cues on behaviour.

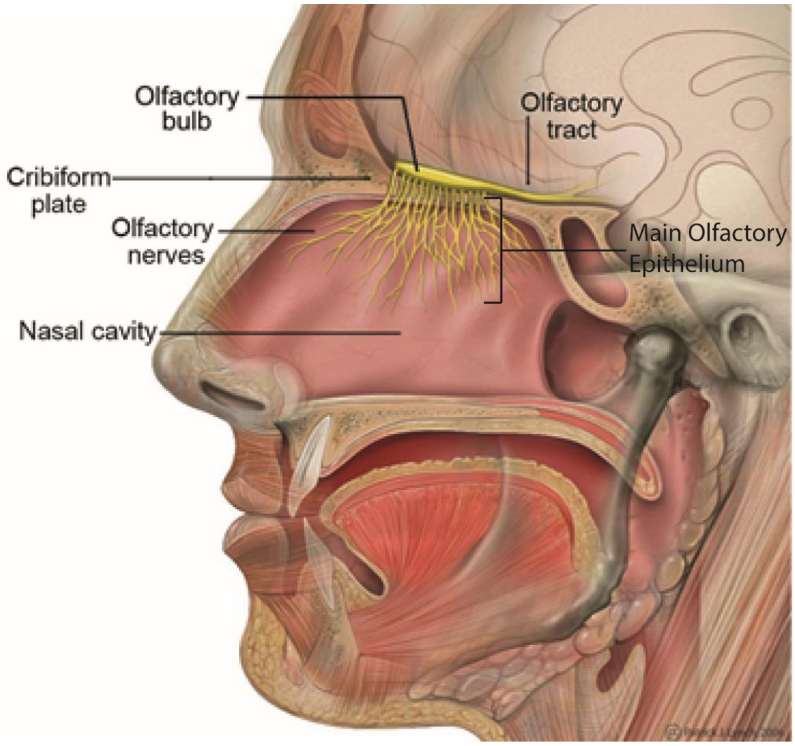


Figure 1.6: Anatomy of human nasal cavity, olfactory epithelium and olfactory bulb. Illustration by Patrick Lynch (medical illustrator; label added).

It has been identified in a number of experimental settings that social odours are a relevant factor in human social communication. However the presence of a human VNO is considered highly unlikely (Trotier, 2011). Electrophysiological responses to 'pheromones' have been associated with a specific region of the human olfactory epithelium (Monti-Bloch and Grosser, 1991) though this work has been highly contested as evidence for a distinct human VNO.

However the notion that human pheromones have an effect on human behaviour that is "slight indeed" (Trotier, 2011) are not reflected in behavioural experiments of human olfaction. Humans are able to detect individuals of the opposite sex and genetic relations (Chen and Haviland-Jones, 2000; Weisfeld *et al.*, 2003) as well as emotional states of individuals from scent cues (Chen and Haviland-Jones, 2000) which is not limited to a sexual context. Negative emotional sates such as aggression and disgust have also been found to be affected by olfactory stimuli. Humans were able to detect disgusted faces more quickly when exposed to aversive olfactory stimuli than a neutral odour (Seubert *et al.*, 2010) and also perceived faces as more

aggressive when presented with a 'fear-exercise' stimuli (Mujica-parodi *et al.*, 2009). Also feelings of anxiety were induced in individuals exposed to odours from aggressively motivated activity (Mutic *et al.*, 2016) suggesting that the perception of negative emotional states is enhanced by odour cues generated by individuals engaged in aggressive or anxiety provoking behaviours.

These findings suggest that olfaction does play a role in social communication and is therefore a factor of interest in the study of disorders such as ASD where one of the key symptoms is a deficit in social communication.

1.3.2 Generality on sensory perception in ASD

Altered social communication is one of the core symptoms of ASD. Communication deficits can be identified in verbal and non-verbal forms of communication such as language, gesturing, eye contact and response to social touch. The severity of these symptoms have a wide range and may be seen to different extents in different patients. For example patients typically have deficits in written and verbal language that can range in severity from individuals who use unusual and restricted use of language to entirely non-verbal individuals (Prelock and Nelson, 2012). Many studies of altered social communication focus on the concept of hyperresponsiveness or hyporesposiveness to sensory stimuli as a possible factor causing altered social communication in ASD patients (Senju and Johnson, 2009; Watson *et al.*, 2011; Prelock and Nelson, 2012). This implies that detection of sensory information can be abnormally enhanced in ASD, leading to increased reactions to sensory cues or that detection could be reduced leading to reduced reaction to sensory cues. This further confirms the need for a more nuanced gene to symptom understanding of ASD symptoms as within the same disorder individuals can be identified with opposite reactions to sensory stimuli that causes communication symptoms in different ways and needs to be addressed differently.

1.3.3 Autism spectrum disorder and social smell

As with other symptoms of ASD increased sensitivity to scent cues (Wicker et al. 2016; Ashwin et al. 2014) and decreased sensitivity to scent cues (Dudova et al. 2011) have both been identified in ASD patients though it is a consistent finding that behavioural responses to olfactory cues are altered in ASD. Endevelt-Shapira et al. (2017) conducted a number of experiments where ASD patients had the opposite reaction to scent cues than that of control individuals. In a spatial location task subjects were presented with two manikins with the same chance of giving correct cues (70%) but were told that one was giving better hints. One manikin secreted a fear scent cue (skydiver's body odour) and the other a neutral scent cue (sport body odour). The fear scent cue induced faster time to target behaviour in ASD participants whereas neutral scent cues had the same effect on the control group which was thought to be an effect of the perceived trustworthiness of one manikin over the other (Endevelt-Shapira *et al.*, 2017). A skin conductance experiment where participants were exposed to odour cues while watching emotional videos showed a similar effect. Increasing levels of Androstadienone (4,16-androstadien-3-one), a molecule associated with arousal found in human sweat, reduce arousal in ASD participants where it increased arousal in the control group (Endevelt-Shapira *et al.*, 2017). Also in an acoustic startle test where participants were exposed to hexadecanal (HEX), a molecule associated with reducing arousal, startle in ASD participants was not reduced though reduction in startle response was identified in the control group (Endevelt-Shapira *et al.*, 2017). This trend was also identified in an automatic imitation experiment where children had to copy the actions of an adult experimenter. Children with ASD were quicker to perform imitation behaviours when exposed to both familiar and unfamiliar female scent cues (mother's body odour or stranger's mother's body odour) which was not found in the control group (Parma *et al.*, 2013). Altered response to odours may reflect misidentification or misinterpretation of scent cues that are found in ASD individuals. This could be a factor contributing to deficit in social interactions. However there is no exploration of any potential mechanisms that may be causing the altered responses

to social odours seen in human ASD patients in either of these studies. As there are few studies addressing this topic the best target for further study is to identify a mouse model of ASD with a social smell phenotype to begin to address the underlying biological mechanism. It is possible that mechanisms causing reduced social memory in mouse models of ASD could reflect a similar issue in ASD patients and so this work could aid the understanding of the causes of social behavioural symptoms in human ASD patients.

1.4 Aims and objectives

The overall aim of this thesis was to identify if changes in social behaviour identified in *Nlgn3*$^{y/-}$ mice involves changes to social odour interest and explore what may be causing this change. As olfaction is one of the most important sensory systems for mouse communication I wanted to identify if social memory phenotypes identified by Radyushkin et al. (2009) could be found in the absence of other social cues. I also wanted to identify if the genetic modification of the mice by knocking out Neuroligin 3 was causing modifications to the VNO that were in turn causing the social memory phenotype. This is important as it could mean that the *Nlgn3*$^{y/-}$ mice are representative of an anosmic phenotype which should be considered when interpreting behaviour. Our aim could be broken down into three separate objectives; is interest and discrimination behaviour for social cues affected in *Nlgn3*$^{y/-}$ mice, is this due to modifications of the VNO in *Nlgn3*$^{y/-}$ mice and if not is social odour detection causing different brain region activation or gene expression in *Nlgn3*$^{y/-}$ mice?

Is interest and discrimination behaviour for social cues affected in *Nlgn3*$^{y/-}$ mice?

Since social memory has been highlighted as a behavioural phenotype in *Nlgn3*$^{y/-}$ mice I wanted to know if they had altered interest for mouse social cues such as MUPs and also discrimination of social cues. This can help us identify if social memory for scent cues alone is affected in *Nlgn3*$^{y/-}$ mice.

Is Neuroligin 3 expressed in the VNO and does it affect social olfaction?

As the VNO has not yet been characterised for genes or proteins associated with ASD I was interested in the Neuroligin 3 mouse I wanted to identify if Neuroligin 3 was present in the VNO. If this was the case I wanted to try to identify what functional role Neuroligin 3 might play in the VNO and if the lack of Neuroligin 3 significantly affects the function of the VNO in social odour detection. This involved attempting to identify the proteins associated with Neuroligin 3 in the VNO and assessing the VNO for physiological differences in response to different scent cues.

Is social odour detection causing different brain region activation or gene expression in *Nlgn3*$^{y/-}$ mice?

As scent cues have been found to affect sustained changes in the brains of mice I was interested to see if I could detect such changes in Neuroligin 3 knockout mice. I wanted to know if I could detect changes in specific brain regions in the *Nlgn3*$^{y/-}$ mice that might indicate why social memory is affected in these mice. I was also interested to identify differences in gene translation in *Nlgn3*$^{y/-}$ mice as this might aid us in developing a functional understanding of the changes to the brain that could be contributing to phenotypes in *Nlgn3*$^{y/-}$ mice. This data could be a useful addition to the development of a model which explains deficits in social behaviour in ASD.

Chapter 2

Materials and Methods

2.1 Animal husbandry and models used

2.1.1 Ethics

All procedures were performed in accordance with the UK Animal (Scientific Procedures) Act 1986, within the appropriate boundaries of associated project and personal licences and in accordance with Cardiff University ethical committee. Animals were housed in standard cages with covering, bedding, wooden chew stick and tunnel with standard mouse chow and water *ad-libitum*. Holding rooms were kept at 21ºC ± 2ºC on a 12 hour day/night cycle. Animals were habituated to handling from weaning (P21-P28 dependent on health and body weight) in preparation for behaviour. Before behaviour animals were moved from the holding room to the behaviour rooms and allowed to habituate for at least 30 minutes to reduce anxiety and/or stress induced by moving the home cage.

2.1.2 Neuroligin 3 knockout mice

The main mouse model used in the following experiments was a Flexible Accelerated STOP Tetracycline Operator (tetO)-knockin (FAST) Neuroligin 3 conditional knock-in model which can re-express Neuroligin 3 in a cre dependent manner, for example cross breeding with mice containing cre-recombinase, as they have a loxP flanked STOP cassette in the promoter region of Neuroligin 3. Otherwise they will not express Neuroligin 3 and are functionally a Neuroligin 3 knock-out mouse (#RBRC05451, Figure 2.1 A) Tanaka et al., 2010). Mating groups were set up to generate male wildtype mice (*Nlgn3*$^{y/+}$), male Neuroligin 3 knockout mice (*Nlgn3*$^{y/-}$), female wildtype mice (*Nlgn3*$^{+/+}$), female heterozygous knockout mice (*Nlgn3*$^{+/-}$) and female homozygous knockout mice (*Nlgn3*$^{-/-}$). These mice were mated to generate groups that were housed with only mice of the same genotype (single genotype

housed: SGH) or in groups that were housed with mice of wildtype and Neuroligin 3 knockout genotype (mixed genotype housed: MGH) using *Nlgn3*$^{+/-}$ females as dams and *Nlgn3*$^{y/+}$ as sires (Figure 2.1 B).

2.1.3 *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ mice

To generate mice with selective re-expression in the olfactory neurons I used B6;129P2-Omp$^{tm4(cre)Mom}$/MomJ (Stock No: 006668 from Jax labs) which express Cre recombinase in Olfactory marker protein expressing cells (OMP). Omp$^{tm4(cre)Mom}$/MomJ females where mating with *Nlgn3*$^{y/-}$ males to generate *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ mice. Due to Cre expression the loxP flanked STOP cassette in the promoter region of Neuroligin 3 is excised in OMP expressing cells.

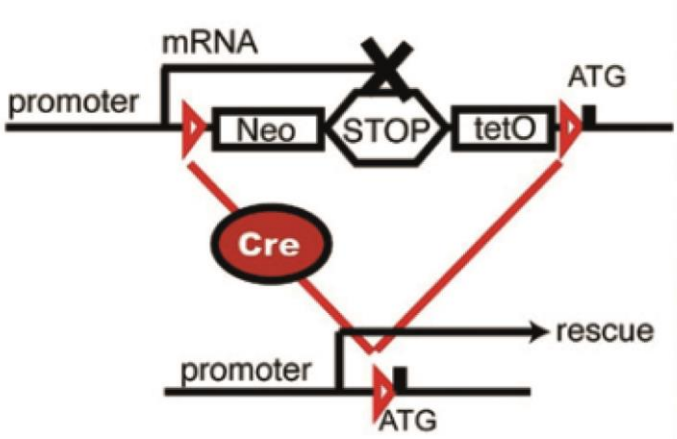
2.1.4 *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ and *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$ mice

To generate mice with selective re-expression in parvalbumin neurons I used B6.129P2-Pvalbtm1(cre)Arbr/J (Stock No: 017320 from Jax labs) mice. *Pvalb*$^{+/+}$ and *Nlgn3*$^{+/-}$ females were used as dams and *Pvalb*$^{Cre/Cre}$ and *Nlgn3*$^{y/+}$ males were used as sires to generate *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ and *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$ mice (Figure 2.1 B). Due to Cre expression the loxP flanked STOP cassette in the promoter region of Neuroligin 3 is excised in parvalbumin expressing cells

2.1.5 *c-Fos-GFP* mice

To map activity of neurons in brain tissue I used B6.Cg-Tg(Fos-tTA,Fos-EGFP)1Mmay/J (Stock No: 018306 Jax labs) mice. These mice are generated with a construct containing the Fos (FBJ osteosarcoma oncogene, minimal promoter) which drives the tetracycline regulated transactivator (tTA) sequence and a construct that contains a two hour half-life green fluorescent protein. So the activity of neurons can be identified by GFP signal and can be inhibited with the use of Doxycycline. These mice cannot be crossed with the *Nlgn3*$^{y/-}$ mice as the expression of tTA in the c-Fos-GFP mice would induce re-expression of Neuroligin 3 in the *Nlgn3*$^{y/-}$ mice (Figrue 2.1 C) Tanaka et al. 2010). Doxycycline cannot be used to resolve this as it would induce Neuroligin 3 knockout but would also inhibit expression of the GFP.

A)



B)

Experiments in males					
Dams	**Sires**	**litters obtained**	**acronym**	**symbol**	**figures**
Nlgn3$^{+/-}$	*Nlgn3*$^{y/+}$	only *Nlgn3*$^{y/+}$	SGH	●	1 to 5
		only *Nlgn3*$^{y/-}$		●	
		Nlgn3$^{y/+}$ and *Nlgn3*$^{y/-}$	MGH	● ○	
Pvalb$^{+/+}$ *Nlgn3*$^{+/-}$	*Pvalb*$^{Cre/Cre}$ *Nlgn3*$^{y/+}$	*Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$ *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$		● ●	3, 4

Experiments in females					
Dams	**Sires**	**litters obtained**	**acronym**	**symbol**	**figures**
Nlgn3$^{-/-}$	*Nlgn3*$^{y/-}$	only *Nlgn3*$^{-/-}$	SGH	●	4, 6
Nlgn3$^{+/-}$	*Nlgn3*$^{y/-}$	*Nlgn3*$^{-/-}$ and *Nlgn3*$^{+/-}$	H-KO	● ●	4, 6
Nlgn3$^{+/-}$	*Nlgn3*$^{y/+}$	*Nlgn3*$^{+/+}$ and *Nlgn3*$^{+/-}$	H-WT	● ○	6

C)

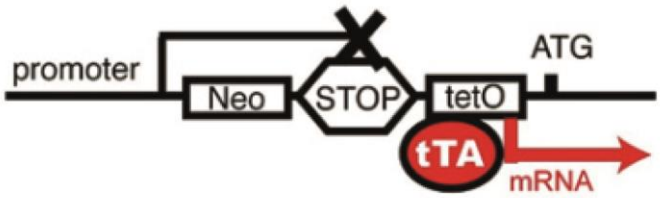


Figure 2.1:Neuroligin 3 knockout mouse construct and breeding. A) FAST system generates an inducible knockin mouse model with cre mediated rescue (Figure 2.A/B from Tanaka et al. 2010). B) Breeding scheme for *Nlgn3*$^{y/+}$, *Nlgn3*$^{y/-}$, *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice with SGH and MGH conditions and *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ and *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$ mice (Figure from Kalbassi et al. 2017). C) FAST system results in re-expression with tTA (Figure 2.C from Tanaka et al. 2010)

2.2 Behaviour

2.2.1 Experimental design of behavioural experiments

Male and female mice used in behavioural experiments were of adult age (postnatal day 60) and had no symptoms of ill health. Male mice were *Nlgn3*$^{y/-}$ or *Nlgn3*$^{y/+}$ and female mice were *Nlgn3*$^{-/-}$ or *Nlgn3*$^{+/+}$. Mice were tested into batches, each of the groups was split by home cage which was randomly assigned to a different order of odour presentation to counterbalance odour effects. This repeated measures design had the advantage of requiring low mouse numbers which is a positive welfare choice and also allows for direct comparison of the different conditions.

2.2.2 Equipment specifications

2.2.2.1 Social interest boxes

Social interest boxes consisted of four plastic 26.5 x 42.5 x 18 cm boxes with infra-red transmitting blocks beneath for dark recording (Figure 2.2). They contain a narrow hole, 11 cm from the sides and 8 cm from the base through which a cotton swab can positioned, held in place by metal clips. An overhead camera was used to record all behaviours and the footage was processed and analysed using EthoVision XT® tracking software (Noldus).

2.2.2.2 Activity boxes

Activity boxes consisted of two plastic 40 x 40 x 40 cm boxes with infra-red transmitting blocks beneath for dark recording. An overhead camera was used to record all behaviours and the footage was processed and analysed using EthoVision XT® tracking software (Noldus).

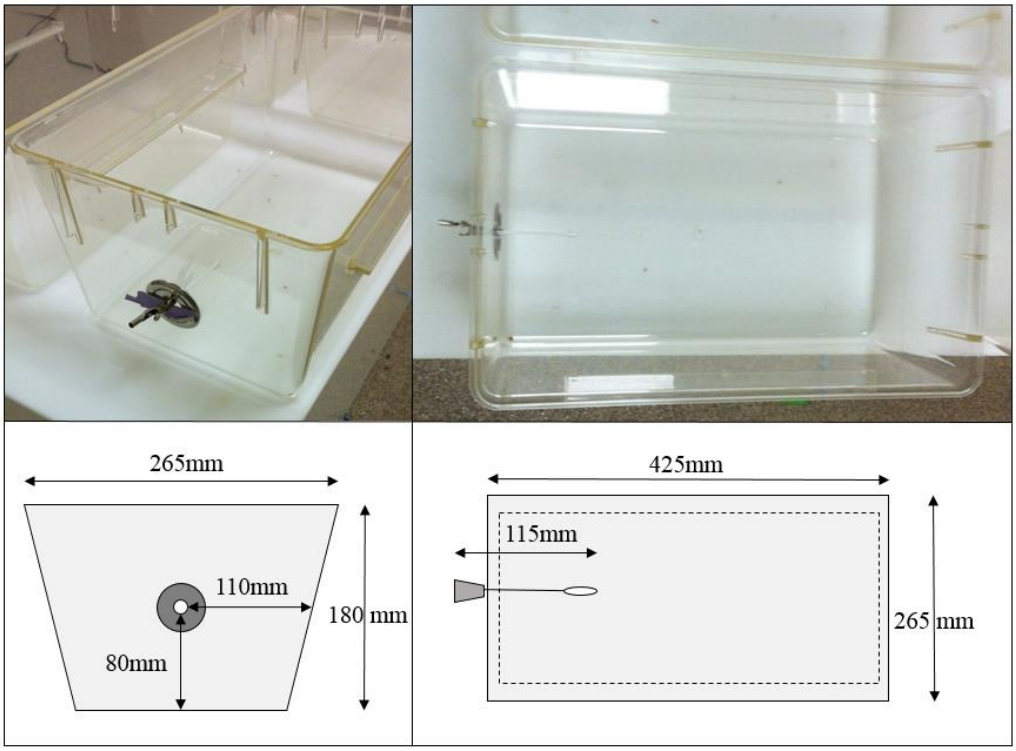


Figure 2.2: Social interest activity boxes with a hole through which cotton swabs with scent cues can be held in place using metal clips.

2.2.3 Social odour interest protocol

In order to assess the interest of mice for social odours I presented them with substances on cotton swabs and measured their interactions with the swab.

Mice were placed into a social interest box and allowed to habituate to the box for two minutes (Figure 2.3a). A clean cotton swab was then introduced to the box through hole in the side of the box as a habituation condition. Mice were recorded for two minutes with the clean cotton swab. Swabs were removed and mice were given one minute in the box. The second swab was then introduced to the box and the mice were recorded for a further two minutes. This swab is the test swab and is stained with 10µl social odours or left clean depending on the testing condition.

To prevent an effect of odour presentation having too much influence on the results the order of exposure to social cues was counterbalanced by randomly assigning the home cages of the

experimental mice to a different order of scent cue presentation. Mice were only tested for social interest once per day in the dark.

Interest was defined as direct physical contact with the cotton not including interaction with the stick such as climbing and pushing. This included sniffing, biting and holding the cotton toward the face (Figure 2.3b). Recordings of the behaviour were viewed and interactions were timed and the total time interacting was taken for each mouse for each exposure. As a control measure a random selection if the recordings were scored by separate individuals and these scores were compared with my scoring. As there were no significant discrepancies between the scores I continued with the manual scoring.

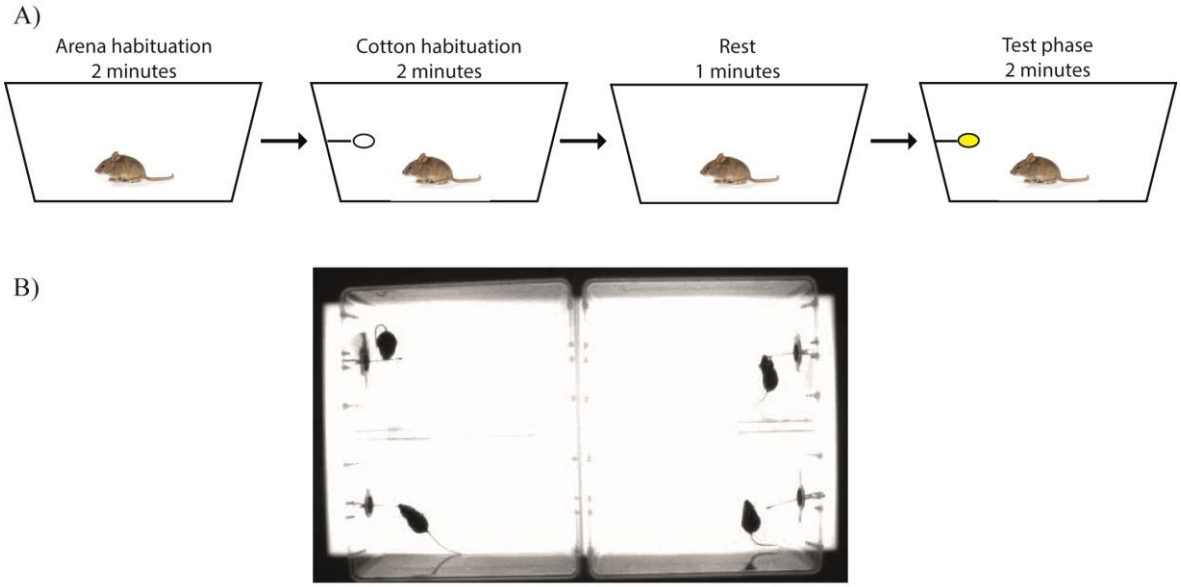


Figure 2.3: A) Social odour interest apparatus set up. Mice are habituated to the arena then exposed to a clean cotton swab. The swab is removed for one minute before the test swab is added and recorded for two minutes. B) Camera view of mice in social odour interest apparatus. Both the mouse in the top right hand courner and the mouse in the bottom left hand corner would be considered to be sniffing the cotton bud but the mouse in the top left hand corner would not as it is chewing the stick rather than interacting with the cotton bud.

2.2.4 Social odour discrimination protocol

In order to begin investigating social memory I assessed the discrimination of mice between two social odours.

Two petri dishes were placed in opposite corners of an activity box (Figure 2.4). The box had a home cage scraping scent cue placed at location 1 (S1) and a clean cotton swab at location 2 (C). Mice were the recorded free roaming in this environment for 10 minutes to habituate, this allowed the mice to become familiar with the scent cue S1. Mice were then returned to their home cage for 30 minutes for memory retention. The box was then set up with the same home cage scraping scent cue in location 1 as in habituation (S1) and a home cage scraping scent cue from a different (novel) cage in the location 2 (S2). Mice were then recorded free roaming for a 4 minute test phase. The arena and the dishes are all cleaned with 70% ethanol and thoroughly dried between each trial to prevent contamination of the stimuli or the arena with scent cues. Fresh scrapings are used for each new trial. Acquisition and test phases took place in darkness.

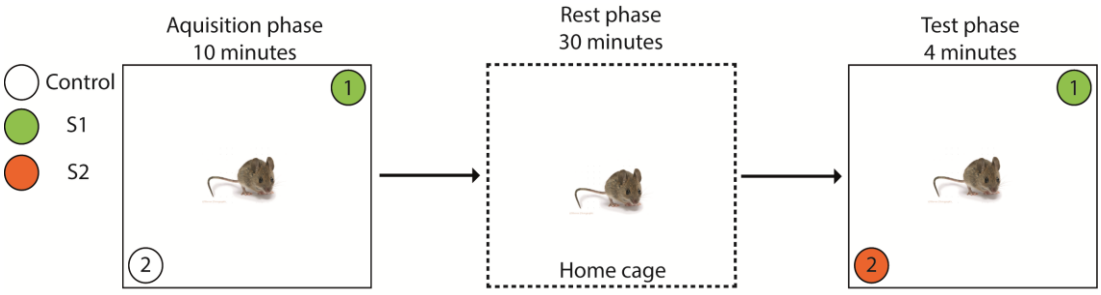


Figure 2.4: Social odour discrimination apparatus set up. Mice are exposed to a scent cue (green, S1) and a control cotton swab (white) for 10 minutes, returned to home cage for 30 minutes and then returned to test box with the previous (now familiar) scent cue (green) and a new scent cue (orange, S2).

2.3 Social odours

2.3.1 Cage scraping and urine scent cues

Social discrimination odours were cage scrapings. A clean cotton bud (white cotton wool on a clear plastic stick) was scraped it around a suitable mouse home cage (depending on the experimental conditions). The cotton bud was cut short and stick in a 3.5cm diameter lidded petri dish with a hole drilled in the top.

Social interest odours were whole urine or HMW urine fraction. Fresh urine was taken from wildtype or knockout stranger males and females by scuffing and gently rubbing abdomen and catching urine flow in a 1.5ml tube. This method has an advantage over cage collection as it prevents contamination of the urine sample and loss of volatiles due but does run the risk of introducing stress cues into the urine. I attempted to reduce this by having long rest periods between collection times and only allowing a short scuffing time for each attempt to collect urine.

Collected urine was flash frozen on dry ice and stored at -20˚C. Samples were then combined by group (e.g. wildtype male) and aliquoted for use in whole urine experiments or were separated by molecular weight using Amicon®Ultra-15 centrifugal filters (Millipore, 30kDa cut off) to produce low molecular weight (LMW) factions and high molecular weight (HMW) fractions. The LMW fraction was collected in the flow through and the HMW fraction was removed from the filter by dilution to original total volume with artificial urine (NaCl 120mM, KCl 40mM, NaH4OH 20mM, CaCl2 4 mM, MgCl2 2.5mM, NaH2PO4 15mM, NaHSO4 20mM, Urea 333mM at pH 7.4) to maintain physiological concentration. On the day of behaviour the social odours were placed in tubes labelled 1 to 4 which indicated one of each of the scent cues, which was decoded for the analysis.

2.3.2 Recombinant scent cues

Exposure to recombinant MUPs for mapping consisted of adding 2µl diluted rMUPs (+/- 0.2ug/ml rMUP20 or rMUP7, provided by Hurst lab, Liverpool University) or control PBS directly to the nose of mice and 5µl to filter paper that was dropped into the home cage for the animal to interact with.

2.4 Dissection

2.4.1 Brain tissue dissection

To gather brain tissue for mass spectrometry or RNA sequencing, mice were sacrificed by cervical dislocation, the brains were removed and transferred to a PBS lubricated metal cutting surface on ice. The brains were separated into olfactory bulb, cortex, striatum, thalamus, hippocampus, cerebellum, brain stem. Each portion was put into individual labelled 1.5ml micro centrifuge tubes and the remaining brain tissue was placed in a 1.5ml micro centrifuge tube marked 'rest of brain'. All tubes are kept on ice until all the tissue is gathered then the tubes are placed in liquid nitrogen to flash freeze the tissue.

2.4.2 Vomeronasal organ (VNO) dissection

To collect the VNO the head of mice (with brains removed as above if required) were submerged in ice cold PBS in a small petri dish and placed onto a cooled metal cutting surface under a dissecting microscope. The jaw was then removed and the soft pallet peeled away to expose the bones beneath. The bones of the upper jaw were then broken to open the space where the VNO sits (Figure 2.5, Mohrhardt et al. 2018), the VNO is then cut out and carefully removed and placed in a labelled 1.5ml micro centrifuge tube and flash frozen in liquid nitrogen or kept for fresh for lysis.

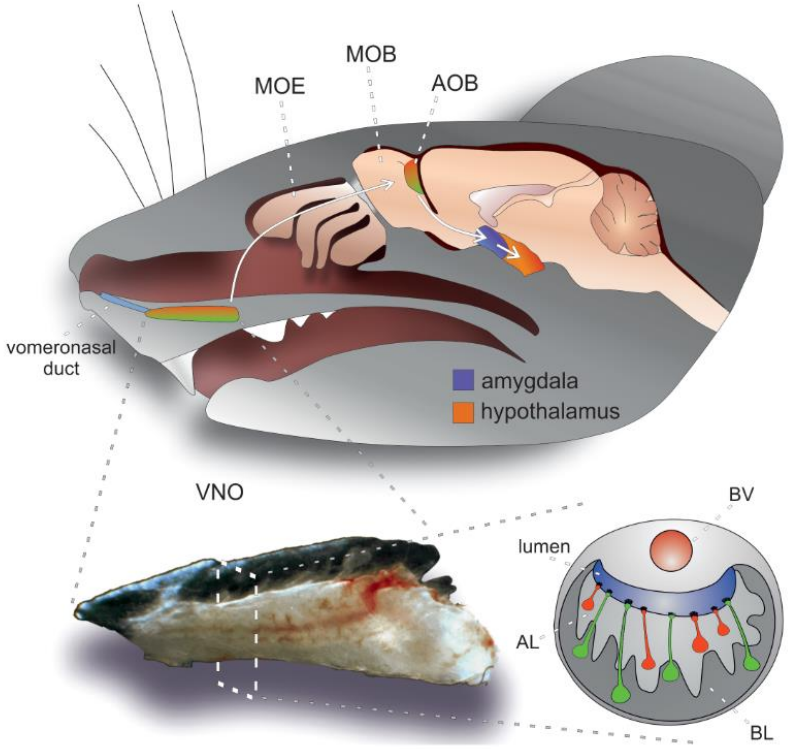


Figure 2.5: Accessory olfactory system anatomy. The vomeronasal organ (VNO) apical layer (AL) and basal layer (BL) are depicted in orange and green indicating where they synapse with the accessory olfactory bulb (AOB). (Taken from Figure 1, Mohrhardt et al. 2018)

2.5 Tissue processing for protein and RNA analysis

2.5.1 Tissue lysis for western blotting

Tissue from dissection is taken and added fresh to lysis buffer (Tris HCL 50mM, 1mM EDTA, 0.1% SDS, NaCl 150mM, 1% triton, 10mM NaF Phosphatase inhibitor, 1mM NaVO4 Phosphatase inhibitor, 1mM DTT) for one hour rotating at 4˚C then centrifuged for 30 minutes at 15000rpm, 4˚C. For western blotting lithium dodecyl sulfate buffer (106mM Tris-HCL, 141mM Tris-base, 2% lithium dodecyl sulfate, 10% glycerol, 0.51mM EDTA, 0.22mM G250 Coommassie Blue, 0.175mM Phenol Red, 10mM DTT; pH 8.5) was added to extracted sample before loading into a 4-12% Bis-Tris polyacrylamide NuPage gel then gel was wet transferred to membrane. After 1 hour blocking in 5% milk, Nlgn3 antibody (Abcam) was added 1/5000 and kept shaking overnight at 4˚C. The membrane was then incubated with secondary

antibody for 1 hour and visualised using horseradish peroxidase and Alexa-488 conjugated secondary antibodies (Thermo Fisher Scientific).

2.5.2 Immunoprecipitation

Samples were lysed in fresh lysis buffer (20mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1% Triton, 10mM NaF, 1mM Na^3VO^4, 0.1% Protease inhibitor (P8340)) at 1ml buffer per 10mg of tissue. Tissue was manually broken up in the lysis buffer and incubated at 4˚C rotating. Separately 10µl of Protein G Sepharose beads (in ethanol) were washed with 500 µl cold PBS. Samples were centrifuged and supernatant was removed and added to the 10µl of washed beads to pre clear for 30 mins rotating at 4˚C. Input was collected by removing 10% of sample. Fresh Protein G Sepharose beads for IP were prepared by washing 20µl of beads with 500µl of lysis buffer then 2µl of Nlgn3 antibody (synaptic systems) was added. Remaining supernatant from the lysis was added and incubate 2 hours rotating at 4˚C. Samples were centrifuged then remove supernatant and beads were washed with lysis buffer to clear unbound proteins. IP was eluted from beads with 50µl of LDS buffer (for 500uL, 250uL of 4X LDS, 50uL of DTT and 150uL of water).

2.5.3 RNA extraction

Tissue from dissection was thawed on ice then broken up in 1ml Trizol in a 1.5ml micro centrifuge tube. The suspended tissue was then separated out but the addition of 200µl chloroform and centrifugation. The clear upper phase was collected and homogenised with 500µl 100% isopropanol per 1ml Trizol. The Qiagen Rneasy mini kit (74104) as par the instructions in the kit was then used for extraction for RNA from the suspension. RNA was stored at -80˚C.

2.5.4 Reverse Transcription

RNA from extraction is diluted to 1250ng in 11µl with dH_2O in a 1.5ml micro centrifuge tube then 1µl of random primers and 1µl of dNTPs were added. The mixture was then incubated for 5 minutes at 65˚C and then put on ice for 1minute. Next 4µl of 5x buffer, 1µl DTT 0.1M,

1µl RNAsin and 1µl superscript III reverse transcriptase were added and then mixture was incubated for 5 minutes at room temperature then for a further 2 hours at 50˚C. To inactivate the enzyme the mixture is heated to 70˚C for 10 minutes. cDNA was stored at -20˚C.

