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Elucidating the neural circuitry underlying social spacing in Drosophila melanogaster through the lens of neuroligin 3

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Abstract

Social interactions among animals can be complex, and abnormal social behaviours may result in negative fitness consequences for both the individual displaying them, and the entire group. To understand the neural basis of complex social behaviour, we can study simpler behaviours that precede and mediate them. Social spacing, the typical distance between individuals in a group, is an easily quantifiable behaviour in *Drosophila melanogaster*. Here, I investigated the neural circuitry underlying social spacing through the lens of Autism-candidate gene *neuroligin 3*. Based on the Nlg3 enrichment pattern in adult fly brains, I hypothesized that *nlg3*-expressing neurons, along with the mushroom bodies and protocerebral bridge, were involved in this behaviour. I determined that all the aforementioned structures are involved, and there is likely sexual dimorphism in this neural circuitry. This research contributes to understanding the role Nlg3 plays in social spacing and reveals more routes of neural connectivity to be investigated.

Keywords

Social behaviour

Behavioural genetics

Neuroligin

Mushroom bodies

Social space

Neural circuitry

Drosophila melanogaster

Summary for Lay Audience

Social interaction among humans has allowed us to create our modern society. Underlying each social interaction are complex patterns of neural signalling that give us the ability to perceive cues and understand each other; this is called neural circuitry. There is a surprising lack of information regarding the neural circuits that govern our behaviour, and because of that we also lack knowledge pertaining to how abnormal social behaviours occur. Abnormal social interaction can lead to negative consequences for an individual and their social group, so why and how might this occur? To investigate this, we can use the vinegar fly (a.k.a. the fruit fly) *Drosophila melanogaster*. Flies display numerous social behaviours, from complex courtship songs to simpler behaviours such as social spacing. To better understand the neural circuitry of complex behaviours, we can study simple ones like social spacing because it often precedes and mediates the complex behaviours. Just as we have a preferred social distance, flies will repeatedly choose a to maintain a specific amount of space from each other. Additionally, about 75% of disease-related genes in humans have a similar gene in the fly, and there is a remarkable amount of similarity in how certain parts of our brains function compared to flies. These similarities allow us to study how genes related to abnormal social interactions, such as those seen in autism spectrum disorders, can affect neural circuitry. To investigate social spacing neural circuitry, I used the fly counterpart to a gene associated with autism (autistic individuals often display abnormal social space) in humans called *neuroligin 3*. This gene is involved in determining how neurons interact with each other, and previous research has shown that mutating this gene affects social spacing. To get a better idea of which neurons are involved in this behaviour, I investigated brain regions that contain the Neuroligin 3 protein. Here I show that brain structures enriched with Nlg3 are involved in social spacing neural circuitry, and that there may be sex differences in the circuitry as well. From here, more studies can be conducted to further specify the neuronal underpinnings of social spacing.

Co-Authorship Statement

This thesis contains data that will be used in an article to be written by myself, J. Wesley Robinson (JWR), Ryley T. Yost (RTY), and Anne F. Simon (AFS). Additionally, much of the introductory section is also to be included in a review to be written by myself, RTY, and AFS.

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Additionally, I would like to thank my lab mates for their continued support and knowledge; especially Ryley and Wes for showing me the ropes and welcoming me into the grad student community. A special thank you to Norman as well for his invaluable help (and many lame jokes) in the last few months of my Master's. You all kept me going in the hardest of times.

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List of Abbreviations

4BL	4 body lengths
AN	all neighbours
ASD	autism spectrum disorder
BPA	Bisphenol-A
CNS	central nervous system
CS	Canton-Special control strain of flies
DAN	dopaminergic neurons
def	*deficient*
dsRNA	double-stranded RNA
EGFP	enhanced green fluorescent protein
FSB	fan-shaped body
GluR1A	glutamate receptor 1A
LAL	lateral accessory lobes
LH	lateral horn
MB	mushroom bodies
MBON	mushroom body output neuron
MiMIC	Minos-Mediated Insertion Cassette
nlg	*neuroligin*
NMJ	neuromuscular junction
NN	nearest neighbour
OL	optic lobes
PB	protocerebral bridge
rg	*rugose*
RISC	RNA-induced silencing complex

RMCE	recombinase mediated cassette exchange
RNAi	RNA interference
shi^{ts}	*shibere temperature-sensitive*
shRNA	short-hairpin RNA
siRNA	short-interfering RNA
Trojan	MiMIC-based construct expressing Gal4 as an artificial exon
Troj	Trojan construct
TrpA1	*Transient receptor channel A1*
UAS	upstream activating sequence

Nomenclature of gene and protein symbols

Drosophila naming conventions based on accepted guidelines established on by the FlyBase Consortium (Gramates et al., 2022).

Genus and species name are italicized, and genus first letter capitalized: *Drosophila melanogaster*

Genus alone in text still italicized and first letter capitalized: *Drosophila*

Gene names: all lowercase and italicized, unless largely accepted and dictated within the scientific community. Ex: *neuroligin 3* (*nlg3)* and *Transient receptor channel A1* (*TrpA)*

Protein names: first letter capitalized, no italicization. Ex: Neuroligin 3 (Nlg3)

Genotypes: if there are deviations from wildtype, gene symbols and other indicators of mutations are written as follows, with semicolons used to separate the three large chromosomes found in *Drosophila*, all italicized, and the fourth chromosome (which is very small) is not included in this notation: *w*;;{GMR15D05-GAL4}attP2*

Human naming conventions based on: Bruford et al., (2020)

Gene symbols: entirely capitalized and italicized. Ex: *NGLN3*

Protein symbols: entirely capitalized but not italicized. Ex: NLGN3

Mouse naming conventions based on: Sundberg & Schofield, (2010)

Gene symbols: the first letter of gene symbol is capitalized, and all letters italicized. Ex: *Nlgn3*

Protein symbols: all letters are capitalized and not italicized. Ex: NLGN3

Chapter 1

1 Introduction to social behaviour and *Drosophila* as a model in neurobiology

1.1 Social behaviour

1.1.1 What is social behaviour?

Social behaviour has long been recognized as an important aspect of survival and reproduction in animals (Couzin, 2009; Kacsoh et al., 2015; Kokko & Monaghan, 2001). Social groups allow increased ability to acquire and defend food sources, stronger defense against predation, help in raising offspring, and increase the likelihood of finding a mate (Esser, 1971; Waser & Wiley, 1979). Abnormal social behaviours may result in negative fitness consequences for both the individual displaying them, and the group as a whole (Székely et al., 2010). In order to exhibit any kind of behaviour, an individual must first properly perceive and integrate cues using their nervous system; in social behaviour, the cues come from other organisms (Couzin, 2009; Szekely et al., 2010). It is theorized that improper integration of cues may contribute to abnormal social behaviours in animals, and in humans can lead to difficulties with social interactions such as those seen in Autism Spectrum Disorders (ASD) and Schizophrenia (Couzin, 2009; Lough et al., 2015). A variety of techniques have been created to study the genetic and neuronal underpinnings of social behaviour and cue integration. Assays for simple behaviours such as aggregation and locomotion are well established among a variety of model organisms including mice, rats, honeybees, zebrafish, and nematodes among many others (Cohen & Denham, 2019; Rittschof & Robinson, 2013; Takahashi & Miczek, 2015; Way et al., 2016). Complex behaviours like aggression and courtship have also been extensively studied in models such as mice and even the vinegar fly, *Drosophila melanogaster* (D'amato, 1991; Ewing, 1983; Vonschilcher, 1976; Lasbleiz et al., 2006; Takahashi & Miczek, 2015). Animal models provide a means to manipulate both genes and environment to explore the underpinnings of social behaviour; and there are many available to choose from, each with varying strengths and weaknesses.

1.1.2 Models of social behaviour

One common model organism used in social behavior studies is the mouse, *Mus musculus*. Many researchers use this model due to its high genetic homology to humans, as well as its expansive displays of social behaviour (Hörnberg et al., 2020; Okada et al., 2015; Radyushkin et al., 2009; Zhu et al., 2019). This model has been used to study many social behaviours including play, repetitive behaviours, social communication, social novelty preference, and much more (Haller et al., 2014; Hörnberg et al., 2020; Okada et al., 2015; Olexová et al., 2012). However, the complexity of the mouse brain and long gestation times limit the productivity of research. A variety of invertebrate models of social behaviour have been studied from an ecological perspective- symbiosis between crustaceans, aggression in crayfish, and aggregation in zebrafish (Patullo et al., 2009; Thiel1 et al., 2001; Way et al., 2016). Interestingly, invertebrate models, such as insects, have also emerged as simpler models of social behaviour.

Many insect models display both simple and complex behaviours, have shorter generation times compared to mammalian models, as well as more inexpensive housing conditions and diets (Keller & Jemielity, 2006; Li et al., 2019; Sokolowski, 2010). For example, insects of the order *Hymenoptera* (bees, ants, and wasps) have been used in diverse experiments to study social behaviours. Using various species of wasps, researchers were able to study the connection between how reproductive conflict is resolved, and how this behaviour is crucial to understanding the evolution of cooperation (Foster & Ratnieks, 2001). *Drosophila melanogaster* has also emerged as a widely used model for social behaviour. This species displays a diverse array of simple and complex behaviours (see section 1.2.2 below). Additionally there exists an extensive assortment of genetic tools to manipulate the fly genome (Adams et al., 2000; Allocca, et al., 2018; Bier, 2005; Brenman-Suttner et al., 2020; Corthals et al., 2017a; Hahn et al., 2013; Hales et al., 2015; Kacsoh et al., 2015; Lee et al., 2018; McNeil et al., 2015; Okray & Hassan, 2013; Simon et al., 2012; Sokolowski, 2001, 2010; Wise et al., 2015; Yost et al., 2020). Such tools and characteristics have allowed researchers to assess the impact of specific genes and environmental conditions on social behaviours in many model organisms.

1.1.3 Genetic and environmental effects on social behaviour

All behaviours are a result of cue integration through an organism's nervous system; but what exactly determines *how* a cue is integrated? Within an individual, the process by which a cue is integrated is dependent on both their genetics and environment. Genetic variation may lead to differential integration within a single species, but an individuals' life experiences also contribute to establishing (or preventing the establishment of) neural circuits that allow proper behavioural responses (Garner & Mayford, 2012; Havekes & Abel, 2009; Levitis et al., 2009; Olexová et al., 2012; Sosa et al., 2021; Székely et al., 2010).

One example of environmental conditions affecting social behaviour is the effect of the gut microbiome in animals, including rats and humans. Recent studies have found that the microbiome affects the development and function of brain regions such as the amygdala, hippocampus, and prefrontal cortex; key contributors to social behaviour and cognition (Bokulich et al., 2016; Sarkar et al., 2020). Additionally, the microbiome helps regulate the availability of biological signaling molecules that influence social behaviour in animals such as sex hormones, neuropeptides, and monoamines (Bokulich et al., 2016; Sarkar et al., 2020). Different microbiome composition between organisms could then lead to differences in cue integration based on how brain development and signaling molecule bioavailability is affected in each individual, thus affecting social behaviour.

In rats, factors such as repeated maternal separation, post-weaning social isolation, and peripubertal stressors have been demonstrated to increase aggressive and violent behaviour in adulthood (Haller et al., 2014). The rats exposed to these early-life stressors also had long-lasting alterations in neurotransmitter, neuropeptide, and hormonal levels due to epigenetic changes induced by those stressors (Haller et al., 2014). This interaction between environment and genetic expression is a great demonstration of how the interplay between life experience and genes can affect behaviour.

There are also examples of single gene knockouts affecting multiple behaviours in model organisms. In male mice, knocking out *Neuroligin 3* expression causes deficiency in social novelty preference, decreased social vocalizations, and increased learning and

memory abilities (Radyushkin et al., 2009) (see Nomenclature of gene and protein symbols). Similarly, honeybees have key genetic differences that allow division of labour; the likelihood of which honeybee will perform many of their behaviours/tasks is biased (Johnson & Jasper, 2016). Researchers have investigated the difference in gene regulation between honeybees that act as "foragers" and "nurses" to help determine the genetic basis for this division of labour. They found many key regulatory genes that have differential expression between the two groups of honeybees, as well as genes that have similar expression between them, but at different ages (Johnson & Jasper, 2016). The difference in gene regulation is hypothesized to be influenced by genetic variation as well as environmental effects that can lead to epigenetic changes (Johnson & Jasper, 2016).

In summary, there is a complex interaction between genes and environment that allows differential development of neural circuits, and therefore differential cue integration among and within species. Much research has been done to determine the genetic and neuronal basis of complex behaviours in a variety of model species, and still there is much to be discovered. To get a better understanding of complex behaviours, we can first establish the genetic and neuronal underpinnings of simple behaviours that mediate and precede them. Social spacing is one such example that can be easily measured in animal models including *D. melanogaster,* which models this behaviour very well.

1.2 *Drosophila* as a model organism in behavioural studies

1.2.1 Overview and history of *Drosophila* as a model organism

The fly has served as a powerful model to learn about the molecular mechanisms governing life, development, and behaviour of animals for almost a century. Thomas Hunt Morgan was the first scientist to earn a Nobel prize for research conducted using *D. melanogaster* in 1933, when he discovered that chromosomes encode genes "like beads on a string" and can often be inherited together through linkage (Das, 2017). Since then, Nobel prizes have been awarded to Drosophilists for many ground-breaking studies, including genetic contributions to embryo development, innate immunity activation, and organization of the olfactory system (Das, 2017). Most recently, in 2017 the Nobel prize for Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash, and

Michael W. Young for their studies uncovering molecular mechanisms that regulate a behavioural response: the circadian rhythm (Das, 2017).

Using *D.* melanogaster, great strides have been taken in the field of behavioural genetics due to the many attributes that make the fly such a powerful model. Flies are inexpensive to house, can be cultured in the lab, have many offspring, and have short generation times (egg to adult in 10 days) (Das, 2017; Hales et al., 2015). In addition to these innate characteristics, the fly has a well-studied brain morphology, a fully annotated genome, and a variety of genetic tools available to manipulate both neurons and genes to study their effects on behaviours (Bier, 2005; Brand & Perrimon, 1993; Hales et al., 2015). It was also reported by the Max Planck Society that about 75% of disease-causing genes in humans have orthologs in *D. melanogaster*; this includes genes that may contribute to abnormal behaviours seen in individuals with developmental disorders such as ASDs (Sokolowski., 2010; Coll-Tane et al., 2019; Hahn et al., 2013; Trobiani et al., 2020; Yost et al., 2020).

1.2.2 *Drosophila* as a model for neurobiology and behaviour

Drosophila melanogaster research has contributed greatly to the foundational understanding of neural function and animal behaviour (Bellen et al., 2010). The straightforward system of behavioural assays paired with genetic screens has allowed us to research all aspects of neurobiology; from the molecular mechanisms governing nervous system development and neuronal plasticity, to the neural circuitry underlying complex behaviours (Bellen et al., 2010)**.** The fly has a very simple brain morphology in comparison to humans, yet the functions of brain structures are highly conserved between these two species (Hirth, 2010). The high level of functional conservation in the fly brain along with the variety of gene orthologs creates many potential avenues of study for researchers investigating diseases and disorders of the nervous system; many researchers have successfully substituted *Drosophila* and human genes involved in evolutionarily conserved mechanisms underlying brain development in both species (Hanks et al., 1998; Leuzinger et al., 1998; Nagao et al., 1998). The fly has been crucial in understanding the molecular pathways underlying nervous system disorders such as Alzheimer's Disease,

ASDs, and Parkinson's Disease (Lu & Vogel, 2009; Sokolowski, 2010; Uchigashima et al., 2021; Wise et al., 2015; Yost et al., 2020).

In addition to studies of human disorders, the neurobiology of simple behaviours in the fly have been studied to understand the most basic neural and genetic underpinnings of behaviour. Locomotion and climbing ability are two simple behaviours that are commonly studied; they represent respectively exploration and escape responses and are also indicative of the overall health and stamina of fly lines. Assays for locomotion and climbing alongside the study of complex behaviours can control for confounding effects due to reduced locomotor ability (Eidhof et al., 2017; Manjila & Hasan, 2018; Nichols et al., 2012; Simon et al., 2012). Some of the more complex behaviours that flies display are courtship, learning and memory, and aggression (Allocca, et al., 2018; Brenman-Suttner et al., 2020; Coll-Tane et al., 2019; Lasbleiz et al., 2006; Mariano et al., 2020; Nichols et al., 2012). Despite the wealth of research dedicated to determining the neural and genetic bases of complex behaviours such as these, much remains to be understood. Studying simpler behaviours that mediate and precede complex behaviours provides a solid first step into understanding how cue integration occurs at the level of neurons and genes (Bhogal & Jongens, 2010; Eidhof et al., 2017; Vonschilcher, 1976; Lasbleiz et al., 2006; Manjila & Hasan, 2018; Nichols et al., 2012; Simon et al., 2012).

1.2.2.1 Social spacing: a simple and quantifiable behaviour

As social behaviour evolved, many animal species developed grouping behaviours as a mechanism that increased their collective fitness. As previously discussed, there are many benefits to forming social groups, and an emerging property of groups is the individual's social spacing. Social spacing behaviour can be defined as the typical or preferred distance between individuals in a group and is established through a balance of attractive and repulsive cues that are communicated between individuals (Couzin, 2009; Kummer, 1970; Waser & Wiley, 1979).

Social spacing varies between species as well as between individuals of the same species; it is largely dependent upon context, previous experience, as well as the ability of an organism to properly perceive and integrate the social cues from other organisms (Lough

et al., 2015; McNeil et al., 2015; Waser & Wiley, 1979). An individual must consider how a specific social space will be perceived by another organism. For example, the interpretation of closeness can vary greatly depending on the context. In some cases, an individual may get close to another as an act of aggression, but the same closeness could also be an aspect of courtship in other cases (Kummer, 1970). Examples of differential social space can be observed in nature, such as in Galeda baboons and Patas monkeys. In these primates, groups of females along with female-male pairs are often found very near to each other- but all male groups and male-male pairs almost always maintain a larger distance. The likelihood of two males being close to each other at all is very low, and usually only occurs during aggressive behaviours (Kummer, 1970). Defined social space where a specific distance between individuals is established and maintained can be seen in many insect species. Variations of self-assemblages with a defined social space are common as a form of defense, such as in the Japanese honeybee *Apis cerana* (Cully & Seeley, 2004). Groups of 400-500 bees will link to each other and swarm intruder hornets; they form a tight ball around the hornet and generate heat to kill it (Ono et al., 1995; Stabentheiner et al., 2007). Many complex behaviours such as self-assemblages are preceded and mediated by social spacing, and for this reason understanding the genetic and neuronal mechanisms underlying social spacing can provide insight into the underpinnings of the more complex behaviours.