2.6 Tissue processing for imaging

2.6.1 Perfusion and tissue preparation

In order to detect immediate early genes in brain and VNO tissue mice were perfuse fixed and selected tissues were cut and stained. If the mice were exposed to a scent cue they were given one hour in the home cage with the scent cue. After exposure mice were culled by intraperitoneal injection of pentobarbatol (Euthatol) overdose. The animal was then pinned on its back by its paws, the chest cavity was opened and the needle of a perfusion pump was clamped into the left ventricle of the heart. The right atrium of the heart was then cut and the animal was flushed with phosphate buffer (PB) till it is running clear from the atrium. The pump was then switched over and 40ml of 4% paraformaldehyde (PFA) was pumped through. Tissue was extracted and fixed in 4% PFA overnight at 4˚C. Tissue was then washed in PB and cryoprotected by submersion in 30% sucrose solution at 4˚C overnight (until the tissue sinks). Brain tissue was placed in a plastic mould which was filled with OCT, frozen in dry ice and stored at -80˚C. Tissue was cut to 16µm sections on cryostat and dry mounted then stored at -20˚C until staining.

2.7.2 Immunohistochemistry

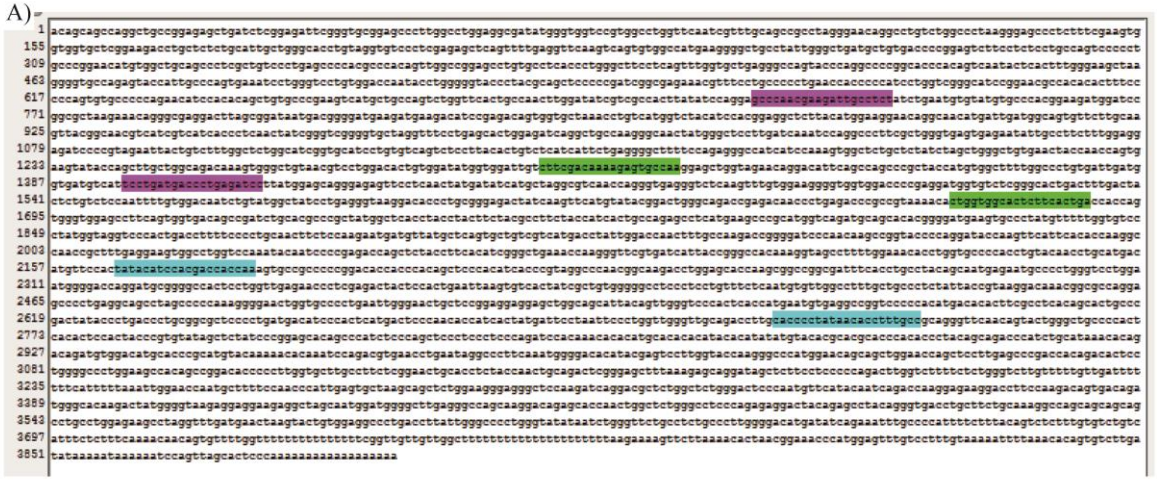
Mounted sections were blocked in 10% normal donkey serum (NDS) for one hour at room temperature then in C-fos primary antibody 1/250 in 2%NDS Tris-Triton (Santa Cruz Biotechnology sc-52 Lot# E3014) overnight at room temperature. After washing in Tris-Triton (3 x 10 mins room temperature) slides were incubated for one hour in secondary antibody (rb 555: 1/1000 in 2% NDS/Tris-Triton) at room temp. After washing slides were stained with DAPI before mounting with Dako mounting medium.

2.7.3 In Situ Hybridisation

2.7.3.1 Generating DNA template for probe generation

DNA template primers were generated manually using ApE and Blast software or copied from the Allen Brain atlas experiment ISPG2 to cover the start, middle and end of the sequence (Figure 2.6a). The subsequent list of primers (Figure 2.6b) were then generated commercially (Sigma) and used in PCR reaction with whole cDNA from RNA extraction and in probe generation for in situ hybridisation experiments.

A)



Probe origin	Probe Title	DNA sequence	Primer sequence	Length bp
Sysy antibody epitope 1849-1868	Probe 1 (A)	TATACATCCACGACCACCAA	TATACATCCACGACCACCAA	576
Sysy antibody epitope 2404-2424	Probe 1 (B)	CACCCCTATAACACCTTTGCC	GGCAAAGGTGTTATAGGGGTG	576
Abcam antibody 399-418	Probe 2 (A)	GCCCAACGAAGATTGCCTCT	GCCCAACGAAGATTGCCTCT	701
Abcam antibody 1080-1099	Probe 2 (B)	TCCTGATGACCCTGAGATCC	GGATCTCAGGGTCATCAGGA	701
Allen brain atlas SS2 & TC 1302 - 1687	Probe 3 (A)	CTTCGACAAAAGAGTGCCAA	CTTCGACAAAAGAGTGCCAA	386
Allen brain atlas SS2 & TC 1302 - 1687	Probe 3 (B)	CTGGTGGCACTCTTCACTGA	TCAGTGAAGAGTGCCACCAG	386

Figure 2.6: Primer pairs generated for Neuroligin 3 in situ hybridisation probe. A) Location of each of the probe pairs in the DNA sequence of NLGN3. Blue highlights represent primer pair 1, purple highlights represent primer pair 2 and green highlights represent primer pair 3. B) Table shows the origin, title, sequence and subsequent product length of the probe pairs.

2.7.3.2 TOPO cloning for probe generation

The DNA template was incubated with pCRII- Blunt-TOPO (New England Biolabs), salt solution and water for 5 minutes at room temperature and then added to OneTop10 competent cells (New England Biolabs) and incubated on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42˚C and transferred to 250µl of LB medium and incubated on 200rpm shaker at 37˚C for 1 hour. The bacteria was then aseptically spread on pre-warmed agarose + 1/1000 kanamycin plates and incubated at 37˚C overnight. Colonies were then picked from the plates and transferred to 3ml LB + 1/1000 kanamycin on a 200rpm shaker at 37˚C overnight. To preserve the bacteria 0.9ml of the bacteria infected broth was added to 0.9ml of 50% glycerol in a 2ml microcentrifuge tube and kept at -80˚C. The rest of the bacteria infected broth was processed using the QIA miniprep kit (27106) to separate the plasmid.

2.7.3.3 Digestion of TOPO product for linearized probe

To check for the insert in the plasmid a restriction digestion was used on the miniprep product. The miniprep product was added to an appropriate restriction enzyme and buffer then incubated for 1 hour at 37˚C. The product of the digestion was then run on a 1.5% agarose gel and measured for expected band size. The colonies that produced a band the expected size of the cut insert were then selected for further processing. At this point the plasmid was sequenced by Eurofins genomics. The selected colonies were then used to inoculate 200ml LB + 1/1000 kanamycin overnight on 200rpm shaker at 37˚C. The plasmid was then separated from the bacteria using the Qiagen maxiprep kit (12362) and re-suspend in 80ul TE buffer. The plasmid DNA was then quantified (25-50µg of DNA required for probe formation) and separately cut by two appropriate restriction enzymes to produce sense and anti-sense probes (in this case with Spe1 and Not1). The linear DNA was then purified with QIA gel extraction kit (28720) before transcription with DIG RNA labelling mix or Flourescin labelling mix (for probes intended for double in situ hybridisation protocol use; Sigma). Probes were then purified using G50 microspin columns (GE Bioscience) and RNase inhibitor is added as a precautionary measure against probe degradation.

2.7.3.4 Hybridisation and probe

An RNase free environment was maintained with Rnase zap and use of sterile disposables. Sections are taken from -20˚C storage and allowed to thaw for 30 minutes at room temperature. Sections were then post-fixed for 15 minutes in 4%PFA at room temperature, then washed 3x3minutes with PBS. To quench endogenous peroxidases the sections were incubated for 15 minutes in 3% hydrogen peroxide at room temperature, then washed 3x3minutes with PBS. Sections were then acetylated for 10 minutes in acetylation buffer (acetic anhydride 50ul + Triethanolamine 234ul + dH_2O 20ml) to allow better probe binding, then washed 3x5 minutes in PBS. A humid chamber was made using a heat resistant lidded tray lined with clean filter paper saturated with humidifying buffer (50% 5xSSC, 50% formamide) and fitted with props to keep slides raised off the filter paper. Sections were then pre-hybridised in the humid chamber for 60 minutes at 65˚C in hybridisation buffer (50% formamide, 0.1% tween-20, 0.25% CHAPS, 250µg/ml yeast tRNA, 50µg/ml herring sperm, denhardts, SSC, 50µg/ml heparin, 2.5mM EDTA). Probes were prepared by adding the required number of probe to a small amount of hybridisation buffer and heat shocked at 80˚C for 5 minutes then cooled on ice for 1 minute before adding hybridisation buffer to the correct total volume for the given probe. The probe mixture was then added to the sections and covered with hybridisation cover slips at 65˚C overnight in the humid chamber. Sections are then washed 3x20 minutes in pre-warmed 0.2x SSC at 65˚C.

2.7.3.5 Probe Detection

Sections are first equilibrated in TN buffer (100 mM Tris-HCl and 150 mM NaCl) for 5 minutes then incubated in Anti-DIG-POD 1:2000 diluted in TNB (0.5% Blocking Reagent of Perkin Elmer in TN buffer) for 30 minutes. Sections were washed 3x5 minutes in TNT (0.01% tween in TN buffer) at room temperature then incubated in Cy3-Tyramide 1:50 in Amplification Reagent (TSA™ kit) for 30 minutes at room temperature. Sections were washed 3x5minutes in TNT then stained with DAPI (1/4000 in PBS) for 5 minutes and washed with PBS before mounting with Dako mounting medium and cover slips.

2.8 c-Fos counting and heat mapping

2.8.1 Image collection and counting

Sections of *c-Fos-GFP* mouse brain were searched manually for signal using a confocal microscope (20x magnification) and images were taken where signal was identified. To map the signal through regions of interest from The Mouse Brain in stereotaxic coordinates (compact second addition, George Paxinos and Keith B.J. Franklin) was used to make an eight section frame of reference and images were taken corresponding to each of the regions. Sections of brains of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice stained with antibody were imaged in the same manner using the same frame of reference. All images were taken at 1024 x 1024 size at 20x magnification in a 3 x 3 tile scan with z-stack. Fiji (imageJ) was used to process the images, z-stacks were merged two sections either side of the midpoint (where the image was clearest) for a total of 5 sections. The images were then divided into sub regions and the points of signal in each region were counted using the Fiji multipoint tool. Regions were measured using the Fiji measure tool.

2.8.2 Heat map generation

Count data for each group was averaged for each of the regions then the image frame of reference generated from The Mouse Brain in stereotaxic coordinates (compact second addition, George Paxinos and Keith B.J. Franklin) were edited using Adobe Illustrator to remove labels and sub-regions were drawn over the top of atlas images. A heat map key was made and then each of the regions was coloured according to the count data, the all the images were assembled in Adobe Illustrator.

2.9 Surgery and AAV injection

The AAV construct glia (AAV5-GFAP(0.7)-EGFP-T2A-iCre, Vector labs) was injected into both hemispheres of the motor cortex of *Nlgn3*$^{y/-}$ and allowed four weeks for re-expression.

Five animals were injected, one of the group was used to validate re-expression (western blot) and the others were used to send to mass spectrometry (immune precipitation for Nlgn3).

In order to inject the AAV the mice underwent stereotactic surgery. Preparation of the animal consisted of inducing surgical plane anaesthesia with isoflurane, positioning the mouse over a heated pad with an isoflurane anaesthesia mask and fixing the head of the animal into the sereotactic frame (Figure 2.7). The scalp of the mouse was shaved and sterilised with iodine before the scalp was cut to reveal the scull of the mouse. After location of the motor cortex using the stereotactic frame, drilling equipment was used to create an opening in the skull of the mouse. A fine needle was used to inject 0.5µl of the AAV into the appropriate site (ML 1.9mm, AP 1.45mm, DV 1.3mm) and then carefully removed. The scalp of the mouse was then sutured and the mouse was removed from the frame to a heated cage where recovery was monitored. Metacam was used as a painkiller and the animals were administered 1ml of saline during the surgery to prevent dehydration. Animals were allowed to recover from the anaesthesia in a pre-warmed cage and were then returned to the home cage with littermates on blue towel/tissue. Animals were monitored and bedding was changed daily until surgery wounds were scabbed over and stitches removed then animals were returned to conventional bedding.

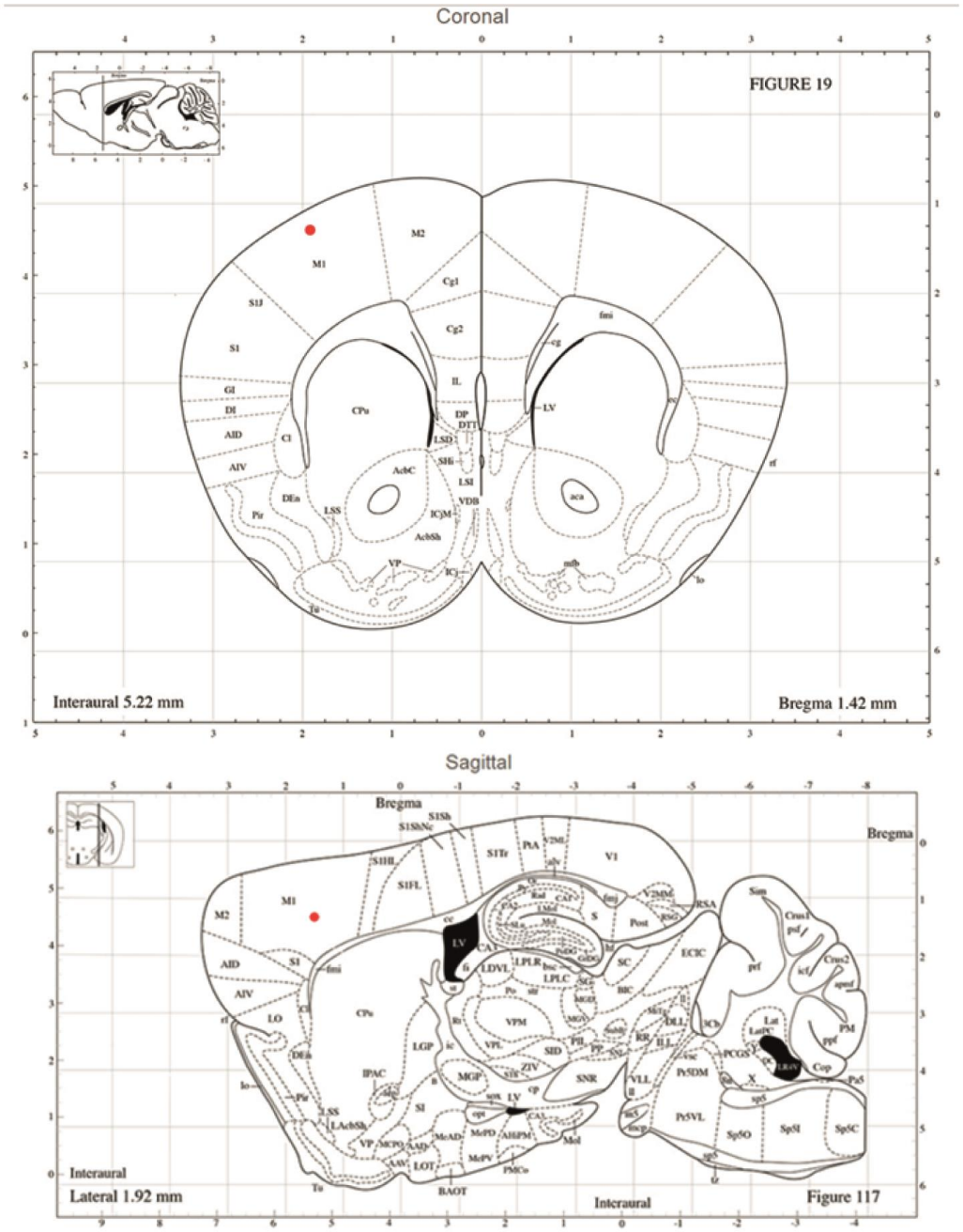


Figure 2.7: Co-ordinates of injection site of AAV (ML 1.9mm, AP 1.45mm, DV 1.3mm). Image generated using Mouse and rat brain atlas: An interactive online tool by Matt Galdica (http://gaidi.ca/weblog/mouse-and-rat-brain-atlas-an-interactive-online-tool).

2.10 Culturing tissue samples and cells

2.10.1 Ex-vivo VNO preparation

VNO samples were extracted and placed fresh and intact into culture medium (10% foetal bovine serum and 1x glutamine in DMEM) and stimulated by pipetting HMW male or female urine fraction or potassium chloride (KCl (1/20)) over the tissue, the sections were then incubated for one hour before fixation with 4% paraformaldehyde. Samples were then prepared for cryostat cutting by embedding in OCT.

2.10.2 Primary cell culture of VNO

Four well plates were prepared by placing 13mm cover slips into each well, each washed with distilled water then coated (in plate) in 0.5mg/ml poly-L-lysine in borate buffer (300µl per well) and dried at 37˚C overnight in a sterile incubator. Excess poly-L-lysine was washed off with dH_2O then laminin (8.33µl in 1ml PBS = 10µl in 1.2ml) was added and warmed at 37˚C, excess laminin was removed just before media was added. Freshly dissected VNOs were kept in Borate buffer (150mM Boric acid in dH_2O (pH 8.3)) on ice then. VNO tissue was put into 500µl papain (0.5U/ml in 5mM l-cysteine-HCL + EDTA + PBS) and cut widthways into 4 pieces with a clean blade then incubated for 20 mins at 37˚C (water bath). Tissue suspension was added to 3ml of media (10% foetal bovine syrum, 1x Glutamax, 1x Penicillin/Streptomycin in DMEM) then centrifuged at 450g for 3mins, re-suspended in 2ml of fresh media then passed through a nylon strainer. Cells were then allowed to stand in 1 ml of media (3 mins at 37˚C in the water bath) before adding to the four well plates. A 50µl aliquot was taken to check for cell density using Nucleocounter® NC-100™ (Chemometec).

2.11 Statistics

Data was recorded and sorted in Excel and statistical analysis was performed in IBM SPSS statistics 20 or RStudio Parametricity of data was analysed in SPSS using Shapiro-Wilk test of normality and Levene's test for equality of variance. Where parametricity was found repeated measure ANOVA or independent samples t-test was used. For post hoc analysis

Bonferroni adjusted pairwise comparison was used. Where parametricity was not found repeated measure ANOVA or Two way ANOVA with Greenhouse-Geisser correction, Friedman test or Kruskal Wallis H test were used. For post hoc analysis Dunn's pairwise comparison was used. All statistics were recorded in a statistics table (Appendix 1). Graphs were generated using R Studio with error bars depicting standard error.

Mass spectrometry data was analysed by hierarchical cluster analysis and two-step cluster analysis in R Studio using the hClust package. First the items (proteins) were grouped together by similarity into small groups by a measure of the difference between them in score, repeat or tissue type. This produces a cluster dendrogram which was then evaluated for Euclidian distance to choose cluster numbers for further analysis. Three clusters were chosen as this number of clusters maintained the largest Euclidian distance while keeping clusters a similar size. A two-step cluster analysis performed using Microsoft Excel allowed the individual properties of each cluster to be identified, such as the identity of proteins in each tissue type in a cluster.

RNA sequencing data was analysed by principal component analysis in R Studio using packages FactoMineR, factoextra, corrplot, ggpubr and plyr. This generated eigen values from which biplots were generated. This data formed the basis for the subsequent cluster analysis.

Chapter 3

Interest and discrimination behaviour of Nlgn3 knockout mice associated with social scent cues

3.1 Introduction

Olfactory cues produced in the tear ducts and liver of mice have been identified as key components in mouse communication leading to modifications in social behaviour. Of these scent cues the MUPs have been associated with many specific behaviours such as territorial behaviour in males (Hurst and Beynon, 2004) and sexual selection in females (Roberts et al. 2012) which is initiated by an interest for major urinary proteins (MUPs) that can be seen in mice regardless of sex. Differences identified in mouse behaviour induced by scent cues could be due to sex dependent difference in the reactivity of VSNs (Herrada and Dulac, 1997; Fu *et al.*, 2015) and/or differences in the pathways activated by scent cues but despite the differences in motivation for scent cues, direct contact with MUPs is required to elicit a behavioural response in both male and female mice.

The social behaviour of *Nlgn3*$^{y/-}$ mice has previously been found to show no differences when measuring interactions with familiar males and strangers in the three chamber test (Radyushkin *et al.*, 2009). However impairment in social memory were identified in *Nlgn3*$^{y/-}$ mice who showed no increased interest for a stranger over a familiar male in the three chamber test (Radyushkin *et al.*, 2009). This could be linked with the reduced interest for social olfactory cues (soiled bedding) found in *Nlgn3*$^{y/-}$ and *Nlgn3*$^{+/-}$ mice (Dere *et al.*, 2018) as reduced sniffing could prevent modifications in the brain normally caused by scent cue interaction which influence future behaviour and since MUPs have been directly linked with such changes (Roberts et al. 2012; Hoffman et al. 2015) I wanted to explore this more directly.

In this chapter I aimed to identify if Neuroligin 3 knockout mice have an altered interest for high molecular weight (HMW) urine fraction which contains MUPs (Chamero *et al.*, 2007; Beynon *et al.*, 2014). I decided to include male and female mice, assessing their interest for male and female HMW urine fraction. As our group was interested in the effect of how the genotype of mice in the home-cage environment affects behaviour I also decided to investigate differences found in the males for housing effects. It has previously been identified that a number of behaviours were affected by housing such as tube test rank and vocalisation and also that testosterone levels in urine were also affected (Kalbassi *et al.*, 2017). I would therefore predict that *Nlgn3*$^{y/+}$ mice housed with *Nlgn3*$^{y/-}$ mice would have a reduced interest for social cues compared to single genotype housed *Nlgn3*$^{y/+}$ mice.

3.2 Interest for high molecular weight urine fractions

I first attempted to identify if there were differences in the interest of *Nlgn3*$^{y/-}$ mice for social odours. As the MUPs have been associated highly with social behaviour in mice I chose to begin with the high molecular weight (HMW) fraction of mouse urine in the experimental conditions. I also decided to begin the behaviour using single genotype housed (SGH) mice to simplify the analysis to begin with. Mice were exposed to scent cue conditions in a randomised order over several days. The scent cue conditions include HMW fractions of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ males and HMW fractions of *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ females as well as a cotton control condition.

3.2.1 Interest of SGH males for HMW urine fraction

Nlgn3$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from SGH housing environments were exposed to HMW urine fraction from unfamiliar wildtype males and a clean cotton swab which acted as a control (Figure 3.1). A repeated measures ANOVA of the resulting sniffing time identified a main effect of scent cue (ANOVA, Within-subjects (Scent cue): $F(1,14)=20.607$, $p=<0.001$) where urine fraction was sniffed more than control. Also there was no main effect of genotype (ANOVA, Between subjects (Genotype): $F(1,14)=2.632$, $p=0.127$) but an interaction between

scent cue and genotype (ANOVA, Scent cue*Genotype: F(1,14)=5.111, p=0.04) driven by the increased HMW urine fraction sniffing of *Nlgn3*$^{y/+}$ compared to *Nlgn3*$^{y/-}$ mice (Pairwise comparisons, WT: Control/HMW, p=<0.001). I concluded from this that SGH *Nlgn3*$^{y/-}$ mice have a reduced interest for male HMW urine fraction compared to SGH *Nlgn3*$^{y/+}$ mice which is consistent with previous work which focused on the social behaviour of these animals (Dere *et al.*, 2018).

Nlgn3$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from SGH housing environments were also exposed to HMW urine fraction from unfamiliar wildtype females and a cotton swab control (Figure 3.2). A repeated measures ANOVA identified main effects of scent cue (ANOVA, Within-subjects (Scent cue): F(1,14)=60.452, p=<0.001) and genotype (ANOVA, Between subjects (Genotype): F(1,14)=22.019, p=<0.001) and an interaction between scent cue and genotype (ANOVA, Scent cue*Genotype: F(1,14)=51.603, p=<0.001). This again was driven by the increased HMW urine fraction sniffing of *Nlgn3*$^{y/+}$ males (Pairwise comparisons, WT: Control/HMW, p=<0.001, HMW: WT/KO, p=<0.001) but whereas previously there was some interest of the *Nlgn3*$^{y/-}$ mice for the male urine fraction there was very little interest for the female urine fraction based on time sniffing. So I also concluded that *Nlgn3*$^{y/-}$ mice have a reduced interest for female HMW urine fraction.

A direct comparison of the interest of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice for HMW urine fractions shows that *Nlgn3*$^{y/-}$ mice have a lowered interest for HMW than *Nlgn3*$^{y/+}$ mice (Figure 3.3). A repeated measures ANOVA shows no main effect of scent cue (ANOVA, Within-subjects (Scent cue): F(1,14)=1.244, p=0.283) but there is a main effect of genotype (ANOVA, Between subjects (Genotype): F(1,14)=24.895, p=<0.001) and an interaction between scent cue and genotype (ANOVA, Scent cue*Genotype: F(1,14)=6.129, p=0.027). The interaction was due to a significant difference in sniffing time between *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice which showed higher interest of *Nlgn3*$^{y/+}$ mice for female scent cue (Pairwise comparisons, Female: WT/KO, p=<0.001). Also an increased interest of *Nlgn3*$^{y/+}$ for female HMW over male HMW (Pairwise comparisons, WT: Male/Female, p=0.024). The reduced interest of *Nlgn3*$^{y/-}$ mice

for female HMW could be due to social behavioural problems caused by the knockout of Neuroligin 3. Our group previously identified that in a free roaming environment *Nlgn3*$^{y/-}$ mice spent significantly less time interacting with females in oestrus than *Nlgn3*$^{y/+}$ mice (Bachmann et al. 2018). Reduced interest of *Nlgn3*$^{y/-}$ mice for females is therefore not restricted to interest for HMW urine fraction but extends to females themselves. However there is a possibility that the reduced interest of *Nlgn3*$^{y/-}$ mice for female HMW is the result of a problem with the detection of MUPs. Female urine has a different MUP profile to males and are typically in less abundance (Mudge *et al.*, 2008) and so female HMW could represent a combination of scent cues that are difficult to detect or that are below the threshold of the altered perception of *Nlgn3*$^{y/-}$ mice. I did not yet have enough data to conclude one hypothesis over the other but could conclude that *Nlgn3*$^{y/-}$ males have a low interest for HMW urine fraction, particularly of female mice.

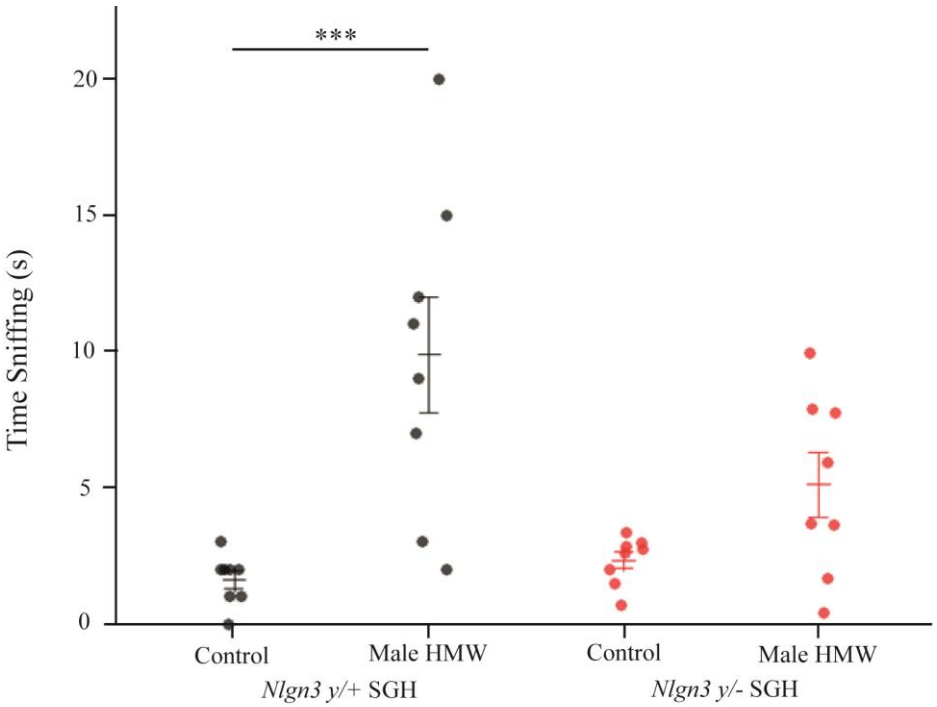


Figure 3.1: SGH male interest for male HMW urine fraction. *Nlgn3y/+* spent significantly more time sniffing HMW male urine fraction than clean cotton control

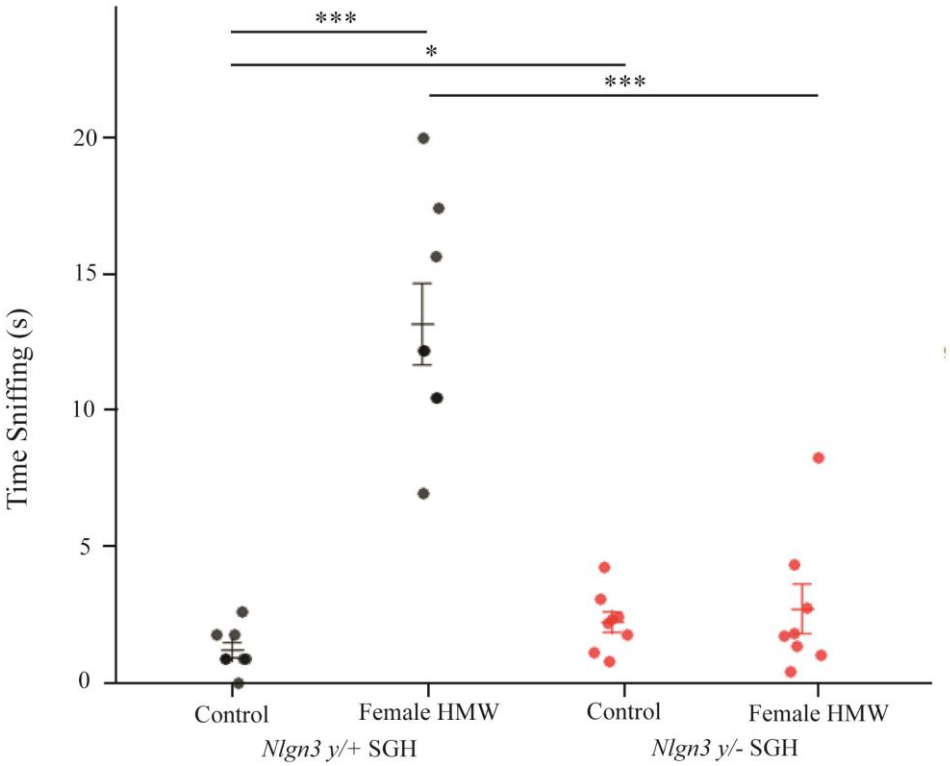


Figure 3.2: SGH male interest for female HMW urine fraction. *Nlgn3y/+* spent significantly more time sniffing HMW female urine fraction than *Nlgn3y/-* or clean cotton control. *Nlgn3y/-* spent significantly more time sniffing clean cotton control than *Nlgn3y/+*

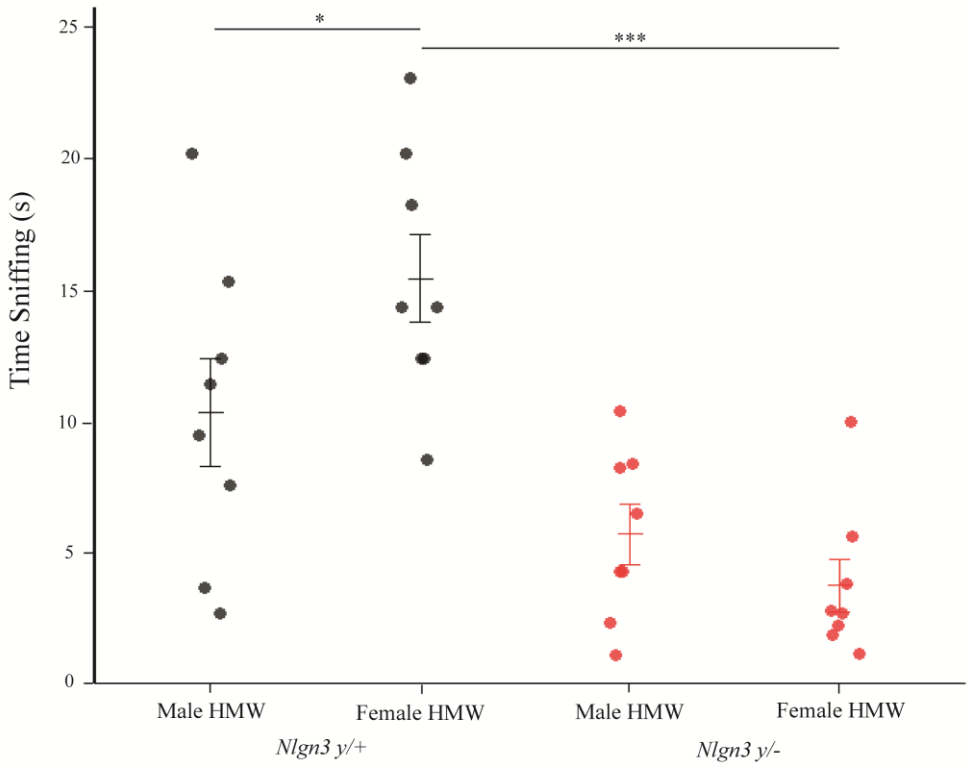


Figure 3.3: Comparison of SGH male interest for male and female HMW urine fraction. *Nlgn3*$^{y/+}$ spent significantly more time sniffing female HMW urine fraction than male HMW urine fraction. *Nlgn3*$^{y/+}$ also spent significantly more time sniffing female HMW than *Nlgn3*$^{y/-}$

3.2.2 Interest of SGH females for HMW urine fraction

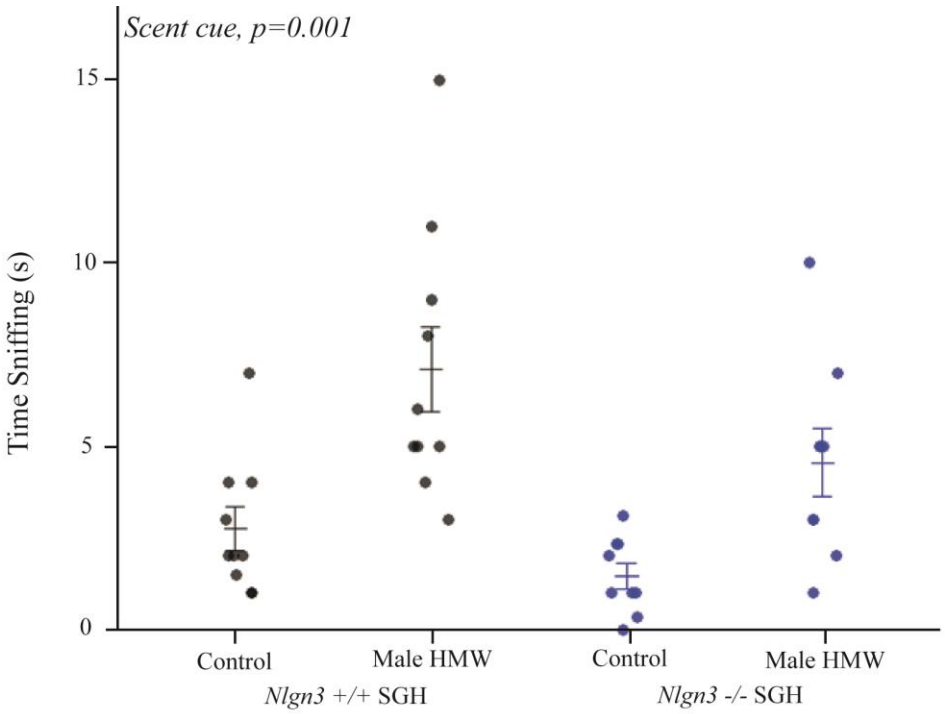
The behaviour of female mice is also affected by social scent cues but the response of males and females to particular urine fractions has been found to differ (Halem, Baum and Cherry, 2001). This gender driven difference could also be reflected in social interest of females for HMW urine fraction compared to males. I wanted to see if the reduced interest found in the *Nlgn3*$^{y/-}$ males was also seen in the *Nlgn3*$^{-/-}$ females or if there is a gender bias in this effect (Figure 3.4). *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice were exposed to HMW urine fraction from unfamiliar wildtype males. A repeated measures ANOVA identified a main effect of scent cue (ANOVA, Within-subjects (Scent cue): $F(1,17)=21.225$, $p=<0.001$) and genotype (ANOVA, Between subjects (Genotype): $F(1,17)=86.533$, $p=0.038$) but there was no interaction between scent

cue and genotype (ANOVA, Scent cue*Genotype: F(1,17)=0.599, p=0.450). This shows that the sniffing behaviour of *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice in response to male HMW urine fraction is similar but that the *Nlgn3*$^{+/+}$ mice spent more time sniffing both the control swab and the HMW urine fraction compared to *Nlgn3*$^{-/-}$ mice. I concluded that *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice have a comparable interest for male HMW urine fraction.

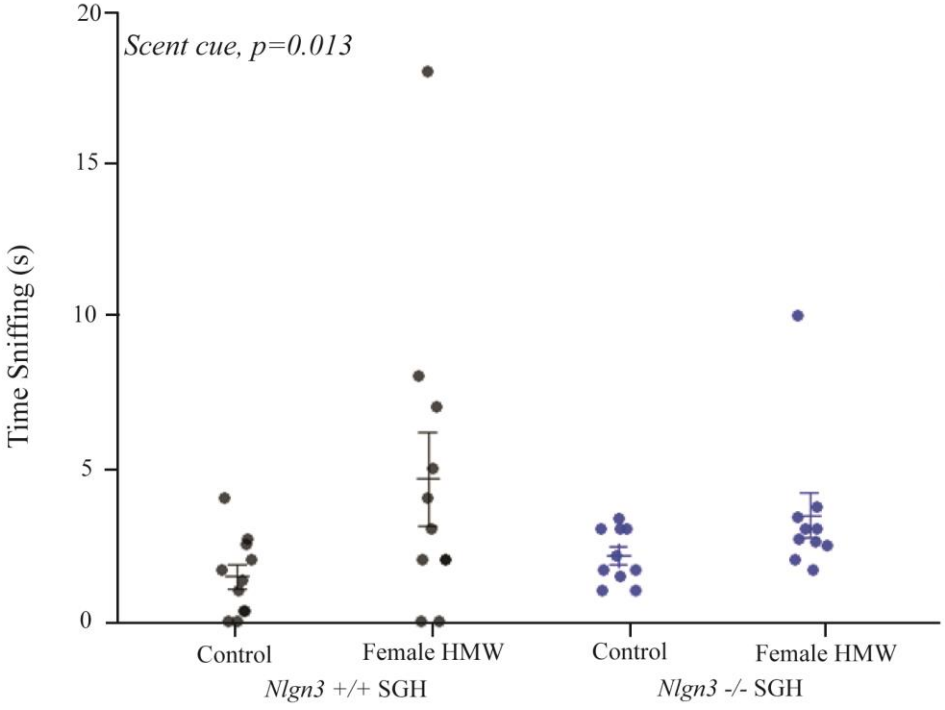
As with the males I also exposed the female mice to female HMW urine fraction and cotton control (Figure 3.5). A repeated measures ANOVA identified a main effect of scent cue (ANOVA, Within-subjects (Scent cue): F(1,19)=7.551, p=0.013) but no main effect of genotype (ANOVA, Between subjects (Genotype): F(1,19)=0.063, p=0.804) or interaction effect of scent cue and genotype (ANOVA, Scent cue*Genotype: F(1,17)=1.294, p=0.269) showing an interest of female HMW over cotton in both *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice. I therefore concluded that the interest of *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice for female HMW urine fraction is also very similar.

3.2.3 Comparison of male and female interest

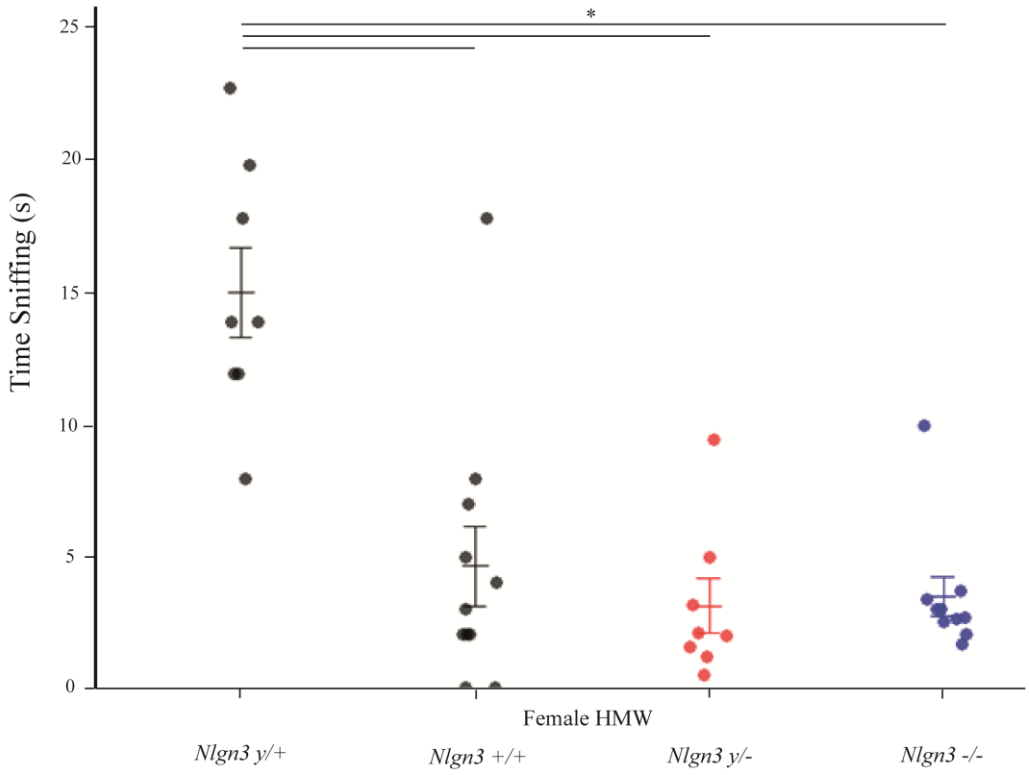
As the female mice in this colony have been found to have a generally low interest for scent cues, I decided to compare the interest of males and females for HMW urine fraction to see how the reduced interest of *Nlgn3*$^{y/-}$ compared to the low interest of females identified here (Figure 3.6). Comparison of male and female interest for female HMW urine fraction using Friedman test (chosen due to non-parametricity of data) identified a difference in the interests of these mice ($\chi 2(3) = 12.3$, $p = 0.006$) that was driven by *Nlgn3*$^{y/+}$ male interest for scent cue (Wilcoxon signed-rank tests, Male WT/Male KO: Z=-2.380, p=0.017, Male WT/Female WT: Z=-2.527, p=0.012, Male WT/Female KO: Z=-2.521, p=0.012) rather than *Nlgn3*$^{y/-}$ interest (Wilcoxon signed-rank tests, Male KO/Female WT: Z=-0.280, p=0.779, Male KO/Female KO: Z=-0.280, p=0.779) or female interest (Wilcoxon signed-rank tests, Female WT/Female KO: Z=-0.415, p=0.678). This showed that *Nlgn3*$^{y/-}$ mice have comparable interest for female HMW urine fraction as *Nlgn3*$^{+/+}$ and *Ngln3*$^{-/-}$ mice.



3.4: SGH female interest for HMW male urine fraction. *Nlgn3+/+* and *Nlgn3-/-* mice spent more time sniffing HMW male urine fraction than control and had a similar interest for HMW male urine fraction



3.5: SGH female interest for HMW female urine fraction. *Nlgn3+/+* and *Nlgn3-/-* mice spent more time sniffing HMW male urine fraction than control and had a similar interest for HMW female urine fraction

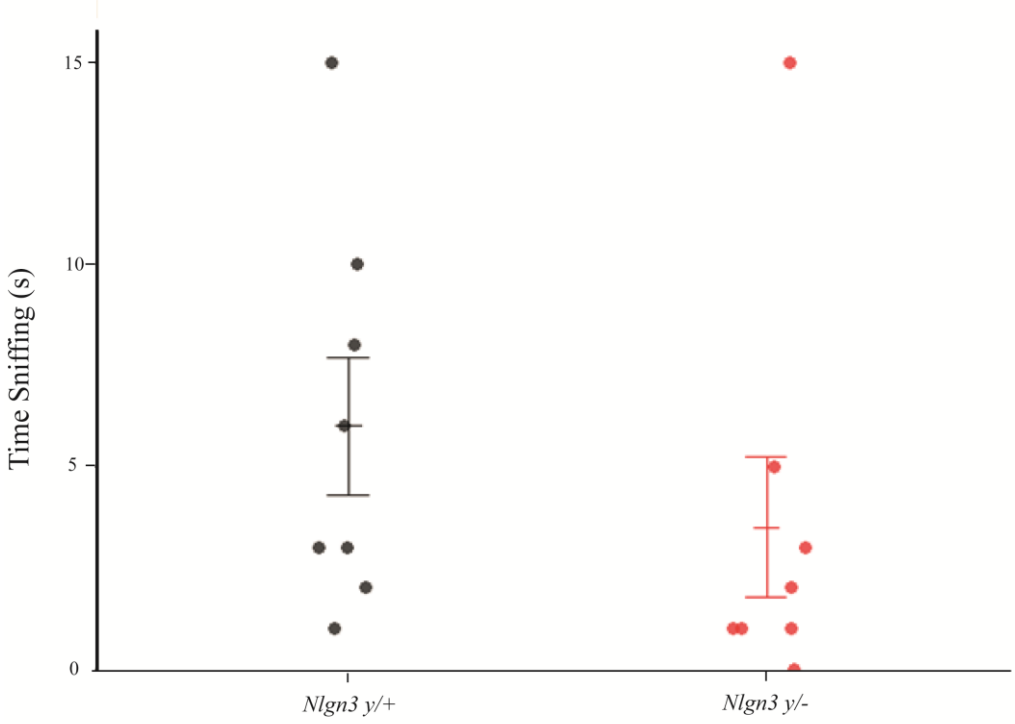


3.6: Comparison of male and female interest for female HMW urine fraction. *Nlgn3*$^{y/+}$ spent significantly more time sniffing female HMW than *Nlgn3*$^{y/-}$, *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$

3.2 Interest of wildtype and *Nlgn3*$^{y/-}$ male for whole urine

To further investigate the possibility that the reduced amount of volatile urine components was affecting the interest of *Nlgn3*$^{y/-}$ mice for HMW urine fraction I exposed *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice to whole urine samples which contain the full assortment of LMW molecules present in collected urine (Figure 3.7). *Nlgn3*$^{y/-}$ males spent a comparable time sniffing the whole urine samples as *Nlgn3*$^{y/+}$ mice (independent samples t-test, $t(14)=1.033$, $p=0.319$). I then concluded that the interest of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice is comparable. The difference between the scent cue of the previous observation and this one is the inclusion of the complete array of LMW molecules usually found in the urine. It was therefore possible that the reduced interest of *Nlgn3*$^{y/-}$ mice was associated particularly with HMW fraction of urine which can be improved when the LMW fraction is included. This could indicate that the attractive elements of the scent cue, the LMW molecules, are detected by the *Nlgn3*$^{y/-}$ mice but that the

HMW molecules such as MUPs are not detected or interpreted in the *Nlgn3*$^{y/-}$ mice in the same way as they are in the *Nlgn3*$^{y/+}$ mice.



3.7: SGH male interest for *Nlgn3*$^{y/+}$ whole urine. No significant difference was identified between the time *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ spent sniffing whole urine.

3.3 Social discrimination

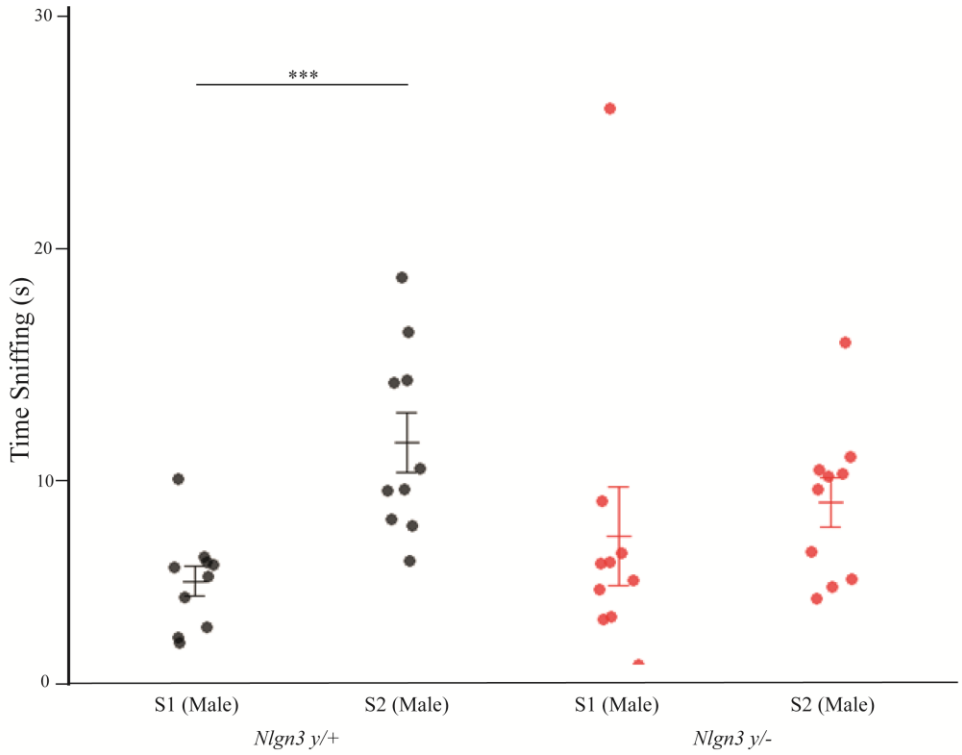
3.3.1 Discrimination of male scent cues

I wanted to identify if *Nlgn3*$^{y/-}$ mice showed an altered social memory for scent cues alone. As urinary scent cues are associated with behaviours that rely on memory such as countermarking I decided to look at discrimination behaviour. I used social cues which contain both HMW and LMW fractions which consisted of scraping home cages of wildtype mice with a cotton swab in an attempt to attract *Nlgn3*$^{y/-}$ mice to the scent cues. *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ males were pre exposed to an unfamiliar cage scraping during an acquisition phase in order to familiarise them with this scent. They were then presented with the familiar cage scraping (S1) and a new

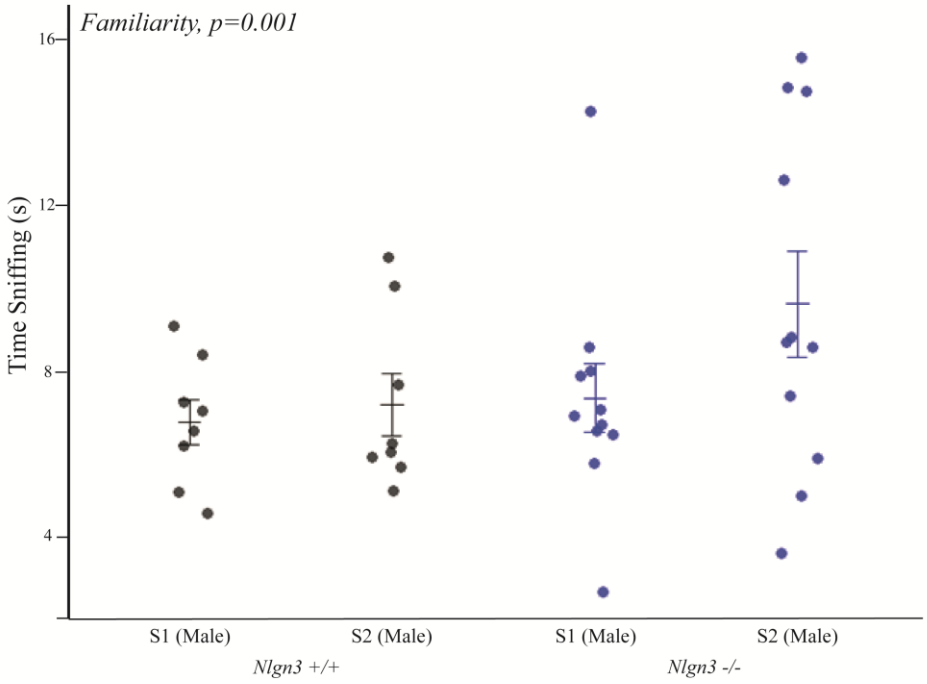
unfamiliar cage scraping (S2) at the same time and interactions with them were recorded (Figure 3.8).

To begin with *Nlgn3*$^{y/+}$ and *Nlng3*$^{y/-}$ mice were exposed to scrapings from male cages. A repeated measures ANOVA identified a main effect of scent cue familiarity (ANOVA, Within-subjects (Familiarity): F(1,18)=19.006, p=<0.001) but not genotype (ANOVA, Between subjects (Genotype): F(1,18)=0.030, p=0.865), though there was an interaction effect of scent cue familiarity and genotype (ANOVA, Familiarity*Genotype: F(1,18)=7.104, p=0.016). This interaction was due to the difference is sniffing time of the *Nlgn3*$^{y/+}$ mice who spent significantly more time sniffing the unfamiliar scent cue than the familiar scent cue (Pairwise comparisons, WT: S1/S2, p=<0.001). Whereas the sniffing times of *Nlgn3*$^{y/-}$ mice did not indicate discrimination behaviour (Pairwise comparison KO: S1/S2, p=0.246) as they did not show a preference for one over the other. I concluded that *Nlgn3*$^{y/-}$ mice have difficulty discriminating male cage scrapings despite having some interest for whole male scent cues. This could indicated that the detection or processing of MUPs is affected in the *Nlgn3*$^{y/-}$ mice as MUPs have been identified as a key factor signalling individual identity in mouse urine (Hurst *et al.*, 2001).

Females exposed to the same behavioural test did not show any discrimination behaviour (Repeated measures ANOVA, Within-subjects (Familiarity): F(1,17)=2.333, p=0.145, Between subjects (Genotype): F(1,17)=1.987, p=0.177, Familiarity*Genotype: F(1,17)=1.108, p=0.307). This could also be a reflection of the low interest of females for scent cues more generally in this cohort (Figure 3.9).



3.8: Discrimination of males between male cage scrapings. *Nlgn3*$^{y/+}$ males spend more time sniffing unfamiliar (S2) scent cue than familiar (S1) scent cue.

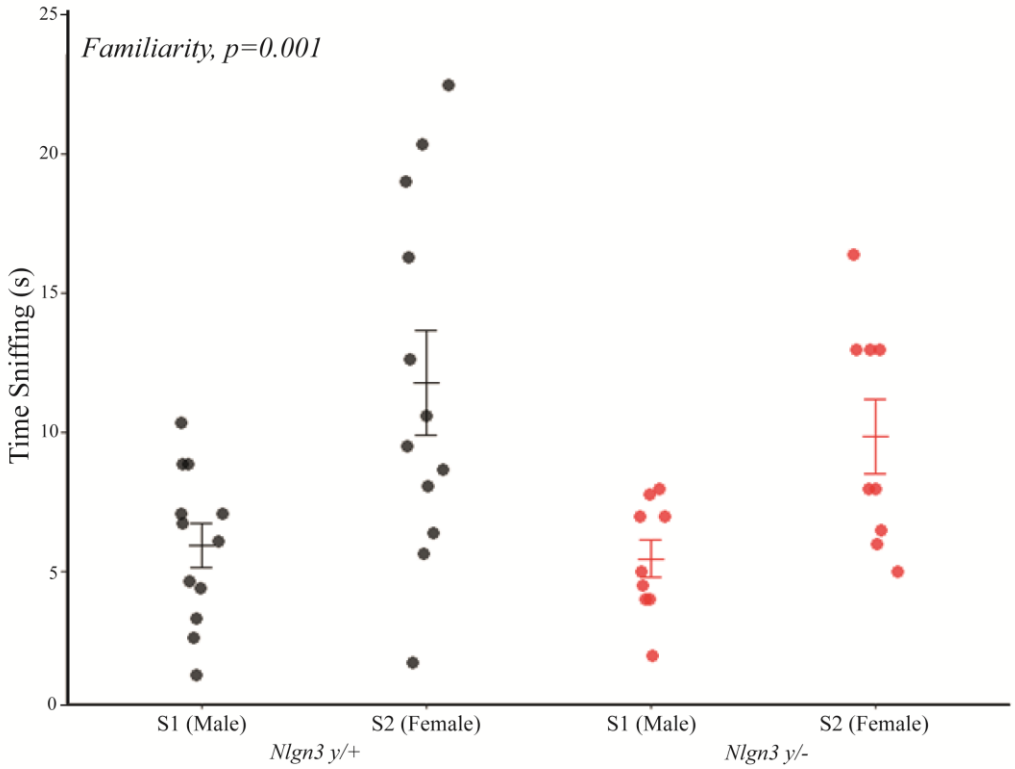


3.9: Discrimination of females between male cage scrapings. Females spent more time sniffing unfamiliar (S2) scent cue than familiar (S1) scent cue.

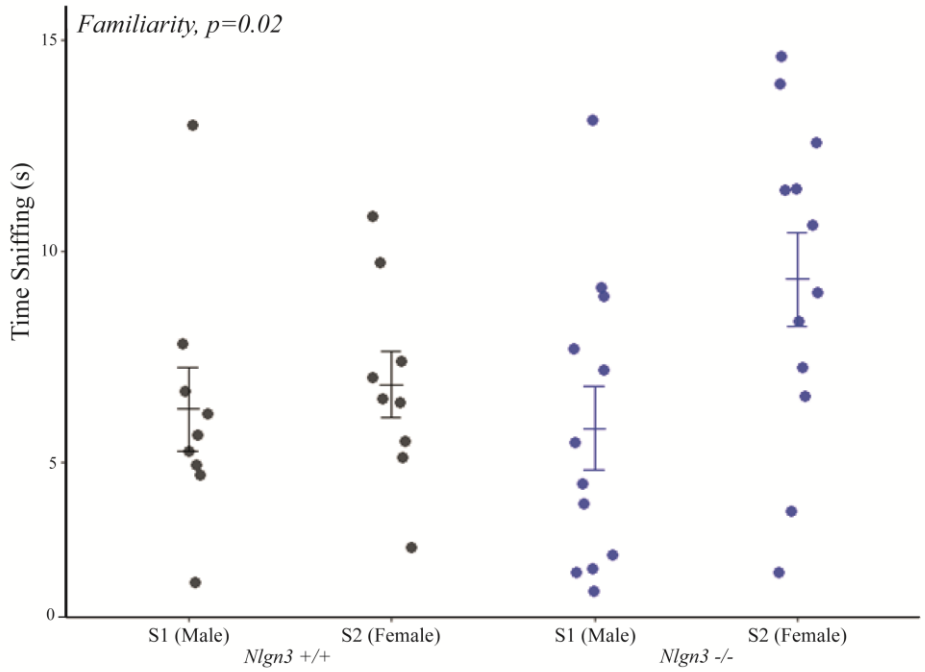
3.3.2. Discrimination of female scent cues from male scent cues

To further investigate the discrimination difficulty of the *Nlgn3*$^{y/-}$ males they were exposed to the same experimental conditions as before but the unfamiliar scent cue presented was a female cage scraping rather than a second male cage scraping as detection of female cues relies more on detection of volatile urinary molecules (Figure 3.10). Under these circumstances *Nlgn3*$^{y/-}$ males were able to discriminate between male and female cage scraping (Repeated measures ANOVA, Within-subjects (Familiarity): $F(1,19)=17.438$, $p=0.001$, Between subjects (Genotype): $F(1,19)=0.687$, $p=0.421$, Familiarity*Genotype: $F(1,19)=0.346$, $p=0.346$). Since female scent cues have less MUPs and detection of female cues is thought to depend more on volatile molecules it is possible that this difference indicates that *Nlgn3*$^{y/-}$ mice have a reduced ability to detect or interpret volatile non-volatile scent cues while their ability to detect and interpret volatile scent cues is less affected. The *Nlgn3*$^{y/-}$ mice showed an interest for female cage scraping that did not reflect their interest for female HMW urine fraction. As with the whole urine scent cues presented before the inclusion of the LMW scent cues which would be present in a cage scraping did seem to renew interest in *Nlgn3*$^{y/-}$ mice for female scent cues.

Female discrimination of female cent cues from male scent cues appears to be present in the *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice (Figure 3.11) as a repeated measures ANOVA shows a main effect of scent cue familiarity (ANOVA, Within-subjects (Familiarity): $F(1,19)=6.392$, $p=0.020$) but not of genotype (ANOVA, Between subjects (Genotype): $F(1,19)=0.740$, $p=0.400$) and no interaction between these factors (ANOVA, Familiarity*Genotype: $F(1,19)=3.316$, $p=0.084$). This suggests that though female interest is low they are still able to discriminate between male and female scent cues.



3.10: Discrimination of males between male and female cage scrapings. Males spent more time sniffing female cage scraping than male cage scraping.



3.11: Discrimination of females between male and female cage scrapings. *Nlgn3*$^{-/-}$ spent more time sniffing female cage scraping than male cage scraping.

3.4 Housing

Another factor I wanted to consider was the peer social environment of the home cage. The influence of the peers within the home cage could be a key factor in the behaviour of adult mice, particularly the social behaviour. Our group identified that housing can affect dominance behaviours seen by tube test wins/losses and interaction time with females (Kalbassi *et al.*, 2017).

There are many factors that can be used to divide the individuals within the cage into groups that may have influence on each other's social behaviour, we decided to focus on genotype. Single genotype housed (SGH) mice were *Nlgn3*$^{y/+}$ or *Nlgn3*$^{y/-}$ mice that were housed with only mice of the same genotype. Mixed genotype housed (MGH) mice were *Nlgn3*$^{y/+}$ or *Nlgn3*$^{y/-}$ mice that were housed in mixed genotype groups. In this way only the mice from the MGH environment could have their behaviour affected by peers of a different genotype. Both of these groups, SGH and MGH mice, were exposed to unfamiliar HMW urine fraction.

3.4.1 Interest of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from SGH and MGH housing conditions for male HMW urine fraction

To establish if the housing environment had an effect on social interest SGH and MGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice were exposed to HMW urine fraction of unfamiliar males (Figure 3.12). A repeated measures ANOVA identified a main effect of scent cue (ANOVA, Within-subjects (Scent Cue): $F(1,32)=39.427$, $p=<0.001$) and genotype (ANOVA, Between-subjects (Genotype): $F(1,32)= 7.039$, $p=0.012$) but not of housing (ANOVA, Between-subjects (Housing): $F(1,32)=2.036$, $p=0.163$. There was an interaction between scent cue and genotype (ANOVA, Scent Cue*Genotype: $F(1,32)=5.865$, $p=0.021$) but not between genotype and housing (ANOVA, Scent Cue*Housing: $F(1,32)=2.032$, $p=0.164$) or genotype and housing (ANOVA, Genotype*Housing: $F(1,32)=<0.001$, $p=0.996$) and no interaction between the three (ANOVA, Scent Cue*Genotype*Housing: $F(1,32)=2.003$, $p=0.167$). *Nlgn3*$^{y/+}$ mice spent significantly more time sniffing HMW urine fraction over control in both housing

conditions (Pairwise comparisons, WT:SGH:Control/HMW, p=<0.001, WT:MGH:Control/HMW, p=0.002). Also the *Nlgn3*$^{y/+}$ MGH mice spent more time sniffing the control than the *Nlgn3*$^{y/+}$ SGH mice (Pairwise comparison, WT:Control:SGH/MGH, p=0.040). The MGH *Nlng3*$^{y/+}$ mice also spent more time sniffing control than the MGH *Nlgn3*$^{y/-}$ mice (Pairwise comparison, MGH:Control:WT/KO, p=0.005). SGH *Nlgn3*$^{y/-}$ mice spent more time sniffing control that MGH *Nlgn3*$^{y/-}$ mice (Pairwise comparisons, KO:Control:SGH/MGH, p=0.030).

A significant difference between the sniffing time of SGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice (Pairwise comparisons, SGH:HMW:WT/KO, p=0.032) was identified.

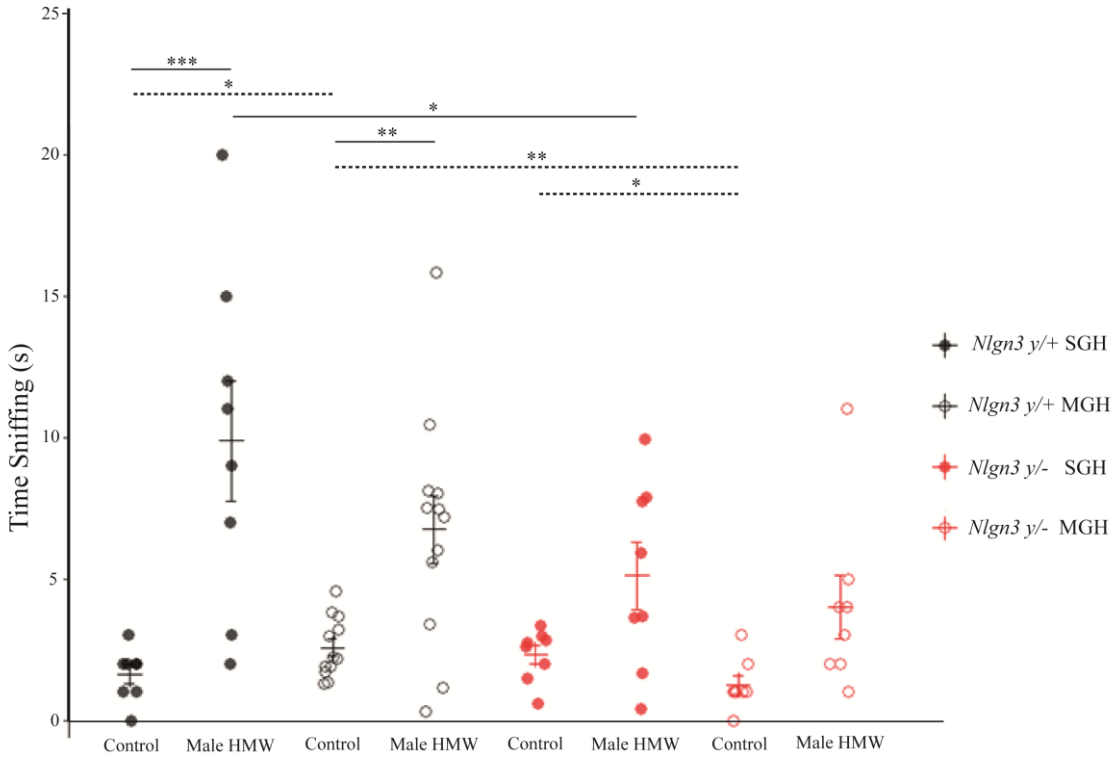
3.4.2 Interest of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from SGH and MGH housing conditions for female HMW urine fraction

SGH and MGH males were also exposed to female HMW urine fractions (Figure 3.13). A repeated measures ANOVA identified a main effect of scent cue (ANOVA, Within-subjects (Scent Cue): F(1,37)=50.267, p=<0.001) and housing (ANOVA, Between-subjects (Housing): F(1,37)=12.731, p=0.001) but not of genotype (ANOVA, Between-subjects (Genotype): F(1,37)= 3.090, p=0.087). There is no interaction between scent cue and genotype (ANOVA, Scent Cue*Genotype: F(1,37)=3.941, p=0.055) but there is an interaction between scent cue and housing (ANOVA, Scent Cue*Housing: F(1,37)=16.078, p=<0.001) and genotype and housing (ANOVA, Genotype*Housing: F(1,37)=6.514, p=0.015) and there is an interaction between the three (ANOVA, Scent Cue*Genotype*Housing: F(1,37)=19.140, p=<0.001).

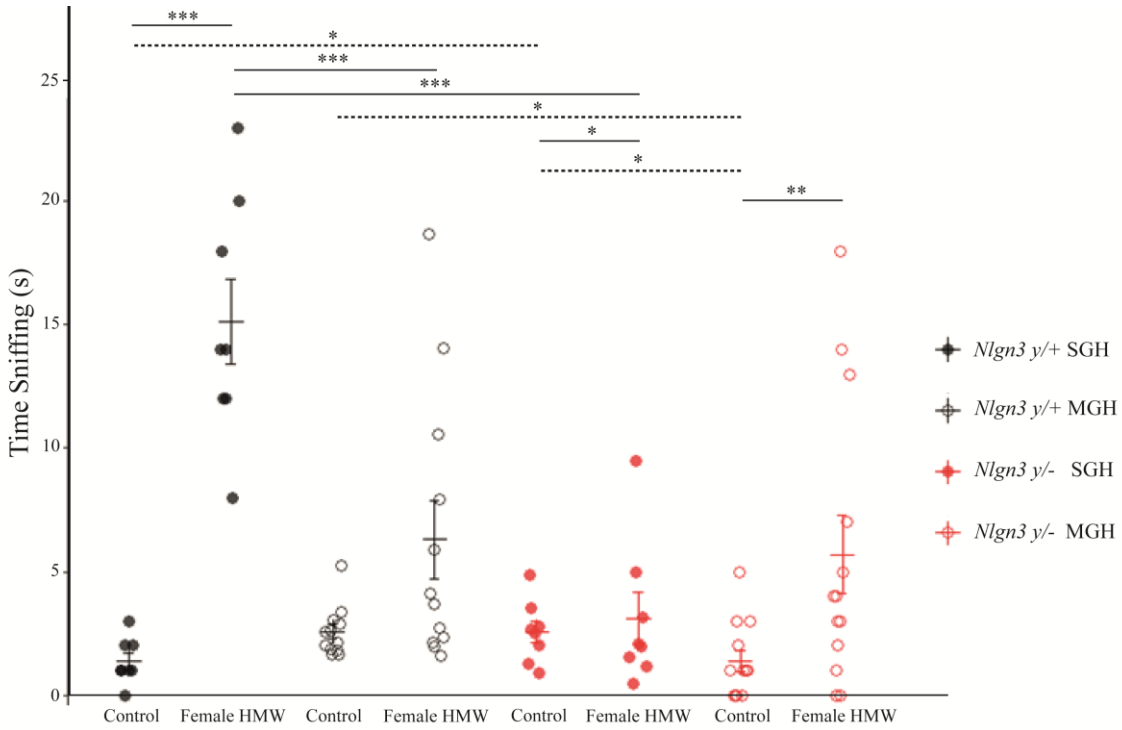
SGH *Nlgn3*$^{y/+}$ mice spent more time sniffing HMW than control (Pairwise comparison, WT:SGH:Control/HMW, p=<0.001) and also spent more time HMW than MGH *Nlgn3*$^{y/+}$ mice (Pairwise comparisons, WT:HMW:SGH/MGH, p=<0.001). SGH *Nlgn3*$^{y/+}$ also spent more time sniffing HMW and control than *Nlgn3*$^{y/-}$ mice (Pairwise comparisons, SGH:Control:WT/KO, p=0.039 and SGH:HMW:WT/KO, p=0.001). MGH *Nlgn3*$^{y/+}$ spent

more time sniffing control than MGH *Nlgn3*$^{y/-}$ mice (Pairwise comparisons, MGH:Control:WT/KO, p=0.039).