1.2.2.2 Social spacing in *Drosophila melanogaster*

Surprisingly, the study of social space determination at the neuronal and genetic level remains poorly understood. To address this, an assay was developed by Dr. Anne Simon and colleagues to study social spacing in *D. melanogaster* (Simon et al., 2012)

In the development of the social spacing assay, many factors were considered to ensure robustness and reproducibility of results. Through a series of experiments researchers determined that: flies tend to settle faster when the chambers are oriented vertically, they mostly remain settled unless perturbed, triangular chambers force group formation and allow flies to choose their preferred social space more efficiently than other shapes, and groups of 12-18 flies display consistent social spacing in chambers of a given size (base: 15.2cm, height: 15.2cm) (McNeil et al., 2015; Simon et al., 2012). It was also confirmed

that social spacing in the fly is not determined randomly by comparing observed experimental results to robust computer models of random social space (Simon et al., 2012). Using these results, the final version of the social spacing chamber was established. The chamber consists of two square glass panes that are separated by 0.47cm using acrylic spacers, which also give the chamber a triangular shape (McNeil et al., 2015; Simon et al., 2012). This set-up creates a pseudo-two-dimensional chamber where the flies can only move in two directions (**Figure 1**). The top of the chamber can be opened to allow insertion of 12-18 flies via aspiration or funnel, and then the chamber is banged onto a soft pad to force flies to the bottom of the chamber while re-sealing the opening (McNeil et al., 2015; Simon et al., 2012). The chambers are held in a vertical position so that flies first try to escape by climbing to the top, forcing them into a tight group, and then quickly dispersing to find their preferred social space. Typically, the flies are left to settle for 20-40 minutes, and a photo is taken to be used in social space analysis (McNeil et al., 2015; Simon et al., 2012)

In the Simon lab, we quantify social space using three different measures: distance to nearest neighbour (NN), distance to all neighbours (AN), and the number of flies within four-body lengths (4BL). Nearest neighbour is a measurement of each fly in the chamber to its closest neighbour in centimetres (cm) and reflects behaviour of a dyad of flies, all neighbours is a measurement of each fly in the chamber to all other flies in the chamber (cm) providing information about the group's structure, and the final method measures the average number of flies within a four-body length radius of each fly in the chamber, which also provides group structure information (**Figure 2**). The initial experiments that were conducted to establish the social spacing assay used the nearest neighbour method of measurement (Simon et al., 2012), but now we use the 4BL method. The 4BL method of measurement leads to an average for each chamber instead of a distribution, which is the output of the other measures, which allows the data to follow a normal distribution. I can conduct statistical analysis that is much more powerful than if I used the nearest neighbour method, which produces data that which is closer to a Poisson distribution. A study I conducted with a lab mate in the summer of 2020 confirmed that the social spacing results measured using the NN method almost always correlated with the 4BL measurement results (Bechard & Zhang, in preparation), and when it did not it was

because the 4BL measurement is more conservative than the NN. The 4BL measurements were also consistent and reproducible across many social spacing assays, providing more reason to use this robust form of measurement (Bechard & Zhang, in preparation.)

Alongside social spacing assays, climbing assays are also conducted as a non-social control behaviour. The climbing assay takes advantage of an innate escape behaviour in flies called negative geotaxis. As alluded to above, when flies are forced to the bottom of a chamber, or "banged down", they will automatically begin to climb upwards after falling in an escape behaviour (Benzer, 1967; Connolly & Tully, 1998). In the climbing assay flies are contained in vertically oriented vials and banged down, then given 15 seconds to climb. After 15 seconds have elapsed, the vials are sealed, and the number of flies that were able to climb into the upper vial within that time are counted. We can then calculate the climbing success rate by dividing the number of flies in the upper vial by the total number of flies tested (Benzer, 1967; Connolly & Tully, 1998). This behaviour is assayed alongside social space as an indicator of each fly line's stamina, to ensure the innate escape response is intact, and to further characterize the behaviour of mutant strains used in our research (Madabattula et al., 2015; Manjila & Hasan, 2018; Simon et al., 2012). This assay along with the social spacing assay have been used extensively to investigate a wide variety of factors that may affect social space.

Environmental factors such as social experience have been demonstrated to affect social spacing in *D. melanogaster*. Virgin flies, as well as flies that undergo a period of social isolation have increased social space (McNeil et al., 2015; Simon et al., 2012; Yost et al., 2020). Bisphenol-A (BPA), a chemical hypothesized to play a causative role in the development of neurodevelopmental disorders, was also found to affect social space of the progeny when mothers were fed BPA: flies settle closer together (Kaur et al., 2015). The sensory modalities involved in social space cue perception have also been investigated. By using mutant fly lines that have deficits in visual or olfactory perception, it was found that vision, but not odor perception plays a role in social space determination (McNeil et al., 2015; Simon et al., 2012). Genetic background, as well as specific genes have been found to affect social space as well. To assess whether genetic background plays a role in social spacing, researchers measured and compared social space of various

D. melanogaster strains. They found that the inbred lab strain Canton-Special (CS), as well as various wild-caught strains have differing social space; all the wild-caught strains had increased social space compared to CS (McNeil et al., 2015). Two Autism candidate genes *neuroligin 3* (*nlg3*) and *rugose* (*rg*) (homologs of human *neuroligin* and *neurobeachin*, respectively) have been demonstrated to affect social space, with the specific effect depending on age and genotype (Wise et al., 2015; Yost et al., 2020). Much has been investigated regarding social spacing in *D. melanogaster*, and there is still much to be discovered. The research that has been conducted thus far is made possible by the variety of genetic tools in the fly that have been developed over the course of decades; some of which I will describe next.

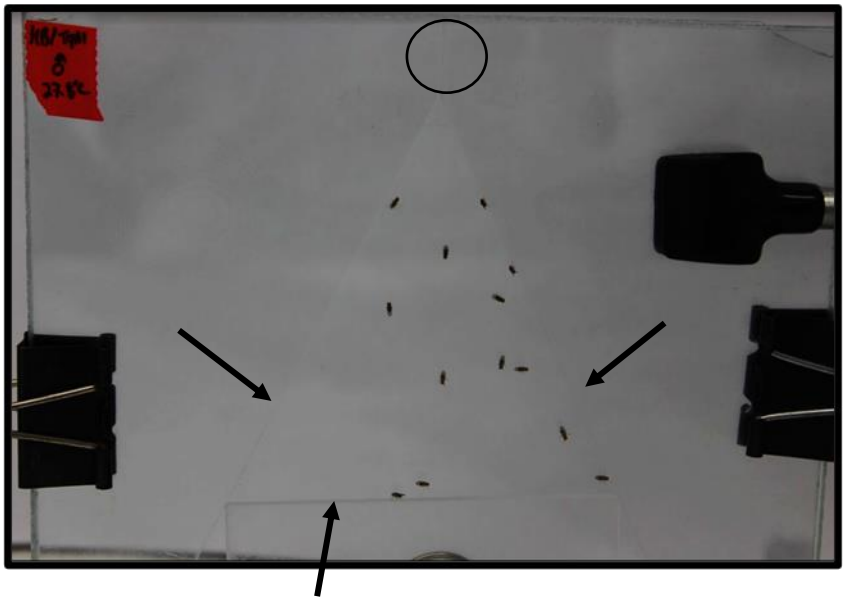

Figure 1. Picture of the social spacing chamber.

An assembled social spacing chamber with flies inserted and settled. Arrows indicate the acrylic spacers which form the triangular shape, and the point where flies can be inserted is circled at the top.

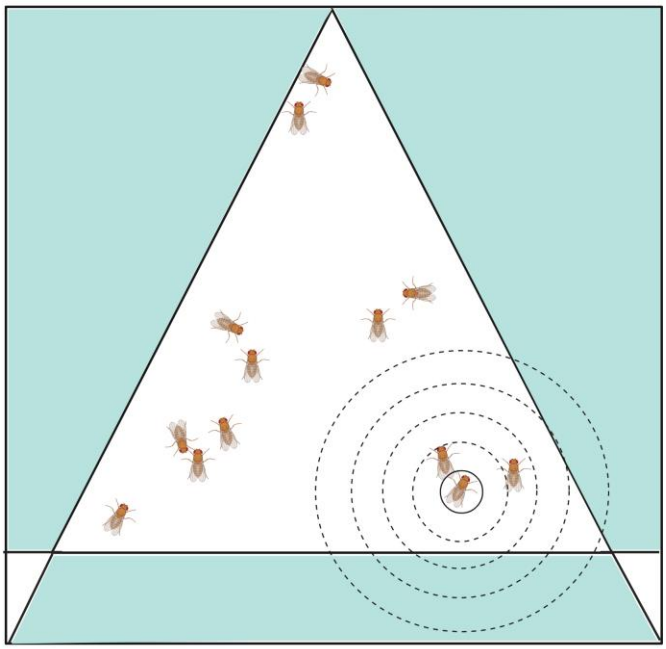

Figure 2. Measuring social space in terms of body-length.

Social space is measured by counting the number of flies within a 4BL radius of a focal fly. In the diagram above, the focal fly (located in the centre of the circle) has a total of two flies within a 4BL radius. This number is measured for each fly in the chamber, and then an average for that chamber is calculated.

1.2.3 Genetic tools in *Drosophila*

1.2.3.1 Gal4/UAS history and applications

The variety of genetic tools available to answer a wide range of questions about the role of genes in *Drosophila melanogaster* behaviour is one of this model's strongest attributes. One such tool that is used extensively in *D. melanogaster* research is the Gal4/UAS system. This system consists of two parts: a driver transgene which allows tissue/brain structure specific expression, and an effector transgene which will only be expressed in the area dictated by the driver (Brand & Perrimon, 1993). For the Gal4/UAS system to be complete, both driver and effector transgenes must be present within a

single organism (**Figure 3**). To achieve a complete system, two fly lines are mated: one parent containing the driver transgene and the other parent containing the effector transgene. The progeny will then contain both driver and effector, and theoretically will express the gene of interest as desired. Fly genotypes that have a complete bipartite Gal4/UAS are described with ">" in genotype abbreviations to indicate the gene listed to the left of ">" is a driver for the effector gene on the right side (i.e., *driver>effector*).

This widely applicable genetic tool was developed by using an activator protein from yeast called Gal4, which was shown to activate transcription in *Drosophila melanogaster*, but only in genes that contain Gal4 binding sites (see next paragraph) (Fischer et al., 1988). These researchers developed two methods to create "drivers" that have different patterns of *Gal4* expression. In their first method, they used existing *Drosophila* promoters that already had defined expression patterns and fused them to *Gal4*. In the second method, they based their work on the "enhancer detection" technique developed by O'Kane and Gehring (1987). A vector was created which included a P-transposase gene promoter that expresses transposase constitutively but cannot transpose itself, and the *Gal4* gene. This vector allowed *Gal4* to be randomly integrated into the fly genome, generating a diverse array of *enhancer-Gal4* combinations.

The expression patterns of the random *Gal4* insertions were assessed by driving the expression of a *lacZ* gene from *E. coli* with each *Gal4*-enhancer combination (henceforth referred to as "driver") and visualizing the results using beta-galactosidase assays. Alongside the creation of driver fly lines, a vector containing five tandem Gal4-optimized binding sites and a transcriptional terminator was created, where a gene of interest could be subcloned between those two elements. The Gal4-optimized binding sites were then named "upstream activating sequences" (UAS), which gave this genetic tool its name: the Gal4/UAS system. The UAS sites allowed *Gal4*-specific transcriptional activation, as well as proper termination of any subcloned genes within the vector, which could be then integrated into fly lines (UAS transgenes henceforth referred to as "effector").

Over time, the Gal4/UAS system has diversified to allow an incredibly wide assortment of expression patterns and experimental manipulations. An ongoing project by the Janelia

research group called “FlyLight” is a website containing the characterizations of thousands of Gal4 drivers, and even gives visualizations of each. This tool is entirely free and is a wonderful resource to help Drosophilists choose drivers that suit their experiments (Jenett et al., 2012) (https://flweb.janelia.org/cgi-bin/flew.cgi). As for the types of experimental manipulations, the Gal4/UAS system can be used in rescue experiments to determine gene function, assess gene expression (driver>*GFP* effector), knock down gene expression (driver>*RNAi* effector), and even manipulate neuron signal transmission.

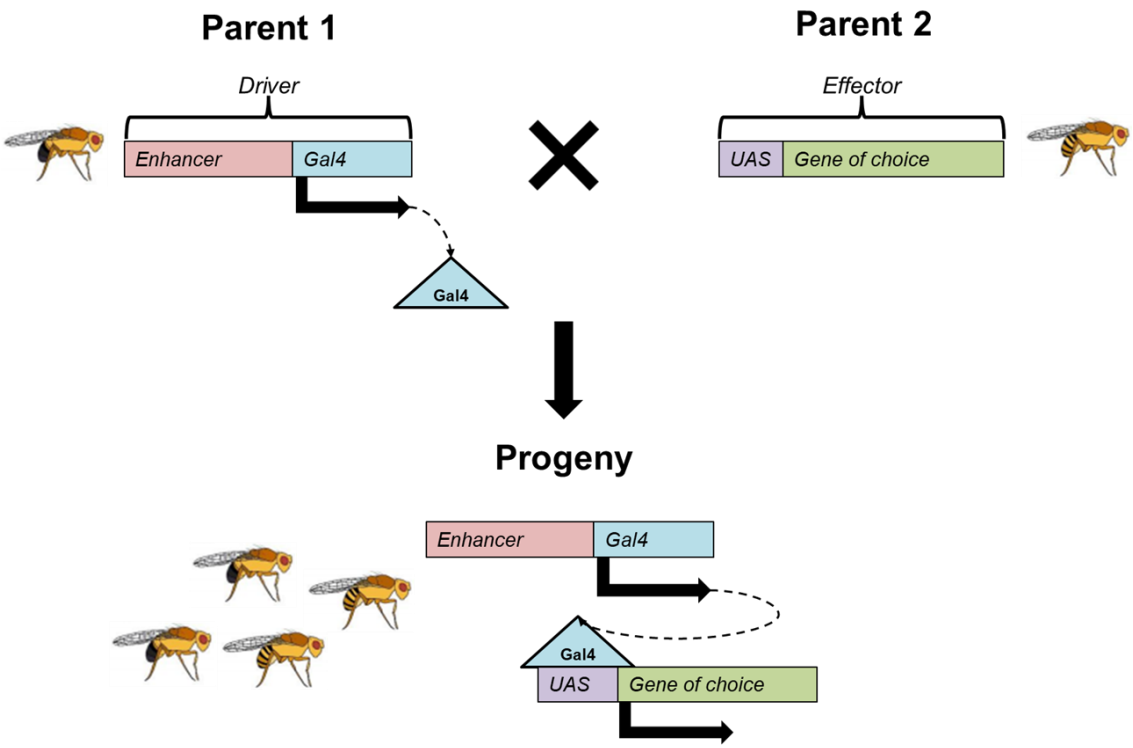

Figure 3. Diagram of the Gal4/UAS system.

The *Gal4* gene is only expressed in areas dictated by the driver sequence. The effector gene is only expressed when its transcription factor (Gal4) binds to the upstream activating sequence (UAS) preceding it. A Gal4/UAS system is only complete when both a driver construct and an effector construct are present within the same genome. To achieve this, two parent lines, one carrying a driver transgene and one carrying an effector transgene are mated. The progeny contains both driver and effector transgenes and therefore have a complete Gal4/UAS system; the effector will be expressed only in areas where the driver is expressed.

1.2.3.2 Transient neuron signal manipulation through temperature

Since the bipartite Gal4/UAS system was developed, many endogenous *D. melanogaster* genes have been adapted into effectors that are used to investigate an assortment of research questions. For example, two genes, *Transient receptor channel A1* (*TrpA1*) and *shibere* (*shi*), have been adapted into effector constructs that allow transient neuron signal manipulation by exposing flies to higher temperatures.

1.2.3.2.1 Neuron hyperactivation

The *TrpA1* gene encodes a cation channel that is expressed in sensory neurons, it opens when warmed and aids in cellular response to heat and chemical stimuli (Howe et al., 2008). This gene has been adapted into an effector fly line where a TrpA1 isoform that opens when exposed to a specific activation temperature, is fused to a UAS sequence (Hamada et al., 2008). When the *TrpA1* effector is expressed in neurons, it allows the influx of cations into cells at its activation temperature and depolarizes them, therefore forcing hyperactivation (**Figure 4**). Hyperactivation via TrpA1 is transient and will only occur while the flies are exposed to temperatures at or above its activation temperature. This effector can be used to investigate the role of specific neuron sets in behaviour as well as development.

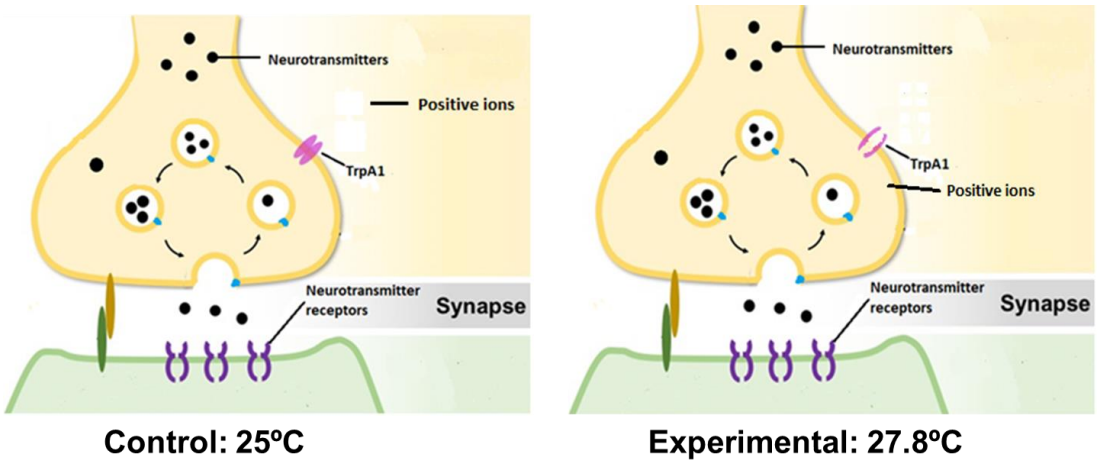

Figure 4. Neuron hyperactivation using TrpA1.

When exposed to its activation temperature (experimentally determined to be 27.8ºC) the TrpA1 channel located on the membrane of the neuron will open. This allows the influx of positive ions into the cell, thus depolarizing it and forcing hyperactivation.

1.2.3.2.2 Neuron inhibition

Another endogenous *Drosophila* gene that has been adapted into an effector for neuron transmission manipulation is *shi*. Dynamin is the protein encoded by *shi*, which aids in the process of endocytosis and is therefore necessary for synaptic vesicle recycling (Kosaka & Ikeda, 1982). A mutant temperature-sensitive *shi* allele (shi^{ts}) exists where Dynamin becomes defective at its specific activation temperature, therefore preventing synaptic vesicle recycling and inhibiting neuron transmission (**Figure 5**) (Kitamoto, 2001). And just like the TrpA1 protein, the mutant phenotype caused by Dynamin is transient and will be rapidly reversed once flies are exposed to temperatures under its activation temperature (Kitamoto, 2001).