SGH *Nlgn3*$^{y/+}$ mice spent more time sniffing HMW than control (KO:SGH:Control/HMW, p=0.012) and also spent more time HMW than MGH *Nlgn3*$^{y/+}$ mice (KO:Control:SGH/MGH, p=0.021). MGH *Nlgn3*$^{y/-}$ mice also spent more time sniffing HMW than control (Pairwise comparisons, KO:MGH:Control/HMW, p=0.003).



3.12: SGH and MGH male interest for HMW male urine fraction. Unbroken lines show significant difference involving HMW, dashed lines show significant difference between control conditions. SGH *Nlgn3*$^{y/+}$ spent more time sniffing HMW male urine fraction than control and also SGH *Nlgn3*$^{y/-}$ MGH *Nlgn3*$^{y/-}$ spent more time sniffing HMW male urine fraction than control.

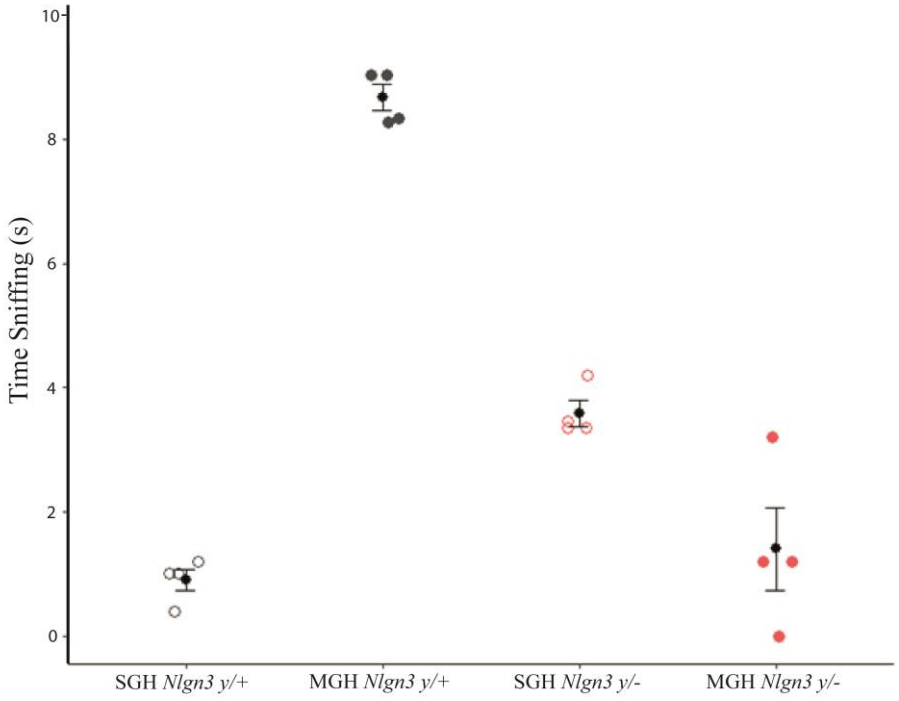


3.13: SGH and MGH male interest for HMW female urine fraction. Unbroken lines show significant difference involving HMW, dashed lines show significant difference between control conditions.

3.4.3 Control sniffing

As a number of the interaction effects of the social interest of males involve significant difference between control conditions I decided to analyse the control sniffing separately. Control conditions from each of the social interest conditions were analysed together (Figure 3.14) and a repeated measures ANOVA identified no main effect of condition, genotype or housing (ANOVA, Within-subjects (Condition): $F(3,96)=0.121$, $p=0.948$, Between-subjects (Genotype): $F(1,32)= 0.181$, $p=0.673$, Between-subjects (Housing): $F(1,32)=0.114$, $p=0.738$). This suggests that the control conditions are not significantly different and the differences between them found in the wider analysis are most likely noise that slight differences in the control conditions are adding. However I decided to leave the control conditions in the previous analysis as the control is an important element and it would be unrealistic to suggest that behavioural data is not inherently noisy. I also decided not to normalise the HMW sniffing

time to the control sniffing as I did not want to risk altering the behaviour in a manner that does not reflect reality in order to make the data cleaner.



3.14: SGH and MGH interest for cotton control across all conditions. Each of the groups had several control exposures and though they did have different baselines there was no significant difference between the interests for control across the groups.

3.5 Discussion

In this chapter I used behavioural experiments to assess the response of *Nlgn3*$^{y/-}$ mice to social scent cues in comparison to *Nlgn3*$^{y/+}$ mice. I identified that *Nlgn3*$^{y/-}$ mice did not show any significant interest for either male or female HMW urine fraction compared to *Nlgn3*$^{y/+}$ mice. The reduction in HMW urine fraction interest is consistent with the findings of Dere et al (2018). This is also reflected in my finding that *Nlgn3*$^{y/-}$ mice are not able to discriminate between male scent cues which could be consistent with Radyushkin et al (2009) as the reduced interest for social cues could be a factor in their reduced social memory. I then decided to further this finding by exploring the social interest of *Nlgn3*$^{y/-}$ mice for female scent cues,

though they did not show an increased sniffing time for female HMW urine fraction they could distinguish between male and female cage scraping scent cues. This suggests that they are able to detect female scent cues when volatile molecules are present and that they have a preference for female over male cues. It is important to consider that the HMW samples are not without volatile urinary molecules but that they have a reduced amount. This is in part due to the binding of volatile molecules to non-volatile molecules which our separation method cannot undo and also the cut-off point of our filters. This means that in the behavioural paradigm the mice will be exposed to some volatile molecules but in a reduced quantity compared to whole urine. It is therefore possible that *Nlgn3*$^{y/-}$ mice are detecting volatile molecules which is enough for them to distinguish and so show an attraction for female scent cues over male scent cues. However, since the discrimination of male urine relies on detecting differences in levels of MUPs then the reaction of *Nlgn3*$^{y/-}$ mice to male scent cues may reflect an inability to detect or interpret MUPs in the same way as *Nlgn3*$^{y/+}$ mice.

I also explored the interest of *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice and identified no significant difference between them. This contradicts the findings of Dere et al (2018) who found *Nlgn3*$^{+/-}$ mice had a reduced interest for soiled male bedding compared to *Nlgn3*$^{+/+}$ mice and based on this I expected a that *Nlgn3*$^{-/-}$ mice would also have a reduced interest for male scent cues. However a consideration I have made is that Dere et al (2018) were using a non-standard experimental procedure and statistical analysis as they were mainly focused methods that utilise the Intellicage equipment. To this end they automate interest scoring so that it reflects the location of the mouse (in the scent cue containing quadrant) rather than behaviour (mouse can be seen sniffing). I therefore have confidence in my finding.

Nlgn3$^{-/-}$ mice showed a comparable interest to *Nlgn3*$^{+/+}$ mice for HMW male and female urine fraction. A comparison of the interest of *Nlgn3*$^{y/+}$, *Nlgn3*$^{y/-}$, *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mouse interest for female HMW fraction showed that the interest of *Nlgn3*$^{y/-}$, *Nlgn3*$^{+/+}$ and *Nlgn3*$^{-/-}$ mice were very similar with only *Nlgn3*$^{y/+}$ mice spending significantly more time sniffing. I concluded from this that the genetic manipulation of Neuroligin 3 knockout seems to affect

the males reducing their interest for HMW urine fractions with was not the case for the females.

This finding suggests that the social memory deficit identified by Radyushkin et al (2009) could be a product of a loss of interest for social cues which have been found to affect social behaviour and memory. What specifically is causing this loss of interest is an important question to pursue. There is the possibility that the VNOs ability to detect scent cues or otherwise function normally could be caused by the genetic manipulation of the animal. As this has not been previously addressed I decided to investigate difference in the VNO of *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{y/+}$ mice.

I was also interested in the effect of housing on the interest of males for social cues. Interest for male scent cues was not affected by housing but there was a significant effect on the interest of *Nlgn3*$^{y/+}$ mice on female interest. This shows that there is not only a significant impact of the genetic manipulation of Neuroligin 3 knockout but also that the social environment can have a significant impact on healthy, genetically normal control mice. An important consideration of this finding for us was the potential influence of maternal behaviour as the *Nlgn3*$^{+/-}$ mouse has been previously found to have a deficit in interest for social cues (Dere *et al.*, 2018) and social cue detection has been found to be an important factor in maternal care (Vaglio, 2009) which is severely affected in mouse models with reduced ability to detect scent cues (Wang and Storm, 2011). This cohort was generated using heterozygous female mice (*Nlgn3*$^{+/-}$ mice) as our breeding dams and since we saw no differences in the behaviour of females due to the social environment we felt that this was unlikely to be the cause of the changes we detect. Though our female mice had a low interest for scent cues in general this did not seem to impact breeding. It is possible that the effect of the Neuroligin 3 knockout is introducing elements to the housing environment that is having an effect on the development on some of the social behaviours of the *Nlgn3*$^{y/+}$mice. I did not see changes in the interest for interaction with females in adult MGH *Nlgn3*$^{y/-}$ mice but I do see this in young MGH *Nlgn3*$^{y/+}$ mice as well as reduced testosterone (Kalbassi et al 2017) showing that long

term changes to physiology are incurred from the housing environment and could make them less good candidates for female mate choice in the long term.

The behavioural experiments in this chapter attempt to cover a number of factors by a repeated measures design where animals were exposed to each of the conditions allowing us to compare each of the conditions directly. This has the benefit that it allows us to utilise less animals to gather more information however it runs the risk of increasing Type I and Type II errors in the interpretation of the data. However due to the limitations of the numbers of mice available the repeated measures design was the best method to be able to explore the factors of importance to this thesis. I attempted to avoid issues such order effects by counterbalancing the exposure of animals to different scent cues. Also mice were only exposed to one scent cue per day to prevent interference of one scent cue with another.

These findings suggest that there might be a difference in the detection or processing of urinary molecules such as MUPs caused by Neuroligin-3 knockout and by the social housing. This could reflect some disruption to the neural pathways involved in processing or recalling scent cue information. However, as I did not know if Neuroligin-3 was expressed in the VNO, I could not be sure if there was disruption to the function of the VNO caused by Neuroligin-3 knockout. This information was not available in the literature so before I could continue to investigate difference in the brain it was important to evaluate the VNO of *Nlgn3*$^{y/-}$ mice compared to *Nlgn3*$^{y/+}$ mice from different housing conditions.

3.6 Summary

- *Nlgn3*$^{y/-}$ mice show reduced interest and lack of discrimination for male cues but have no significantly less interest for whole male urine and can discriminate male from female scent cues
- Female mice in this colony have a low interest for scent cues

Chapter 4

Investigation of Nlgn3 in the VNO

4.1 Introduction

In the previous chapter I identified that *Nlgn3*$^{y/-}$ mice had a reduced interest for HMW urine fraction and they did not show a preference for unfamiliar male odours over familiar male odours which aligns with previous data and allows us to narrow down these behaviours to a difference in response to HMW urine fraction from the whole mouse or soiled bedding (Radyushkin *et al.*, 2009; Dere *et al.*, 2018). However I did not know if the difference in response to HMW was the result of detection problems for social cues associated with Neuroligin 3 knockout.

I decided that it was important to establish if Neuroligin 3 could be playing a role in the function of the VNO. The VNO contains VSNs which project long axons to the AOB though there are no synapses present in the VNO Neuroligin 3 is expressed in glial cells as well as neurons and is involved in a number of biological functions (Shen, Huo and Zhao, 2015). It was an important question to address because if the VNO is physically affected by Neuroligin 3 knockout then this could be the reason for the reduced interest for HMW urine fraction that I observed. I therefore would begin by investigating the VNO to identify if Neuroligin 3 was expressed.

As for the function of the VNO, a number of different methods have been used to assess effect of different scent cues on the response of particular cell types. Whole urine has been found to stimulate cells across both layers of the VNO though many of the individual components have been isolated to the stimulation of particular cell types (Review: Liberles 2014). I know from studies assessing the detection of individual identity in mice that the MUP profile can be recognised (Hurst *et al.*, 2001; Roberts *et al.*, 2018) and that the V2R positive VSNs in the

basal VNO bind with MUPs specifically (Leinders-zufall *et al.*, 2004) where as volatile components are detected through V1R positive VSNs (Boschat *et al.*, 2002)

Immediate early genes have been used to analyse scent cue exposed VNOs, in response to male bedding Egr1 was the highest expressing immediate early gene (Isogai et al. 2011, Figure 4.1).

Electrophysiology and calcium imagining have also been used to identify that individual cell response is specific to the sex, familial status and even markers of individual identity in urine samples (Dulac and Torello, 2003; Fu *et al.*, 2015).

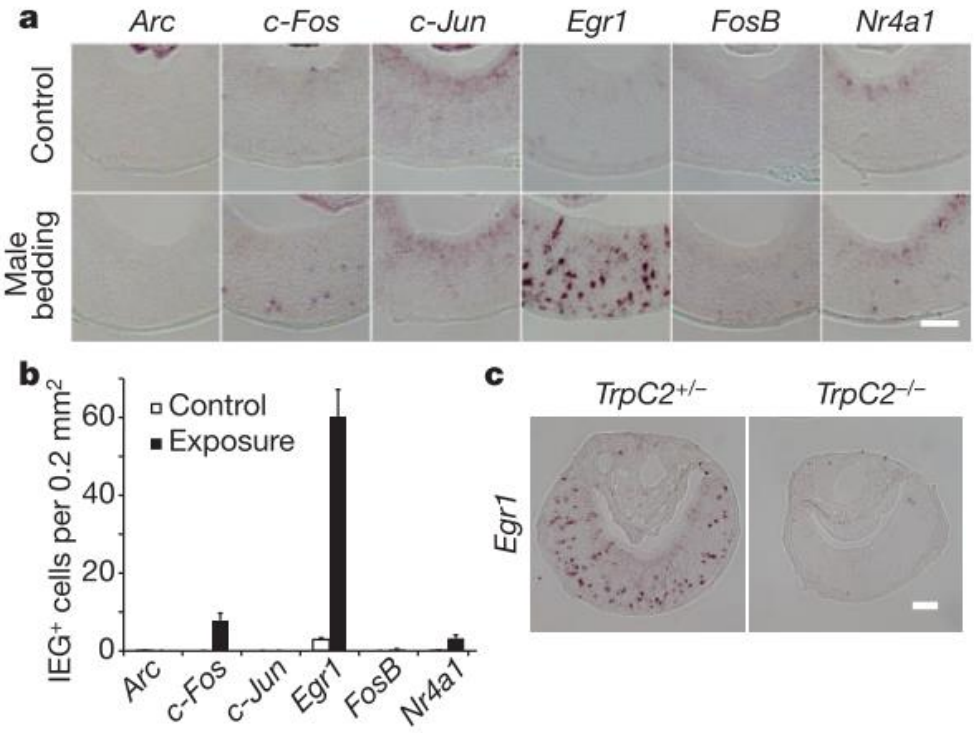


Figure 4.1: Immediate early genes in the VNO. Egr1 staining shows the most signal in the VNO after exposure to male bedding, disruption of VNO activation by TrpC2 abolished Egr1 signal implying that Egr1 signal can only be seen in active, stimulated VSNs in the VNO (Figure 1 from Isogai et al. 2011)

4.2 Nlgn3 in the Vomeronasal Organ

4.2.1 Initial screening for Nlgn3 in the VNO

As I wanted to investigate social odour interest in *Nlgn3*$^{y/-}$ mice I decided to identify first if Neuroligin 3 was expressed in the vomeronasal organ (VNO). I began with a western blot analysis using the cerebellum as a control region (Figure 4.2) to see if Neuroligin 3 was detected at all. I detected bands in the western blot of VNO tissue that were the appropriate molecular weight (measured against a protein ladder) to suggest that Neuroligin 3 was present in the VNO. There were equivalent sized bands detected in the cerebellum though they were of much greater intensity and the *Nlgn3*$^{y/-}$ control showed comparable bands for VNO and cerebellum. Also the beta tubulin control showed comparable bands across each tissue type indicating the absence of a band of Neuroligin 3 size in the control was not due to lysis issues.

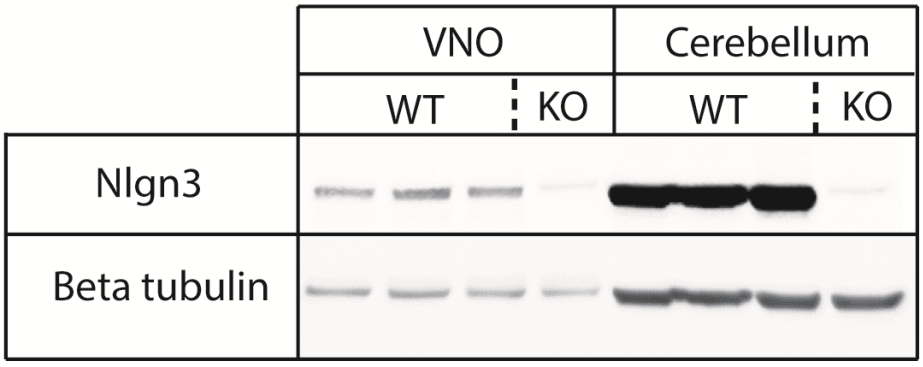


Figure 4.2: Experiments identifying Neuroligin 3 in the VNO. Western blot of *Nlgn3*$^{y/+}$ (WT) and *Nlgn3*$^{y/-}$ (KO) tissue from VNO and cerebellum with beta tubulin control.

In addition to western blot analysis, I used situ hybridisation staining methods to confirm the expression of Nlgn3 in the VNO. I designed three primer pairs, each targeting a different region of the Neuroligin 3 RNA as it is unknown if the Neuroligin 3 protein or RNA is modified in the VNO. All primer pairs produce bands from the VNO RNA that are comparable in size with the cerebellum control bands so each of the primer pairs was used to generate a probe. Each of the probes was tested on control tissue and the probe that gave the clearest

signal was used in subsequent in situ hybridisation experiments. The tissue was exposed to either the sense or the anti-sense probe so that unspecific signal could be identified (Figure 4.3a) however some points were very close, forming large areas of signal that were difficult to count so the intensity across the image was measured (Figure 4.3b) to clarify the difference in the signal. The in situ hybridisation obtained showed signal in the main body of the VNO which was not limited to a specific layer or region and could be present in the neurons, glia and/or supportive cells.

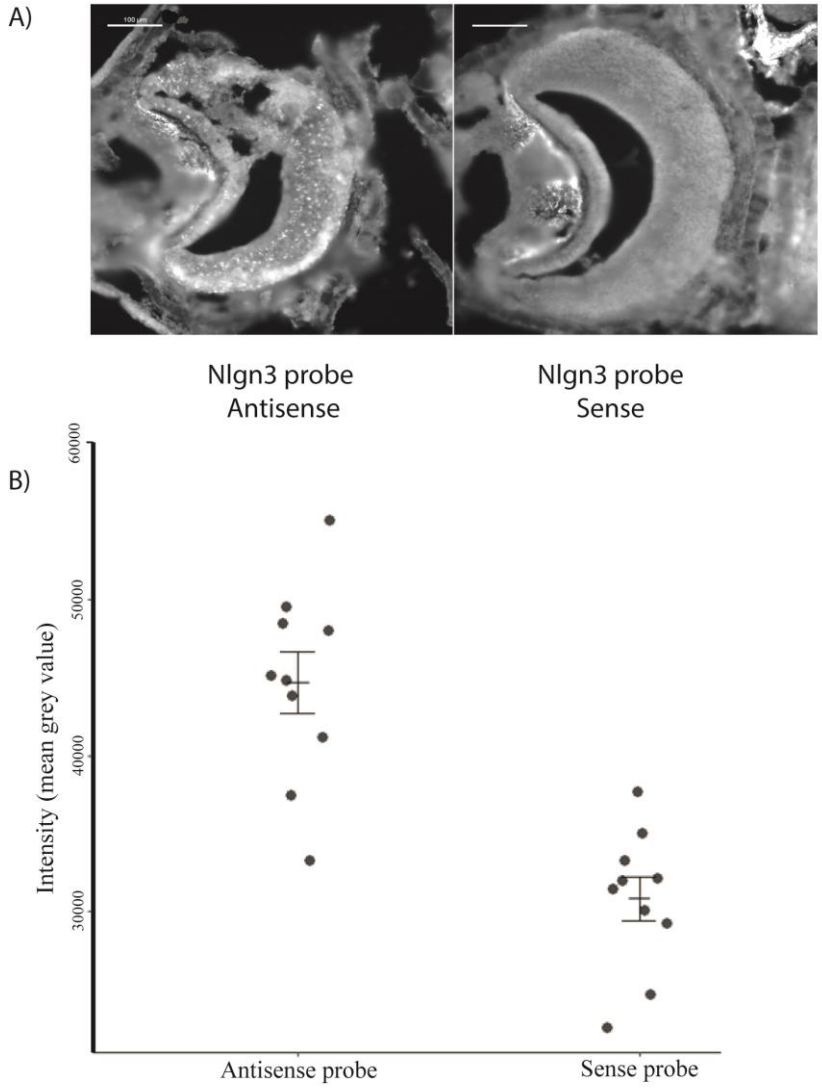


Figure 4.3: Neuroligin 3 mRNA is detectable in the VNO by in situ hybridisation. A) In situ hybridisation of *Nlgn3*$^{y/+}$ VNO for Neuroligin antisense (complimentary) and sense (matching) probes, scale bar = 100µm. B) Plot of intensity of image signal of antisense and sense probe.

4.2.2 Re-expression experiment using *OMP*$^{Cre/+}$ mouse models

To begin I decided use a method with a higher spatial resolution that the western blot and so I prepared samples of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ VNO and brain for immunohistochemistry using Nlgn3 antibodies which could allow us to localise Neuroligin 3 to specific cell types. Unfortunately I could not identify any Neuroligin 3 signal in the brain or in the VNO of these mice and was unable to make conclusions based on this method.

I wanted to identify if Neuroligin 3 was expressed in neurons in the VNO and so I reared Olfactory Marker protein (OMP) Cre mice and crossed them with the *Nlgn3*$^{y/-}$ mice *(OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$). As Olfactory Marker protein (OMP) is specifically expressed in olfactory epithelial cells, Cre expression the would cause the loxP flanked STOP cassette in the promoter region of Neuroligin 3 to be excised in OMP expressing cells only. This generated mice with Neuroligin 3 expressed only in the OMP expressing cells.

I assessed re-expression in the VNO with western blot using the cerebellum and *Nlgn3*$^{y/+}$ VNO as control (Figure 4.4). Western blot for Neuroligin 3 did not show a band in the VNO of *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ mice though bands were identified in the control VNO and cerebellum. This suggested that Neuroligin 3 was not present in neurons in the VNO so I concluded that it could be present in glial cells or other supportive cells instead.

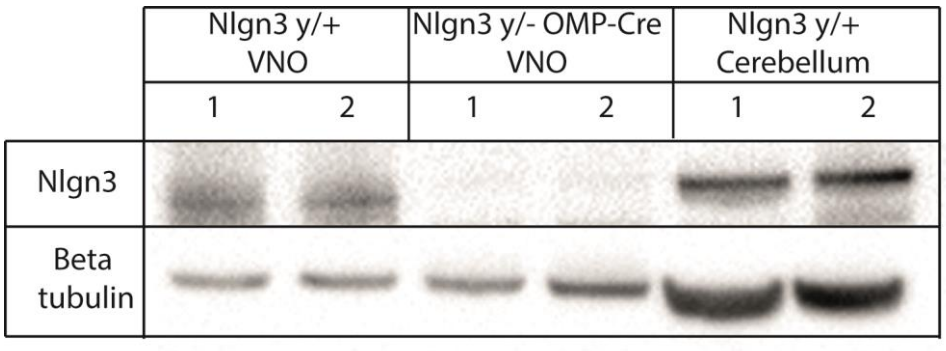


Figure 4.4: Western blot of re-expression on Neuroligin 3 in *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ VNO.

4.3 Mass spectrometry analysis of VNO

4.3.1. Interacting partners with Neuroligin 3 in the VNO

The outcome from the *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ experiment showed that Neuroligin 3 was not likely to expressed by neurons in the VNO so I did not continue to use the *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ mouse to answer questions about the role of Neuroligin 3 in the VNO. As I did not have a clear indication of the role of Neuroligin 3 in the VNO I decided to identify which proteins were associated with Neuroligin 3 in the VNO. The previous detection methods such as situ hybridisation detected mRNA but not protein and using western blots relies on the choice of proteins to look for, so I decided to use mass spectrometry as an unbiased measure to detect Neuroligin 3 and proteins that were associated.

4.3.1 Immunoprecipitation of VNO tissue

The next step was to assess the output of Neuroligin 3 immunoprecipitated tissue. I dissected and lysed VNO tissue the, using beads primed with Neuroligin 3 antibody, immunoprecipitated (IP) the sample for Neuroligin 3. The output was then assessed for known associated proteins of Neuroligin 3 by western blot (Figure 4.5). The western blot showed a band the appropriate size for Neuroligin 3 (compared to protein ladder) in the *Nlgn3*$^{y/+}$ sample but no band was present in the *Nlgn3*$^{y/-}$ sample which showed that the IP method was able to bind Neuroligin 3 specifically.

The western blot also showed no band for PSD95 in the VNO IP output in the *Nlgn3*$^{y/+}$ mouse sample which suggests that these proteins are not associated with each other in the VNO. It could be that these proteins are present in different cells or that these proteins could be in the same cells but are not associated with each other. Therefore an unbiased analysis of the proteins associated with Neuroligin 3 in the VNO was required to begin to understand the role of Neuroligin 3 in this tissue.

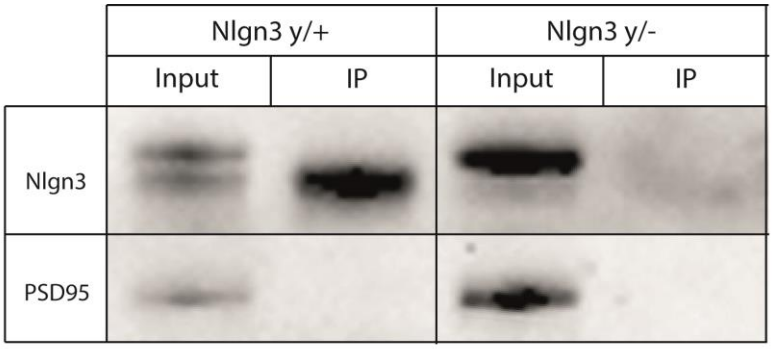


Figure 4.5: Western blot of IP VNO tissue from *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice. Neuroligin 3 (Nlgn3, lower band) was present in the *Nlgn3*$^{y/+}$ IP but not in the *Nlgn3*$^{y/-}$ IP. PSD95 was not detected in the IP of Neuroligin 3.

4.3.2 Tissue processing

Tissue lysis from cerebellum, striatum and VNO were obtained with dissection and lysis techniques described in the general methods. The cerebellum was chosen as a control region Neuroligin 3 has been identified in large quantities in cerebellum tissue (Kalbassi *et al.*, 2017). The striatum was chosen as a region which has been specifically linked to dysfunction and ASD type symptoms with Neuroligin 3 mutations (Rothwell *et al.*, 2014) and so might have a protein profile of interest in this investigation.

I also wanted to obtain a sample of *Nlgn3*$^{y/+}$ glial cells which I chose to generate using a selective re-expression using a AAV-GFAP construct (AAV-GFAP(0.7)-EGFP-T2A-iCre serotype, Vector Biolabs) generating *GFAP-Cre Nlgn3*$^{y/-}$ cells in the tissue. Adult (P60) male mice were injected bilaterally in the motor cortex and allowed to recover for four weeks before tissue was harvested and lysed in the same manner as the other brain regions. Before sending the tissue for mass spectrometry I checked for re-expression using western blotting (Figure 4.6). For the VNO I collected samples of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice to provide a comparative sample with which to focus on Neuroligin 3. Three samples of each of our chosen tissue types (each from different animals) were sent to Bristol Proteomics for mass spectrometry analysis.

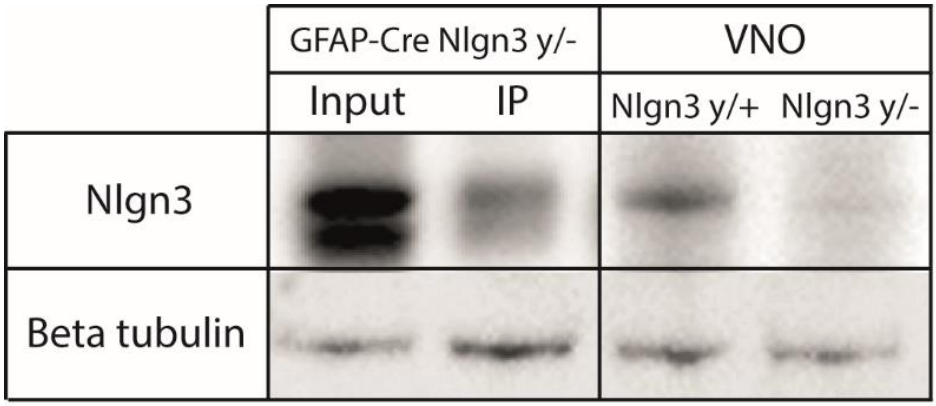


Figure 4.6: Western blot of IP motor cortex from GFAP-AAV *Nlgn3*$^{y/-}$ mouse showing re-expression of Neuroligin 3 in the tissue.

4.3.3 Exploration of Neuroligin 3 IP data

The output of the mass spectrometry was a list of proteins that were identified in each sample (Figure 4.7). In an initial analysis I identified the proteins in the output that were associate with each tissue type and the VNO. I identified that there were a number of proteins associated with the ESCRT III complex (Chmp2a, Chmp4b, Crbn), more so in the GFAP-AAV *Nlgn3*$^{y/-}$ mouse cells. The ESCRT III complex mediates membrane remodelling including transporting membrane proteins to the plasma membrane (McCullough, Frost and Sundquist, 2018) and Neuroligin 3 has been associated with activity dependent remodelling at the synapse, which is thought to contribute to ASD (Singh and Eroglu, 2013). This association of proteins could suggest a role for Neuroligin 3 in membrane remodelling in the VNO in glial cells but I do not have enough data to conclude this, though it could provide a basis for future experiments.

4.3.4 Data processing and hierarchical cluster analysis

The output of the mass spectrometry has a number of values associated with each protein identified in each sample. The 'Score' value reflects how closely the detected protein matched the full protein in the Bristol proteomics database, the 'Coverage' value reflects how much of the protein was represented with the peptides detected and the 'Number of Peptides' value reflects the number of peptides associated with the protein that were detected. There could be cause to argue the relevance of choosing one of these values over another as the basis for

analysis depending on the question that the mass spectrometry data is generated to address. I was interested in how associated the list of VNO proteins was with the lists of proteins from other tissue types so I decided to focus our analysis on comparisons.

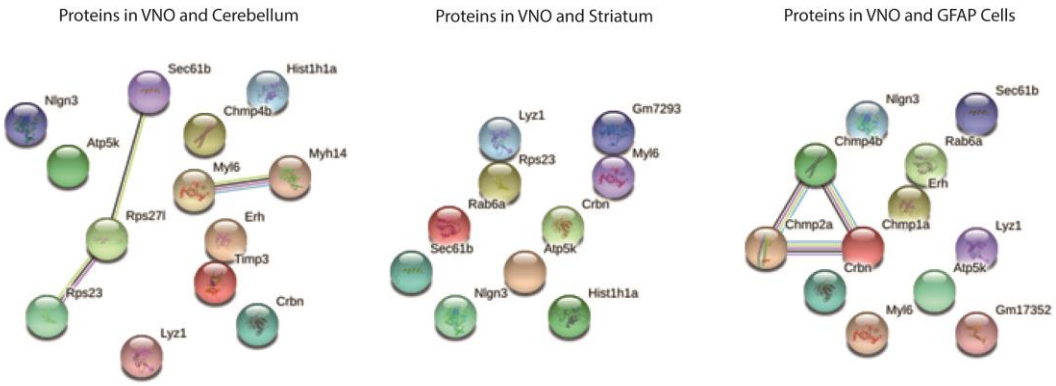


Figure 4.7: Individual proteins shared between VNO and cerebellum, striatum and GFAP-AAV *Nlgn3*$^{y/-}$ cells. Images were generated using STRING (string-db.org)

To begin I sorted the data using RStudio so that the lists of different proteins were all combined by the 'Score' value. I then converted any null values to 0 representing the absence of that protein in the output from that tissue. In order to identify a list of proteins that are associated with Neuroligin 3 I compared the protein lists produced by mass spectrometry of the chosen tissue types to the list generated by the analysis of *Nlgn3*$^{y/-}$ VNO tissue to create a list of proteins that could be eliminated from subsequent analysis. I then inputted this data into a hierarchical clustering analysis using RStudio to assess the number of clusters that the data could be split into while maintaining clusters of similar size (Figure 4.8).

I identified that three clusters best fit the criteria and then used this information to perform a two-step cluster analysis in SPSS generate groups from the data. Each of the clusters contained different of proteins for each of the brain regions. The number of proteins shared between the VNO and other tissues did not show a clear distinction as it was quite similar in each tissue.

As the list of proteins were still a considerable size I decided to break down each cluster further by adding a score associated with the detection of each protein in the repeated samples of each brain region. These modified lists in each of the clusters was used to compare the VNO to the other tissue types. A comparison of the protein list reveals no particular association of the proteins found in the VNO with any individual tissue type. I felt that the cluster analysis alone was insufficient to make specific conclusions about the function of Neuroligin 3 in the VNO.

I decided to use the cluster analysis as a basis from which to begin a more targeted analysis where I would take specific biological functions in the VNO and look for associated proteins in the mass spectrometry output. A well-known biological role of Neuroligin 3 is as a synaptic adhesion molecule where it has been found to contribute to synapse stability, function and maturation (Rawson *et al.*, 2006) and disruption in this function has been associated with ASD (Ebert and Greenberg, 2013). However I concluded from the VNO Neuroligin 3 IP western blotting that PSD95 was not associated with Neuroligin 3 in the VNO and also since I did not identify re-expression in the $OMP^{Cre/+}$ $Nlgn3^{y/-}$ mouse I was interested in looking at possible non-synaptic, non-neuronal roles of Neuroligin 3. Neuroligin 3 has been identified in the olfactory ensheathing cells (OECs) which are glial cell that surround the axons of olfactory receptor neurons between the MOEs and the olfactory bulb and is thought to be involved in the formation of glial sheath (Gilbert et al. 2001) and could be playing a supportive role in the VNO. Analysis of the VNO mass spectrometry data for markers of OEC identity suggests that the Neuroligin 3 positive cells are probably not related to OECs. For example S100β, GFAP, NCAM and vimentin are not present in the data which are known markers of OECs (Vincent, West and Chuah, 2005). In order to understand what the role of Neuroligin 3 in the VNO could be it was clear to us that analysis of proteins was insufficient though it can be a very useful tool when combined with other methods.

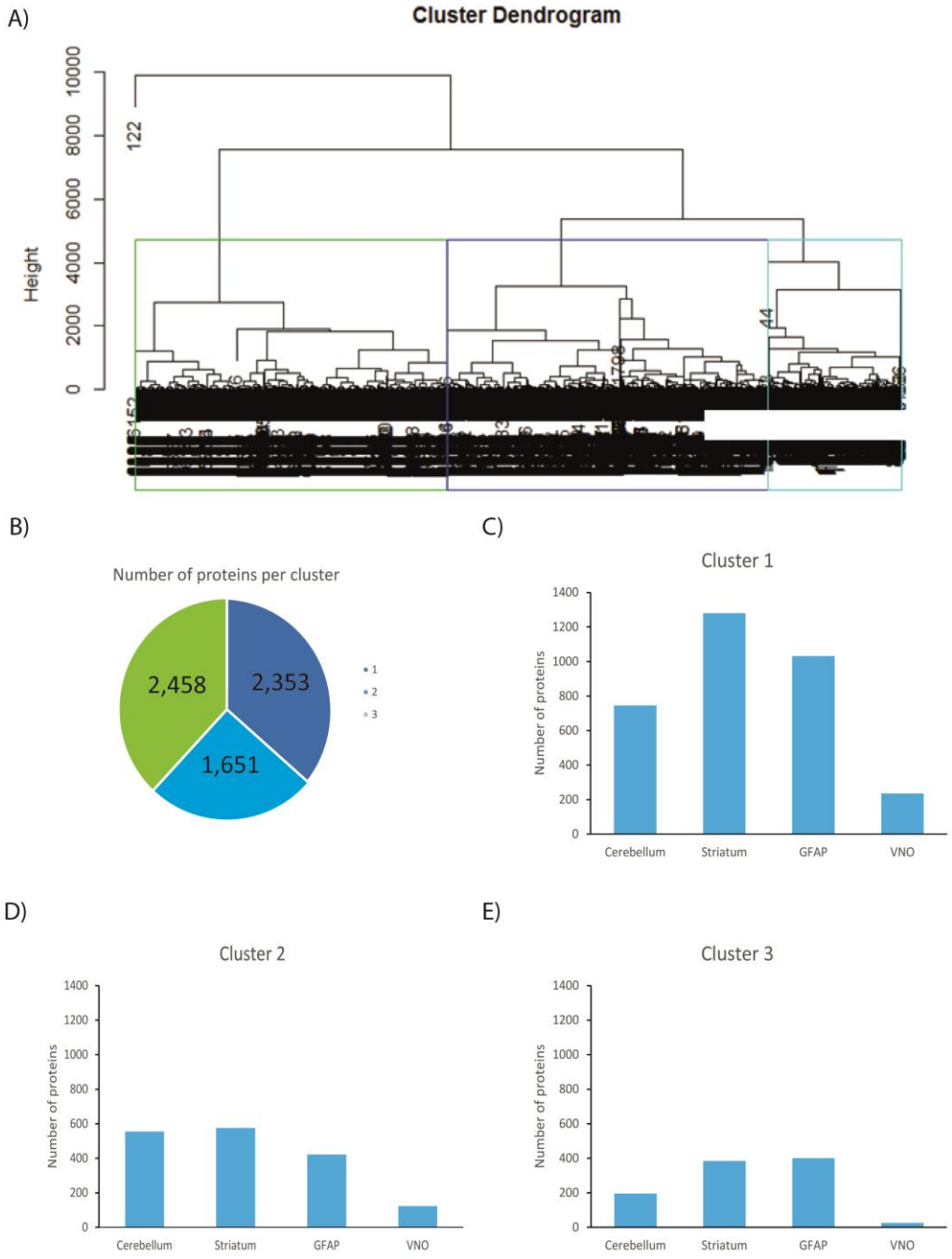


Figure 4.8: Cluster analysis of proteins from mass spectrometry of Neuroligin 3 IP tissues. A) Cluster dendrogram of hierarchical clustering analysis showing the three clusters. B) Number of proteins in each of the clusters. C-E) Number of proteins in each cluster by tissue type.

4.4 Nlgn3 involvement in cell activity

4.4.1 Ex-vivo experiment

I wanted to identify if *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice had different reactivity in the VNO when exposed to scent cues. I began by exposing *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice to HMW scent cues and then processing the VNO for immunohistochemistry with EGR1 antibody but was unable to detect any signal in the VNO. As with the Neuroligin 3 staining of the VNO tissue I changed to in situ hybridisation staining method and generated an Egr1 probe. However I still did not detect Egr1 in any of the samples. I considered that this might be due to the variability in the sniffing of mice so decided to use an ex-vivo culture model where I had more control over the exposure of the VNO to the scent cues. VNOs were dissected and placed directly in culture medium then HMW urine fraction was applied directly to the tissue which was incubated for 1 hour. The tissue was then fixed and processed for staining. I achieved some signal using the Egr1 probe so I continued with this method. I was hoping to be able to detect if there was any regional specificity to the expression of Neuroligin 3 so decided not to use a broader method such as qRT-PCR at this point.

Nlgn3$^{y/+}$ male VNOs were exposed in culture to whole male urine, whole female urine, a solution of potassium chloride (positive control) and water (negative control). Tissue was then stained using the Egr1 in situ antisense and sense probes (Figure 4.9). The signal was slightly dispersed rather than forming clear, countable points so I measured the intensity of the sections at ten randomised points per sample in each of the three repeats of the experiment (Figure 4.10).

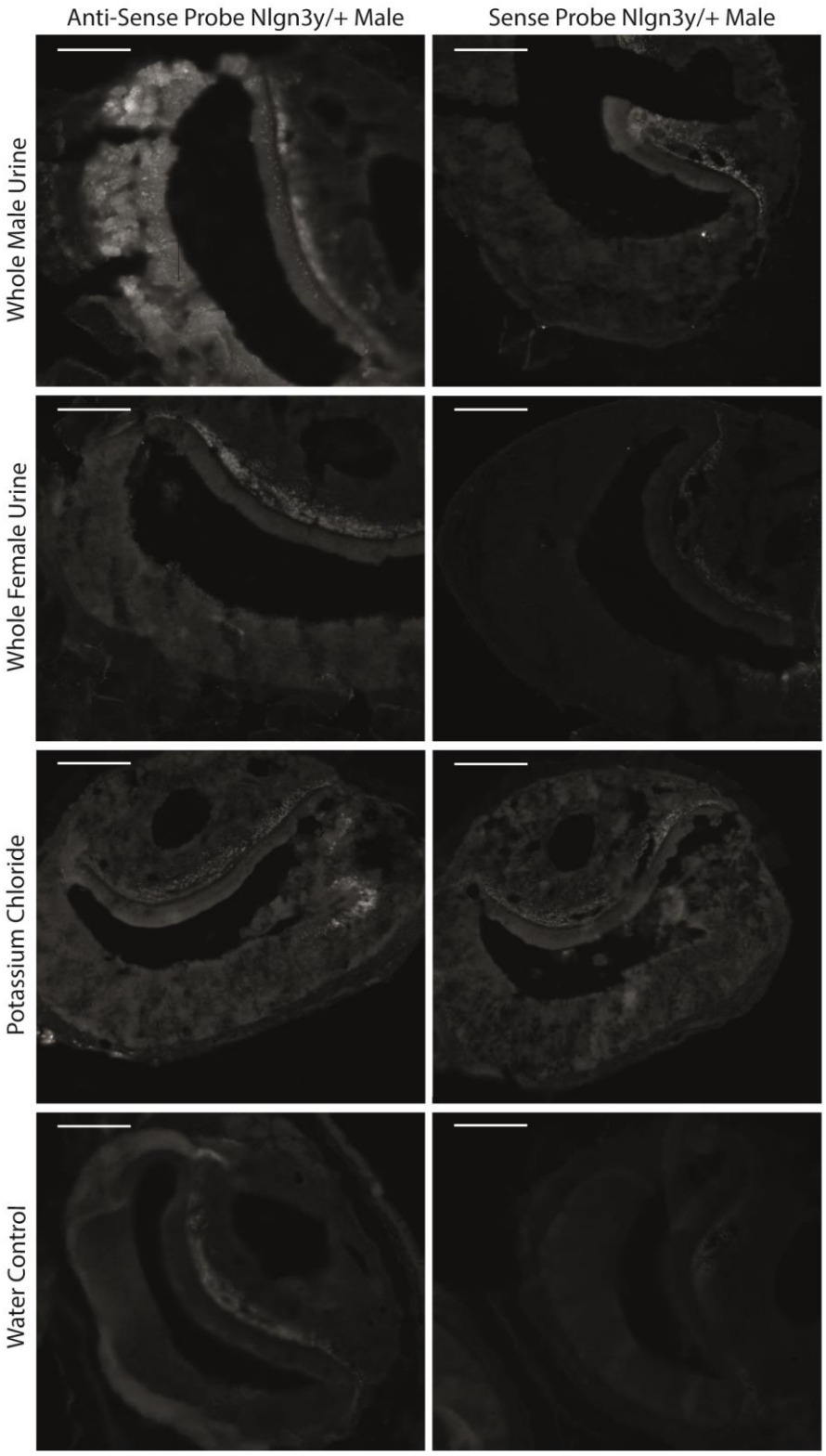


Figure 4.9: In situ hybridisation with Neuroligin 3 antisense and sense probe on *Nlgn3*$^{y/+}$ VNO exposed to whole urine (male and female), KCl as positive control and water as a negative control. Scale bar = 100µm.

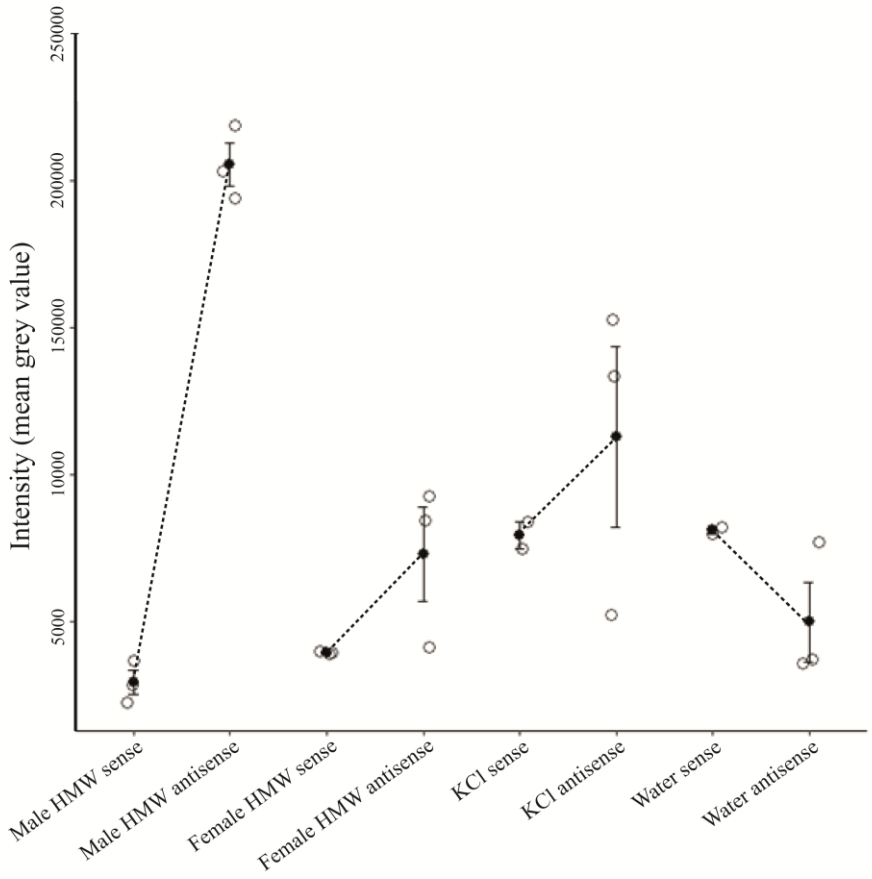


Figure 4.10: Intensity of Egr1 sense and antisense probe of *Nlgn3*$^{y/+}$ male VNO

I observed a higher signal in the VNO post exposure to HMW male urine and to a lesser extent to those exposed to female HMW and KCl but not to water. However there was a lot of variation in the amount of signal detected in the sense probe which is intended to show the background signal and assure us of the specificity of the probe. I had a similar difficulty in concluding on the difference between *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mouse VNO reaction to HMW male urine fraction where the variance in the signal detected in the antisense probe of *Nlgn3*$^{y/-}$ mouse was very high (Figure 4.11).

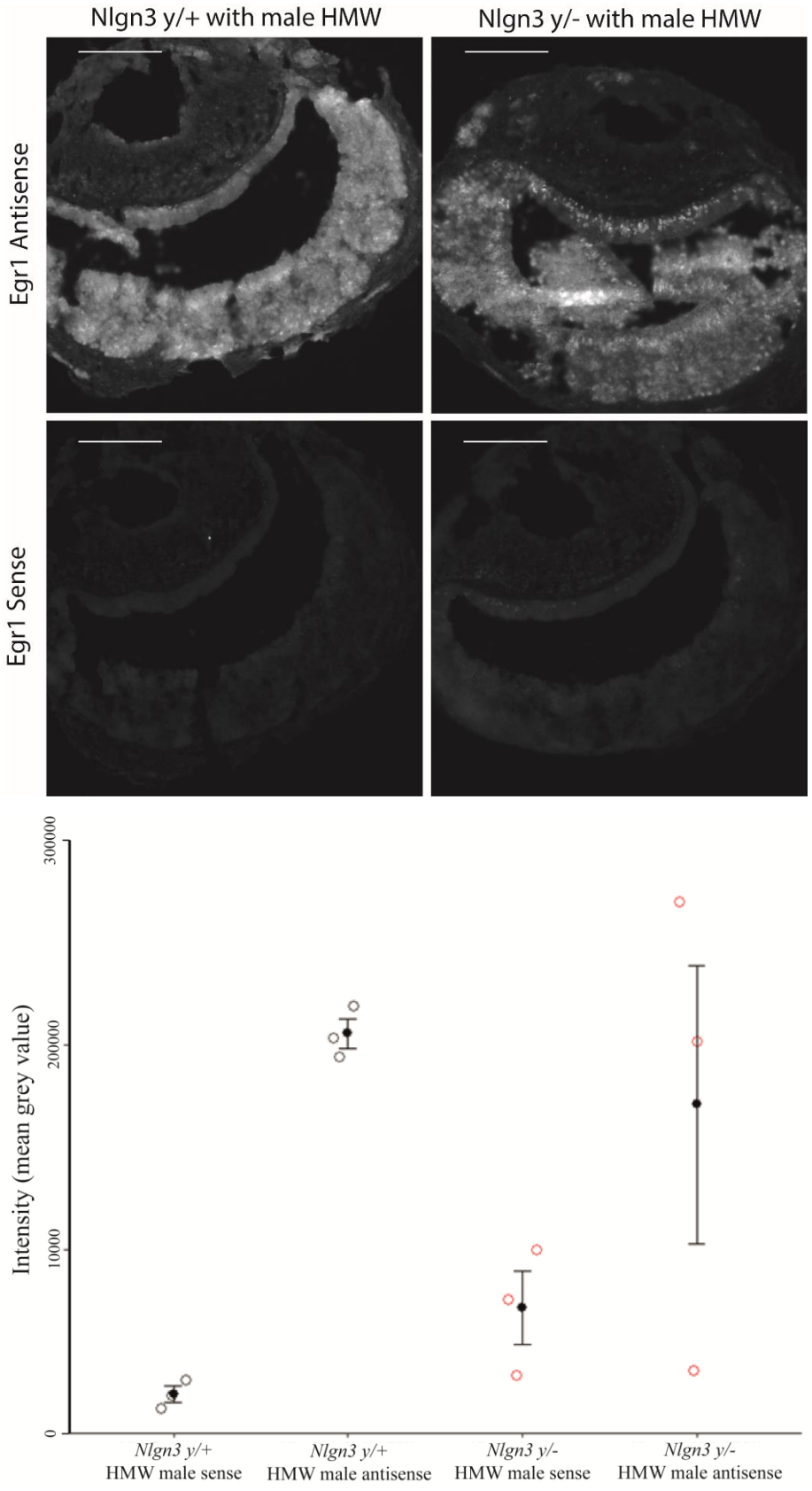


Figure 4.11: Comparison of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ VNO reaction to HMW male urine fraction. Scale bar = 100µm.

4.4.2 Cell culture

Another method I attempted in order to detect differences in VNO cell activity was to create primary cultures from VNO tissue, preferably including VSNs and OECs. I intended to analyse differences in the neuronal and glial responses to stimuli and possibly separate and analyse different cell types. I was able to grow individual cells from dissociated VNO for up to five days at which point I was able to identify some living cells that resembled the morphology of OECs from the examples found in the literature (Figure 4.12) but none of the cells that resembles VSNs lasted longer than a few hours.

Initially I attempted to use calcium signalling to identify responses of cultured cells to scent cues but this did not yield any identifiable signal that gave consistent results. I then changed technique to patch clamping which, due to the short life span of the cells also did not yield any significant results. The main difficulty I encountered was then the survival of the VNO cells which was typically very short and also the yield of cells that had the appearance of neurons was very low. There was also quite a lot of inconsistency in the quality of the cultures from one to the next despite using the same method which suggested that the cells were very sensitive to change.

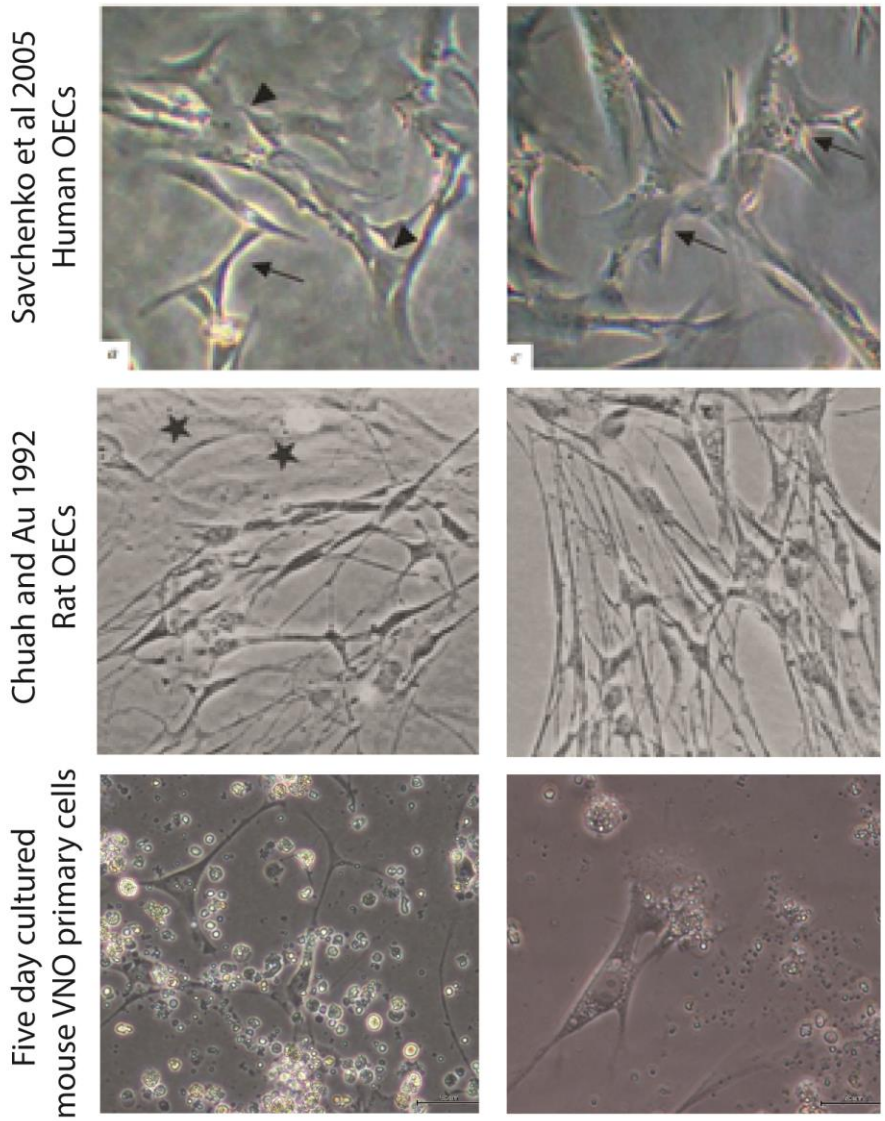


Figure 4.12: Comparison of cell culture from human OEC culture (Savchenko et al 2005), rat OEC culture (Chuah et al 1992) identified in the literature and mouse primary VNO cells from my culture experiment.

4.5 Re-expressing Nlgn3

4.5.1 *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ re-expression does not result in re-expression in the VNO

As I was unable to determine the changes in the VNO between *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{y/+}$ mice I decided to identify if re-expression in the brain could cause any improvements to behavioural deficit. In this way I could potentially identify if Neuroligin 3 in the VNO is affecting interest

for scent cues and discrimination as it should not be possible to rescue behaviour by re-expressing Neuroligin 3 in the brain but not in the VNO.

Our group has identified that social behaviours such as tube test rank and interactions with females that are affected by Neuroligin 3 knockout and/or social housing can be rescued with the re-expression of Neuroligin 3 in parvalbumin expressing neurons in a number of brain regions (Kalbassi et al. 2017). This suggested that parvalbumin expressing neurons are involved in controlling territorial behaviours which are affected by social odours. I was interested to identify if the interest for social scent cues was also affected in these mice.

To begin with I assessed if there was any re-expression of Neuroligin 3 in the VNO of *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mice by lysis of VNO tissue and western blot. I identified no band associated with Nlgn3 in the VNO of *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mice, though I did identify an unspecific band that was not present in the *Nlgn3 y/+* VNO (Figure 4.13). As I had seen this unspecific band in western blot when using this antibody before I concluded that Neuroligin 3 was not present in the VNO of *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/}$ mice.

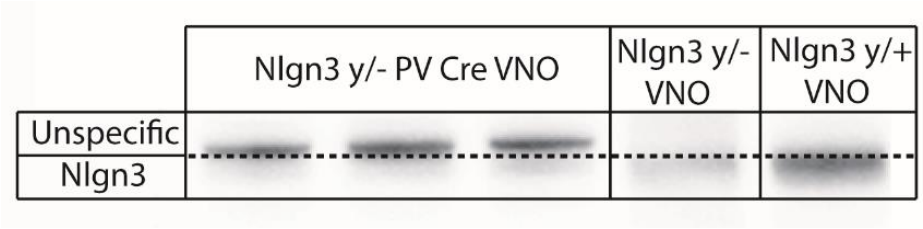


Figure 4.13: Western blot of *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ VNO with *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ VNO control.

4.5.2 *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ behaviour

Given the lack of re-expression in the VNO of the *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mouse I decided to assess the social interest of these mice (Figure 4.14). As the breeding conditions of the *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mice necessitated a mixed genotype housing environment I used MGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice as the control animals on which to base the comparison of

behavioural rescue. *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$ mice were included as a genetic control for the *Pvalb*$^{Cre/+}$ mouse model and housing control for the *Pvalb*$^{Cre/+}$mouse mixed housing environment. I identified an interest for social cues in the *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ and *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$ which, as there was no re-expression in the VNO of these mice, suggests that the role of Neuroligin 3 in the VNO may not be crucial to these behaviours (Figure 4.13, ANOVA, Within subjects (sniffing):F(1,38)=9.037, p=0.005, Between subjects (genotype): F(1,38)=1.412, p=0.254, Sniffing*Genotype: F(3,38)=8.874, p<0.001, pairwise comparison: *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$, p=>0.001, *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$, p=0.001). It is possible that the effect of the Neuroligin 3 knockout on the function of the VNO is ether not responsible for this behaviour specifically or that the negative effect of the Neuroligin 3 knockout on the VNO is not sufficient to prevent the rescue of this behaviour with the knock-in of Nlgn3 in parvalbumin positive neurons.

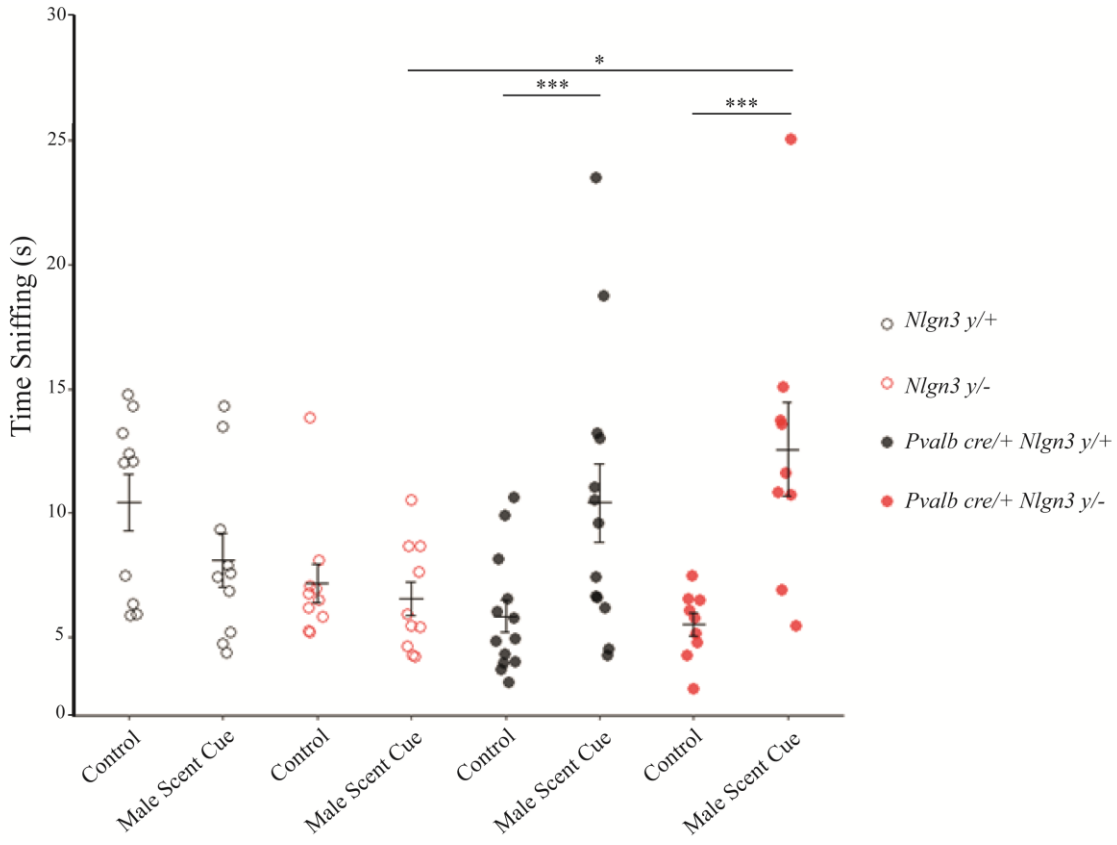


Figure 4.14: Comparison of MGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ with *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/+}$ and *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$

4.6 Discussion

I attempted in this chapter to identify more about the functional differences, if any were to be found, in the VNO of the *Nlgn3*$^{y/-}$ compared to *Nlgn3*$^{y/+}$. I did identify that Neuroligin 3 was present in the VNO and that it did not seem to be associated with PSD95 as I would expect based on what is known about the function of Neuroligin 3 in the synapse. Due to the lack of re-expression in the VNO of *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ mice I considered it possible that Neuroligin 3 was present in glial cells rather than neurons and wanted to try to look at the activity of individual regions and cells of the VNO in response to different scent to gain some understanding of Neuroligin 3 in the VNO. However, despite the use of several method I found in the literature I was unable to generate comparable results that could be used to give this information. With the in situ hybridisation I was unable to get a consistent signal which, although the signal I could see seems to give theoretically feasible data it is not data that I could trust enough to form clear conclusions. The cell culture was unable to provide healthy cells that could be patch clamped or collected. Also I did not have access to the resources to perform electrophysiology or calcium signalling experiments on VNO slices.

However, despite this set back I was able to identify, with the use of the *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mice, that reduced functioning of the VNO is not the sole factor in the reduced interest of *Nlgn3*$^{y/-}$ mice for some social cues. This suggests that Neuroligin 3 knockout was damaging VNO function, which may well be the case, but it does not appear that the reduction in function cannot be compensated by other rescue knock-in of Neuroligin 3. This gave me an incentive to move away from the VNO at this point and explore changes in the *Nlgn3*$^{y/-}$ mouse brain in response to scent cues and return to the VNO in the future when I have new strategies to approach this.

4.7 Summary

- Neuroligin-3 protein and RNA were identified in VNO tissue

- Attempts to assess the function of Neuroligin-3 in the VNO or to detect differences between *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{y/+}$ mice from different housing conditions were unsuccessful
- Re-expressing Neuroligin-3 in the central nervous system in parvalbumin cells rescued some interest for male scent cues in *Nlgn3*$^{y/-}$ mice (*Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mice)

Chapter 5

Mapping c-Fos signal to identify regions of interest associated with MUP20 detection

5.1 Introduction

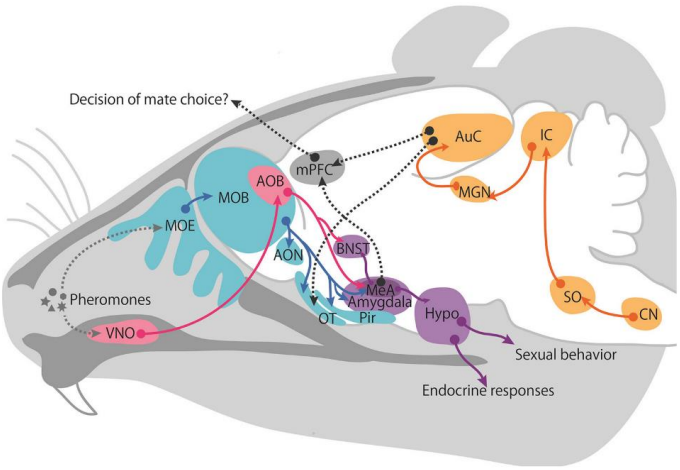
The broad aim of this work was to explore social memory deficit in *Nlgn3*$^{y/-}$ mice. I identified that *Nlgn3*$^{y/-}$ mice have a reduced interest for social cues such as urine HMW fraction and also that the do not discriminate scent cues of males. However, since this could be improved by altering expression in a small selection of neurons in the brain despite a lack of re-expression in the VNO I realised it was important to identify what differences could be identified in the brain of *Nlgn3*$^{y/-}$ mice compared to *Nlgn3*$^{y/+}$ mice after exposure to scent cues. I was particularly interested to identify if differences in the brain of *Nlgn3*$^{y/-}$ mice could be identified for some scent cues and not others which could help to begin to understand the behaviour of *Nlgn3*$^{y/-}$ mice. I decided to first focus on MUPs as they have been found to affect social behaviour and have been found to stimulate specific brain regions in wildtype mice.

MUP20, also known as Darcin (Roberts *et al.*, 2010), is detected specifically by the V2R positive VSNs in the basal VNO. MUP20 is produced only in the male urine and increases sexual attraction in females altering spatial memory (Roberts *et al.*, 2010) and stimulating neurogenesis in the hippocampus (Hoffman *et al.*, 2015). This finding was not limited to females, however the reaction of males was dependent on the perceived competitiveness of the scent cue. Males presented with rMUP20 or the urine of an unfamiliar male showed a conditioned place preference and adding rMUP20 to familiar male urine could induce a conditioned place preference (Roberts et al. 2012). Neurogenesis was not identified in the male hippocampus but it is a possible region of interest. Neither MUP7 nor MUP11 induced a similar response in males or females indicating not just that individual MUPs can be detected but also that they have different effects on behaviour (Hoffman *et al.*, 2015).

MUPs in general and MUP20 in particular, are able to induce sex specific behaviour in mice. Territorial behaviours in males are based on the identification of individuals based on the MUP profile of the individual (Hurst and Beynon, 2004) which shows that the detection of individual MUPs is important to mouse social behaviour. This suggests that brain regions associated with aggression such as the amygdala could be activated due to MUP20 exposure in males.

A number of brain regions have also been associated with female mate choice (Figure 5.1 (Asaba *et al.*, 2014) including the amygdala and hypothalamus which make good candidate regions to examine. For example the posterodorsal medial amygdala of female rats was found to have increased Fos signal when they were exposed to increasing concentrations of male MUPs (Kumar *et al.*, 2014).

Taken together these factors make MUP20 a good scent cue to use to identify differences between activation in the brains male mice due to Neuroligin 3 knockout and/or housing conditions which is the aim of this chapter. I hypothesised that there would be differences in the signal due to genotype which may allow further investigation of the differences I have identified in the behavioural investigation of *Nlgn3*$^{y/-}$ mice.



5.1: A proposed pathway for male olfactory and auditory cues in the female mouse leading to mate choice (Taken from Figure 2, Asaba et al. 2014) Abbreviations: accessory olfactory bulb (AOB), anterior olfactory nucleus (AON), auditory cortex (AuC), bed nucleus of the stria terminalis (BNST), cochlear nucleus (CN), Hypothalamus (Hypo), inferior colliculus (IC), medial amygdala (MeA), medial geniculate nucleus (MGN), main olfactory bulb (MOB), main olfactory epithelium (MOE), medial prefrontal cortex (mPFC), olfactory tubercle (OT), piriform cortex (Pir), superior olivary nucleus (SO), vomeronasal organ (VNO)

5.2 Social interest for HMW scent cues with the addition of MUP20

5.2.1 Social interest of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ males for HMW male urine fraction with additional MUP20

As I was interested in difference in brain region activation associated with MUP20 I first decided to see if *Nlgn3*$^{y/-}$ mice have altered interest for MUP20. *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ males were exposed to rMUP20 added to HMW male urine fraction which was used as a buffer since mice do not seem to be attracted to rMUP20 by itself and I wanted to ensure that they would perform sniffing behaviours. A repeated measures ANOVA of the resulting sniffing time identified a significant main effect of scent cue and genotype but not of housing (ANOVA, Within subjects (sniffing): $F(1,35)=30.137$, $p=<0.001$, Between subjects (genotype): $F(1,35)=4.412$, $p=0.043$, Between subjects(housing): $F(1,35)=0.051$, $p=0.823$). An interaction effect of sniffing and genotype was identified (Sniffing*Genotype: $F(1,35)=8.375$, $p=0.007$) though no interaction was identified between scent cue and housing or genotype and housing

and there was no interaction between the three factors (Sniffing*Housing: F(1,35)=0.154, p=0.697, Genotype*Housing: F(1,35)=0.253, p=0.618, Sniffing*Genotype*Housing: F(1,35)=2.086, p=0.158). This effect was driven by the difference between *Nlgn3*$^{y/+}$ mice interest for control and HMW with MUP20 exposure as both SGH and MGH *Nlgn3*$^{y/+}$ spent more time sniffing HMW male urine fraction with MUP20 than control (WT:SGH:Control/HMW, p=0.001, WT:MGH:Control/HMW, p=<0.001) and also the difference between MGH *Nlgn3*$^{y/+}$ and MGH *Nlgn3*$^{y/-}$ mice (MGH:HMW:WT/KO, p=0.016). I therefore concluded that *Nlgn3*$^{y/+}$ males regardless of housing had greater interest for male HMW with MUP20 as I predicted. It is possible then that the interest identified in the *Nlgn3*$^{y/-}$ mice for whole urine was due to volatile elements of urine and so addition of another HMW element did not stimulate their interest like whole urine did but I did not have enough information to conclude from this data.