As an effector, shi^{ts} can be used to transiently inhibit specific subsets of neurons to assess their involvement in behaviour and development. The shi^{ts} effector can also be used in conjunction with the *TrpA1* effector to gauge whether a set of neurons is normally inhibited or activated during different behaviours. For example, if a behaviour does not change while a subset of neurons is hyperactivated by *TrpA1*, then perhaps those neurons are normally hyperactivated during this behaviour. Thus, hyperactivating using *TrpA1*

would be ineffective, as those neurons already have rapid transmission which cannot be activated further.

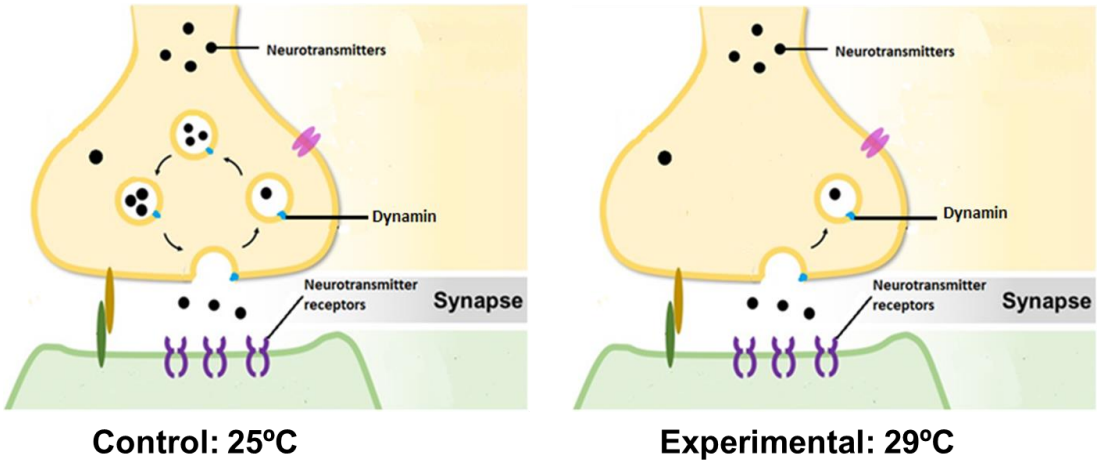

Figure 5. Neuron inhibition using a temperature sensitive Dynamin (*shi^{ts}*).

When exposed to its activation temperature (experimentally determined to be 29ºC) the Dynamin protein can no longer function, and thus vesicles cannot be formed to encapsulate neurotransmitters. Without vesicle formation neurotransmitters cannot fuse to the neuronal membrane to be released, thus forcing inhibition of signals.

1.2.3.3 Newly developed Gal4/UAS tools

1.2.3.3.1 UAS-RNAi constructs

Additional genetic constructs have been developed for use in the Gal4/UAS system, including UAS-RNAi effectors that allow knockdown of gene expression in specific areas dictated by the Gal4 driver. RNAi is a method used to reduce the expression of a specific gene within a live organism. In this method, a double stranded RNA (dsRNA) that has identical sequence to the mRNA of the gene to be knocked down is inserted into the cytosol of many cells. Once there, an endogenous enzymatic pathway called the RNA-induced silencing complex (RISC) allows the degradation of the target mRNA. The dsRNA, once inserted, is referred to as a short-interfering RNA (siRNA) duplex. The guide strand of the siRNA duplex (sequence identical to the target mRNA) is loaded into the RISC with the help of Argonaute proteins and other RNA-binding proteins. The RISC

then guides the siRNA to the complementary mRNA, where it binds and allows Ago to cleave the mRNA. After cleavage, the mRNA is then completely degraded by endogenous nucleases, thus reducing expression of the target gene (Moore et al., 2010).

In an RNAi effector, an RNAi construct is fused to a UAS, and then integrated into the fly genome, which can then be mated to a fly line containing a Gal4 driver to get a complete system. In my project, I use an RNAi effector that consists of a UAS sequence fused to a short hairpin RNA (shRNA) construct. The shRNA functions similarly to the siRNA that I described previously, except shRNAs can be integrated into the DNA, and have the two complementary RNA sequences connected by a short loop of 4-11 nucleotides (Moore et al., 2010). Through the use of shRNA we can endogenously express RNA, allow the integration of UAS-shRNA constructs into the fly genome, which we can then selectively activate using various drivers.

1.2.3.3.2 Trojan-Gal4 constructs

Another valuable tool that was made possible by the Gal4/UAS system are Trojan constructs. The creation of Trojan began with a group of researchers that created a Minos-Mediated Insertion Cassette (MiMIC), which is an artificially designed transposable element that has been inserted into over 17,500 areas of the *D. melanogaster* genome (Venken et al., 2011). The MiMIC cassette is flanked by inverted repeats of DNA sequence from the transposable element *Minos*, which allow the insertion of this cassette randomly through the fly genome (Venken et al., 2011). Also within the MiMIC are a splice acceptor site to ensure transcription of the cassette, followed by a stop codon to halt translation of the native gene, the coding sequence of enhanced green fluorescent protein (*EGFP*) with a *polyA* tail, and a *yellow+* marker that causes flies with a MiMIC insertion to have a darker body colour (Venken et al., 2011). The *EGFP* component of the cassette allows visualization of where that MiMIC is being expressed using fluorescent microscopy, and the darker body colour allows confirmation of MiMIC insertion visually.

Also within the cassette there are two additional inverted flanking sites called *attP*, which is DNA sequence that is recognized by an integrase ϕC31; this sequence and protein were

originally found in the ϕC31 phage within *Streptomyces* bacteria (Groth et al., 2000). The phage uses this system to insert its own DNA into bacterial genomes by recognizing *attB* sites which are commonly found in bacteria and swapping it with its own DNA which is flanked by *attP* sites (Groth et al., 2000). The inner flanking *attP* sites allow recombination within that cassette in the presence of DNA that is flanked by *attB* sites; researchers can create genetic constructs that are *attB* flanked, and then use ϕC31integrase to recombine their transgene into the position of the MiMIC (**Figure 6**) (Venken et al., 2011). This method of exchanging DNA cassette via recombination is called Recombinase Mediated Cassette Exchange (RMCE).

Many groups of researchers took advantage of fly lines that contained MiMIC sites inserted in a "coding intron" that is between two exons in a gene. Inserting a DNA cassette into this "coding intron" makes an artificial exon which would be expressed as the endogenous coding gene is also expressed. The insertion of a gene in a coding intron would also terminate transcription of the native gene early, so the native gene would either not be expressed, or its expression would produce a truncated protein (Venken et al., 2011). This idea was used by a group of researchers to create transgenic flies that have a *Gal4* that would be expressed as the native gene of insertion is expressed (Diao et al., 2015). This *Gal4* construct could then be used to express various effectors to learn about the native gene. To create a functional *Gal4* cassette to be inserted into a MiMIC site via RMCE, the DNA cassette must be flanked by *attB* sites, and within it contain: a splice acceptor site, a linker DNA sequence, a viral gene *T2A*, *Gal4* with a *polyA* tail, and a splice donor site. The splice acceptor and donor sites ensure that the construct with be included in the mRNA transcript. The linker DNA sequence maintains the native gene reading frame. The *polyA* tail arrests the transcription of the inserted DNA cassette, resulting in a full *Gal4* mRNA and halting the transcription of the rest of the native gene. To ensure that the translation of the *Gal4* mRNA does not include the upstream attached mRNA of the native gene, a *T2A* site is used. A *T2A* site is a DNA sequence derived from viruses that causes the ribosome to "skip" over it and reinitiate translation starting with the *Gal4* portion of the mRNA. This allows the creation of Gal4 protein that has a proper conformation, as it does not include any portion of the native protein (**Figure 7**) (Diao et al., 2015; Diao & White, 2012).

The final product, which is the "Trojan" construct, results in a *Gal4* that is expressed just as the endogenous coding gene is normally expressed, as all the regulatory factors that normally act on that gene will be activating *Gal4* transcription. Also, the coding gene will have either extremely reduced, or no detectable expression because the inserted DNA cassette interrupts transcription of that gene (**Figure 8**) (Diao et al., 2015; Diao & White, 2012). Trojan differs from typical *Gal4* constructs because it is transcribed as an "artificial exon" therefore more accurately captures native gene expression patterns.

Trojan allows us to investigate brain regions that are hypothesized to be involved in behaviour due to the genes that are expressed within that region. For example, the *neuroligin* family of genes within *D. melanogaster* have been associated with various social behaviours, including social spacing (Corthals et al., 2017; Li et al., 2013; Sun et al., 2011; Yost et al., 2020). In the Simon Lab, our interest is mainly in the role of *neuroligin 3* (*nlg3)* in behaviour. To assess the involvement of *nlg3* neurons in behaviour, we can mate a *nlg3-Trojan* driver line of flies to effector fly lines that allow us to manipulate neuron transmission (i.e. *TrpA1* and shi^{ts}), and use their progeny in behavioural assays. This method allows us to use pre-existing knowledge of genes involved in behaviour as steppingstones to elucidate the neural circuitry underlying these behaviours.

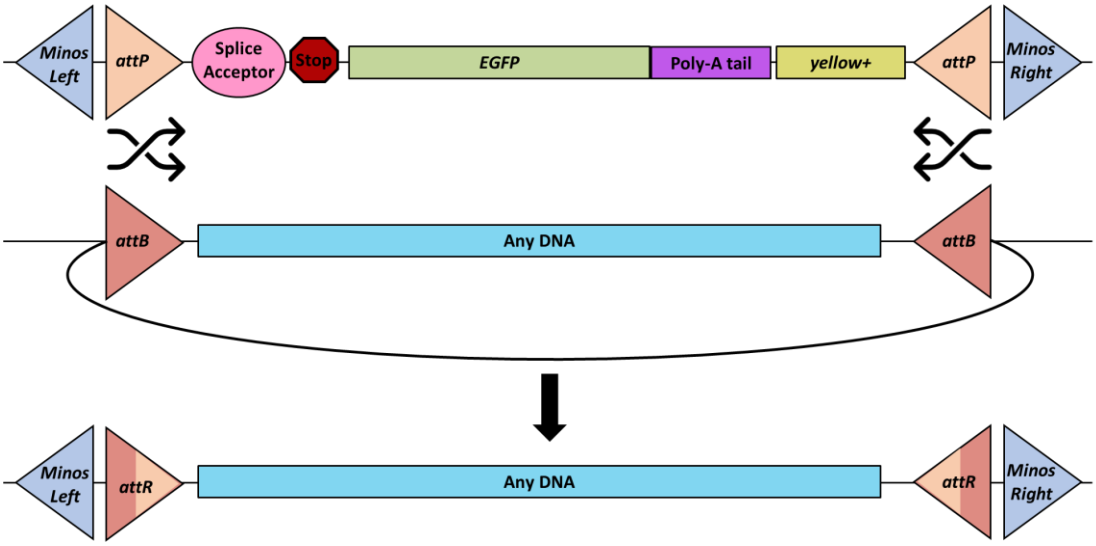

Figure 6. MiMIC construct and recombinase mediated cassette exchange diagram.

The MiMIC cassette is flanked by inverted repeats of DNA sequence from the transposable element *Minos* indicated by the blue triangles; they allow the insertion of this cassette randomly through the fly genome. Also included are a splice acceptor (pink circle), *EGFP* to allow visualization via fluorescent microscopy (green box), a *polyA* tail (purple box), a stop codon to inhibit translation of the native gene (red octagon), and a *yellow+* marker that causes flies with a MiMIC insertion to have a darker body colour. There are two additional inverted flanking sites called *attP* (light brown triangles), which is DNA sequence that is recognized by integrase ϕC31. This integrase also recognizes *attB* sites (darker bown/red triangles) on DNA that is not yet integrated into the genome. The *attP* and *attB* sites allow recombination within that cassette in the presence of DNA that is flanked by *attB* sites (blue box). Researchers can create genetic constructs that are *attB* flanked, and then use ϕC31integrase to recombine their transgene into the position of the MiMIC. This method of exchanging DNA cassette via recombination is called Recombinase Mediated Cassette Exchange (RMCE). Figure adapted from Venken et al., 2011.

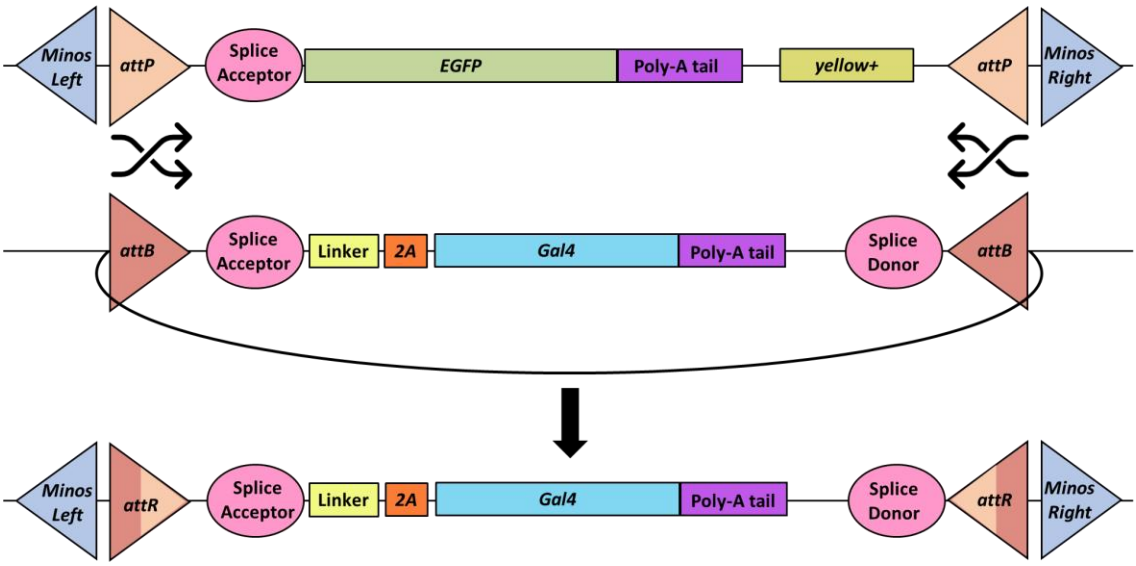

Figure 7. Trojan construct and RMCE diagram.

Using the MiMIC insertions that are scattered throughout the fly genome, researchers can conduct RMCE with a Trojan construct. This construct allows the expression of *Gal4* that is the same as the native gene where the transgene is inserted; it as described as an artificial exon within that gene. Included in the Trojan construct are a splice acceptor and splice donor (pink circles), a linker (yellow box) which maintains the reading frame of the native gene, a viral DNA sequence *2A* (orange box) to allow initiation of translation at the *Gal4* gene (blue box), a *polyA* tail (purple box), and *attB* sites to allow RMCE with the Trojan construct. Figure adapted from Diao et al., 2015; Venken et al., 2011.

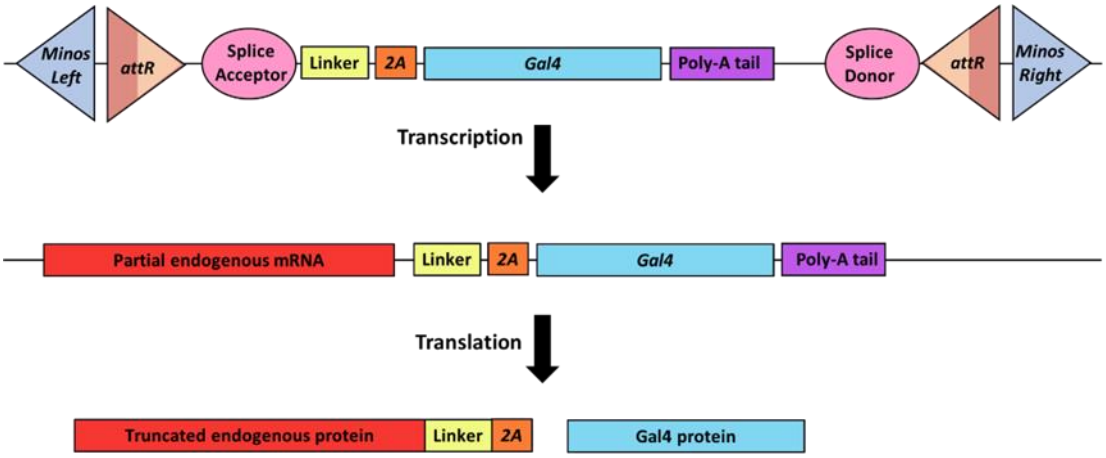

Figure 8. Depiction of transcription and translation of Trojan constructs.

Trojan constructs that are inserted into the *D. melanogaster* genome (top) are transcribed as "artificial exons" within the gene that they are inserted into. The linker DNA and *2A* sequence allow the proper translation of the *Gal4* gene, although the native protein often becomes truncated. Figure adapted from Diao et al., 2015; Venken et al., 2011.

1.3 Neuroligin and social behaviour

1.3.1 Neuroligin function and evolutionary ancestry

Proper brain function in all organisms underlies their ability to properly perceive cues, integrate them, and formulate an appropriate behavioural response (Couzin, 2009; Szekely et al., 2010). Knowing that proper brain function depends on the precise connectivity of individual neurons within it, we can investigate proteins that regulate this connectivity as possible modulators of behaviour. Two proteins that are known to be involved in the formation, maturation, and specialization of neuron connections in humans and *Drosophila* are Neuroligin and Neurexin (Biswas et al., 2010; Chen et al., 2012; Ichtchenko et al., 1996; Knight et al., 2011; Liu et al., 2022; Maćkowiak et al., 2014; Sun et al., 2011; Uchigashima et al., 2021). These proteins function in synapses, which are the areas where neurons connect and interact with each other (Chen et al., 2012; Knight et al., 2011; Maćkowiak et al., 2014; Uchigashima et al., 2021). The synapse consists of a pre-synaptic junction where signals from other neurons can be passed on, and the post-synaptic junction where the signals released by the pre-synapse are received (**Figure 9**). Neuroligins were first identified as the post-synaptic binding partners for the more extensively studied neurexin proteins, which are in pre-synaptic compartments (Knight et al., 2011; Maćkowiak et al., 2014). These trans-synaptic binding partners work together, and independently, to regulate the function and maturation of the synapse.

Neuroligins are of particular interest in research on social behaviour, due to their well-researched roles in behaviour among animals. In mammals there are four neuroligin homologs, and only in humans there is an additional homolog on the Y-chromosome (Bolliger et al., 2001, 2008; Ichtchenko et al., 1995, 1996). Homologs of neuroligin have also been identified in many invertebrates including *C. elegans, Aplysia* (sea slugs), mosquitos (*Anopheles gambiae*), honeybees (*Apis mellifera*), and as previously mentioned, *D. melanogaster* (Banovic et al., 2010; Biswas et al., 2008, 2010; Choi et al., 2011; Gilbert et al., 2001; Hunter et al., 2010; Sun et al., 2011). However, there is much evidence suggesting that neuroligins in vertebrates evolved independently from

neuroligins in arthropods and nematodes from a common ancestor (Knight et al., 2011; Thomas et al., 2022).