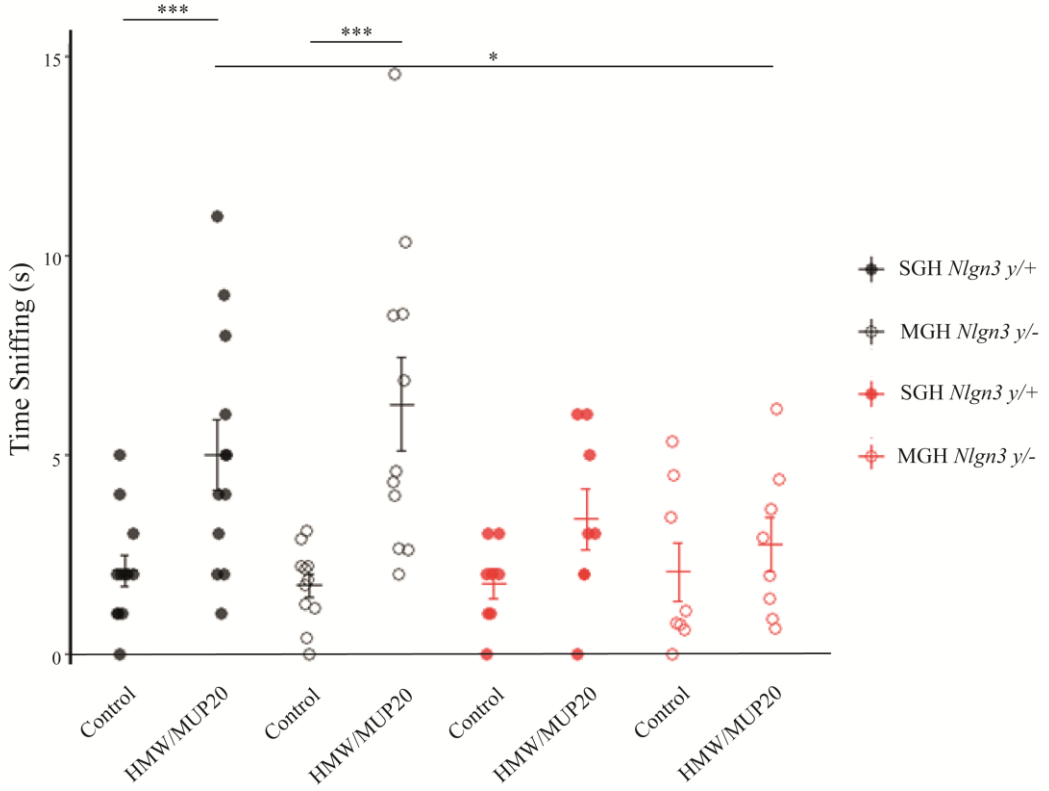


Figure 5.2: Interest of SGH and MGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ for male HMW urine fraction with the addition of rMUP20.

5.2.2 Social interest of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ males for HMW female urine fraction with additional MUP20

As the female HMW does not naturally contain MUP20 I tried using it as an alternative buffer to see if I could increase the interest of *Nlgn3*$^{y/-}$ mice for female HMW to the level of their interest for the HMW male implying a threshold for interest based on MUP content. A repeated measures ANOVA of the sniffing times showed a significant main effect of scent cue (ANOVA, Within subjects (sniffing):F(1,39)=16.336, p=<0.001) but not genotype or housing (ANOVA, Between subjects (genotype): F(1,39)=2.461, p=0.125, Between subjects(housing): F(1,39)=0.328, p=0.570). I identified an interaction effect between scent cue, genotype and housing but not between any two of the factors alone (Sniffing*Genotype: F(1,39)=2.910, p=0.096, Sniffing*Housing: F(1,39)=1.154, p=0.289, Genotype*Housing: F(1,35)=3.818, p=0.058, Sniffing*Genotype*Housing: F(1,35)=8.595, p=0.006). However this was not due to any increase of interest of the *Nlgn3*$^{y/-}$ mice but rather a reduction of interest in the MGH *Nlgn3*$^{y/+}$ mice resulting in a significant difference between MGH and SGH *Nlgn3*$^{y/+}$ (WT:SGH:Control/HMW, p=<0.001). There was also a significant difference between the time SGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ spent sniffing HMW+MUP20 (SGH:HMW:WT/KO, p=0.008) and a significant difference between MGH *Nlgn3*$^{y/-}$ (KO:MGH:Control/HMW, p=0.019). I concluded that MUP20 induces less interest from *Nlgn3*$^{y/-}$ mice and MGH *Nlgn3*$^{y/+}$ mice than SGH *Nlgn3*$^{y/+}$ mice which may yield difference in brain activation.

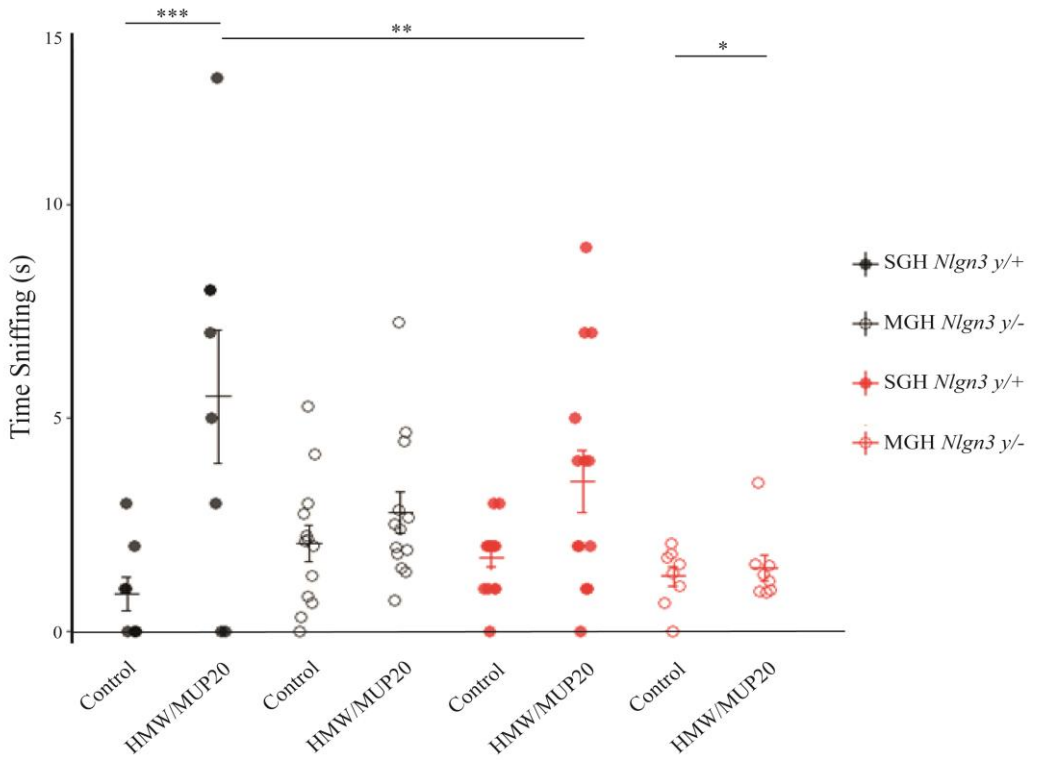


Figure 5.3: Interest of SGH and MGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ for female HMW urine fraction with the addition of rMUP20.

5.3 GFP signal initial mapping

5.3.1 Identifying Cfos-GFP signal in MUP20 exposed mice

The initial question driving this experiment was if there is an identifiable reactivity difference in the CNS due to genotype or housing when mice are exposed to MUP20 so I decided to use c-Fos as a marker for activity and map difference that I see in the brain of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from SGH and MGH housing.

To begin with the c-Fos-GFP mouse was used for a screening experiment where males were exposed directly to the rMUP20 or to water which acts as a control group by the application of a drop of the scent cue applied directly to the nose of each mouse and a scent cue stained filter paper added to the home cage. I did not mix rMUP20 with HMW urine fraction in this experiment as I wanted to look at the signal due to MUP20 separately from other scent cues. I applied the scent cue directly to the nose of the mice as I had concerns about the mice sniffing

the filter paper. The mice were perfused one hour after exposure and the brains were processed for confocal imaging. Observations of the sections directly, by viewing slides in the confocal microscope, to assess the prevalence of the c-Fos-GFP signal identified the dentate gyrus as the only region of the brain with a visibly high difference in signal between the rMUP20 exposed animals and the control. This was somewhat unexpected as there is a known association of brain regions such as the accessory olfactory bulb and the hypothalamus which showed little staining in ether condition (Figure 5.3).

5.3.2 MUP7 does not show the same pattern of c-Fos activation in the dentate gyrus as MUP20

I decided to investigate if the signal that I observed in the dentate gyrus was the result of MUP detection in general or MUP20 more specifically. It has been previously shown that MUP20 induces spatial learning and neurogenesis is the dentate gyrus of female mice but MUP7 did not (Hoffman *et al.*, 2015). This suggests that the dentate gyrus is an important brain region in MUP recognition so I decided to make a comparison between c-fos expression due to MUP20 or MUP7 exposure in the dentate gyrus.

Very little signal in the dentate gyrus of MUP7 exposed males compared with MUP20 exposed males which is consistent with behavioural findings (Roberts et al. 2012). In order to quantify the signal eight sections were selected across the dentate gyrus using a mouse brain atlas for each of the mice in each of the conditions to try to minimise a biased selection of sections to count. I decided to focus on the dentate gyrus at this time due to the high signal (Figure 5.4). An analysis of the counts using Friedman test (due to non parametricity of data) identified a significant difference ($X2(2)=16.020$, $p=<0.001$) with mean ranks of 27 for control, 18.59 for MUP7 and 40.31 for MUP20 which was due to increased signal count in MUP20 exposed mice compared to control and MUP7 exposed mice (Dunn's pairwise comparison, MUP20/Control: $p=0.030$, MUP7/MUP20: $p=<0.001$) though no significant difference was identified between MUP7 and control (MUP7/Control: $p=0.390$, Figure 5.5). The low counts

identified in the MUP7 exposed mice were comparable to control implying that the increase in signal in the dentate gyrus I identified with MUP20 exposure is not associated with the detection of MUP7.

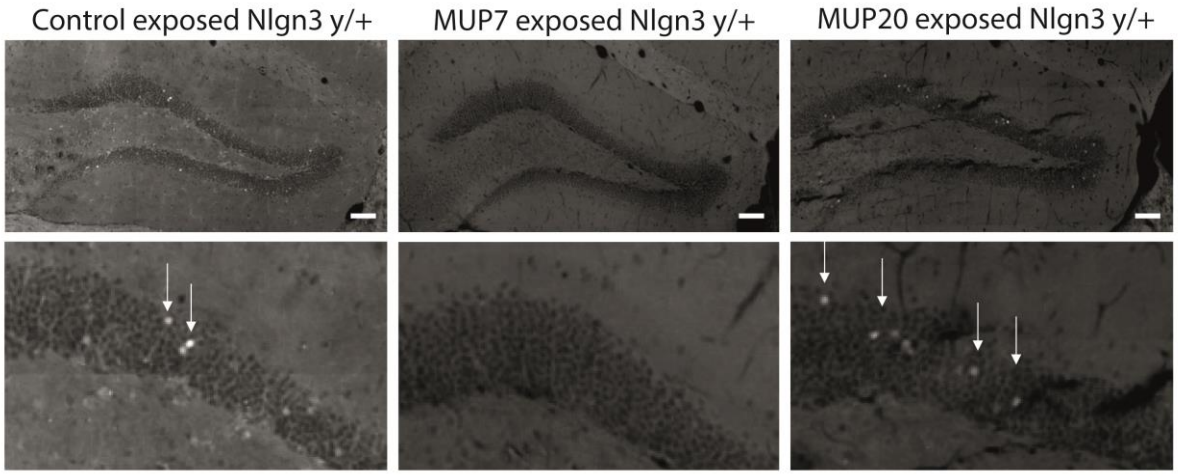


Figure 5.4: GFP signal in the dentate gyrus of c-Fos-GFP mice exposed to control cues, MUP7 and MUP20 (arrows indicate the signal which have the appearance of bright dots of a comparable size to the dapi signal). Scale bar = 100µm.

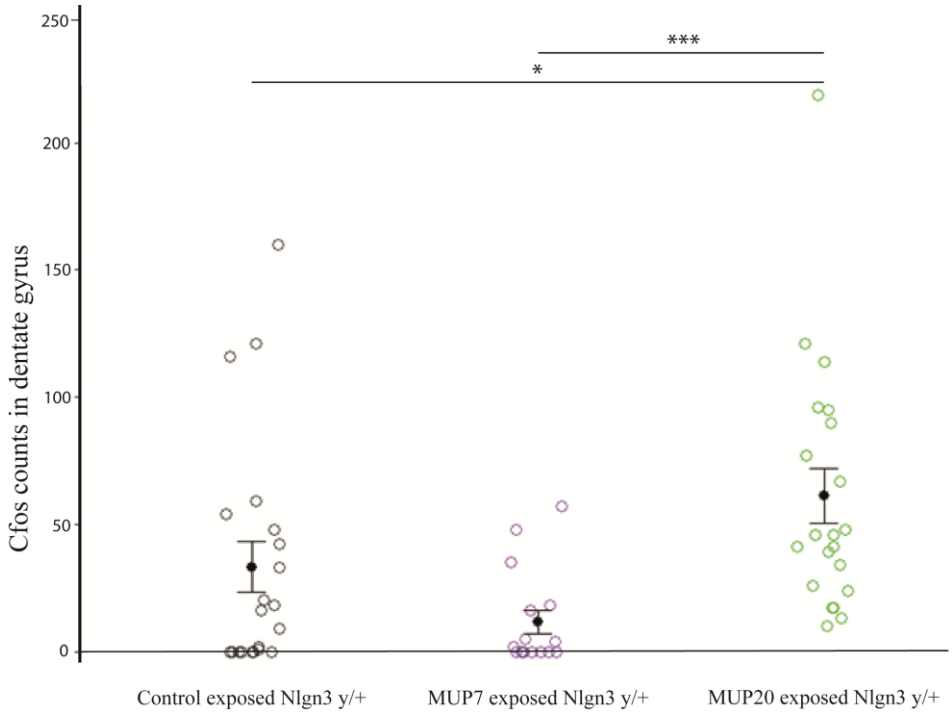


Figure 5.5: c-Fos signal in the dentate gyrus of c-Fos-GFP mice exposed to control, MUP7 and MUP20

5.4 GFP signal vs antibody

With the dentate gyrus identified as a region of interest I wanted to explore if there was any difference identifiable in *Nlgn3*$^{y/-}$ compared with *Nlgn3*$^{y/+}$ in SGH and MGH housing. However there was a conflict between the c-Fos-GFP mouse model and the Neuroligin 3 knockout mouse model that I used meant was it is not possible to breed them to create *Nlgn3*$^{y/-}$ *c-Fos-GFP* mice (see methods section). Therefore an antibody would have to be used to detect c-Fos signal when assessing the differences between *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ c-Fos signal in brain tissue. In order to see if the signal from the *c-Fos-GFP* mice matches the signal from the antibody, sections of *c-Fos-GFP* mouse brain were stained with the c-Fos antibody and the two types of signal were compared to each other. Eight sections across the dentate gyrus were selected as previously and were analysed for both the GFP and the antibody signal.

The antibody signal visibly overlapped with the GFP signal in the dentate gyrus and was verified in counts of GFAP and antibody as no main effect of signal between these two was found in a repeated measures ANOVA though as expected due to our pervious analysis there was a main effect of scent cue and no resultant interaction was identified (Repeated measures ANOVA (Greenhouse-Geisser correction) Within subjects (Signal): $F(1,50)=3.232$, $p=0.074$, Between subjects (Scent cue):, $F(1,50)=9.527$, $p=<0.001$, Signal*Scent Cue:, $F(2,50)=1.037$, $p=0.362$). From this result, I concluded that the antibody signal was analogous with the GFP signal despite the difference in absolute values (Figure 5.6 and 5.7). This allowed me to continue the experiment using the c-Fos antibody in the *Nlgn3*$^{y/-}$.

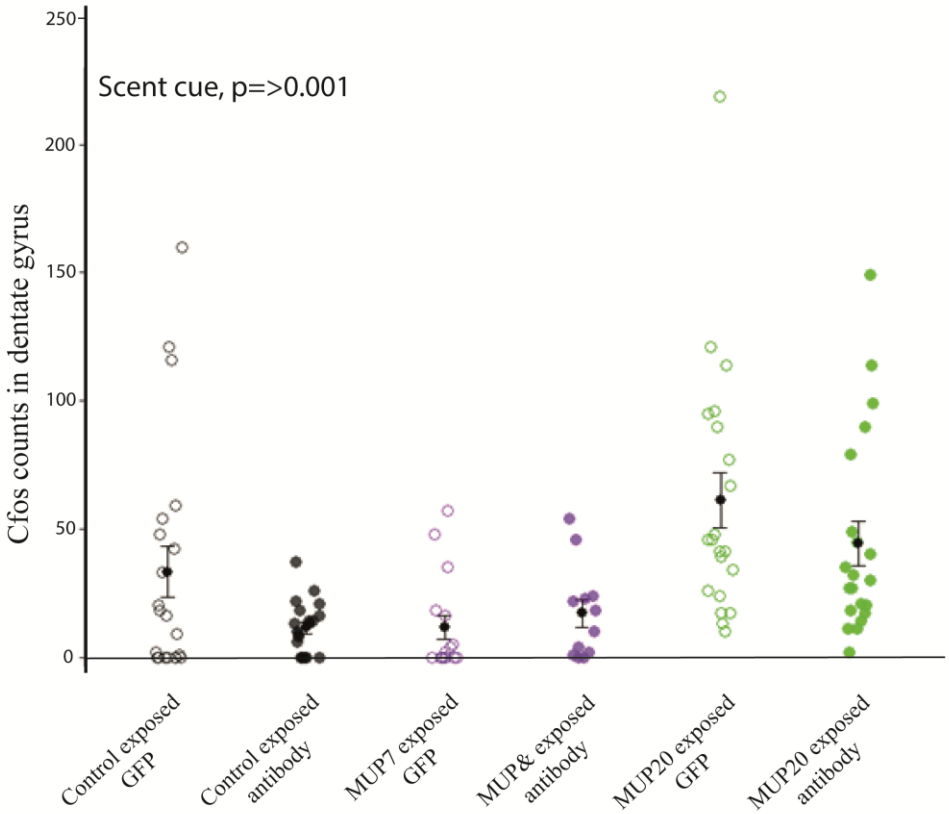


Figure 5.6: Comparison of the GFP and antibody signal in the dentate gyrus of c-Fos-GFP mice exposed to control, MUP7 and MUP20.

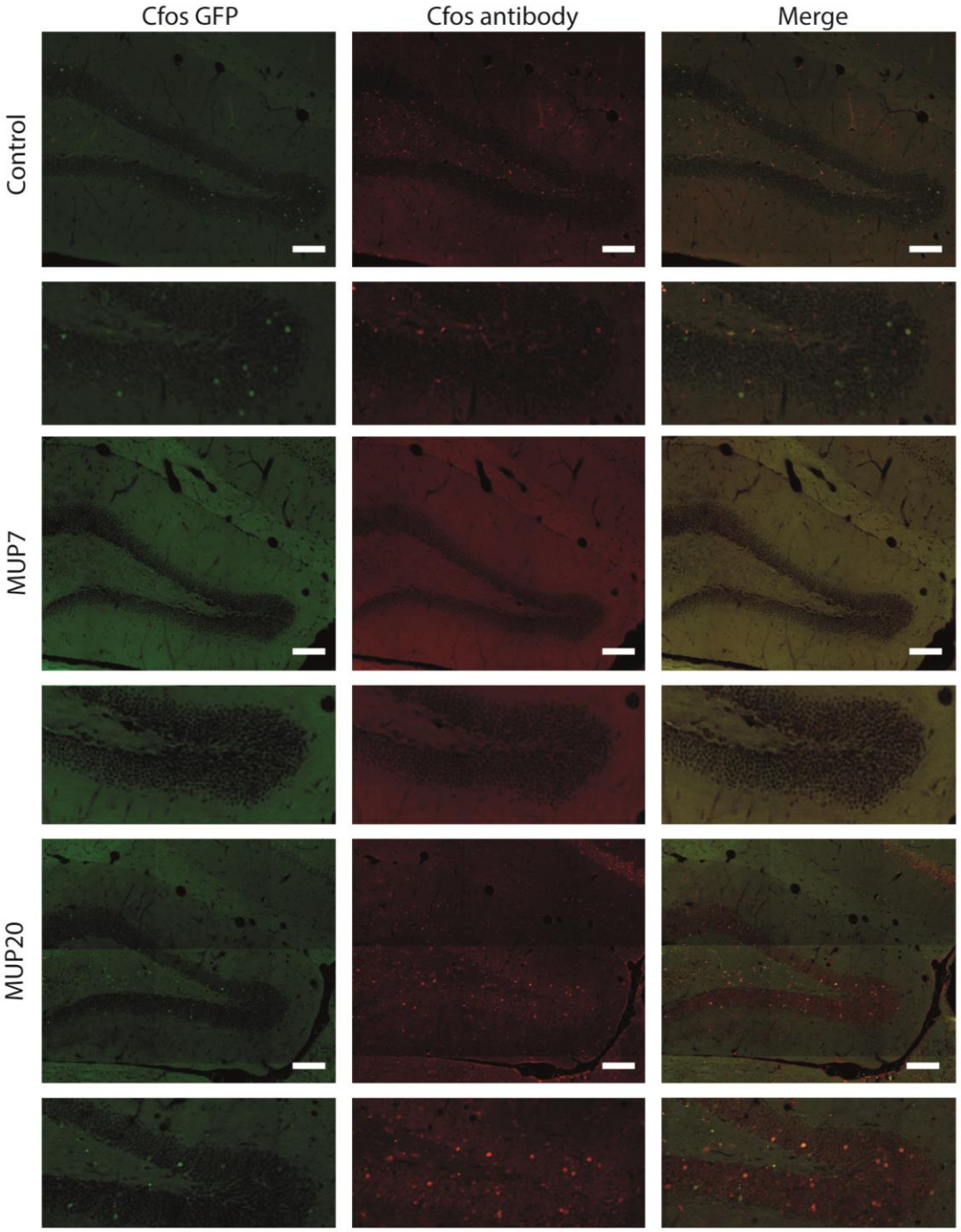


Figure 5.7: Sections of dentate gyrus showing the overlap of signal between GFP and antibody of c-Fos-GFP mice exposed to control, MUP7 and MUP20. Scale bar = 100µm.

5.4 Counts by region (genotype and housing)

Groups of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ males from SGH and MGH home environments were exposed to MUP20 and control and then analysed for c-Fos antibody signal. The olfactory bulb, dentate gyrus and hypothalamus were identified as regions with GFAP signal previously so I focused my analysis in these regions with the cortex as a control region and so the antibody counts reflected this. The regions of interest were imaged and divided in their anatomical sub regions by a frame of reference and visual inspection of regional landmarks to prevent inaccuracy. Heat maps of the signal count were manually generated comparing SGH and MGH *Nlgn3*$^{y/+}$ mice (Figure 5.8) and *Nlgn3*$^{y/-}$ mice (Figure 5.9).

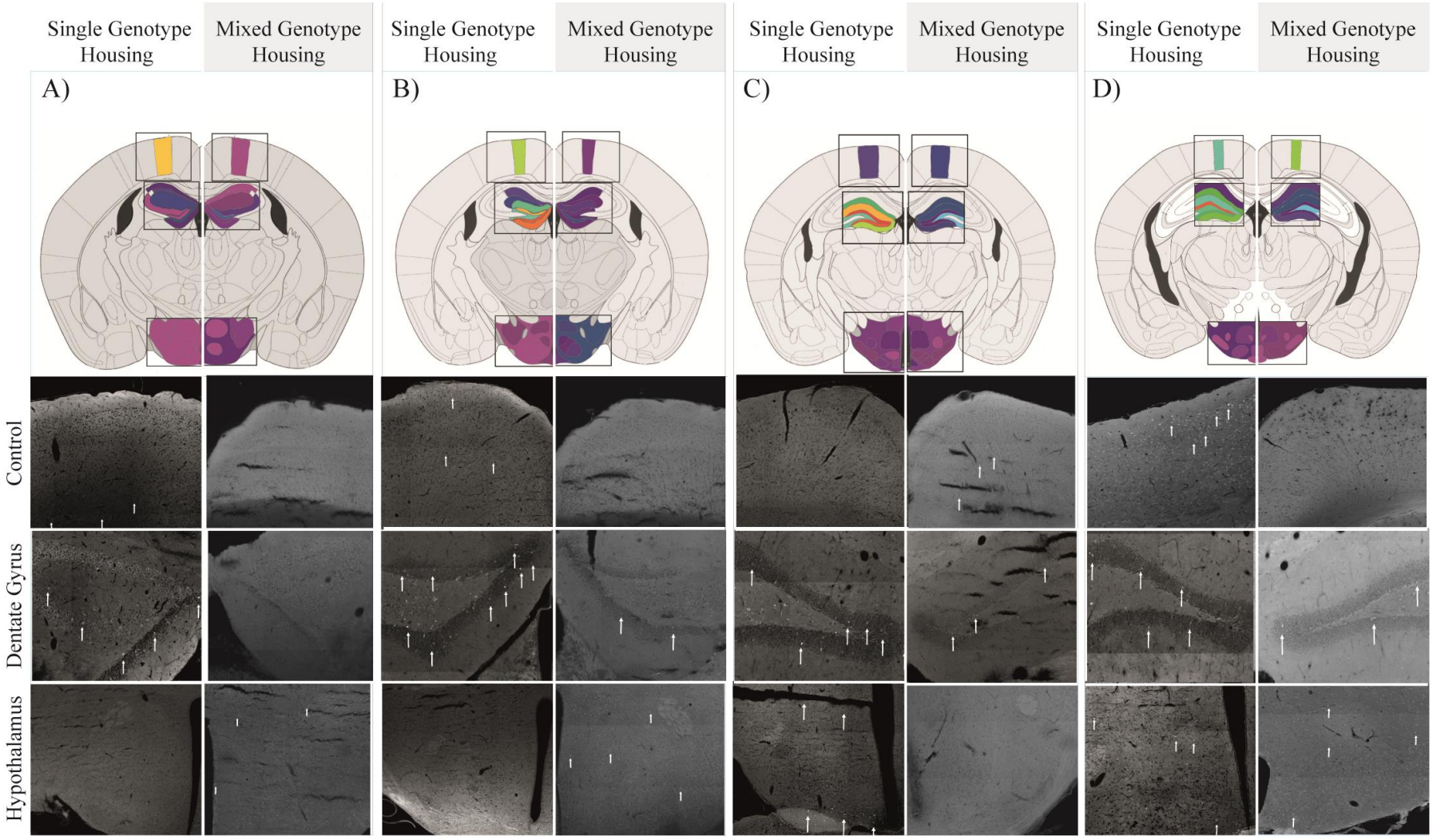
The resultant counts were analysed by sub region within the regions of interest. The olfactory bulb was divided into the glomerular layer, the external plexiform layer, the metrial cell layer, the granual layer and the anterior commissure (Figure 5.10 A). I analysed the regions separately with a two way ANOVA and in each region I identified there was a main effect of housing but no other significant difference (ANOVA(Greenhouse-Geisser correction): Glomerular layer: Genotype: F(1,91)=<0.001, p=1, Housing: F(1,91)=5.927, p=0.017, Genotype*Housing: F(1,91)=0.178, p=0.674, Anterior commissure: Genotype: F(1,83)=0.238, p=0.627, Housing: F(1,83)=11.158, p=0.001, Genotype*Housing: F(1,83)=0.013, p=0.911, External plexiform layer: Genotype: F(1,90)=0.344, p=0.559, Housing: F(1,90)=27.408, p=<0.00, Genotype*Housing: F(1,90)=<0.001, p=1, Metrial cell layer: Genotype: F(1,81)=<0.001, p=1, Housing: F(1,81)=24.480, p=<0.001, Genotype*Housing: F(1,81)=0.394, p=0.532: Granual layer, Genotype: F(1,82)=1.541, p=0.218, Housing: F(1,82)=13.456, p=<0.001, Genotype*Housing: F(1,82)=0.114, p=0.737). This is due to significantly higher signal in the MGH over the SGH mice.

My counts of the hypothalamus have a less consistent trend so I divided the hypothalamus into separate nuclei (Figure 5.10 B) but only identified significant differences in two. The lateral hypothalamic area showed a main effect of housing (ANOVA (Greenhouse-Geisser

correction) Genotype: F(1,37)=1.311, p=0.260, Housing: F(1,37)=8.146, p=0.007, Genotype*Housing: F(1,37)=0.120, p=0.731) where counts are higher in the MGH and the ventromedial hypothalamic nucleus showed a main effect of genotype (ANOVA (Greenhouse-Geisser correction), Genotype: F(1,38)=7.889, p=0.008, Housing: F(1,38)=1.263, p=0.268, Genotype*Housing: F(1,38)=<0.001, p=1) where counts are higher in the *Nlgn3*$^{y/-}$ mice.

The dentate gyrus was divided into the polymorph layer, the granular layer and the molecular layer (Figure 10 C). No significant difference between genotype or housing were found in the polymorph layer (ANOVA (Greenhouse-Geisser correction) Genotype: F(1,40)=1.814, p=0.186, Housing: F(1,40)=1.671, p=0.204, Genotype*Housing: F(1,66)=2.104, p=0.155), a main effect of housing was found in the molecular layer (ANOVA (Greenhouse-Geisser correction), Genotype: F(1,284)=0.230, p=0.880, Housing: F(1,284)=4.388, p=0.037, Genotype*Housing: F(1,284)=0.004, p=0.948) and in the granular layer I identified an interaction effect between housing and genotype (ANOVA (Greenhouse-Geisser correction) Genotype: F(1,207)=27.750, p=<0.001, Housing: F(1,207)=38.864, p=<0.001, Genotype*Housing: F(1,207)=19.770, p=<0.001) where SGH *Nlgn3*$^{y/+}$ mice had significantly more signal (SGH: WT/KO, p=<0.001, WT: SGH/KO, p=<0.001).

Due to time restrictions, I unfortunately could not complete this study and obtain a powerful statistical analysis. Nevertheless, I could observe some trends in our data. For example, in this analysis, I identified a number of potential differences in our groups, particularly between SGH and MGH mice though I did not see as much signal as I expected in the brains as a whole. This could be due to the exposure of mice to rMUP20 rather than HMW urine fraction with a large quantity of MUP20 which may have been a more salient cue to use as it is more biologically relevant so it would be useful to add an additional comparison in a future experiment where HMW+MUP20 is compared to these groups.


Single Genotype Housing
Mixed Genotype Housing
Single Genotype Housing
Mixed Genotype Housing
Single Genotype Housing
Mixed Genotype Housing
Single Genotype Housing
Mixed Genotype Housing
A)
B)
C)
D)
Control
Dentate Gyrus
Hypothalamus

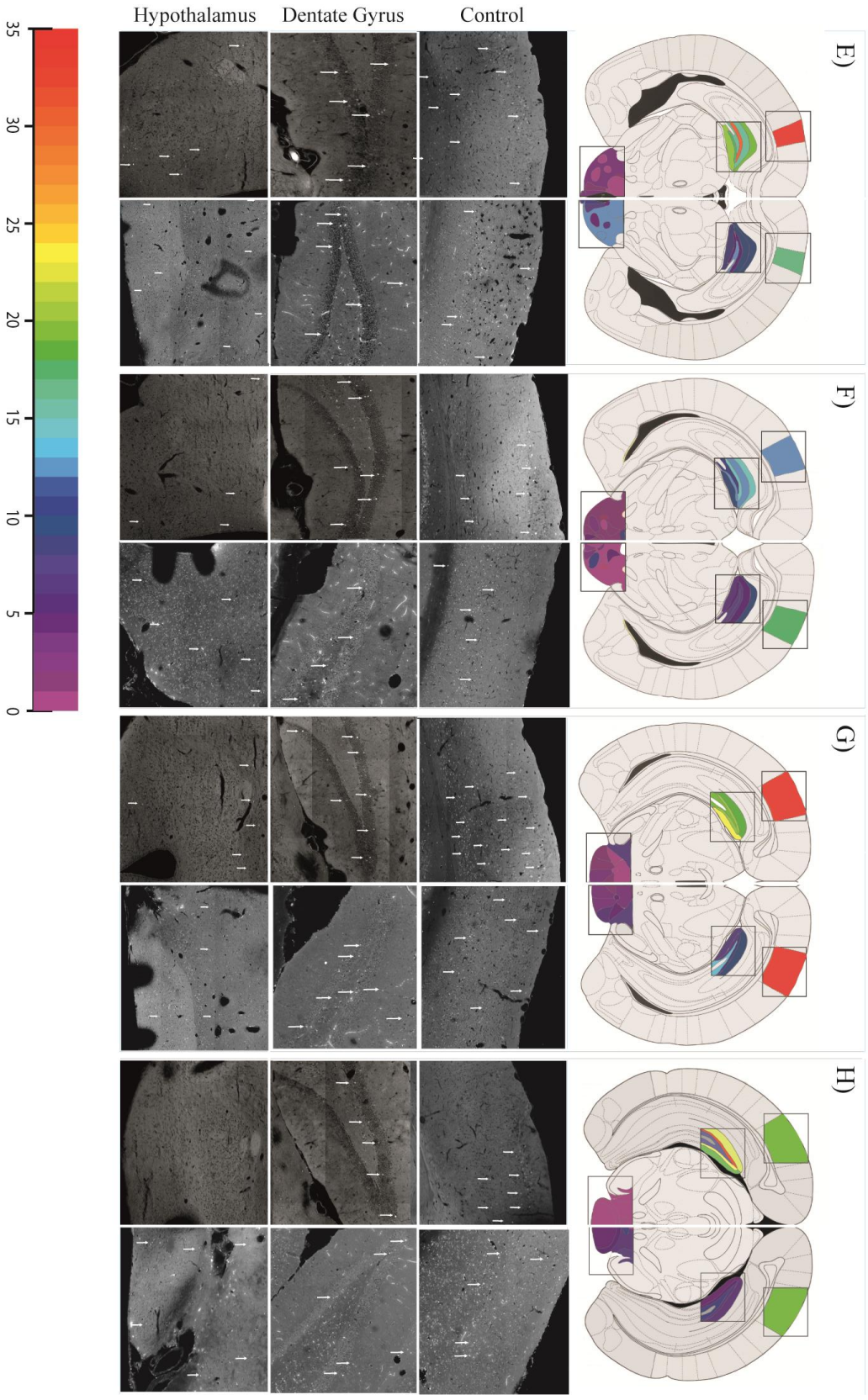
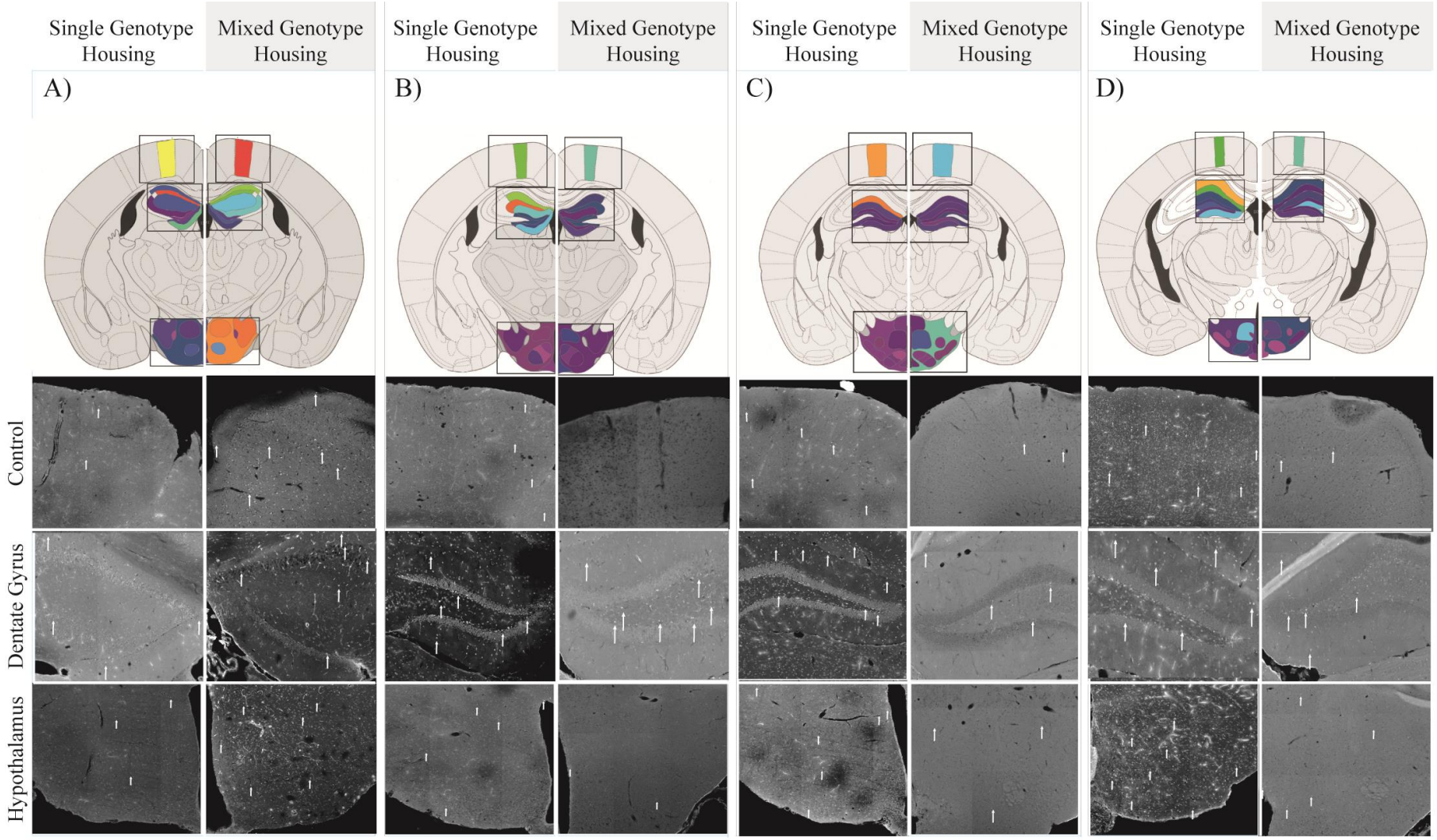


Figure 5.8: Heat map comparing c-Fos antibody signal found in SGH and MGH *Nlgn3*$^{y/+}$ mice exposed to MUP20


Single Genotype Housing
Mixed Genotype Housing
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Mixed Genotype Housing
A)
B)
C)
D)
Control
Dentate Gyrus
Hypothalamus

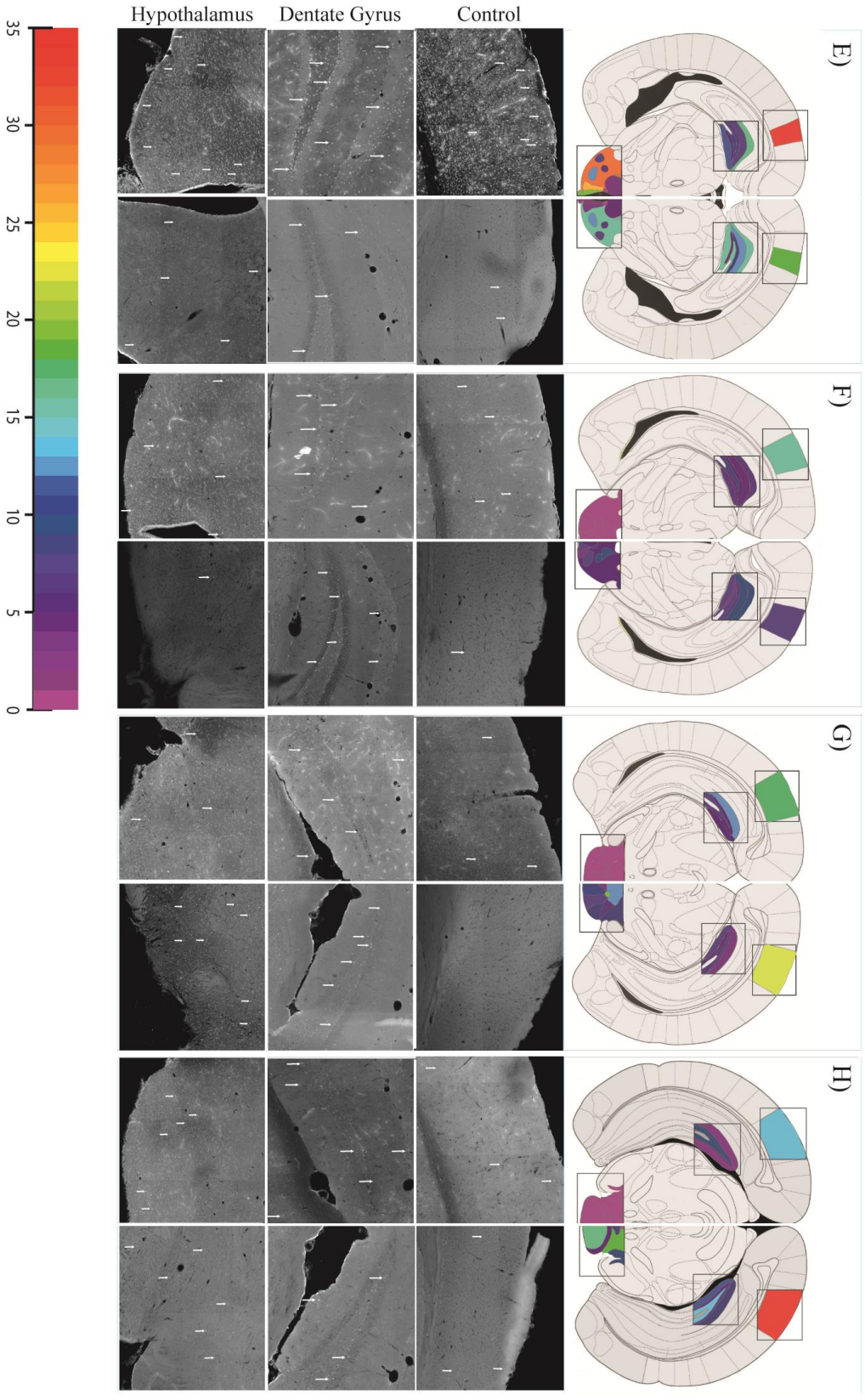

Figure 5.9: Heat map comparing c-Fos antibody signal found in SGH and MGH *Nlgn3*$^{y/-}$ mice exposed to MUP20.

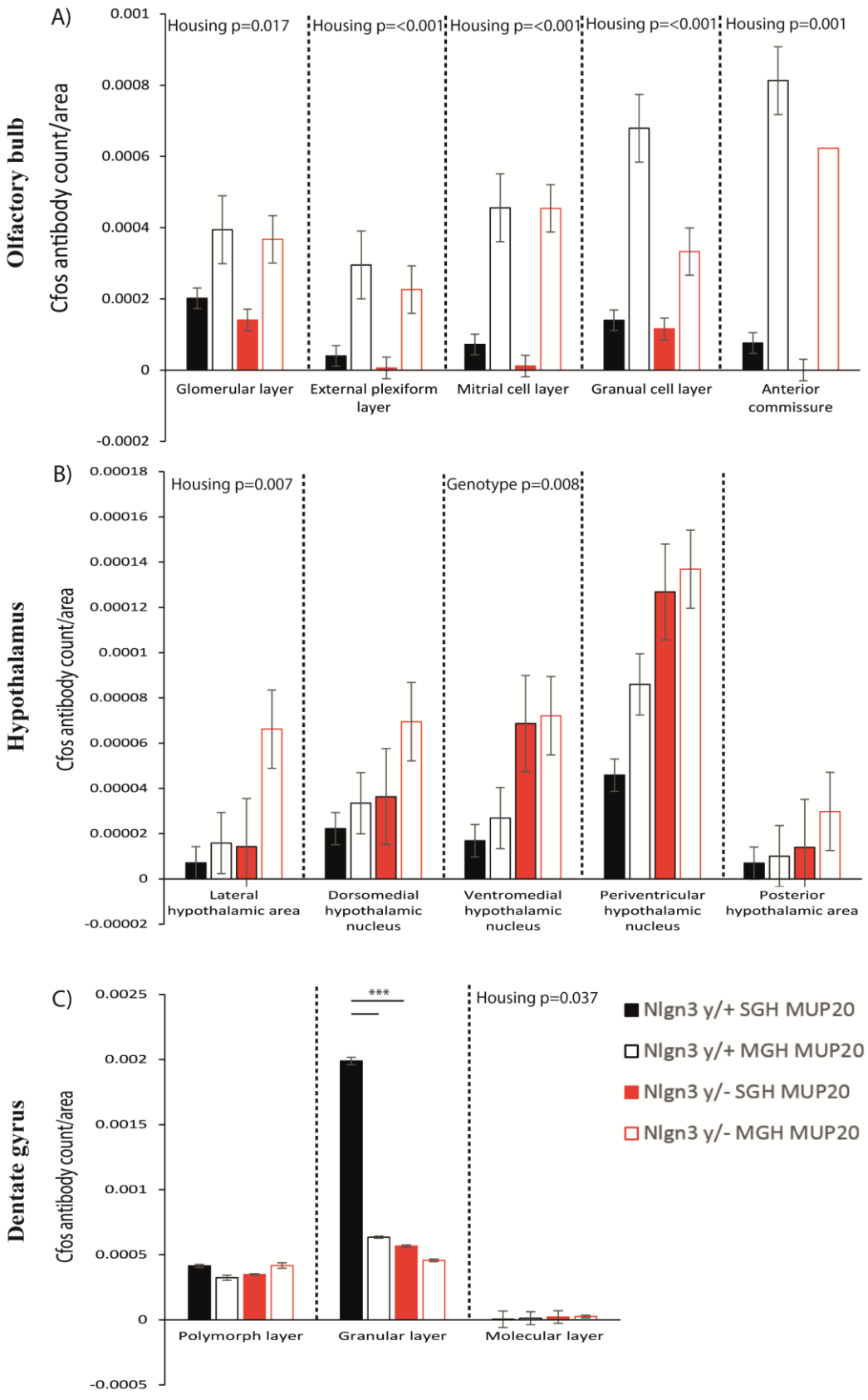

Figure 5.10: c-Fos antibody counts in different brain regions of MUP20 exposed *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice from SGH and MGH.

5.5 RNA sequencing

Another method to look at differences in the brain of mice due to housing and genotype could be to look for genetic changes.

In order to try and understand if additional genetic changes between SGH and MGH groups could be identified and if additional genetic changes could be detected between *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice in the hippocampus analyse RNA for differences between our groups.

I extracted RNA from the hippocampus of three each SGH and MGH *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice. These samples were then sequenced to produce a list of genes which I analysed using a principal component analysis. Data from each of the samples (1-3 SGH *Nlgn3*$^{y/+}$, 4-6 SGH *Nlgn3*$^{y/-}$, 7-9 MGH *Nlgn3*$^{y/+}$ and 10-12 MGH *Nlgn3*$^{y/-}$) were correlated against the gene list to generate a series of factors that account for the highest remaining variability, without being correlated, called principal components (PCs). These PCs were then assessed for how well the samples and variables fit using correlation plots and bi-plots were generated for the most significant PCs to analyse the differences between the samples.

In the hippocampus the first PC accounts for 41.5% of the variability and the second accounts for 30.4% (Figure 5.11 A) and these two PCs show the best fit for most of the samples (Figure 5.11 B) and variables (Figure 5.11 C). I plotted PC1 and PC2 against each other in a bi-plot (Figure 5.12) and identified that each of the repeats of the samples clustered more closely together than samples from other experimental groups showing a lower variation within the groups than between them. I also identified that the SGH *Nlgn3*$^{y/+}$ and SGH *Nlgn3*$^{y/-}$ clusters were spaced disparately compared to MGH *Nlgn3*$^{y/+}$ and MGH *Nlgn3*$^{y/-}$ which were spaced more closely showing higher variation between SGH groups than MGH groups.

Of the genes identified Drd1 which encodes the D1 dopamine receptor could be of interest as this receptor has been associated with spatial learning and memory in the hippocampus (Kempadoo *et al.*, 2016) and may be associated with the social memory phenotype I have identified.

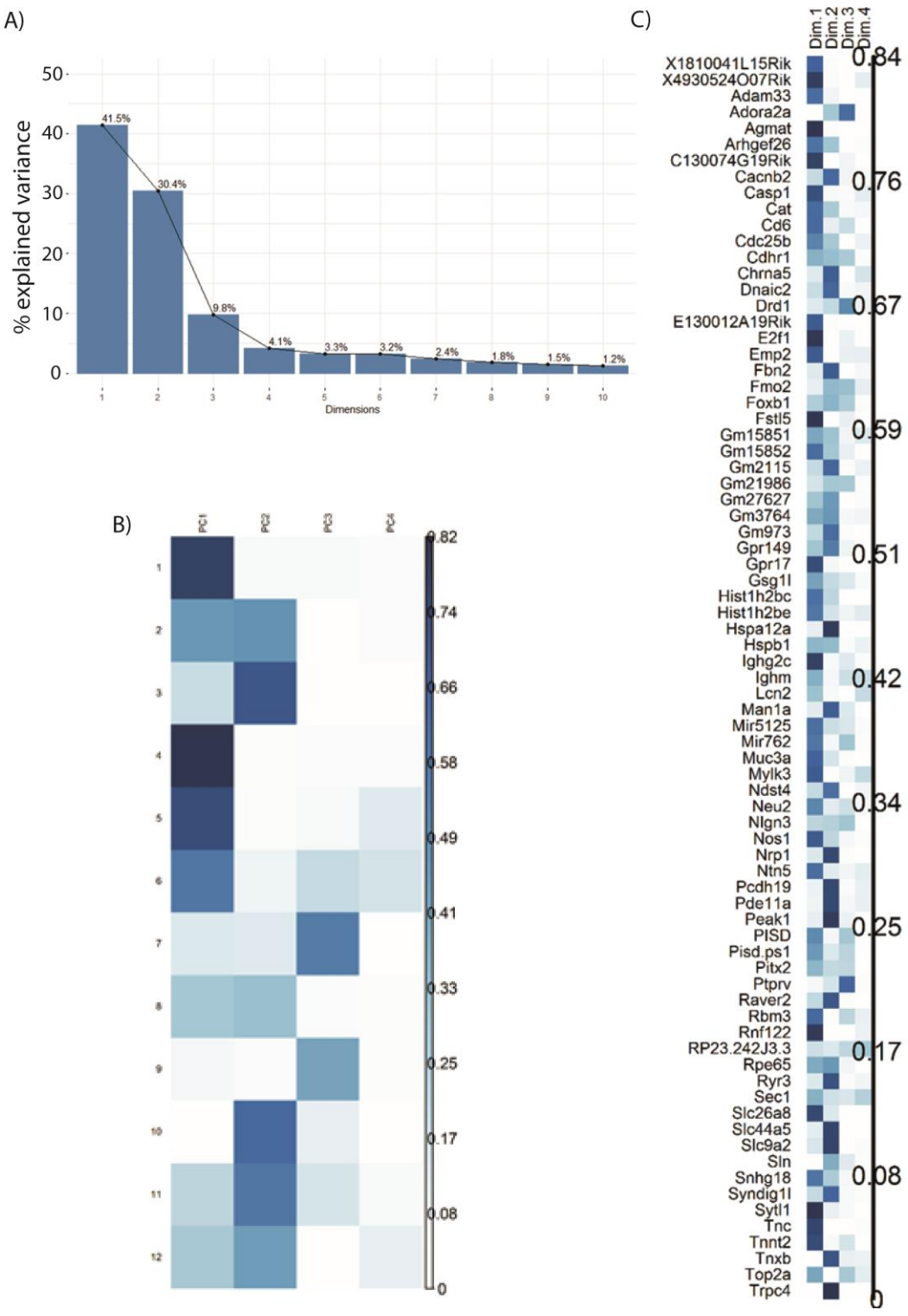

Figure 5.11: RNA sequencing analysis of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice form SGH and MGH. A) Scree plot showing variance explain by each PC. B) Fit of samples to PCs. C) Fit of variables to PCs

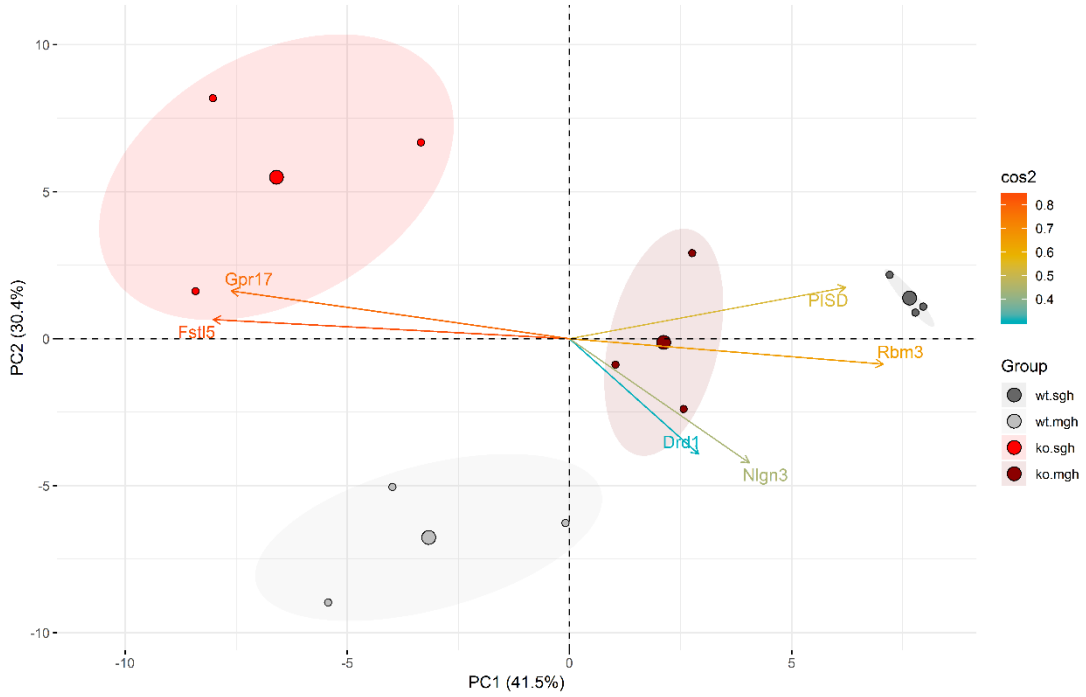

Figure 5.12: Bi-plot of PC1 and PC2 showing clustering of each of the samples (mean value of each group represented by the largest circle in each group colour).

5.6 Discussion

In this chapter I was interested in the differences between the brains of SGH and MGH *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice that had been exposed to MUP20.

To begin I had to identify if genotype or housing had an effect on the interest of mice for MUP20 in particular. To do this I tested the interest of SGH and MGH *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice for HMW urine fraction with rMUP20 added in high concentration. I identified that when MUP20 was added to male HMW *Nlgn3*$^{y/-}$ mice had less interest than *Nlgn3*$^{y/+}$ mice.

When MUP20 was added to female urine MGH *Nlgn3*$^{y/+}$ mice showed a reduced interest, which was similar to *Nlgn3*$^{y/-}$ mice. This suggests that the housing conditions of the mice have some effect on the interest of mice for MUP20. When I directly exposed to MUP20 to the mice and looked at the c-Fos signal I could see a number of differences in the quantification of the signal between SGH and MGH housed mice. C-Fos signal in the olfactory bulb was significantly higher in MGH than SGH mice as was signal in the lateral hypothalamic area.

I also detected some differences in signal quantification that were due to differences between the genotypes, such as the signal detected in the ventromedial hypothalamic nuclei. The ventromedial hypothalamic nuclei has been found to mediate aggressive behaviour of males particularly in reaction to male social cues such as MUPs which show experience dependent changes that reflect social experience (Review Hashikawa et al. 2017). When considered with the increase in signal identified in the dentate gyrus of SGH *Nlgn3*$^{y/-}$ mice compared to *Nlgn3*$^{y/-}$ mice it is possible that the signal identified in these regions could be linked to the reduced social memory found in the *Nlgn3*$^{y/-}$ mice (Radyushkin *et al.*, 2009) which could be pursued with further experiments.

However since the finding that the dentate gyrus signal increase is limited to SGH *Nlgn3*$^{y/+}$ mice it is possible that social interactions and environment could also be an important factor affecting social memory. As there was generally not a lot of signal detected in future

experiments the use of female urine as a buffer with rMUPs may increase the signal that can be detected which might improve the analysis of c-Fos in different brain regions.

Many of the limitations in interpreting the data of this experiment are due to the small sample sizes of each of the groups. In attempting to make the most of the tissue and measuring a lot of different brain regions and making so many comparisons there is more chance of introducing error resulting in false conclusions. This was mainly due to time limitations, which were unavoidable and skill limitations, which was improved as the experiment continued. However I think this experiment lays down the groundwork for a much improved future experiment that would involve a larger cohort in each group.

In this future experiment I would also slightly change the approach to identifying individual MUP pathways. This is because the outcomes of this experiment were quite surprising as I anticipated more signal in the brain and particularly to identify signal in more brain regions. For example I did not detect signal in the amygdala in any of the different groups which I would have defiantly expected to see in *Nlgn3*$^{y/+}$ mice which could be compared with the signal in *Nlgn3*$^{y/-}$ mice. This is based on the finding that MUP20 causes aggression in male mice (Roberts et al., 2010) and that aggression is associated with the amygdala which has been identified as a brain region involved in olfactory cue detection (Dulac and Wagner, 2006). It is possible that MUP20 alone is not an effective molecule to transmit social information and has to be combined with other urinary molecules or that there is a short time window or very specific pathway associated with MUP20 that is difficult to detect by this method. In a future experiment it might be more effective to analyse the signal that could be identified in response to HMW with and without the addition of rMUPs. This would be more biologically relevant as mice would not be exposed to rMUPs naturally and also allow us to identify if activity in some brain regions could be downregulated by particular MUPs. Overall this could help us to more clearly identify brain regions of interest associated with particular MUPs that are affected by Neuroligin-3 knockout or housing.

The RNA sequencing data implies that the MGH mice are more similar to each other genetically than the SGH mice despite the same genetic manipulation affecting *Nlgn3*$^{y/-}$ mice from both housing conditions. As one of the genes I identified, Drd1, it is possible that the dopamine system could be involved. It is already known that dopamine neurons are involved in habituation to novel stimuli (Bariselli *et al.*, 2018) and to spatial learning (Kempadoo *et al.*, 2016) so the dopamine system is an important target for investigating social behaviour differences in our SGH and MGH *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice. This finding requires replication to ensure that it is a robust finding as there is a possibility of identifying false positives with cluster analysis, this could be achieved by looking more directly at levels of Drd1 with methods such as qRT-PCR. Also I cannot conclude from this experiment if Drd1 is relevant to social odour processing it would be interesting to continue this line of enquiry with further experiments. For example attempting to rescue behavioural modifications caused by mixed genotype housing by upregulating Drd1.

5.7 Summary

- The dentate gyrus is activated by exposing *Nlgn3*$^{y/+}$ mice to rMUP20 which is not evident in *Nlgn3*$^{y/-}$ mice
- There is otherwise a low amount of c-Fos signal that is detectable by exposing *Nlgn3*$^{y/+}$ mice to rMUP20
- RNA analysis highlighted Drd1 as a target for future experiments

Chapter 6

General Discussion

6.1 Summary of results

I was interested in the effect of Neuroligin 3 knockout and housing environment on specific reactions to social cues. In chapter 3 I used behavioural experiments to detect interest for social cues. *Nlgn3*$^{y/-}$ mice showed reduced interest for male and female HMW urine fraction compared to *Nlgn3*$^{y/+}$ mice. Female *Nlgn3*$^{+/+}$ and *Nlgn3* $^{-/-}$ mice showed comparable low interest to male and female HMW urine fraction. Interest of *Nlgn3*$^{y/+}$ mice for female scent cues was dependent on housing condition as the MGH *Nlgn3*$^{y/+}$ mice spent significantly less time sniffing female scent cues than SGH *Nlgn3*$^{y/+}$ mice. *Nlgn3*$^{y/-}$ mice also did not show discrimination behaviour between male cage scrapings though they did discriminate between male and female cage scrapings. *Nlgn3*$^{-/-}$ mice showed a comparable interest to *Nlgn3*$^{+/+}$ mice for HMW male and female urine fraction. A comparison of the interest of *Nlgn3*$^{y/+}$ and *Nlgn3*$^{y/-}$ mice, females had a similar interest to *Nlgn3*$^{y/-}$ mice.

I was also interested to identify if Neuroligin 3 was present in the VNO. Using a number of methods I did detect Neuroligin 3 in the VNO but it did not seem to be associated with PSD95 so I was curious about where Neuroligin 3 was located in the VNO and what proteins it was associated with. I analysed the proteins that were detected in mass spectrometry analysis of Neuroligin3 IP VNO tissue but were unable to form strong conclusions about the function of Neuroligin 3 in the VNO. I also could not detect re-expression of Neuroligin 3 in the VNO of *OMP*$^{Cre/+}$ *Nlgn3*$^{y/-}$ mice. To look at the function of the VNO in our different groups I exposed VNOs to scent cues ex-vivo which did show more signal in the *Nlgn3*$^{y/+}$ mice but unfortunately the signal was very inconsistent so I felt I could not form conclusions based on this data. I also attempted to make culture of VNO cells but was unable to generate healthy cultures that could

be used in experiments. However despite this I was able to determine that Neuroligin 3 in the VNO is not the sole factor causing reduced interest in the *Nlgn3*$^{y/-}$ mice. This is partially due to the findings of reduced interest in the MGH *Nlgn3*$^{y/+}$ mice but also the rescue of interest for social cues found in the *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mice.

I then decided to look for differences in the brains of SGH and MGH *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ exposed to scent cues to see if the responses were the same. I began with behavioural experiments to look at the social interest of SGH and MGH *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice for HMW urine fraction with MUP20 added. *Nlgn3*$^{y/-}$ mice had less interest than *Nlgn3*$^{y/+}$ mice when male urine was used as the buffer but when female urine was used MGH *Nlgn3*$^{y/+}$ mice also showed reduced interest though SGH *Nlgn3*$^{y/+}$ mice retained their interest. I then exposed the mice to rMUP20 directly and identified c-Fos signal in the brain. C-Fos signal in the olfactory bulb the lateral hypothalamic area and was significantly higher in MGH than SGH mice and c-fos signal in the ventromedial hypothalamic nuclei was significantly higher in *Nlgn3*$^{y/+}$ than *Nlgn3*$^{y/-}$ mice. In the dentate gyrus there was higher signal in the granular layer of SGH *Nlgn3*$^{y/+}$ mice. I also identified with our analysis of RNA sequencing data that genes in MGH mice cluster more closely than mice of the same genotype. Also Drd1 was a gene of particular interest identified in this analysis.

6.2 Is interest and discrimination behaviour for social cues affected in *Nlgn3*$^{y/-}$ mice?

It has been previously identified that social memory is affected in *Nlgn3*$^{y/-}$ mice (Radyushkin et al. 2009) which is a phenotype that could be of relevance to ASD. Social behaviours of mice rely on the detection of social cues and so I wanted to identify if *Nlgn3*$^{y/-}$ mice have altered responses to social cues. There were a number of potential outcomes, first there could have been no altered response of *Nlgn3*$^{y/-}$ mice to social odours. This could indicate a more memory specific problem rather than a social deficit and so may be more associated with ASD

symptoms such as intellectual disability. Secondly there could have been an altered response due to the effect of the genetic manipulation on the olfactory system. If Neuroligin-3 was a protein of importance to the proper functioning of the VNO, for example, then the ability of the *Nlgn3*$^{y/-}$ mice to smell might be giving the appearance of social memory deficit in certain behavioural tests. I wanted to identify if there was a social memory phenotype by attempting to eliminate some of these possibilities.

To begin I had to identify if there was an altered response of *Nlgn3*$^{y/-}$ mice for social odours. *Nlgn3*$^{y/-}$ mice showed reduced interest for HMW urine fraction but were attracted to whole male urine and female cage scrapings to a similar degree as *Nlgn3*$^{y/+}$ mice. This shows that *Nlgn3*$^{y/-}$ mice can detect social cues, though it doesn't rule out the possibility that olfaction is affected. The simplicity of these experiments have the benefit of replicability as there are few resource and skill requirements. However it is difficult to make specific conclusions about how social olfaction behaviour is affected beyond an interest of the mice. For example it would be difficult to identify the difference between a lack of motivation to sniff and a reduced ability to detect scent cues since the outcome in both would be less sniffing. However as it had not yet been established that *Nlgn3*$^{y/-}$ mice have altered responses to social cues this experiment does make a useful contribution to the study of *Nlgn3*$^{y/-}$ mice.

These experiments could also have provided information about *Nlgn3*$^{-/-}$ mice which are usually not the subject of study. This would be valuable information as there is an established gender bias in ASD diagnosis and also difference in responses of male and female mice to scent cues, and so there might be gender specific effects which could be protective to females. This could then be investigated in other genetic models used to study ASD and might show differences in ASD that are due to gender. However the female mice in this cohort had a strangely reduced response to social cues, the *Nlgn3*$^{+/+}$ mice had a comparable interest to *Nlgn3*$^{y/-}$ mice. It is uncertain what might be the cause of this as the mice had reached sexual maturity and were naive to males. It could have been beneficial to test female mice from other colonies to identify if the housing environment was responsible but due to time restrictions

this work was not undertaken. If future work was to focus on the female mice it would be important to understand this issue first. This is of particular importance as our group identified environmental factors affecting the male mice in this cohort.

Housing *Nlgn3*$^{y/+}$ mice with *Nlgn3*$^{y/-}$ mice altered the behavioural response of both geneotypes in a number of behavioural tests (Kalbassi et al. 2014). I identified that interest for social cues was affected by housing condition. This could be a factor affecting a number of different animal models and in the study of ASD where environmental factors have been found to have a causative factor investigation of these factors in different mouse models could help us to understand if there are genes which indicate more risk from environmental factors or indicate which are most significant.

Despite the number of further questions raised by these experiments I feel that I was able to determine that interest and discrimination for social cues was affected in *Nlgn3*$^{y/-}$ mice and that it is important to further investigate these mice to try and identify why.

6.3 Is Neuroligin 3 expressed in the VNO and does it affect social olfaction?

This question was quite challenging to answer and despite trying a number of different methods I would say it was not adequately answered. I was able to identify that Neuroligin-3 protein is present in the VNO tissue using mass spectrometry which was a novel finding as the VNO is not widely studied. I didn't identify any function related information from the mass spectrometry data but much of that could be due to the limitations of my skills to work with this data but also due to prioritising other methods as I felt the mass spectrometry data alone was not sufficient to identify function. Attempts to culture VNO cells was also unsuccessful which I thought might be due to the sensitivity of VNO cells and so I decided to try exposing whole VNOs. Dissected VNOs of perfused mice exposed to scent cues did not show any Egr1 signal and the signal identified in cultured whole VNOs exposed to scent cues did not produce conclusive data. Rather than attempt these methods again a more beneficial

experiment could be to use slices of VNO and measure responses to social odour molecules using calcium signalling methods which we were not equipped to do at the time. This could show us if there are differences in the function of VNO due to genotype, housing and gender and might even have the spatial resolution to help us make hypotheses about the nature of the differences if any were there.

However, despite the difficulty working with the VNO directly, I was able to go some way toward addressing the question of the effect of Neuroligin 3 in the VNO on social olfaction. Social interest behaviour can be rescued without re-expressing Neuroligin 3 in the VNO if it is re-expressed in Parvalbumin neurons. This implies that the altered behaviour of *Nlgn3*$^{y/-}$ mice to social cues could be due to central nervous system modifications rather than peripheral nervous system modifications. This may mean that the VNO is functionally not affected by Nlgn3 knockout, however I think this is still an important factor to confirm, especially since a rigorous investigation of the rescue was not completed for this thesis (e.g. exposing *Pvalb*$^{Cre/+}$*Nlgn3*$^{y/-}$ mice to all of the social cues that *Nlgn3*$^{y/-}$ mice were exposed to in these experiments.

6.4 Is social odour detection causing different brain region activation or gene expression in *Nlgn3*$^{y/-}$ mice?

This objective represents the beginning of an exploration into the differences in central nervous system that are due to Neuroligin-3 knockout and housing. As the interest of *Nlgn3*$^{y/-}$ mice in the social interest experiment was still reduced with the addition of rMUP20 I proceeded to use MUP20 as the stimuli to identify if c-Fos signal (used as an allegory for neuron activation) differed in brain regions associated with scent cue detection in *Nlgn3*$^{y/-}$ mice or as a result of housing. However I decided to expose the mice involved in the c-Fos experiment to rMUP20 alone which I thought would result in less signal, showing only MUP20 specific activation and make differences between the groups clearer. Unfortunately

the signal was much more reduced than expected resulting in identifiable signal in only a few regions.

The low signal could be due to timing factors, the brain regions we associate with MUP20 related behaviours could have more transient signal that can only be detected in a specific time window. The low signal could also reflect a low response to rMUP20 separate from other olfactory cues, possibly the behavioural response of mice to MUP20 is more significant when it is combined with other scent cues. Recombinant MUPs are completely stripped of all volatile molecules whereas MUPs in HMW urine fraction will still bind normally with volatile molecules so even when filtered they will still contain some. This could be key activating certain brain regions in response to scent cues.

Regardless of this we identified signal in the granule layer of the dentate gyrus that was significantly higher in SGH *Nlgn3*$^{y/+}$ mice. This reflected the behaviour that we saw when mice were exposed to rMUP20 with female HMW urine buffer and so we identified that both Neuroligin 3 knockout and social housing affect c-Fos signal in the dentate gyrus.

Considering this in the context of Hoffman et al. (2015) finding that MUP20 has been found to induce neurogenesis and social memory in female mice it is possible that this signifies that MUP20 could be involved in social memory in male mice. A future experiment would be to selectively re-express Neuroligin 3 in the dentate gyrus to see if it is possible to rescue interest for social odours, discrimination of social odours and social memory. More broadly it would be important to repeat the mapping experiment undertaken in this thesis with more complex social odour such as HMW urine fraction as this might highlight more regions of interest between the groups that were missed by using rMUP20 alone.

Differences between the groups were also identified in the RNA analysis. MGH samples clustered more closely than SGH samples suggesting that they have more in common in the factors explaining the highest variance (principal components) in the mRNA data. Finding the gene *Drd1* was highlighted as having a similar relation to the sample clusters as Neuroligin 3

was suggestive as dopaminergic neurons in the ventral tegmental area (VTA) with specific Neuroligin 3 knockout have been found to show reduced exploration of novel mice and did not habituate to the location of a familiar mouse (Bariselli *et al.*, 2018). This findings aligned with our behavioural findings that *Nlgn3*$^{y/-}$ mice have reduced interest for social odours and do not discriminate between familiar and unfamiliar male social odours. However it is important to note that cluster analysis is by no means conclusive and can only be used as a tool to begin forming further hypotheses. Further experiments identifying if the dopaminergic neurons of the VTA are modulating this social odour interest and discrimination by selective re-expression of Neuroligin 3 in these cells are required.