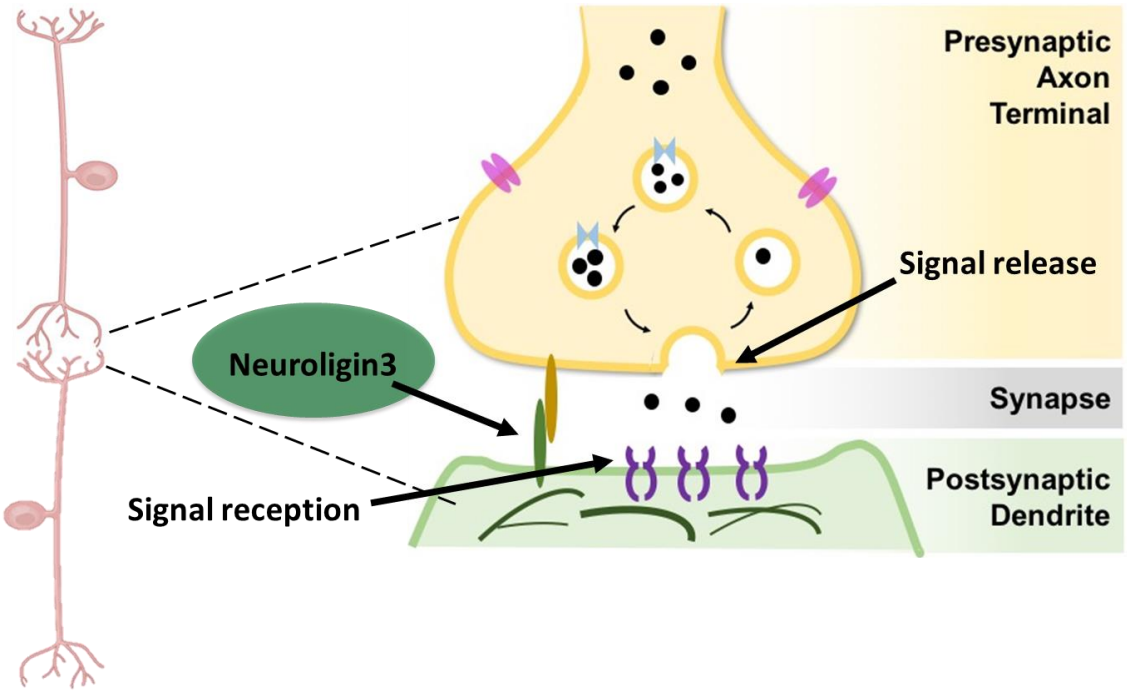

Figure 9. Diagram depicting a typical *D. melanogaster* neuron.

Pictured above is a zoomed in depiction of the synapse between two neurons. The location of Nlg3, a protein of interest, is indicated, as well as the pre-synaptic terminal which releases signals, and the post-synaptic terminal (also called dendrite) which receives signals. Adapted from Tawfik & Flood (2016).

1.3.2 Neuroligin in vertebrates and invertebrates

Despite their independent evolutionary origins, neuroligins in both vertebrates and invertebrates retain many structural and functional characteristics. Both vertebrate and invertebrate neuroligins mainly localize to post-synaptic compartments and are key regulators in synaptic maturation (Biswas et al., 2010; Knight et al., 2011). Studies of *neuroligins* (*nlg*) in honeybees have found that expression of *nlg1* and *nlg3* increase significantly after associative scent training, and *nlg1* expression seems to increase with sensory stimulation in general (Biswas et al., 2010). Similarly in *C. elegans*, the only *neuroligin* gene is involved in sensory modulation, and studies in sea slugs provided evidence that they are involved in long-term synaptic modulation (Knight et al., 2011).

In humans, *neuroligins* have been associated with Autism Spectrum Disorders, Alzheimer's disease, and Schizophrenia; *Neuroligins* (*NLGN*) *3* and *4* specifically have been commonly implicated in ASDs (see Nomenclature of gene and protein symbols) (Arons et al., 2012; Lough et al., 2015). Neuroligins and mutations within their associated genes have been extensively studied in mice, especially as models of ASD. Due to recently shared ancestry, the naming of each neuroligin gene in mice is based on its homology to the human neuroligins, so mouse *Neuroligin 1* (*Nlgn1)* shares most sequence similarity and function to human *Nlgn1*, and this trend is the same in the other three neuroligin genes (Knight et al., 2011). Knockout or overexpression of *Nlgn1* in mice led to learning and memory deficits and enhanced repetitive behaviour (Maćkowiak et al., 2014). *Neuroligin 2* knockout reduced social calls, decreased pain sensitivity, and increased anxiety; overexpression impaired social interaction in addition to increasing anxiety (Maćkowiak et al., 2014). Also in mice, a *Nlgn3* knockout causes a behavioural phenotype reminiscent of ASD attributes including impaired social interaction, reduced social calls, and also produced hyperactivity alterations in learning and memory processes. Researchers created a mutant line of mice that contain a *Nlgn3* mutation similar to a *NLGN3* mutation that is commonly found in Autistic people. These mice displayed impaired social interaction, increased social calls, and enhanced learning ability (Radyushkin et al., 2009). *Neuroligin 4* knockout mice also display behaviours that

closely resemble the main ASD characteristics: impaired social interaction, reduced social calls, and repetitive behaviours (Maćkowiak et al., 2014).

1.3.3 Overview of *neuroligin* in *Drosophila melanogaster*

In *D. melanogaster*, the *neuroligin* (*nlg*) gene family consists of four paralogs named *nlg1*, *nlg2*, *nlg3*, and *nlg4*. Each *neuroligin* paralog arose due to a gene duplication event after the divergence of arthropods from vertebrates, and thus the naming of *Drosophila neuroligins* does not indicate homology to the similarly named human neuroligins. However, specific *Drosophila neuroligins* are orthologous to some human *neuroligin* genes, meaning they may function similarly (Hirth, 2010; Howe et al., 2008; Gramates et al., 2022). In some cases, a specific *neuroligin* gene in *Drosophila* can be considered the "most" orthologous to a human neuroligin, meaning its function is the *most* similar. In other cases a certain *Drosophila neuroligin* is equally orthologous to various human neuroligin genes. The status of how orthologous each *Drosophila neuroligin* gene is in comparison to human neuroligin genes is constantly updated based on new research. A score for comparison of *Drosophila* and human orthologs is calculated by using various ortholog comparison tools, and determining how many of those tools produced the same results (via Flybase.org)(Hirth, 2010; Howe et al., 2008; Gramates et al., 2022). For example, *Drosophila nlg1* has its highest score of 7/15 when compared to human *NLGN1*, *NLGN3*, and *NLGN4X*, meaning seven of the 15 ortholog tools found *nlg1* function to be most similar to all three of the aforementioned human orthologs (Hirth, 2010; Howe et al., 2008; Gramates et al., 2022). When comparing *nlg2*, it has its highest score of 10/15 for *NLGN1*, *NLGN3*, and *NLGN4X* (Hirth, 2010; Howe et al., 2008; Gramates et al., 2022). For *nlg3*, by coincidence its highest score is 12/15 for the similarly named *NLGN3* (Howe et al., 2008; (Hirth, 2010; Howe et al., 2008; Gramates et al., 2022). Interestingly, the human neuroligin that *Drosophila nlg4* is the most orthologous to is *NLGN3* with a high score of 9/15 (Howe et al., 2008; Gramates et al., 2022).

Some of the four paralogs are expressed at different synapse "types", while others are expressed similarly. The two synapse "types" are excitatory and inhibitory synapses. Excitatory synapses, when activated, lead to depolarization of the cell which will

continue to pass along the signal they received; inhibitory synapses will prevent the signal from being passed on when activated, as the cell becomes hyperpolarized. In *D. melanogaster*, Nlg1, Nlg2, and Nlg3 are abundant in predominantly excitatory synapses, and Nlg4 in inhibitory synapses; Nlg3 in has yet to be fully characterized but has been found in excitatory synapses. Only Nlg1 is has been reported specifically within the neuromuscular junction (NMJ), whereas *nlg(2-4)* have been found to express in both the central nervous system (CNS) and NMJ. Only Nlg4 has been more specifically characterized: it is located in neurons involved circadian rhythm (Li et al., 2013; Wu et al., 2018; Xing et al., 2014; Corthals et al., 2017). The differential expression of *neuroligin* paralogs may underlie some of the mutant behavioural phenotypes that are observed when these genes are knocked out or mutated.

1.3.4 Role of *Drosophila neuroligin* in social and non-social behaviours

Abnormal behavioural phenotypes associated with mutations in *neuroligin* genes in mammals have also been observed in mutations of their orthologs in *D. melanogaster*. Mutant social behavioural phenotypes have been observed in fly lines with mutations in *nlg2, nlg3*, and *nlg4*. Deletion of the *nlg4* gene affects social and non-social behaviours including reduced sleep time, longer sleep onset latency, reduced open-space avoidance, decreased social space, and courtship songs with higher sine song frequency (Corthals et al., 2017; Li et al., 2013). Flies with mutations in *nlg2* display similar and/or opposite behavioural abnormalities to *nlg4* mutants; reduced open-space avoidance, shorter sleep episodes, lower tendency to form groups, courtship songs with lower sine song frequency, and reduced social interactions in male-female courtship (Corthals et al., 2017; Li et al., 2013). The *nlg3* gene is significantly less characterized compared to the other *Drosophila neuroligins*. Considering its high ortholog score with the human NLGN3, which is the neuroligin most associated with non-syndromic ASD, studying *Drosophila nlg3* in particular may provide transferable insight into the underlying mechanisms of ASD in humans (Uchigashima et al., 2021).

Studies involving *nlg3* have found that male flies with a full deletion of the *nlg3* gene (*nlg3-def*) have reduced locomotor activity in larvae and adults, decreased climbing

ability at in 3-4 day old adults, increased social spacing throughout their lifespan and after being socially isolated, as well as increased aggression in old age (Wu et al., 2018; Xing et al., 2014; Yost et al., 2020). Female *nlg3-def* flies have reduced climbing ability at 3-4 and 7-10 days old, increased social space when old or after experiencing social isolation, as well as decreased avoidance of an dour released by stressed flies called *Drosophila* stress odorant (Brenman-Suttner et al., 2020; Fernandez et al., 2017; McNeil et al., 2015). At the molecular level, Nlg3 is cleaved by a protease called Tumor necrosis factor a-converting enzyme (Wu et al., 2018). This cleavage happens only in neurons and not muscle, and results in two protein variants: Nlg3-Full length (Nlg3-FL) and Nlg3-Short (Nlg3-S). Using a mutant fly line that has an uncleavable form of Nlg3 ($Nlg3^{\Delta 10}$), researchers discovered that cleavage is necessary for proper locomotor behaviour. Furthermore, this behaviour was only rescued when Nlg3-S was co-expressed in the $Nlg3^{\Delta 10}$ mutants, meaning it is the short variant of Nlg3 that is necessary for proper locomotor activity (Wu et al., 2018).

Recently, the elucidation of the *nlg3* expression pattern has begun. In the Simon lab, we used immunocytochemistry to image *nlg3* expression in the adult fly brain, which revealed enrichment of the protein in three brain structures that are currently of interest: the mushroom bodies (MB) and the protocerebral bridge (PB), as well as in the optic lobes (OL) (**Figure 10**) (Robinson & Bechard, in preparation).

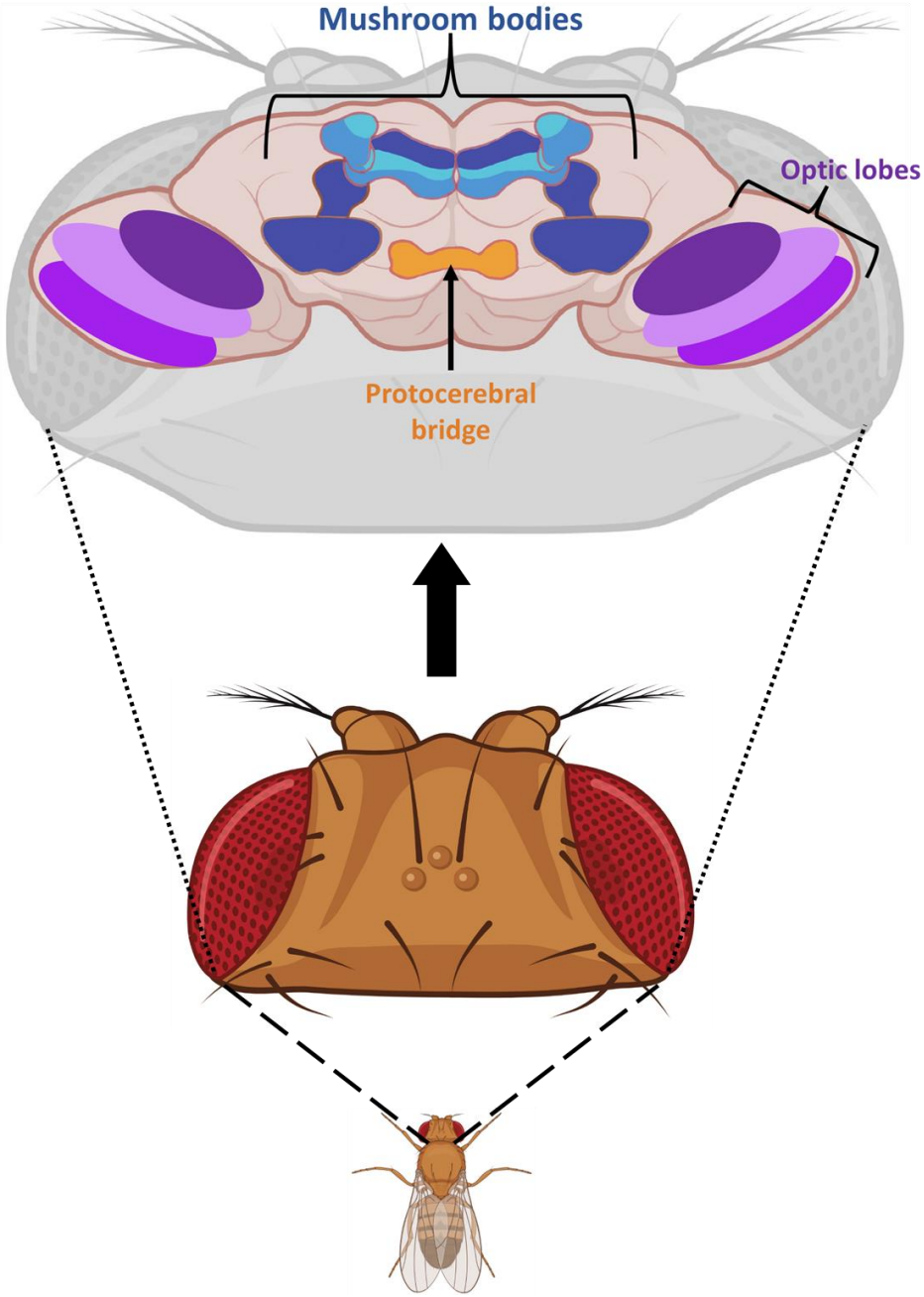

Figure 10. Structures of interest within the adult *D. melanogaster* brain.

Overhead view of the adult fly brain. The mushroom bodies and protocerebral bridge are situated at the central area of the brain. The mushroom bodies are symmetrical, and do not connect to each other directly. Image created using Biorender.

1.4 *Drosophila* neural circuitry

The adult *D. melanogaster* brain consists of approximately 200,000 neurons divided into various interconnected structures to allow the perception and interpretation of information from the outside world (Bargmann, 2006; Raji & Potter, 2021). The number of neurons in male versus female brains is almost identical, with the males having slightly more variation (Raji & Potter, 2021). Despite the similarity in neuron number, the male and female brains function in a dimorphic manner which allows the vast array of complex behavioural differences between them (Raji & Potter, 2021). It is hypothesized that observed differences in behaviour between sexes, as well as in mutants, can be attributed to variations in their underlying neural circuitry (Lawton et al., 2014; Raji & Potter, 2021). Neural circuitry can be described as interconnected neurons that allow a specific function when activated. The complete neural circuitry of the *Drosophila* brain has yet to be characterized, but various research groups have begun to map the connections of all neurons in the adult fly brain; these maps are known as the "connectomes". One research group used machine learning to analyze 3-D reconstructions of the fly brain, combining the efforts of over 50 years of research that contributed to the creation of those models (Scheffer et al., 2020). This group of scientists was able to create the most comprehensive connectome model of any animal to date, comprising approximately 25,000 neurons and 20 million connections; they have made this map publicly available (Scheffer et al., 2020). Most of the research to date has focused on discerning the connectivity of the central brain, which refers to the brain area between the optic lobes (**Figure 10)**. Included in the central brain are two of the structures that were found to be enriched with Nlg3: the MB and the PB (Robinson & Bechard, in preparation).

The central brain of the adult fly is broadly organized into three sections called the protocerebrum, deutocerebrum, and tritocerebrum, which parallel the forebrain, midbrain, and hindbrain of humans, respectively (O'Kane, 2011)**.** Within the protocerebrum lies the central complex (which contains the PB), and the MB; these brain structures are known as the integration centres for a variety of sensory inputs (Strauss, 2014; Wolff & Rubin, 2018). Brains are constantly sensing and integrating information from the environment.

In insects, including the fly, the general flow of information starts with the sensory neurons which allow perception of environmental cues and generate signals that are passed on to the central complex and MB (Biswas et al., 2010; Lin et al., 2013; O'Kane, 2011). Within the central complex and MB, the signals are integrated and assessed; this step is where the *meaning* of the incoming cue is determined. After integration via these central brain structures, signals are then relayed to the appropriate structures to formulate a physical behaviour response (**Figure 11**) (Aso et al., 2014; Li et al., 2020; Menzel, 2014; Scheffer et al., 2020; Strauss, 2002).

Neuroligin 3 helps to regulate and mature synapses which may affect how cues are integrated; Nlg3 is also enriched in the MB and PB which are structures known to be involved in integrating signals from sensory neurons and determining outputs (Budreck & Scheiffele, 2007; Knight et al., 2011; Li et al., 2020; Lin et al., 2013; Menzel, 2014; Robinson & Bechard, in preparation; Tomita et al., 2021). There is also evidence that *nlg3* and the MB play roles in social space determination in *D. melanogaster* (Burg et al., 2013; Yost et al., 2020). In my research project, I looked to connect the roles of the MB, PB, and *nlg3* in determining social space.

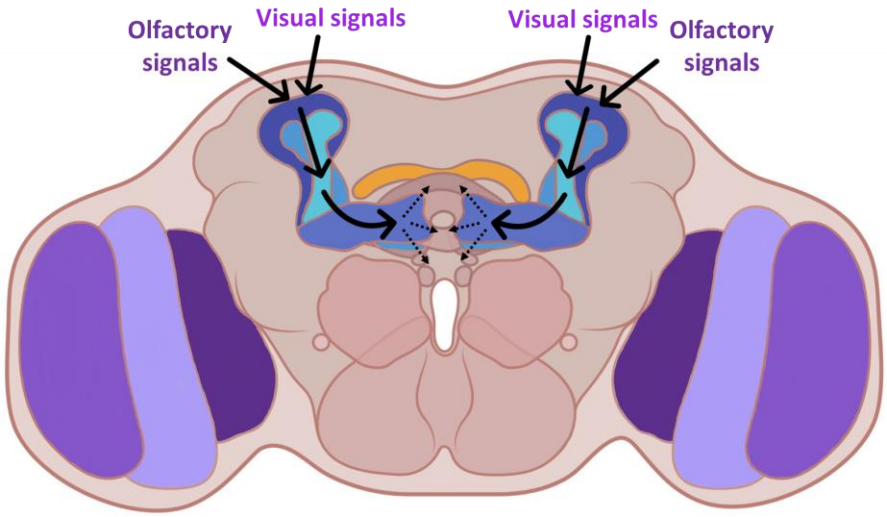

Figure 11. Direction of signal integration through the mushroom bodies.