6.5 Future work

The work in this thesis has highlighted a number of factors which indicate interesting lines of inquiry for future work. A highly interesting follow up experiment would be to measure the activity of brain regions of interest during sniffing behaviour of *Nlgn3*$^{y/-}$ and *Nlgn3*$^{y/+}$ mice from both housing groups by use of in vivo electrophysiology. This would negate the issue of identifying specific time windows of activation as the mice could be recorded before, during and after sniffing behaviour. Brain regions with marked differences in activation could then be targeted in re-expression experiments to attempt to recover function. In this way we could accurately target the brain regions from which social memory deficit originates in *Nlgn3*$^{y/-}$ mice.

However, to begin with it is highly important to further the mapping work of chapter 5 by adding HMW urine fractions from males and females as well as HMW with individual rMUPs added with increased group sizes to find robust regions of interest. With this data it is likely that not only the dentate gyrus would be highlighted. Also it is important to establish that the VNO of *Nlgn3*$^{y/-}$ mice is not affected by the genetic manipulation so calcium imaging of VNO exposed to different scent cues is a must. I also think it would be important to include female

mice in these experiments, however it would be important to first establish why there is a low interest for social cues in the females of this particular cohort.

This could be an opportunity to explore more environmental factors affecting social behaviour in mouse models since we identified that the social environment modified behaviours of *Nlgn3*$^{y/-}$ mice and *Nlgn3*$^{y/+}$ littermates. It would be of great interest to know if this effect is also found in other mouse models of ASD risk genes as this may allow for a greater understanding of the impact of environmental factors on particular risk genes. Other factors such as stress, enrichment, density of housing etc. could be explored in different genetic models which could allow us to begin linking genes, environmental factors and social behaviour phenotypes and so generate a more individual understanding of different presentations of ASD.

6.6 Concluding remarks

This experimental work was intended to address the social olfaction of *Nlgn3*$^{y/-}$ mice. We were able to conclude that deficits in social odour interest and discrimination behaviours were caused by Neuroligin 3 knockout and also by social housing and that social interest could be rescued by re-expression in parvalbumin neurons. We were unable to make a clear assessment of the changes in the activity of the VNO due to these factors but were able to identify the granular layer of the dentate gyrus as a region of interest and Drd1 as a gene of interest for future work.

As mentioned there is much more work required to fully explore this topic, after which there are a number of other mouse models which could be analysed in a similar manner. However the role of mouse models in research is to provide information about disease and disorder and though none of the experiments performed in this thesis were directly translational it does raise some interesting concepts that can inform future work. An analysis of social behavioural symptoms against specific mutations could provide more understanding about the different presentations of ASD. Do patients who express a *NLGN3* mutation always show hyporesposiveness to olfactory cues? It would be worthwhile to make this assessment if only

to have a measure of the predictive value of mouse models for social olfaction behaviours in humans.

Also the differences we identified in the mice that were due to housing condition suggest that there is a lot to be considered in the use of mouse models about the social environment in which they are raised. Environmental factors play a significant role in the presentation of ASD and should not be ignored in the assessment of our mouse models. It is possible that phenotypes could have been missed in mouse models because of the use of wildtype littermates as controls in behavioural experiments.

More broadly it is difficult to identify the impact of this work on human ASD as we have not been able to clearly establish if the effect of Neuroligin-3 knockout is entirely centrally-mediated due to modifications to the brain or if there is a peripheral influence in the VNO. Though social smell has been identified to transmit information in humans and is affected in ASD patients the impact of olfaction on social behaviour is subtle and requires much more exploration.

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Appendix 1

Figure	Shapiro-Wilk test of normality	Test for Equality of variances	Type of test and result	Observed power	Post Hoc test	Sample size
3.1	WT/Control: W(8)=0.906, p=0.324 WT/HMW: W(8)=0.972, p=0.911 KO/Control: W(8)=0.909, p=0.345 KO/HMW: W(8)=0.959, p=0.345	Control: Levene's test F(1,14)=1.979 p=0.181 HMW: Levene's test F(1,14)=2.687 p=0.123	Repeated measures ANOVA Within-subjects (Scent cue): F(1,14)=20.607, p=<0.001 Between subjects (Genotype): F(1,14)=2.632, p=0.127 Scent cue*Genotype: F(1,14)=5.111, p=0.04	0.988 0.327 0.557	Bonferroni adjusted pairwise comparison WT: Control/HMW, p=<0.001 KO: Control/HMW, p=0.129 Control: WT/KO, p=0.138 HMW: WT/KO, p=0.069	Nlgn3 y/+ n=8 Nlgn3 y/- n=8 (repeated measure scent cue)
3.2	WT/Control: W(8)=0.906, p=0.324 WT/HMW: W(8)=0.960, p=0.810	Control: Levene's test F(1,14)=0.280 p=0.606	Repeated measures ANOVA Within-subjects (Scent cue): F(1,14)=60.452, p=<0.001	1	Bonferroni adjusted pairwise comparison WT: Control/HMW, p=<0.001	Nlgn3 y/+ n=8 Nlgn3 y/- n=8 (repeated measure scent cue)

	KO/Control: W(8)=0.960, p=0.808 KO/HMW: W(8)=0.812, p=0.038	HMW: Levene's test F(1,14)=2.687 p=0.123	Between subjects (Genotype): F(1,14)=22.019, p=<0.001 Scent cue*Genotype: F(1,14)=51.603, p=<0.001	0.992 1	KO: Control/HMW, p=0.682 Control: WT/KO, p=0.049 HMW: WT/KO, p=<0.001	
3.3	WT/HMW male: W(8)=0.972, p=0.911 WT/HMW female: W(8)=0.960, p=0.810 KO/HMW male: W(8)=0.959, p=0.796 KO/HMW female: W(8)=0.812, p=0.038	Male HMW: Levene's test F(1,14)=1.979 p=0.181 Female HMW: Levene's test F(1,14)=2.687 p=0.123	Repeated measures ANOVA Within-subjects (Scent cue): F(1,14)=1.244, p=0.283 Between subjects (Genotype): F(1,14)=24.895, p=<0.001 Scent cue*Genotype: F(1,14)=6.129, p=0.027	 0.180 0.996 0.635	Bonferroni adjusted pairwise comparison WT: Male/Female, p=0.024 KO: Male/Female, p=0.352 Male: WT/KO, p=0.069 Female: WT/KO, p=<0.001	Nlgn3 y/+ n=8 Nlgn3 y/- n=8 (repeated measure scent cue)
3.5	WT/Control: W(10)=0.845, p=0.051 WT/HMW: W(10)=0.889, p=0.166 KO/Control:	Control: Levene's test F(1,17)=1.449 p=0.245 HMW:	Repeated measures ANOVA Within-subjects (Scent cue): F(1,17)=21.225, p=<0.001	 0.991 0.565	N/A	Nlgn3 +/+ n=10 Nlgn3 +/- n=9 (repeated measure scent cue)

	W(9)=0.940, p=0.579 KO/HMW: W(9)=0.889, p=0.536	Levene's test F(1,17)=0.858 p=0.367	Between subjects (Genotype): F(1,17)=86.533, p=0.038 Scent cue*Genotype: F(1,17)=0.599, p=0.450	0.113		
3.6	WT/Control: W(11)=0.930, p=0.410 WT/HMW: W(11)=0.793, p=0.003 KO/Control: W(10)=0.880, p=0.129 KO/HMW: W(10)=0.615, p=<0.001	Control: Levene's test F(1,19)=1.006 p=0.328 HMW: Levene's test F(1,19)=2.682 p=0.118	Repeated measures ANOVA Within-subjects (Scent cue): F(1,19)=7.551, p=0.013 Between subjects (Genotype): F(1,19)=0.063, p=0.804 Scent cue*Genotype: F(1,17)=1.294, p=0.269	0.741 0.057 0.191	N/A	Nlgn3 +/+ n=11 Nlgn3 +/- n=10 (repeated measure scent cue)
3.7	WT: W(19)=0.926, p=0.146 KO: W(18)=0.750, p=<0.001 Male: W(16)=0.916, p=0.143 Female: W(21)=0.734, p=<0.001	Genotype: Levene's test F(1,35)=22.580 p=<0.001 Gender:	Friedman test $\chi^2(3) = 12.3$, $p = 0.060$ Wilcoxon signed-rank tests Male WT/Male KO: Z=-2.380, p=0.017	N/A	N/A	Nlgn3 y/+ n=8 Nlgn3 y/- n=8 Nlgn3 +/+ n=8 Nlgn3 +/- n=8

		Levene's test F(1,35)=11.452 p=0.002	Male WT/Female WT: Z=-2.527, p=0.012 Male WT/Female KO: Z=-2.521, p=0.012 Male KO/Female WT: Z=-0.280, p=0.779 Male KO/Female KO: Z=-0.280, p=0.779 Female WT/Female KO: Z=-0.415, p=0.678			
3.8	WT: W(8)=0.907, p=0.333 KO: W(8)=0.688, p=0.002	WT urine: Levene's test F(1,17)=0.203, p=0.659	Independent samples t-test t(14)=1.033, p=0.319	N/A	N/A	Nlgn3 y/+ n=8 Nlgn3 y/- n=8
3.9	WT/S1: W(10)=0.909, p=0.272 WT/S2: W(10)=0.925, p=0.402	S1:	Repeated measures ANOVA	0.984	Bonferroni adjusted pairwise comparison	Nlgn3 y/+ n=10 Nlgn3 y/- n=10

	KO/S1: W(10)=0.639, p=<0.001 KO/S2: W(10)=0.904, p=0.245	Levene's test F(1,18)=1.161, p=0.296 S2: Levene's test F(1,18)=0.791 p=0.385	Within-subjects (Familiarity): F(1,18)=19.006, p=<0.001 Between subjects (Genotype): F(1,18)=0.030, p=0.865 Familiarity*Genotype: F(1,18)=7.104, p=0.016	0.053 0.713	WT: S1/S2, p=<0.001 KO: S1/S2, p=0.246 S1: WT/KO, p=0.393 S2: WT/KO, p=0.140	(repeated measure scent cue)
3.10	WT/S1: W(12)=0.973, p=0.940 WT/S2: W(12)=0.953, p=0.688 KO/S1: W(9)=0.920, p=0.394 KO/S2: W(9)=0.894, p=0.220	S1: Levene's test F(1,19)=0.746, p=0.399 S2: Levene's test F(1,19)=2.244, p=0.151	Repeated measures ANOVA Within-subjects (Familiarity): F(1,19)=17.438, p=0.001 Between subjects (Genotype): F(1,19)=0.687, p=0.421 Familiarity*Genotype: F(1,19)=0.346, p=0.346	0.977 0.123 0.086	N/A	Nlgn3 y/+ n=12 Nlgn3 y/- n=10 (repeated measure scent cue)
3.11	WT/S1: W(8)=0.975, p=0.932 WT/S2: W(8)=0.829, p=0.058	S1:	Repeated measures ANOVA		N/A	Nlgn3 +/+ n=8 Nlgn3 -/- n=11

	KO/S1: W(11)=0.833, p=0.026 KO/S2: W(11)=0.916, p=0.287	Levene's test F(1,17)=0.354, p=0.560 S2: Levene's test F(1,17)=5.247, p=0.035		0.302 0.265 0.169		(repeated measure scent cue)
3.12	WT/S1: W(9)=0.868, p=0.118 WT/S2: W(9)=0.962, p=0.824 KO/S1: W(12)=0.916, p=0.251 KO/S2: W(12)=0.961, p=0.795	S1: Levene's test F(1,19)=1.091, p=0.309 S2: Levene's test F(1,19)=3.023, p=0.098	Repeated measures ANOVA Within-subjects (Familiarity): F(1,19)=6.392, p=0.020 Between subjects (Genotype): F(1,19)=0.740, p=0.400 Familiarity*Genotype: F(1,19)=3.316, p=0.084	0.670 0.129 0.409	Bonferroni adjusted pairwise comparison WT: S1/S2, p=0.645 KO: S1/S2, p=0.004 S1: WT/KO, p=0.756 S2: WT/KO, p=0.101	Nlgn3 +/+ n=9 Nlgn3 -/- n=12 (repeated measure scent cue)
3.13	WT/Control: W(20)=0.962, p=0.578	Scent Cue: Control:	Repeated measures ANOVA		Bonferroni adjusted pairwise comparison	SGH Nlgn3 y/+ n=8 SGH Nlgn3 y/- n=8

	WT/HMW: W(20)=0.958, p=0.504 KO/Control: W(16)=0.915, p=0.140 KO/HMW: W(16)=0.926, p=0.212 SGH/Control: W(16)=0.942, p=0.369 SGH/HMW: W(16)=0.946, p=0.428 MGH/Control: W(20)=0.946, p=0.309 MGH/HMW: W(20)=0.942, p=0.266	Levene's test F(1,34)=0.176, p=0.678 KO urine: Levene's test F(1,34)=1.737, p=0.196 Housing: Control: Levene's test F(1,34)=1.002, p=0.324 KO urine: Levene's test F(1,34)=1.210, p=0.279	Within-subjects (Scent Cue): F(1,32)=39.427, p=<0.001 Between-subjects (Genotype): F(1,32)= 7.039, p=0.012 Between-subjects (Housing): F(1,32)=2.036, p=0.163 Scent Cue*Genotype: F(1,32)=5.865, p=0.021 Scent Cue*Housing: F(1,32)=2.032, p=0.164 Genotype*Housing: F(1,32)=<0.001, p=0.996 Scent Cue*Genotype*Housing: F(1,32)=2.003, p=0.167	1 0.730 0.283 0.651 0.282 0.050 0.279	SGH:Control:WT/KO, p=0.148 SGH:HMW:WT/KO, p=0.032 MGH:Control:WT/KO, p=0.005 MGH:HMW:WT/KO, p=0.168 WT:Control:SGH/MGH, p=0.040 WT:HMW:SGH/MGH, p=0.119 KO:Control:SGH/MGH, p=0.030 KO:HMW:SGH/MGH, p=0.611 WT:SGH:Control/HMW, p=<0.001 WT:MGH:Control/HMW, p=0.002 KO:SGH:Control/HMW, p=0.073 KO:MGH:Control/HMW, p=0.075	MGH Nlgn3 y/+ n=12 MGH Nlgn3 y/- n=8 (repeated measure scent cue)

3.14	WT/Control: W(16)=0.941, p=0.363 WT/HMW: W(16)=0.916, p=0.143 KO/Control: W(25)=0.935, p=0.115 KO/HMW: W(25)=0.843, p=0.001 SGH/Control: W(20)=0.938, p=0.223 SGH/HMW: W(20)=0.921, p=0.105 MGH/Control: W(21)=0.912, p=0.060 MGH/HMW: W(21)=0.812, p=0.001	Scent Cue: Control: Levene's test F(1,39)=0.174, p=0.679 KO urine: Levene's test F(1,39)=2.902, p=0.096 Housing: Control: Levene's test F(1,39)=3.113, p=0.086 KO urine:	Repeated measures ANOVA Within-subjects (Scent Cue): F(1,37)=50.267, p=<0.001 Between-subjects (Genotype): F(1,37)= 3.090, p=0.087 Between-subjects (Housing): F(1,37)=12.731, p=0.001 Scent Cue*Genotype: F(1,37)=3.941, p=0.055 Scent Cue*Housing: F(1,37)=16.078, p=<0.001 Genotype*Housing: F(1,37)=6.514, p=0.015 Scent Cue*Genotype*Housing: F(1,37)=19.140, p=<0.001	1 0.402 0.935 0.490 0.974 0.700 0.989	Bonferroni adjusted pairwise comparison SGH:Control:WT/KO, p=0.039 SGH:HMW:WT/KO, p=0.001 MGH:Control:WT/KO, p=0.039 MGH:HMW:WT/KO, p=0.265 WT:Control:SGH/MGH, p=0.060 WT:HMW:SGH/MGH, p=<0.001 KO:Control:SGH/MGH, p=0.021 KO:HMW:SGH/MGH, p=0.766 WT:SGH:Control/HMW, p=<0.001 WT:MGH:Control/HMW, p=0.756	SGH Nlgn3 y/+ n=8 SGH Nlgn3 y/- n=8 MGH Nlgn3 y/+ n=12 MGH Nlgn3 y/- n=13 (repeated measure scent cue)

		Levene's test F(1,39)=4.769, p=0.035			KO:SGH:Control/HMW, p=0.012 KO:MGH:Control/HMW, p=0.003	
3.15	WT: Control 1: W(20)=0.871, p=0.012 Control 2: W(20)=0.962, p=0.578 Control 3: W(20)=0.871, p=0.012 Control 4: W(20)=0.938, p=0.223 KO: Control 1: W(16)=0.863, p=0.021	Genotype: Control 1: Levene's test F(1,34)=1.012, p=0.322 Control 2: Levene's test F(1,34)=0.176, p=0.678 Control 3:	Repeated measures ANOVA Within-subjects (Sniffing): F(3,96)=0.121, p=0.948 Between-subjects (Genotype): F(1,32)= 0.181, p=0.673 Between-subjects (Housing): F(1,32)=0.114, p=0.738 Sniffing*Genotype: F(3,96)=0.257, p=0.856 Sniffing*Housing: F(3,96)=0.267, p=0.849	0.071 0.070 0.062 0.097 0.099	N/A	SGH Nlgn3 y/+ n=4 SGH Nlgn3 y/- n=4 MGH Nlgn3 y/+ n=4 MGH Nlgn3 y/- n=4 (repeated measure scent cue)

	Control 2: W(16)=0.915, p=0.140 Control 3: W(16)=0.863, p=0.021 Control 4: W(16)=0.933, p=0.272 SGH: Control 1: W(16)=0.884, p=0.044 Control 2: W(16)=0.942, p=0.369 Control 3: W(16)=0.884, p=0.044 Control 4: W(16)=0.941, p=0.363 MGH:	Levene's test F(1,34)=1.012, p=0.322 Control 4: Levene's test F(1,34)=2.803, p=0.103 Housing: Control 1: Levene's test F(1,34)=0.004, p=0.947 Control 2: Levene's test F(1,34)=1.002, p=0.324 Control 3:	Genotype*Housing: F(1,32)=7.816, p=0.009 Sniffing*Genotype*Housing: F(3,96)=0.120, p=0.948	0.774 0.071		

	Control 1: W(20)=0.830, p=0.003 Control 2: W(20)=0.946, p=0.309 Control 3: W(20)=0.830, p=0.003 Control 4: W(16)=0.940, p=0.241	Levene's test F(1,34)=0.004, p=0.947 Control 4: Levene's test F(1,34)=0.104, p=0.749				
4.13	Control: WT: W(10)=0.843, p=0.048 KO: W(10)=0.696, p=0.001 PVwt: W(13)=0.881, p=0.073 PVko: W(9)=0.975, p=0.931 Scent Cue: WT: W(10)=0.878, p=0.123 KO: W(10)=0.897, p=0.203 PVwt: W(13)=0.888, p=0.092	Control: Levene's test F(3,38)=4.154, p=0.012 Scent Cue: Levene's test F(3,38)=1.513, p=0.227	Repeated measures ANOVA Within subjects (sniffing): F(1,38)=9.037, p=0.005 Between subjects (genotype): F(1,38)=1.412, p=0.254 Sniffing*Genotype: F(3,38)=8.874, p<0.001	0.834 0.344 0.991	Bonferroni adjusted pairwise comparison Control:WT/KO, p=0.046 Control:WT/PVwt, p=0.001 Control: WT/PVko, p=0.001 Control: KO/PVwt, p=1 Control: KO/PVko, p=1 Control: PVwt/PVko, p=1	Nlgn3 y/+ n=10 Nlgn3 y/- n=10 PV Nlgn3 y/+ n=13 PV Nlgn3 y/- n=9

	PVko: W(9)=0.888, p=0.191				ScentCue: WT/KO, p=1 ScentCue: WT/PVwt, p=1 ScentCue: WT/PVko, p=0.242 ScentCue: KO/PVwt, p=0.307 ScentCue: KO/PVko, p=0.040 ScentCue: PVwt/PVko, p=1 WT: Control/ScentCue, p=0.119 KO: Control/ScentCue, p=0.674 PVwt: Control/ScentCue, p=0.001 PVko: Control/ScentCue, p=<0.001	
5.2	Control/WT: W(23)=0.942, p=0.195 HMW/WT: W(23)=0.939, P=0.078	Genotype: Control: Levene's test F(1,37)=2.122, p=0.154	Repeated measures ANOVA Within subjects (sniffing): F(1,35)=30.137, p=<0.001 Between subjects (genotype):	1.0 0.533	Bonferroni adjusted pairwise comparison SGH:Control:WT/KO, p=0.600 SGH:HMW:WT/KO, p=0.241	SGH Nlgn3 y/+ n=12 SGH Nlgn3 y/- n=11 MGH Nlgn3 y/+ n=8 MGH Nlgn3 y/- n=8

	Control/KO: W(16)=0.916, p=0.144 HMW/KO: W(16)=0.939, P=0.339 Control/SGH: W(20)=0.918, p=0.89 HMW/SGH: W(20)=0.948, p=0.339 Control/MGH: W(19)=0.932, p=0.191 HMW/MGH: W(19)=0889, p=0.031	HMW: Levene's test F(1,37)=4.417, p=0.42 Housing: Control: Levene's test F(1,37)=0.859, p=0.360 HMW: Levene's test F(1,37)=0.559, p=0.460	F(1,35)=4.412, p=0.043 Between subjects(housing): F(1,35)=0.051, p=0.823 Sniffing*Genotype: F(1,35)=8.375, p=0.007 Sniffing*Housing: F(1,35)=0.154, p=0.697 Genotype*Housing: F(1,35)=0.253, p=0.618 Sniffing*Genotype*Housing: F(1,35)=2.086, p=0.158	0.056 0.803 0.067 0.780 0.290	MGH:Control:WT/KO, p=0.602 MGH:HMW:WT/KO, p=0.016 WT:Control:SGH/MGH, p=0.523 WT:HMW:SGH/MGH, p=0.317 KO:Control:SGH/MGH, p=0.668 KO:HMW:SGH/MGH, p=0.672 WT:SGH:Control/HMW, p=0.001 WT:MGH:Control/HMW, p=<0.001 KO:SGH:Control/HMW, p=0.102 KO:MGH:Control/HMW, p=0.482	(repeated measure scent cue)
5.3	Control/WT: W(21)=0.908, p=0.049	Genotype: Control:	Repeated measures ANOVA	0.976	Bonferroni adjusted pairwise comparison	SGH Nlgn3 y/+ n=9 SGH Nlgn3 y/- n=12

	HMW/WT: W(21)=0.880, P=0.014 Control/KO: W(22)=0.923, p=0.089 HMW/KO: W(22)=0.833, P=0.002 Control/SGH: W(17)=0.915, p=0.121 HMW/SGH: W(17)=0.825, p=0.005 Control/MGH: W(26)=0.928, p=0.071 HMW/MGH: W(26)=0897, p=0.013	Levene's test F(1,41)=7.802, p=0.008 HMW: Levene's test F(1,41)=1.259, p=0.268 Housing: Control: Levene's test F(1,41)=0.447, p=0.507 HMW: Levene's test F(1,41)=2.300, p=0.137	Within subjects (sniffing): F(1,39)=16.336, p=<0.001 Between subjects (genotype): F(1,39)=2.461, p=0.125 Between subjects(housing): F(1,39)=0.328, p=0.570 Sniffing*Genotype: F(1,39)=2.910, p=0.096 Sniffing*Housing: F(1,39)=1.154, p=0.289 Genotype*Housing: F(1,35)=3.818, p=0.058 Sniffing*Genotype*Housing: F(1,35)=8.595, p=0.006	0.334 0.086 0.182 0.384 0.478 0.816	SGH:Control:WT/KO, p=0.635 SGH:HMW:WT/KO, p=0.008 MGH:Control:WT/KO, p=0.456 MGH:HMW:WT/KO, p=0.508 WT:Control:SGH/MGH, p=0.045 WT:HMW:SGH/MGH, p=0.053 KO:Control:SGH/MGH, p=0.389 KO:HMW:SGH/MGH, p=0.099 WT:SGH:Control/HMW, p=<0.001 WT:MGH:Control/HMW, p=0.351 KO:SGH:Control/HMW, p=0.834 KO:MGH:Control/HMW, p=0.019	MGH Nlgn3 y/+ n=14 MGH Nlgn3 y/- n=8 (repeated measure scent cue)

5.6	Control: W(21)=0.750, p=<0.001 MUP7: W(16)=0.684, p=<0.001 MUP20: W(21)=0.834, p=0.002	Sniffing: Levene's test F(2,55)=3.181, p=0.049	Kruskal Wallis H test $X^2(2)=16.020$, p=<0.001 Mean ranks: Control = 27.00 MUP7 = 18.59 MUP20 = 40.31	N/A	Dunn's pairwise comparison (adjusted) MUP7/Control: p=0.390 MUP7/MUP20: p=<0.001 MUP20/Control: p=0.030	Control n=21 MUP7 n=16 MUP7 n=21
5.7	Scent cue: Control: W(41)=0.654, p=<0.001 MUP7: W(28)=0.768, p=<0.001 MUP20: W(42)=0.837, p=<0.001 Signal: GFP: W(58)=0.793, p=<0.001	Scent cue: Levene's test F(2,108)=4.502, p=0.013 Signal: Levene's test F(1,109)=6.687, p=0.011	Repeated measures ANOVA (Greenhouse-Geisser correction) Within subjects (Signal): F(1,50)=3.323, p=0.074 Between subjects (Scent cue): F(2,50)=9.527, p=<0.001 Signal*Scent cue:	0.432 0.974 0.221	N/A	Control n=41 MUP7 n=28 MUP20 n=42

	Antibody: W(53)=0.745, p=<0.001		F(2,50)=1.037, p=0.362			
5.11 A	**Glomerular layer** WT: W(45)=0.791, p=<0.001 KO: W(50)=0.728, p=<0.001 SGH: W(40)=0.635, p=<0.001 MGH: W(55)=0.831, p=<0.001 **Anterior commissure** WT: W(36)=0.624, p=<0.001	**Glomerular layer** Genotype: Levene's test F(1,93)=0.103, p=0.749 Housing: Levene's test F(1,93)=6.778, p=0.011 **Anterior commissure** Genotype:	Two way ANOVA (Greenhouse-Geisser correction) **Glomerular layer** Genotype: F(1,91)=<0.001, p=1 Housing: F(1,91)=5.927, p=0.017 Genotype*Housing: F(1,91)=0.178, p=0.674 Anterior commissure Genotype: F(1,83)=0.238, p=0.627		N/A	Glomerular layer SGH Nlgn3y/+ n=22 MGH Nlgn3y/+ n=23 SGH Nlgn3y/- n=18 MGH Nlgn3y/- n=32 External plexiform layer SGH Nlgn3y/+ n=22 MGH Nlgn3y/+ n=17 SGH Nlgn3y/- n=18 MGH Nlgn3y/- n=32 Metrial cell layer SGH Nlgn3y/+ n=19

	KO: W(51)=0.669, p=<0.001 SGH: W(25)=0.267, p=<0.001 MGH: W(62)=0.749, p=<0.001 **External plexiform layer** WT: W(45)=0.696, p=<0.001 KO: W(49)=0.712, p=<0.001 SGH: W(39)=0.366, p=<0.001 MGH: W(55)=0.828, p=<0.001	Levene's test F(1,85)=0.006, p=0.938 Housing: Levene's test F(1,85)=26.463, p=<0.001 **External plexiform layer** Genotype: Levene's test F(1,92)=0.269, p=0.605 Housing:	Housing: F(1,83)=11.158, p=0.001 Genotype*Housing: F(1,83)=0.013, p=0.911 **External plexiform layer** Genotype: F(1,90)=0.344, p=0.559 Housing: F(1,90)=27.408, p=<0.001 Genotype*Housing: F(1,90)=<0.001, p=1 **Metrial cell layer** Genotype: F(1,81)=<0.001, p=1			MGH Nlgn3y/+ n=16 SGH Nlgn3y/- n=20 MGH Nlgn3y/- n=30 Granual layer SGH Nlgn3y/+ n=14 MGH Nlgn3y/+ n=11 SGH Nlgn3y/- n=22 MGH Nlgn3y/- n=39 Anterior commissure SGH Nlgn3y/+ n=14 MGH Nlgn3y/+ n=11 SGH Nlgn3y/- n=22 MGH Nlgn3y/- n=39

	Metrial cell layer WT: W(39)=0.740, p=<0.001 KO: W(46)=0.726, p=<0.001 SGH: W(35)=0.474, p=<0.001 MGH: W(50)=0.869, p=<0.001 **Granual layer** WT: W(36)=0.804, p=<0.001 KO: W(50)=0.861, p=<0.001	Levene's test F(1,92)=25.145, p=<0.001 **Metrial cell layer** Genotype: Levene's test F(1,83)=1.106, p=0.296 Housing: Levene's test F(1,83)=51.664, p=<0.001 **Granual layer** Genotype:	Housing: F(1,81)=24.480, p=<0.001 Genotype*Housing: F(1,81)=0.394, p=0.532 **Granual layer** Genotype: F(1,82)=1.541, p=0.218 Housing: F(1,82)=13.456, p=<0.001 Genotype*Housing: F(1,82)=0.114, p=0.737			

	SGH: W(25)=0.701, p=<0.001 MGH: W(61)=0.872, p=<0.001	Levene's test F(1,84)=3.003, p=0.087 Housing: Levene's test F(1,84)=7.132, p=0.009				
5.11B	**Lateral hypothalamic area** WT: W(24)=0.712, p=<0.001 KO: W(17)=0.700, p=<0.001	**Lateral hypothalamic area** Genotype: Levene's test F(1,39)=0.765, p=0.387	**Lateral hypothalamic area** Genotype: F(1,37)=1.311, p=0.260 Housing: F(1,37)=8.146, p=0.007		N/A	Lateral hypothalamic area SGH Nlgn3y/+ n=12 MGH Nlgn3y/+ n=12 SGH Nlgn3y/- n=9 MGH Nlgn3y/- n=9

	SGH: W(20)=0.757, p=<0.001 MGH: W(21)=0.824, p=0.002 **Dorsomedial hypothalamic nucleus** WT: W(21)=0.617, p=<0.001 KO: W(18)=0.826, p=0.004 SGH: W(18)=0.665, p=<0.001 MGH: W(21)=0.791, p=<0.001 **Ventromedial hypothalamic nucleus**	Housing: Levene's test F(1,39)=13.129, p=0.001 **Dorsomedial hypothalamic nucleus** Genotype: Levene's test F(1,37)=4.206, p=0.047 Housing: Levene's test F(1,37)=3.393, p=0.073	Genotype*Housing: F(1,37)=0.120, p=0.731 **Dorsomedial hypothalamic nucleus** Genotype: F(1,35)=3.614, p=0.066 Housing: F(1,35)=3.066, p=0.089 Genotype*Housing: F(1,35)=1.364, p=0.251 **Ventromedial hypothalamic nucleus** Genotype: F(1,38)=7.889, p=0.008 Housing: F(1,38)=1.263, p=0.268			Dorsomedial hypothalamic nucleus SGH Nlgn3y/+ n=9 MGH Nlgn3y/+ n=9 SGH Nlgn3y/- n=12 MGH Nlgn3y/- n=9 Ventromedial hypothalamic nucleus SGH Nlgn3y/+ n=9 MGH Nlgn3y/+ n=9 SGH Nlgn3y/- n=12 MGH Nlgn3y/- n=9 Periventricular hypothalamic nucleus SGH Nlgn3y/+ n=15

	WT: W(21)=0.662, p=<0.001 KO: W(21)=0.843, p=0.003 SGH: W(18)=0.586, p=<0.001 MGH: W(24)=0.817, p=0.001 **Periventricular hypothalamic nucleus** WT: W(35)=0.620, p=<0.001 KO: W(35)=0.724, p=<0.001 SGH: W(30)=0.633, p=<0.001	**Ventromedial hypothalamic nucleus** Genotype: Levene's test F(1,40)=6.539, p=0.014 Housing: Levene's test F(1,40)=2.198, p=0.146 **Periventricular hypothalamic nucleus** Genotype:	Genotype*Housing: F(1,38)=<0.001, p=1 **Periventricular hypothalamic nucleus** Genotype: F(1,66)=2.725, p=0.104 Housing: F(1,66)=0.395, p=0.532 Genotype*Housing: F(1,66)=0.141, p=0.708 **Posterior hypothalamic area** Genotype: F(1,36)=2.504, p=0.122 Housing: F(1,36)=2.336, p=0.135			MGH Nlgn3y/+ n=20 SGH Nlgn3y/- n=15 MGH Nlgn3y/- n=20 Posterior hypothalamic area SGH Nlgn3y/+ n=8 MGH Nlgn3y/+ n=12 SGH Nlgn3y/- n=8 MGH Nlgn3y/- n=12

	MGH: W(40)=0.689, p=<0.001 **Posterior hypothalamic area** WT: W(20)=0.655, p=<0.001 KO: W(20)=0.801, p=<0.001 SGH: W(16)=0.711, p=<0.001 MGH: W(24)=0.763, p=<0.001	Levene's test F(1,68)=6.313, p=0.014 Housing: Levene's test F(1,68)=1.631, p=0.206 **Posterior hypothalamic area** Genotype: Levene's test F(1,38)=7.784, p=0.008 Housing:	Genotype*Housing: F(1,36)=1.480, p=0.232			

		Levene's test F(1,38)=8.198, p=0.007				
5.11 C	**Polymorph layer** WT: W(35)=0.620, p=<0.001 KO: W(35)=0.724, p=<0.001 SGH: W(30)=0.633, p=<0.001 MGH: W(40)=0.699, p=0.001 **Granular layer** WT: W(106)=0.710, p=<0.001	**Polymorph layer** Genotype: Levene's test F(1,68)=6.313, p=0.014 Housing: Levene's test F(1,68)=1.631, p=0.206 **Granular layer** Genotype:	**Polymorph layer** Genotype: F(1,40)=1.814, p=0.186 Housing: F(1,40)=1.671, p=0.204 Genotype*Housing: F(1,66)=2.104, p=0.155 **Granular layer** Genotype: F(1,207)=27.750, p=>0.001 Housing: F(1,207)=38.864, p=>0.001		**Granular layer** SGH: WT/KO, p=<0.001 MGH: WT/KO, p=0.532 WT: SGH/KO, p=<0.001 KO: SGH/MGH, p=0.209	Polymorph layer SGH Nlgn3y/+ n=24 MGH Nlgn3y/+ n=30 SGH Nlgn3y/- n=24 MGH Nlgn3y/- n=40 Granular layer SGH Nlgn3y/+ n=45 MGH Nlgn3y/+ n=56 SGH Nlgn3y/- n=45 MGH Nlgn3y/- n=60 Molecular layer

	KO: W(105)=0.812, p=<0.001 SGH: W(91)=0.746, p=<0.001 MGH: W(120)=0.825, p=0.001 **Molecular layer** WT: W(143)=0.271, p=<0.001 KO: W(145)=0.178, p=<0.001 SGH: W(121)=0.240, p=<0.001 MGH: W(167)=0.324, p=0.001	Levene's test F(1,209)=19.736, p=<0.001 Housing: Levene's test F(1,209)=32.199, p=>0.001 **Molecular layer** Genotype: Levene's test F(1,286)=0.003, p=0.959 Housing:	Genotype*Housing: F(1,207)=19.770, p=>0.001 **Molecular layer** Genotype: F(1,284)=0.230, p=0.880 Housing: F(1,284)=4.388, p=0.037 Genotype*Housing: F(1,284)=0.004, p=0.948			SGH Nlgn3y/+ n=66 MGH Nlgn3y/+ n=57 SGH Nlgn3y/- n=77 MGH Nlgn3y/- n=92

		Levene's test F(1,286)=4.119, p=0.043				