Visual and olfactory signals are received at the calyces and relayed down the lobes to MBONs which pass on appropriate signalling to numerous other brain regions in order to form a behavioural response. Blue coloured regions are the mushroom bodies, orange is the protocerebral bridge, and the purple are the optic lobes. Image created using Biorender.

1.4.1 The mushroom bodies and protocerebral bridge

1.4.1.1 Characteristics and function of the mushroom bodies

Given that most responses to sensory stimuli are experience-dependent, an organism must be capable of consolidating memories and learning to respond appropriately under most contexts (Aso et al., 2014). In *D. melanogaster*, the brain structure most associated with learning and memory consolidation are the MB. The MB are comprised of a pair of symmetrical neuronal structures which have been well established as a centre for integrating olfactory sensory stimuli, learning, and memory (Baltruschat et al., 2021; Keleman et al., 2012; Li et al., 2020; Lin, 2023; Menzel, 2014). Recent studies have uncovered a diverse array of roles that the MB play; they are involved in sleep regulation, appetitive memory, social attraction, and innate courtship drive, as well as many other

complex behaviours (Joiner et al., 2006; Lim et al., 2018; Senapati et al., 2019; Sun et al., 2020).

Broadly, the symmetric MB are each composed of three structures: the calyx which receives signals from other neurons, the lobes which incorporate signals from the calyx and convey them to the third structure, the mushroom body output neurons (MBON) which send signals to structures other than the MB, as well as back to the MB (Li et al., 2020; Lin, 2023; Scaplen et al., 2021). The lobes and calyces are comprised of parallel axonal fibres called Kenyon cells; the calyces are the ends of the Kenyon cells that allow them to receive signals from other neurons (dendrites). The lobes are the long, parallel axonal fibres that relay signals to the MBONs (**Figure 11; Figure 12**) (Li et al., 2020; Lin, 2023). The calyces/lobes are comprised of approximately 2000 Kenyon cells which synapse onto only 34 different MBONs (Modi et al., 2020). Using the recently established connectome data, it was determined that of the approximately 25,000 newly mapped neurons, about 2600 neurons were likely to send signals to the MB, and about 1500 neurons receive signals directly from MBONs (Li et al., 2020; Scheffer et al., 2020). This means that about 2600 neurons connect and send signals to the 2000 Kenyon cells, then these signals are disseminated to only 34 MBONs, and the MBONs then pass the signals on to about 1500 other neurons; this requires a whole lot of organization and integration within the structure. Even more impressive, is that a portion of the 2600 neurons that converge on the MB, called dopaminergic neurons (DAN), receive signals from approximately 3200 neurons before sending signals to the MB (Li et al., 2020; Scheffer et al., 2020).

All of this is to say that it is no surprise that the MB are involved in regulating a wide variety of complex behaviours. The MB have also been shown to play a role in social space (Burg et al., 2013). Researchers found that mutations in a gene encoding a synaptic ion channel, *narrow abdomen*, reduced sociality in *Drosophila*, and suppressing expression of this gene in the MB decreased social space (Burg et al., 2013). Many of the neural circuits involving the MB are modular which can change how cues are integrated, and thus the behaviour of flies in response to the environment (Li et al., 2020; Modi et al., 2020; Scaplen et al., 2021). Modulation can occur through the connection of MBONs

to the DANs that are innervating the calyces (Aso et al., 2014; Modi et al., 2020; Scaplen et al., 2021). The DANs are dopaminergic, meaning the signals they send and receive are mainly regulated by the neurotransmitter dopamine (Lim et al., 2018; Lin, 2023). Dopamine is known to play roles in associative learning; some DANs and dopamine receptors allow the fly to create positive or negative associations with certain stimuli such as courtship pheromones, and can affect behavioural responses such as social space (Fernandez et al., 2017; Keleman et al., 2012).

Neuroligin 3 has yet to be directly associated with dopaminergic synapses, but there is evidence suggesting that both *nlg3,* the MB, and dopamine play roles in social spacing (Burg et al., 2013; Fernandez et al., 2017; Yost et al., 2020). However, *nlg3* has been associated with glutamatergic synapses; these kinds of synapses are also present in MBONs and therefore can be regulated upstream by DANs (Aso et al., 2014; Li et al., 2020; Modi et al., 2020; Xing et al., 2014). There is also evidence that glutamatergic MBONs connect and send signals to the MB lobes, thereby creating another route for modulation of behaviour specifically through glutamatergic activity (Aso et al., 2014).

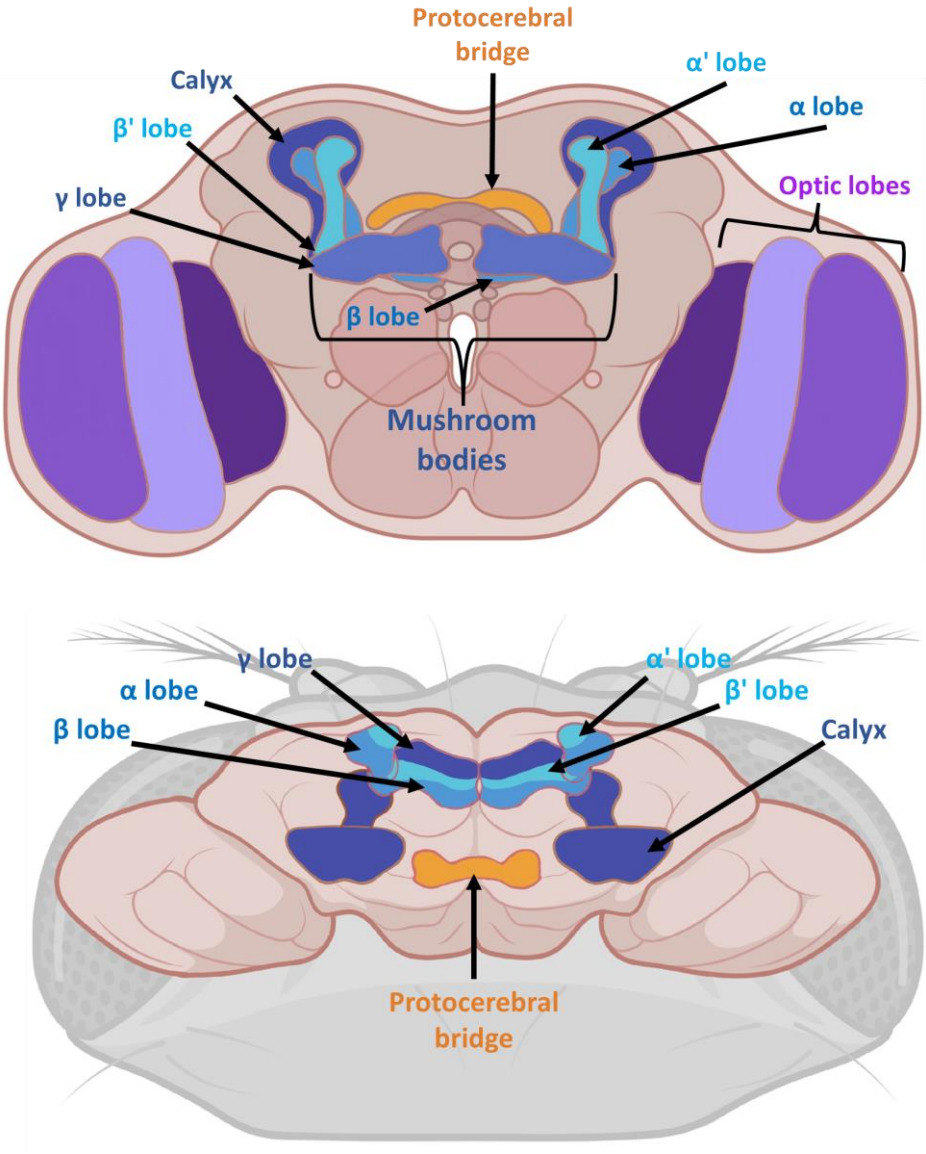

Figure 12. Detailed diagram of the mushroom bodies.

The top diagram depicts the adult *D. melanogaster* brain from an anterior view, and the lower diagram depicts an overhead view. All brain regions coloured in a shade of blue are part of the mushroom bodies, the orange is the protocerebral bridge, and the purple are the optic lobes. Created using Biorender.

1.4.1.2 Characteristics and function of the protocerebral bridge

The PB is a brain structure that has been historically characterized by its involvement in the integration of light cues from the optic lobes, proprioception, and locomotor behaviour (Eidhof et al., 2017; Lin et al., 2013; Triphan et al., 2010; Wolff & Rubin, 2018). Described as having a handlebar-like structure, the PB has connectivity to other structures within the central complex such as the fan-shaped body and ellipsoid body, which are also involved in locomotor behaviours (Eidhof et al., 2017; Scheffer et al., 2020; Strauss, 2002; Triphan et al., 2010). Behavioural analysis of flies that have structural deficits in the PB have demonstrated the importance of this structure in visual targeting of motor actions (Triphan et al., 2010). When walking flies approach gaps, they can attempt to cross the gap by reaching their forelimbs in front of them to contact the other side. Healthy flies have a small range of motion when reaching across the gap; they can see the size of the gap and accurately adjust their forelimbs to reach the other side (Triphan et al., 2010). Male flies that have impairments in PB structure have significantly reduced accuracy when adjusting their front limbs to reach a target, such as when they are crossing a gap (Triphan et al., 2010).

The connectivity of PB neurons has received less attention compared to other areas in the *Drosophila* brain as it was mainly associated with involvement in simple behaviours, but newer research has demonstrated that this is not the case. It was recently discovered that male flies can learn and remember the size of their bodies through visual feedback while walking (Krause et al., 2019). When small flies approach a large gap, they will often not attempt to cross it and turn around; larger flies that approach the same gap will often attempt (and succeed at) crossing the gap, which indicates that flies can understand the limits of their reach (Krause et al., 2019). When researchers repressed the expression of signaling molecules involved in memory formation within the PB, they found that flies could no longer accurately assess or remember their body sizes; small flies would often attempt and fail to cross gaps that were too large (Krause et al., 2019). This research provides evidence of PB involvement in memory consolidation. Interestingly, much of the research regarding neural circuits underlying motor-targeting based on visual cues has only been conducted in male flies. Other recent research has also discovered PB neurons

that are involved in the regulation of sleep in the fly (Tomita et al., 2021). The potential involvement of the PB in social spacing has yet to be thoroughly investigated, however its enrichment of Nlg3 may indicate a role in the neural circuitry underlying this behaviour.

1.4.1.3 Knockdown of *neuroligin 3* in the mushroom bodies and protocerebral bridge

A graduate student in the Simon lab, J. Wesley Robinson, also investigated how *nlg3*, the MB, and the PB may be connected in social space determination. He used *MB>nlg3-RNAi*, and *PB>nlg3-RNAi* flies in social spacing assays to determine whether *nlg3* in those structures individually may affect social space. Interestingly, knockdown of *nlg3* in only the MB or only the PB did not result in social space changes (Robinson, 2019; Robinson & Bechard, in preparation). However, *nlg3* knockdown in the PB did result in locomotor and climbing deficits (Robinson, 2019; Robinson & Bechard, in preparation).

1.5 Hypothesis, objectives, rationale, and predictions

1.5.1 Hypothesis

Previous research has demonstrated *nlg3* deletion affects social spacing behaviour in *D. melanogaster*. Neuroligin 3 is enriched in the MB and PB which indicates that these brain structures may play a role in the neural circuitry involved in social spacing. However, *nlg3* knockdown in the MB or PB individually did not affect social space. Based on this, I hypothesize that MB, PB, and *nlg3*-expressing neurons are involved in social space cue integration, and *nlg3* expression must be knocked down within the MB and PB simultaneously to affect social spacing.

1.5.2 Objective 1

Objective: Determine if the MB, PB, and *nlg3*-expressing neurons are involved in social spacing.

Rationale: Mutations of genes in the MB and mutations in *nlg3* have been shown to affect social spacing. The Nlg3 protein was found to be enriched in the MB and PB. Previous RNAi knockdown of *nlg3* in the MB or PB individually did not affect social

spacing. We know that Nlg3 regulates the function and maturation of synapses, so it can likely affect whether a synapse is excitatory or inhibitory; but perhaps it does not determine the type of synapse on its own. It is possible that knocking down *nlg3* in the MB or PB was not sufficient to change neuron transmission in these structures, and thus social space was not affected. To test whether *nlg3*-neurons, the MB, or PB are broadly involved in social space, I have chosen to measure social space while transiently manipulating neuron transmission in those neurons using the Gal4/UAS system. If differences in social space are observed while these neurons are artificially hyperactivated or inhibited, this indicates involvement in social spacing neural circuitry (**Figure 13**).

Prediction: The MB, PB, and *nlg3*-neurons are involved in social space determination. Neuron transmission is tightly regulated within the MB and PB, so I predict that inhibiting or hyperactivating neurons in either of these structures will affect social space. Following that logic, *nlg3*-neurons are present in the MB and PB (along with other structures), so manipulating neuron transmission in any way will affect social space. I will use the Gal4-UAS system with *MB, PB,* and *nlg3-Trojan* drivers and *TrpA1* and shi^{ts} effectors to hyperactivate or inhibit neurons, respectively, within those neurons and assess the effects on social space.

1.5.3 Objective 2

Objective: Determine if knocking down *nlg3* expression in both the MB and PB simultaneously affects social spacing.

Rationale: Social spacing was not affected when *nlg3* was knocked down in the MB or PB individual; perhaps Nlg3 in one brain structure could be compensating for the loss in the other. And so, it is possible that *nlg3* must be knocked down in both the MB and PB simultaneously to affect social space. This set of experiments will confirm whether *nlg3* expression in both structures is necessary for proper social spacing. The *nlg3-Trojan* driver causes expression of effectors in the MB, PB, and likely other brain structures such as the optic lobes which also display enriched Nlg3. If discrepancies are seen between the observed phenotypes in this objective versus objective 1, this will indicate that other

neurons/brain structures may be involved in social spacing. These results will provide direction for future studies to determine the complete social spacing neural circuitry (**Figure 13**).

Prediction: Previous research has demonstrated that *nlg3-def* mutants have differential social space depending on their sex. Based on this information, I predict that knocking down *nlg3* expression in the MB and PB simultaneously will affect social spacing in a sexually dimorphic manner. I will use the Gal4-UAS system in combination with *nlg3-RNAi* and the *nlg3-Trojan* driver to knock down nlg3 expression within these structures.

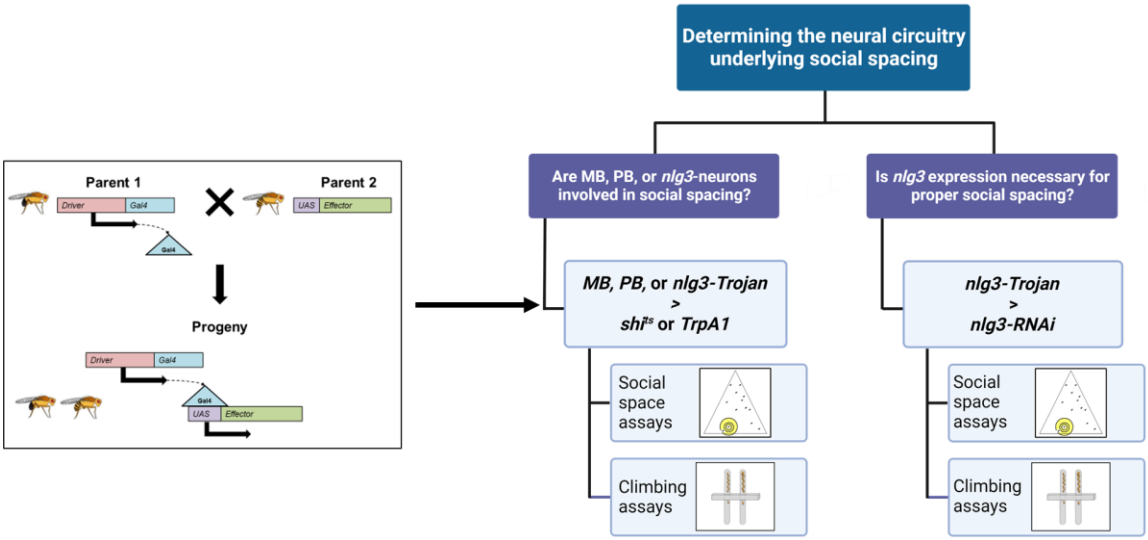

Figure 13. Objectives flow chart.

The flow chart above outlines how I will investigate each of my objectives. Fly lines will be crossed to generate testable progeny for social spacing and climbing assays. Created using Biorender.

Chapter 2

2 Methods

2.1 Fly lines and husbandry

All fly lines used for the purposes of this thesis were either ordered from Bloomington *Drosophila* Stock Centre (Department of Biology, Indiana University, Bloomington, IN, https://bdsc.indiana.edu/), or generated by collaborators Joshua Issacson (Western University, Moehring Lab) or Dr. Brian Mozer (Office of Research Integrity, MD, Rockville USA)(Yost et al., 2020), see **Table 1.**

The *nlg3-RNAi* line was generated by our collaborator Joshua Isaacson in the laboratory of Dr. Moehring, as described below in section 2.2.3.1.

To control for genetic variation between fly lines, we outcrossed new fly lines to our laboratory control line *Canton-Special* (*CS*). The process of outcrossing consists of mating the mutant lines to *CS*, collecting the progeny of that cross, and once again mating them to *CS* for five successive generations. Mutant fly lines have visible phenotypic markers, such as different eye colours, which can be used to ensure that all progenies still contain their respective transgenes. This process allows us to generate more genetic similarity between all our fly lines so that potential effects on behaviour can be attributed to the intended experimental manipulations.

All *Drosophila melanogaster* stocks and crosses were reared in mixed sex bottles with homemade fly food containing brown sugar, yeast, agar, cornmeal, benzoic acid, methyl paraben, and propionic acid (Fisher Scientific, Whitby, ON, Canada). Rearing conditions were set to 50% humidity, 25 ºC, on a 12:12 hour light:dark cycle.

For all crosses, 20 male and 20 female parents were collected and transferred into bottles to mate for 1 week, and then removed before progeny emerged. Female flies store the sperm of all male mates to fertilize their eggs, so only virgin females (distinguishable by a large black dot on their undersides) were used in crosses to ensure accuracy of the progeny genotype (Fowler, 1973). All flies from each fly line were transferred into new

bottles every two weeks, and then the flies were dumped seven days later. This results in a maximum parental age of 14 days to control for behavioural variations seen in progeny of older flies (Brenman-Suttner et al., 2020; Corthals et al., 2017). All fly lines and crosses with full genotypes are listed in **Table 1**, **Table 2,** and **Table 3**.

Driver fly lines were mated with effector fly lines to generate progeny with a complete Gal4/UAS system. An MB driver line (hereafter *MB*), a PB driver line (hereafter *PB*) and a *nlg3* driver (hereafter *nlg3-Trojan*) were mated with effector lines *TrpA1*, and shi^{ts}; the progeny of which were used in behavioural assays (see section 1.2.3.2 for details on each of these lines) (**Table 2**). Only *nlg3-Trojan* was mated to the *nlg3-RNAi* effector, as *nlg3* knockdown in either the MB or the PB has been previously investigated (Robinson, 2019). Temperature controls were also generated by mating mutant lines to our control strain, CS and then testing their social space at increased temperatures (**Table 3**).

Table 1. List of all fly lines used for crosses.

The full genotypes for each fly line along with their purpose and source are listed. Fly line abbreviations created for simplicity in text. Flylight driver expression visualization is not yet available for all drivers (Jenett et al., 2012).

Reference	Source	Stock Number	Flylight Driver Expression Data	Fly line abbreviation	Purpose	Genotype
Jenett et al., 2012	Bloomington Stock Centre	50422	R55G08	*PB*	Driver	*w[1118]; P{y[+t7.7] w[+mC]=GMR55G08-GAL4}attP2*
Jenett et al., 2012	Bloomington Stock Centre	49265	R15E01	*MB*	Driver	*w[1118]; P{y[+t7.7] w[+mC]=GMR15E01-GAL4}attP2*
Jenett et al., 2012	Bloomington Stock Centre	48686	R15D05	*MB:PB*	Driver	*w*;;{GMR15D05-GAL4}attP2*
Lee et al., 2018	Bloomington Stock Centre	76134	-	*Trojan*	Driver	*y[1] w*; Mi{Trojan-GAL4.2}Nlg3[MI00445-TG4.2]*
Koushika et al., 1996	Dr. Jamie Kramer (Bloomington Stock Centre)	8765	-	*elav*	Driver	*P{w[+mC]=GAL4-elav.L}2/CyO*
Koenig et al., 1983	Bloomington Stock Centre	44222	-	*shi^{ts}*	Effector	*w*; UAS-shi(TS1)*
Hamada et al., 2008	Bloomington Stock Centre	26263	-	*TrpA1*	Effector	*w*; UAS-TrpA1(TS)*
Robinson & Bechard, in preparation.	Moehring Lab	-	-	*nlg3-RNAi*	Effector	*w-/w-; UAS-Nlg3-shRNA/CyO; MKRS/TM6B*
Lee & Luo, 2001	Bloomington Stock Centre	6314	-	*GFP*	Effector	*y,w, UAS-mCD8::GFP*

Table 2. Table of crosses for flies used in behavioural assays.

Full genotypes for each line are listed in **Table 1**. Parental strains must be crossed to get progeny with a complete Gal4/UAS system. The purpose of each driver/effector combination and progeny genotypes are listed.

Driver parent genotype	**Effector parent genotype**	**Progeny genotype**	**Manipulation**
MB/MB	*TrpA1/TrpA1*	*MB>TrpA1*	MB neuron hyperactivation
MB/MB	*shi^{ts} /shi^{ts}*	*MB>shi^{ts}*	MB neuron inhibition
PB/PB	*TrpA1/TrpA1*	*PB>TrpA1*	PB neuron hyperactivation
PB/PB	*shi^{ts} /shi^{ts}*	*PB>shi^{ts}*	PB neuron inhibition
Trojan/Trojan	*TrpA1/TrpA1*	*Trojan>TrpA1*	Trojan neuron hyperactivation
Trojan/Trojan	*shi^{ts} /shi^{ts}*	*Trojan>shi^{ts}*	Trojan neuron inhibition
Trojan/Trojan	*nlg3-RNAi/ nlg3-RNAi*	*Trojan>nlg3-RNAi*	Trojan neuron *nlg3* expression knockdown

Table 3. Table of crosses for temperature control flies used in behavioural assays.

Full genotypes for each parental line are listed in **Table 1.** Each of the experimental fly lines was crossed with CS and progeny were used in behavioural assays. Canton-Special fly line is represented by (+). Progenies' genotypes are heterozygous for each driver and effector transgene.

Parent 1 genotype	Parent 2 genotype	Progeny genotype	Purpose
+/+	*TrpA1/TrpA1*	*+/TrpA1*	Assess potential *TrpA1* transgene leakiness on social space
+/+	shi^{ts} /shi^{ts}	+/shi^{ts}	Assess potential shi^{ts} transgene leakiness on social space
+/+	+/+	+/+	Assess the effect of temperature on social space

2.2 Testing driver and effector efficacy

2.2.1 Driver expression using immunocytochemistry

To assess driver efficacy, each driver was crossed with a *UAS-mcD8:GFP* (hereafter *GFP*) effector fly line to visualize their expression patterns in the fly brain. The *GFP* fly line contains a transgene with a green fluorescent protein gene fused to *mcD8*; a mouse-derived gene that localizes to cell membranes. It is possible to visualize the expression by viewing the *driver>GFP* brains under a fluorescent microscope directly or using immunocytochemistry (**Figure 14**). In the immunocytochemistry method, a primary antibody binds to GFP protein and then the fluorescent secondary antibody will bind to the primary antibody, which can be seen with the fluorescent microscope. In the direct method, the GFP protein as expressed by the driver is visualized using a fluorescent microscope. For this thesis, both methods were used.

Whole brains of 3–4-day old *driver>GFP* flies were dissected in 1X phosphate buffered saline (PBS). Brains that are to be imaged using *GFP* expression alone were mounted on a glass slide using FluoroshieldTM mounting media. Brains imaged using

immunocytochemistry were immediately placed in Bouin solution for 5 minutes for fixation, and then transferred into 1X PBS with 0.5% Triton X-100 (henceforth 1X PBT). They were transferred to new 1X PBT solutions four times for 10 minutes each to remove the fixative. Then brains were placed into a blocking solution (1X PBT and 5% GS) for 1.5 hours at room temperature. After blocking, the brains were transferred to new blocking solution which also contained the primary antibody (1:200 anti-GFP polyclonal antibody) for 4 hours at room temperature, then 48 hours at 4 °C. Then, the brains were washed again with 1X PBT three times, 10 minutes each. Next, brains were incubated with fluorescent secondary antibodies (1:500, diluted in blocking solution) for 4 hours at room temperature, and then 72 hours at 4 °C. Brains were washed again in 1X PBT, 3 times for 30 minutes, and then mounted on glass slides using FluoroshieldTM mounting media for viewing under the fluorescent microscope. The imaging was performed using an Imager Z1 Zeiss compound fluorescent microscope. All images were captured at the either 10X or 20X magnification and exposure times between 180-220 ms. For capturing images of brains via fluorescent antibody, excitation wavelength was 450-490 nm, and emission wavelength 515-565 nm. For directing imaging of GFP in the brains, excitation wavelength 390 nm and emission wavelength 460 nm were used.

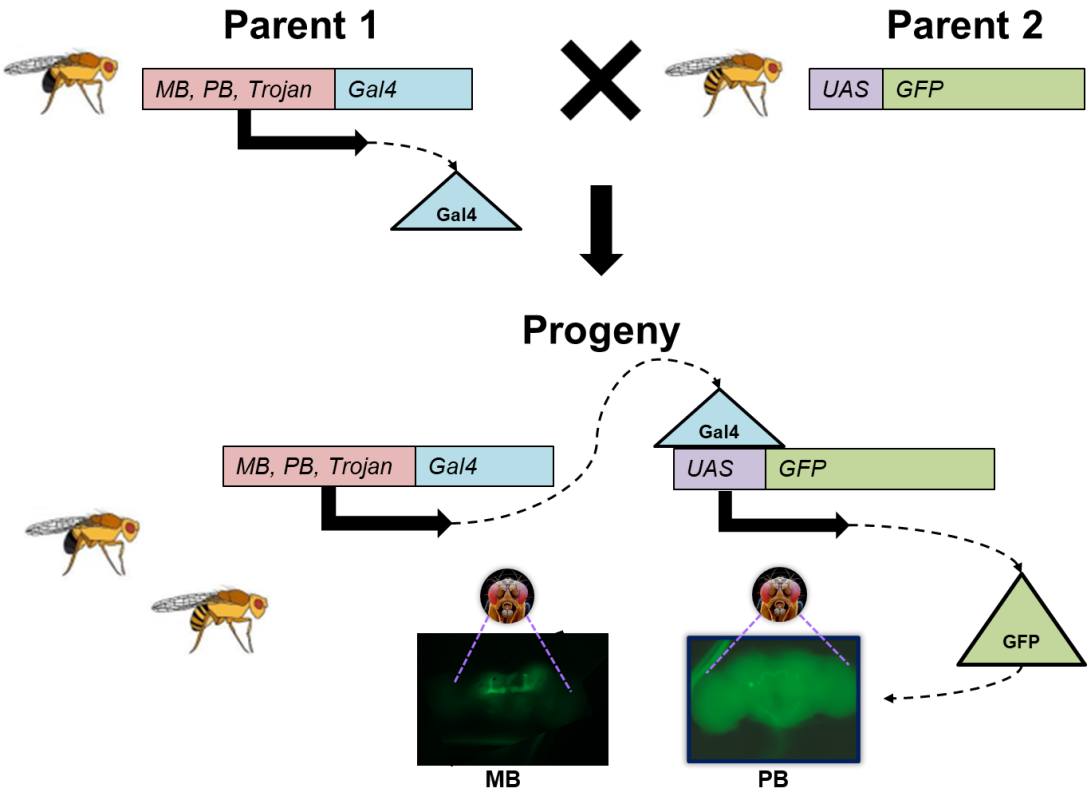

Figure 14. Driver confirmation using Driver>GFP.

To visualize driver expression patterns, the driver lines are crossed with *GFP* flies, and the progeny contain GFP in the brain regions dictated by the driver, which can then be seen using fluorescent microscopy.

2.2.2 Temperature-controlled neuron manipulation

To ensure that the *TrpA1* and *shi^{ts}* genetic constructs are functioning properly, these lines were crossed with a pan-neuronal driver *elav* and the progenies were assessed at their respective activation temperatures (*TrpA1*:27.8 ºC and *shi^{ts}*:29 ºC, versus their normal growth temperature 25 ºC). The *elav* driver is expressed in every neuron in the fly brain, so using it in conjunction with *TrpA1* and *shi^{ts}* effectors allows one to either hyperactivate or inhibit the entire brain transiently. If the genetic constructs are functioning properly, the *elav>TrpA1* flies are expected to seize due to pan-neuronal hyperactivation, whereas the *elav>shi^{ts}* flies are expected to faint. The *elav>TrpA1* and *elav>shi^{ts}* progenies were

collected then tested at 3-4 days old at 50% humidity and under uniform lighting. They were exposed to their activation temperatures until seizing or fainting were observed, which occurred within 30 s of exposure.

2.2.3 *nlg3*-RNAi line generation and efficacy confirmation

2.2.3.1 Creation of the *nlg3-RNAi* fly line

The *nlg3-RNAi* fly line was created by a lab mate, J. Wesley Robinson, in collaboration with a fellow graduate student named Joshua Isaacson from the Moehring laboratory at Western University. They worked together to create a transgenic hairpin UAS-RNAi construct that targets the first exon of *nlg3* mRNA when driven (**Figure 15**). The construct was cloned into a vector and injected into fly embryos, which were mated back to the parental line after reaching adulthood (Chang et al., 2014; Ni et al., 2011; Vert et al., 2006). These flies were then mated to another line which contained genes that generated phenotypic markers to incorporate visible markers into the progeny. Finally, those progenies were outcrossed to our control line *CS* over 5 generations to establish a final *nlg3-RNAi* line that shared 98.88% genetic similarity to *CS*. Then, this effector was mated to a pan-neuronal driver line *elav,* to generate progeny where *elav>nlg3-RNAi* to check the efficacy of the RNAi construct. A lab mate, Ryley Yost, extracted protein from the brains of those flies and used a western blot to assess whether *nlg3* had been knocked down- which it was, indicated by a lower amount of *nlg3* in *elav>RNAi* flies compared to *CS* and other genotypic controls.

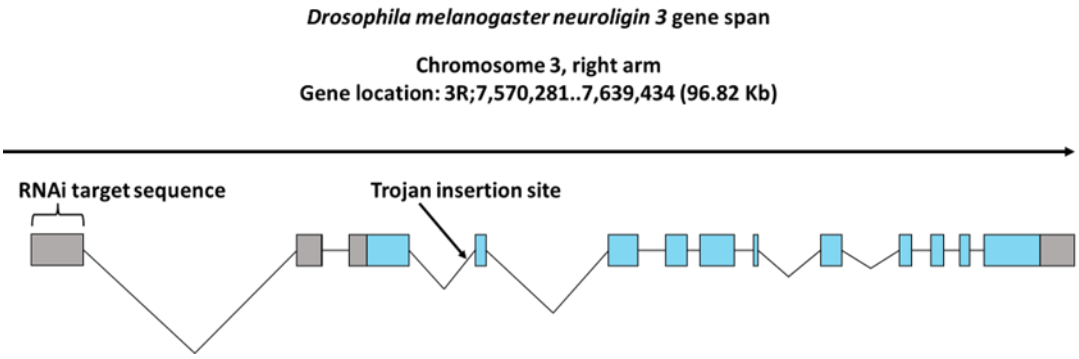

Figure 15. *Neuroligin 3* gene map.

The location of the Trojan construct insertion is indicated, along with the target sequence of the RNAi construct. Boxes represent exons; blue areas are translated and grey areas are the 5′ and 3′ UTRs. Longer lines between exons indicate a greater distance between them. Information adapted from Flybase (Gramates et al., 2022).

2.2.3.2 Confirming *nlg3-RNAi* efficacy through RT-PCR

2.2.3.2.1 RNA extraction

To confirm *nlg3-RNAi* efficacy, I used a protocol established by Dr. Amanda Moehring, that was since adapted by Liam Brown and Ryley Yost from the Simon Lab. I extracted RNA from the heads flies of *nlg3-Troj>nlg3-RNAi, nlg3-Troj/+, CS,* and *+/nlg3-RNAi* flies using the following methods: for each genotype 20 fly heads of each sex were removed and transferred into Eppendorf tubes. Then 20 µL of TRIzol reagent was added to each tube, and a pestle gun was used multiple times to homogenize the head tissue. Samples were then centrifuged at 8000 *g* for 30 seconds at 4 ºC. The process of homogenizing and centrifuging was then repeated 2 more times to ensure tissue was well broken down. Next, 480 µL of TRIzol reagent was added to each sample, then allowed to incubate for 5 minutes at room temperature. Following incubation, 200 µL of chloroform was added to each sample, then mixed vigorously by inverting the tubes, and once again allowed to incubate at room temperature for 2-3 minutes. All samples were then transferred to the centrifuge where they were spun at 10,000 *xg* for 18 minutes at 4 ºC.

Then, only the aqueous phase was transferred to a new Eppendorf tube and 250 µL of 100% isopropanol, and then 0.5 µL of glycogen was added to the new tubes containing the aqueous phase. The samples were incubated at room temperature for 10 minutes, chilled on ice for 3 minutes, followed by centrifugation at max speed at 4 ºC for 10 minutes. Supernatant was discarded from the samples and then 500 µL of 75% ethanol was added and gently mixed by pipetting the sample up and down. Again, samples were chilled for 3 minutes and then centrifuged at max speed and 4 ºC for 5 minutes; this was repeated 2 more times. After the final centrifugation, ethanol was removed by carefully pipetting it out, while taking care to not touch the RNA pellet at the bottom of the tube. Next, samples were placed in the fume hood for 10 minutes with the lids of the tubes open to allow the rest of the ethanol to evaporate. Finally, the dry RNA pellet was rehydrated using 20 µL of nuclease-free water and incubated at 55-60 ºC for 10-15 minutes and mixed every 2-3 minutes while incubating to dissolve the pellet.

2.2.3.2.2 gDNA removal

After extracting the RNA from fly heads, I then removed the genomic DNA from those samples before conducting cDNA synthesis. I used a TURBO DNA removal kit (Invitrogen, catalog number: AM2238) to remove the genomic DNA: first, 1 µL of TURBO DNase and 2.5 µL of TURBO DNase buffer was aliquoted to new Eppendorf tubes, one for each RNA sample. After measuring the concentrations of each RNA sample using a Nanodrop Spectrophotometer, I then calculated the liquid volume equivalent to 320 ng of RNA for each sample and added that to the new Eppendorf tubes containing the TURBO reagents. I then added nuclease-free water to each tube to get a final volume of 25 µL. The samples were briefly centrifuged and then incubated at 37 ºC for 25 minutes. After incubation, 2.5 µL of DNase inactivation reagent was added to each tube and mixed via pipet. The samples were then incubated at room temperature for 5 minutes, and flicked occasionally to ensure that all reagents were still suspended. Next, the samples were centrifuged at 10,000 *xg* for 1.5 minutes. Then, 10 µL of supernatant from each sample was transferred into a PCR microtube and incubated at 70 ºC for 5 minutes, followed by chilling on ice. These samples were then used in cDNA synthesis.

2.2.3.2.3 cDNA synthesis

After gDNA removal, an iScript cDNA kit (Bio-Rad, catalog number: 1708891) was used to reverse transcribe the clean RNA into cDNA. A master mix that specified 4 µL of 5X iScript reaction mixture, 1 µL of iScript reverse transcriptase, and 5 µL of nuclease-free water for each sample was created aliquoted to new PCR tubes, along with 10 µL of the clean RNA samples. All samples were placed in the thermocycler and incubated at temperatures suggested by the iScript kit: 5 minutes at 25 ºC for priming, 20 minutes at 46 ºC for reverse transcription, and 1 minute at 95 ºC to inactivate the reverse transcriptase. The newly formed cDNA was then used as a template in polymerase chain reaction (PCR).

2.2.3.2.4 PCR

A master mix that specifies 2.5 µL of 10X PCR buffer (containing 1.5 mM $MgCl_2$), 0.5 µL of dNTPs, 0.5 µL for the *nlg3* forward and reverse primers, 0.125 µL of Taq DNA polymerase, 20.875 µL of water and 1 µL of cDNA template were added to new PCR microtubes (one per cDNA sample). For each cDNA sample, one control sample was also run that used primers for a *Drosophila* reference gene, *rpl32* (**Table 4**). All samples, including a no DNA template control, were then transferred into the thermocycler. The samples were incubated at 95ºC for 30 seconds to denature DNA, then 30 seconds at 60 ºC for annealing, and 9 minutes at 68 ºC for extension, which was repeated 34 times. Samples were then incubated at 68 ºC for 10 minutes after the final cycle, and then removed from the thermocycler to be stored at 4ºC until agarose gel electrophoresis.

I also conducted multiplex PCR so that I could use a semi-quantitative method to assess *nlg3* expression in each genotype as well. This method was the exact same as the PCR described above, except both sets of primers were used in a single PCR reaction.

Table 4. Primers used in RT-PCR reactions.

Primer sets designed by Liam Brown, a previous honours thesis student in the Simon lab.

Gene Name	Forward or Reverse	Sequence (5'-3')	Expected Amplicon size
nlg3	F	ACTGGTCCAACTTTGTGCGA	153
nlg3	R	GCTTCGGCTTGGTGTCAAAA	
rpl32	F	AAGCGGCGACGCACTCTGTT	133
rpl32	R	GCCCAGCATACAGGCCCAAG	

2.2.3.2.5 Agarose gel electrophoresis

A 2% agarose gel was prepared using 1X TBE buffer and agarose, along with SYBR green. One sample to test for the presence for *nlg3* mRNA, and one sample for the reference gene *rpl32* mRNA was run for each genotype separated by sex, along with a no template control and a 50 kb ladder. The gel was run for 90 minutes at 90 V and then imaged using the gel documentation system for analysis.

2.3 Behavioural assays

2.3.1 Fly handling prior to behavioural assays

Progeny from crosses were kept in mixed-sex bottles containing food to ensure all flies used for behavioural assays are mated and sated. Twenty-four hours prior to assaying, 3–4-day old flies were sexed under cold anesthesia, divided into treatment groups (12-18 flies for social space assays, 20-50 for climbing assays), and transferred into vials containing food. The number of flies in a limited amount of space (density) affects social spacing, as well as their sex. Thus, in social space assays groups of 12-18 flies of the same sex are tested within one chamber; this range has been shown to reduce variation in this behaviour leading to more reproducible results (McNeil et al., 2015). For the climbing assay flies are separated into groups of 20-50 per treatment based on previously

established protocols (Fernandez et al., 2017; Madabattula et al., 2015). Flies were then left overnight to recover from potential confounding effects due to cold anesthesia.

Two hours prior to experimentation, flies were transferred into new vials and placed in the designated "behaviour room" to acclimate to testing conditions: 25ºC and 50% humidity. All behavioural assays took place in the behaviour room under uniform lighting and between 12:00-4:00 pm as flies are less active at that time of day, and are more likely to settle (Brenman-Suttner et al., 2020; McNeil et al., 2015)

For temperature-sensitive experiments involving *TrpA1* and *shi*^{ts}, plastic sheets were set up to divide the behaviour room so that multiple testing temperatures could be concurrently maintained. After the initial 2-hour environmental acclimation, flies were acclimated to their activation temperatures for 15 minutes and then inserted in social spacing chambers. To ensure consistent neuronal hyperactivation (*TrpA1* at 27.8ºC) or inhibition (*shi*^{ts} at 29ºC), all experiments involving temperature- sensitive flies were conducted at their respective activation temperatures. For each of these treatments, control assays containing flies of the same sex and genotype were run concurrently at 25ºC to act as a temperature control.

2.3.2 Social space assay

In the social spacing assay, flies were gently aspirated into vertically oriented 2-dimensional triangular chambers and first forced into a tight group at the top of the chamber, then allowed to explore freely until settled at their preferred social space (**Figure 1**) The temperature-sensitive treatment groups were assayed at their respective activation temperatures to ensure proper neuronal manipulation, while their controls were simultaneously assayed at 25ºC within the same room.

Once the flies have settled (approximately 20-40 minutes after insertion), a photo of the chamber was taken for social space analysis. To measure social space, the number of flies within the distance of four body lengths to each fly (4BL) was counted. The average 4BL value for each chamber was then calculated to serve as a single replicate. Three replicates

for each treatment were run once a week and repeated on three independent weeks for a summation of nine replicates (108-162 flies per treatment for each sex).

2.3.3 Climbing assay

Flies have a natural tendency to climb upwards after falling, a behaviour known as negative geotaxis, which can be measured as a socially independent behaviour (Ganetzky & Flanagan, 1978). Grouped flies were inserted into testing tubes and connected to the climbing assay apparatus (**Figure 16**). To force flies to the bottom of the tube, I strongly tapped the apparatus down and then gave the flies 15 seconds to climb upwards. After the elapsed time, the flies that climbed upwards into a second vial are separated and the number of flies in both the top and bottom vial were counted (Fernandez et al., 2017; Madabattula et al., 2015; Nichols et al., 2012). For each experiment, the baseline climbing ability is established by its associated control group. Flies that have reduced climbing ability are less likely to reach the top vial and this will be reflected in the climbing success rate. Three replicates were run once a week and repeated three times on independent weeks for a total of nine replicates (180-450 flies per treatment for each sex).

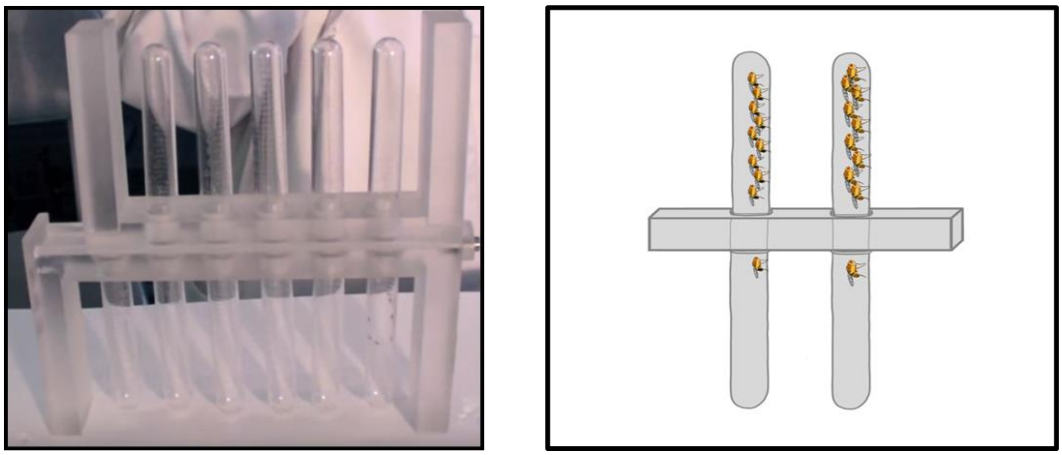

Figure 16. Picture and diagram of the counter-current climbing apparatus.

The image on the left shows a fully assembled climbing apparatus, which includes multiple tubes so that multiple replicates can be run at once. The diagram on the right depicts the behaviour of flies in the climbing apparatus after being banged downwards. Diagram created using Biorender.

2.4 Quantification and statistical analysis of behavioural assay data using ImageJ and Graphpad Prism 8

2.4.1 Social spacing quantification

Social spacing photos were analyzed using the free open access imaging software ImageJ (National Institute of Health, Bethesda, Maryland, United States) and new routines developed for our approach (Yost et al., 2020). The data was imported into GraphPad Prism 9 (Prism version 9.4.1 for PC, GraphPad Software, La Jolla California, USA, www.graphpad.com) for statistical analysis and graphical representation. 4BL data consists of mean social space measurements and thus has been found to follow a normal distribution.

2.4.2 Climbing quantification

The climbing success rate was calculated by determining the percentage of flies that were able to climb into the top vial within the elapsed time. Normality for each data set was tested, and significance was calculated using Welch's unpaired t-test (alpha level 0.05).

Mean climbing success rate values were inputted into GraphPad Prism 9 to generate bar graphs and which display the mean and standard error to the mean.

2.4.3 Statistical analysis

The normality of all data sets were assessed using the Shapiro-Wilke test, which is based on data correlation and corresponding normal scores (Peat & Barton, 2005). This normality test has been demonstrated to show more robust power in comparison to other tests and has been recommended by various researchers as the best choice for testing data normality (Ghasemi & Zahediasl, 2012; Thode, 2002).

After ensuring normality of the data sets, Welch's unpaired t-test with an alpha level of 0.05 was used to compare if there were significant differences in social space between control and treatment groups. All social space and climbing measurements were plotted on bar graphs which display the mean as well as the standard error to the mean.

3 Results

To investigate the role of the MB, PB, and *nlg3* neurons in social spacing, I selectively hyperactivated or silenced neuron transmission in those regions during social spacing assays. To do this, I used drivers targeted to the MB, PB, or *nlg3* neurons in conjunction with inducible neuron-hyperactivating or silencing effectors, *TrpA1* and shi^{ts}, respectively. The results of these experiments indicate whether these structures are involved in social spacing neural circuitry, and if they may play an excitatory or inhibitory role. I conducted climbing assays alongside the social spacing assays to act as a socially independent control behaviour and further characterize the mutant lines.

Before performing these experiments, I confirmed the efficacy of the driver and effector transgenes respectively using behavioural assays and fluorescence microscopy, as described previously in the methods section. Control crosses were conducted to generate progeny for shi^{ts} and *TrpA1*; they were tested at room temperature and activation temperatures to assess possible effects of increased temperature on the effectors without a driver.

3.1 Controlling experimental conditions

3.1.1 Mushroom bodies and protocerebral bridge drivers produce sufficient and accurate effector expression

To assess the expression pattern of the drivers I used in my study, I crossed each of them with an effector line that expressed *GFP* in the cell membrane and then visualized the results using fluorescence microscopy. I compared the brain image results to detailed diagrams of the *D. melanogaster* brain to determine whether the drivers were expressing in the appropriate regions (Jenett et al., 2012). I also visually assessed the degree of fluorescence as a measure of how strongly the UAS is expressed by each driver to determine if there was sufficient expression for my experiments. I was unsure whether using immunocytochemistry versus imaging the brain directly would produce a better image, so I tried both methods with the first driver test: *MB>GFP*.

From both methods clear images were produced, and it can be seen through the *GFP* expression levels that the *MB* line drives accurate and sufficient UAS expression (**Figure 17**). After this set of experiments, I realized that using immunocytochemistry to image the brains did not result in a more accurate/detailed image, so I decided to visualize all future drivers with direct GFP fluorescence. When imaging the brains of *PB>GFP* flies, I found that the *PB* line also drove sufficient and accurate UAS expression (**Figure 18**).

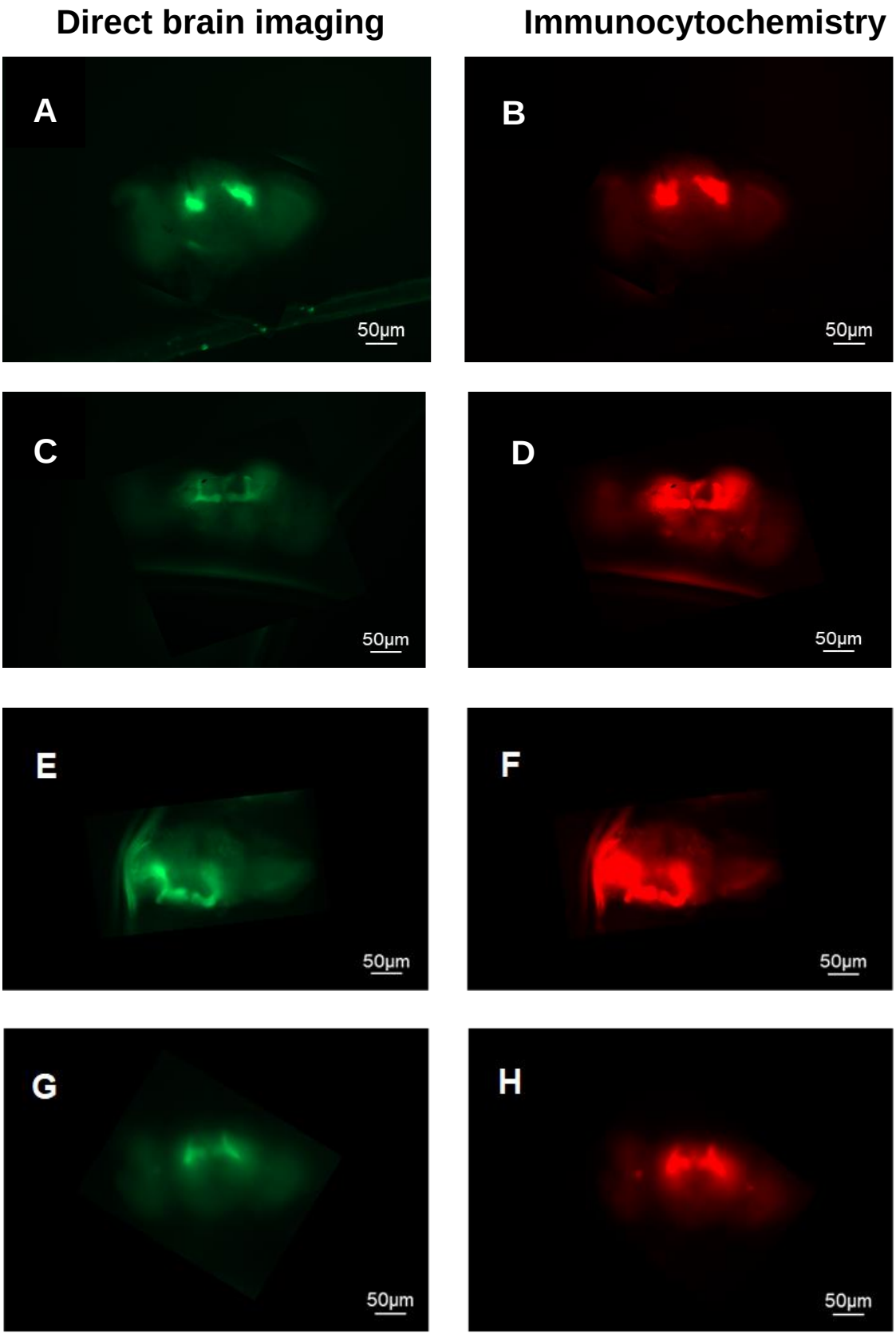
Direct brain imaging
Immunocytochemistry
A
B
C
D
E
F
G
H
50μm

Figure 17. Images of *MB>GFP D. melanogaster* brains.

Whole brains of 3–4-day old flies were dissected and imaged. Images were taken at 10X magnification and a 180 ms exposure time using a fluorescent microscope as described in 2.2.1. Each pair of brain images depict the same brain, which was first imaged by assessing GFP directly, and then imaged again using the fluorescent antibody. (A) and (B) display the posterior side of the brain where the calyces of the mushroom bodies can be seen clearly enriched with GFP. (C-F) show the anterior side of the brain with the pedunculus enriched with GFP. (G) and (H) show a slightly overhead view, with both the calyces and pedunculus visibly enriched with GFP. Images on the left (A, C, E, G) are the result of direct imaging and images on the right (B, D, F, H) are images captured after using the immunocytochemistry protocol (see 2.2.1.).

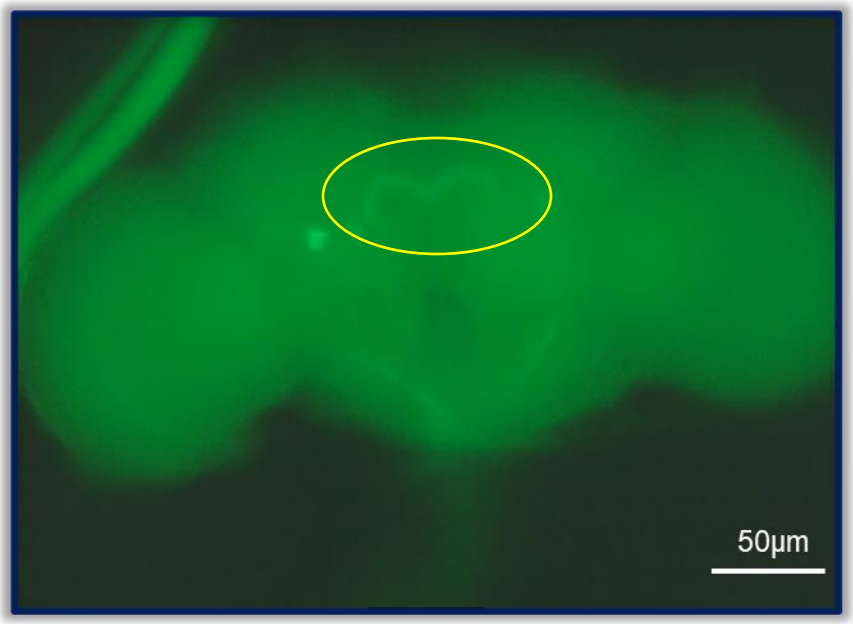

Figure 18. Image of *PB>GFP* adult *D. melanogaster* brain.

Whole brains were dissected and directly imaged from 3–4-day old flies. This image was captured at 20X magnification and 210 ms exposure time using a fluorescent microscope. The yellow circle indicates the GFP enrichment within the PB.

3.1.2 Mushroom bodies : protocerebral bridge driver did not produce sufficient effector expression

The brains of *MB:PB>GFP* flies showed little to no enrichment of GFP, making it difficult to visualize (**Figure 19**). This is likely because the *MB:PB* driver itself does not promote strong gene expression. I decided to change to a different driver that also encapsulated the MB and PB, as well as promoted stronger effector expression: *nlg3-Trojan*. The *nlg3-Trojan* drives effector expression in the same pattern as the *Drosophila melanogaster nlg3* gene, and thus is a great tool for determining whether the MB and PB, as well as *nlg3* neurons, are involved in social spacing.

Imaging *Trojan>GFP* fly brains proved to have similar issues to the *MB:PB>GFP* brains, as there was weak *GFP* expression. However, A fellow graduate student in the lab

(J. Wesley Robinson) is currently establishing a confocal imaging technique to produce clearer brain images and has also confirmed *nlg3-Trojan* expression in the MB and PB.

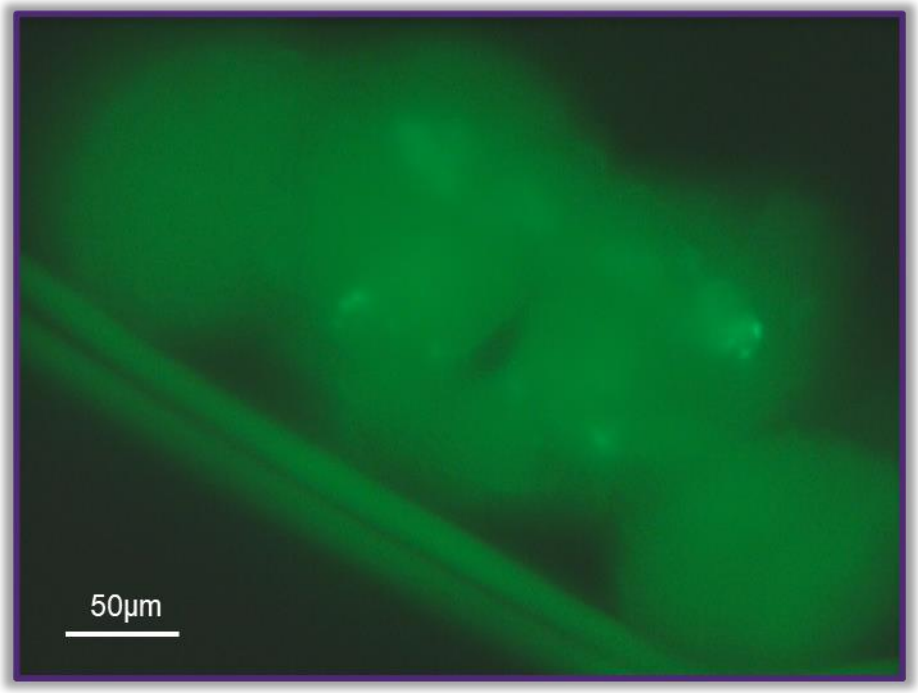

Figure 19. Image of *MB:PB>GFP D. melanogaster* brain.

Whole brains were dissected and imaged from 3–4-day old flies. Image captured at 20X magnification and 220 ms exposure time. Some low GFP enrichment can be seen near the central complex, but it is not specific to the MB or PB.

3.1.3 *TrpA1* and *shi^{ts}* effectors alter neuron transmission as intended

To assess whether the *TrpA1* and *shi^{ts}* effectors were altering neuron transmission as expected, and to identify ideal activation temperatures, behavioural assays using *elav>TrpA1* and *elav>shi^{ts}* flies were conducted. If *TrpA1* is functioning properly, then expressing it using an *elav* driver will cause the flies have seizures when exposed to the activation temperature due to pan-neuronal hyperactivation. To test this, I placed *elav>TrpA1* flies in a chamber and slowly increased the heat to observe for seizing behaviour. Once the temperature reached 27.8 ºC all flies within the chamber began to seize which indicated that *TrpA1* was functioning properly (**Figure 20**). This testing also

established 27.8 ºC as the activation temperature for my future experiments with *TrpA1*. A previous lab mate (Soliman, 2020) used the same principle to test whether *shi*^{ts} was functioning properly, except that *elav>shi*^{ts} flies are expected to faint when exposed to the activation temperature due to pan-neuronal inhibition. The *elav>shi*^{ts} flies began to faint once the temperature reached 29 ºC, indicating that the *shi*^{ts} effector was also functioning properly, and establishing the activation temperature for the *shi*^{ts} effector.

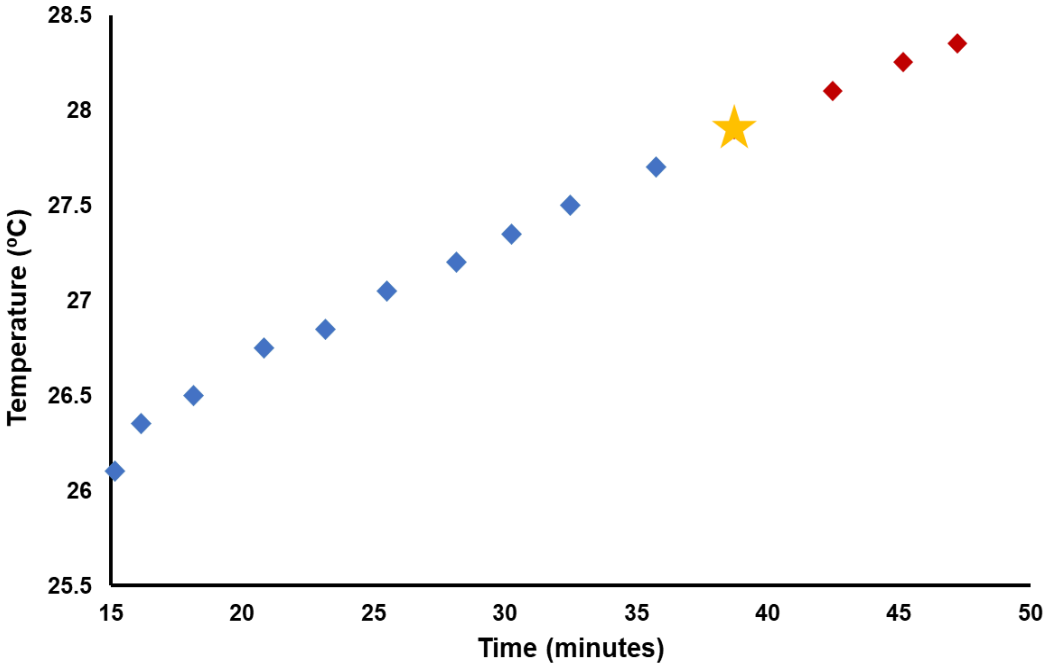

Figure 20. Testing *TrpA1* efficacy.

elav>TrpA1 flies were exposed to increasing temperature and observed for seizing behaviour. The flies began to seize at 27.8 ºC (yellow star) and continued to seize at higher temperatures (red diamonds). When the same flies were exposed to temperatures below 27.8 ºC, they stopped seizing and resumed typical behaviour after a period of recovery.

3.1.4 Social space is not affected by increased temperature

To ensure that changes in social space are due to the intended neuronal manipulations and not a side-effect of increased temperature, I compared social space results of the progeny of each effector mated with our lab control strain *CS*. These flies are heterozygous at the effector loci just as they would be when one of the parents is a driver. I tested *+/TrpA1*, *+/shi^{ts}*, and *CS* flies at 25 ºC and at their respective activation temperatures. Testing the *+/TrpA1* and *+/shi^{ts}* flies at their activation temperatures also indicates whether the effector is "leaky", meaning it tests if the effector gene is expressed in the absence of a driver. If the social space differs for each of these genotypes at their activation temperature versus 25 ºC, then it is possible that the effector is leaky or there is an effect of temperature on social space. When I tested males and females of *+/TrpA1*, *CS*, and *+/shi^{ts}* flies at 25 ºC and at their respective activation temperatures, there was no difference in social space (**Figure 21**) (Welch's two-tailed unpaired t-test; males for each genotype: p=0.2827, p=0.9308, p=0.8563, females for each genotype: p=0.6353, p=0.9219, and p=0.9915, respectively.) The same tests were conducted using a climbing assay by a lab mate (J. Wesley Robinson) and there was no effect of temperature on climbing for *+/TrpA1*, *+/shi^{ts}*, and +/+ (*CS*) flies.

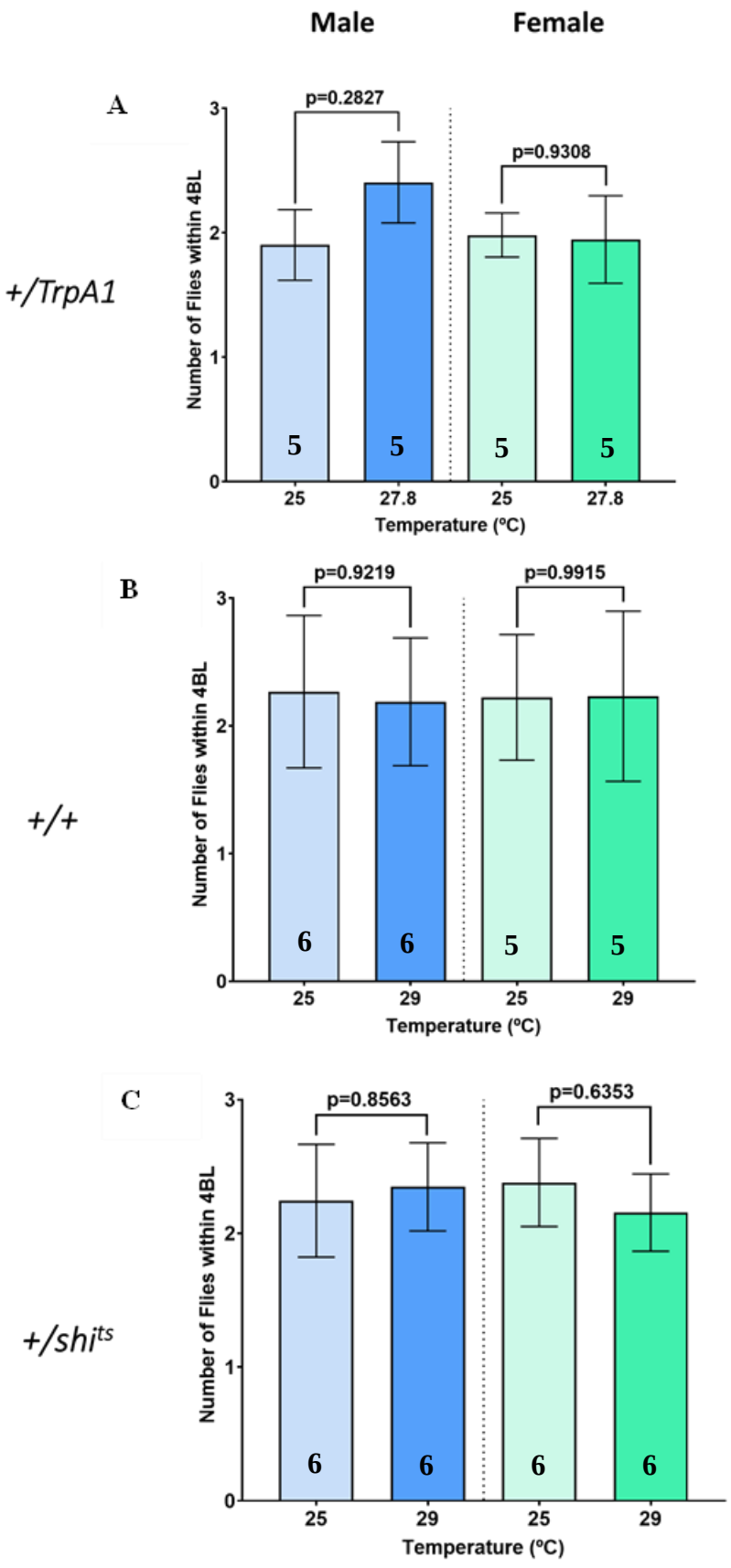
Male
Female
A
+/TrpA1
p=0.2827
p=0.9308
Number of Flies within 4BL
5
5
5
5
25
27.8
25
27.8
Temperature (°C)
B
+/+
p=0.9219
p=0.9915
Number of Flies within 4BL
6
6
5
5
25
29
25
29
Temperature (°C)
C
+/shi^ts
p=0.8563
p=0.6353
Number of Flies within 4BL
6
6
6
6
25
29
25
29
Temperature (°C)

Figure 21. Temperature does not affect social space in *+/TrpA1* (A), +/+ (*CS*) (B), or *+/shi^{ts}* (C) flies.

Bars represent the average number of flies within 4BL of each fly at 25 ºC versus activation temperature for each genotype, separated by sex. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 12-18 flies per genotype for each sex).

3.1.5 Manipulating neuron transmission in the mushroom bodies increases social space

Using *MB>TrpA1* flies, I determined whether hyperactivating the MB affected social space. I found that both males and females exhibited a significant increase in social space when this structure was hyperactivated (Welch's two-tailed unpaired t-test; p=0.0064, p=0.0583, respectively). When I selectively inhibited the MB using *MB>shi^{ts}* flies, I found that social space was also increased in both males and females (Welch's two-tailed unpaired t-test; p=0.0040, p=0.0678, respectively); although not to a statistically significant degree in females, there is a clear biological difference (**Figure 22**).

3.1.6 Climbing ability is not affected by altering neuron transmission in the mushroom bodies

Using *MB>TrpA1* and *MB>shi^{ts}* flies, I found that neuron manipulation in the MB does not affecting climbing ability. Both males and females' climbing ability was unaffected when the MB were hyperactivated (Welch's two-tailed unpaired t-test; p= 0.1096, female p=0.0623, respectively) or inhibited (Welch's two-tailed unpaired t-test; p=0.8975, p=0.9358, respectively) (**Figure 23)**.

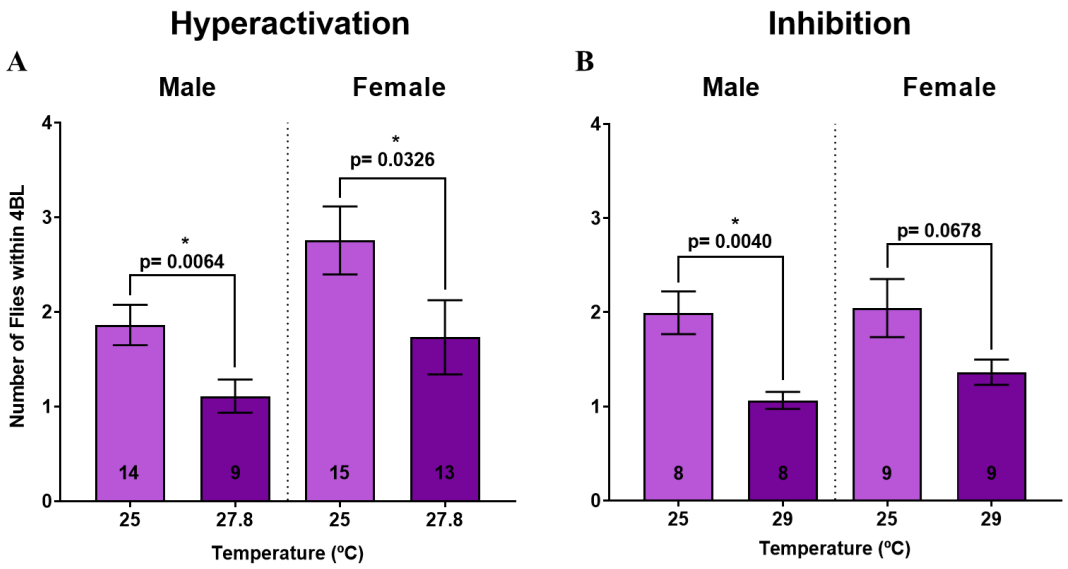

Figure 22. Altering neuron transmission in the mushroom bodies increases social space.

Bars represent the average number of flies within 4 BL of each fly at 25 ºC versus activation temperature for *MB>TrpA1* (A) and *MB>shi^{ts}* (B) flies, separated by sex. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 12-18 flies per genotype for each sex).

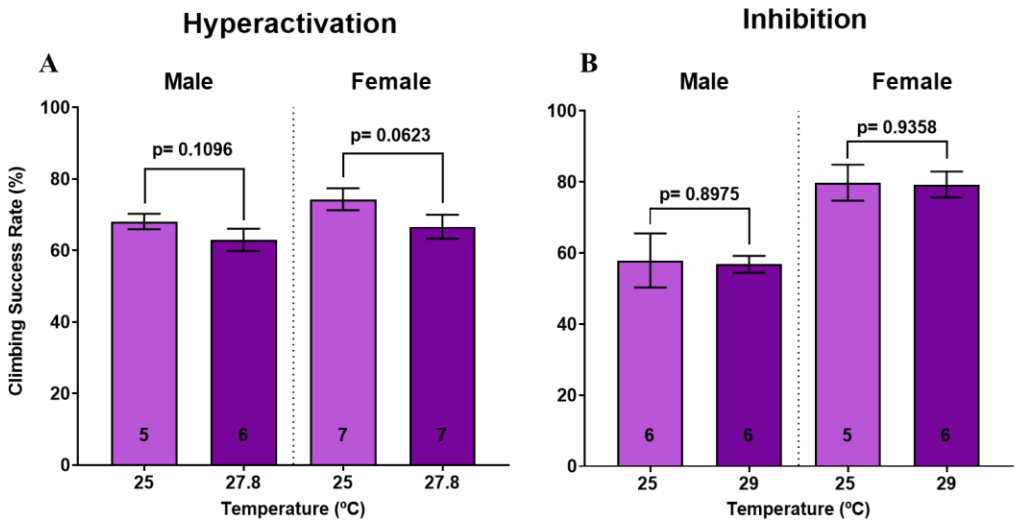

Figure 23. Climbing ability is not affected by MB neuron manipulation.

Bars represent the percentage of flies that climbed successfully within a 15 second timespan at 25 ºC versus activation temperature for *MB>TrpA1* (A) and *MB>shi^{ts}* (B) flies, separated by sex. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 20-60 flies per genotype for each sex).

3.1.7 Manipulating neuron transmission in the protocerebral bridge affects social space, but only in females

After assessing the role of the MB in social spacing, I then moved on to investigating whether the PB is involved in social spacing neural circuitry. Using *PB>TrpA1* flies, I transiently hyperactivated this structure while conducting social space assays and found that there was no effect on social spacing in males, but females displayed decreased social space (Welch's two-tailed unpaired t-test; p=0.9868, p=0.0992, respectively). Interestingly, inhibiting the PB neurons using *PB>shi^{ts}* flies caused an increase in social space, but again, only in females (Welch's two-tailed unpaired t-test; male p= 0.7057, female p=0.0221) (**Figure 24**).

3.1.8 Climbing ability is not affected by altering neuron transmission in the protocerebral bridge

To investigate whether altering PB neuron transmission affects climbing ability, I transiently hyperactivated or inhibited this structure during climbing assays using *PB>TrpA1* and *PB>shi^{ts}* flies. I found that hyperactivating (*TrpA1*) or inhibiting (*shi^{ts}*) the PB did not affect climbing ability in both males and females (Welch's two-tailed unpaired t-test; hyperactivation: males p= 0.2938 and females p= 0.4307, inhibition: males p= 0.6060 and females p= 0.7405)(**Figure 25**).

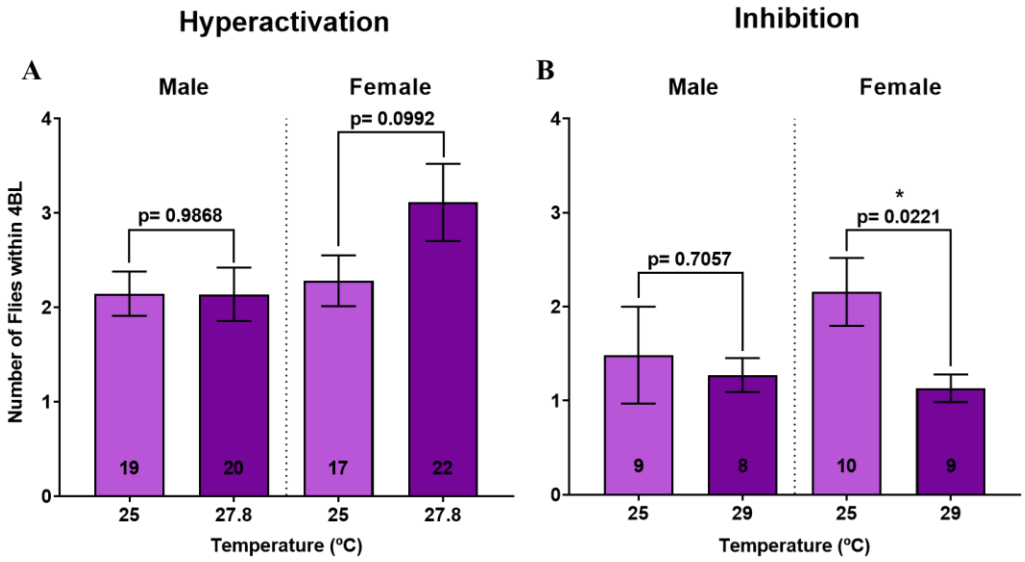

Figure 24. Altering neuron transmission in the protocerebral bridge affects social space in females.

Bars represent the average number of flies within 4 BL of each fly at 25 ºC versus activation temperature for *PB>TrpA1* (A) and *PB>shi^{ts}* (B) flies, separated by sex. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 12-18 flies per genotype for each sex).

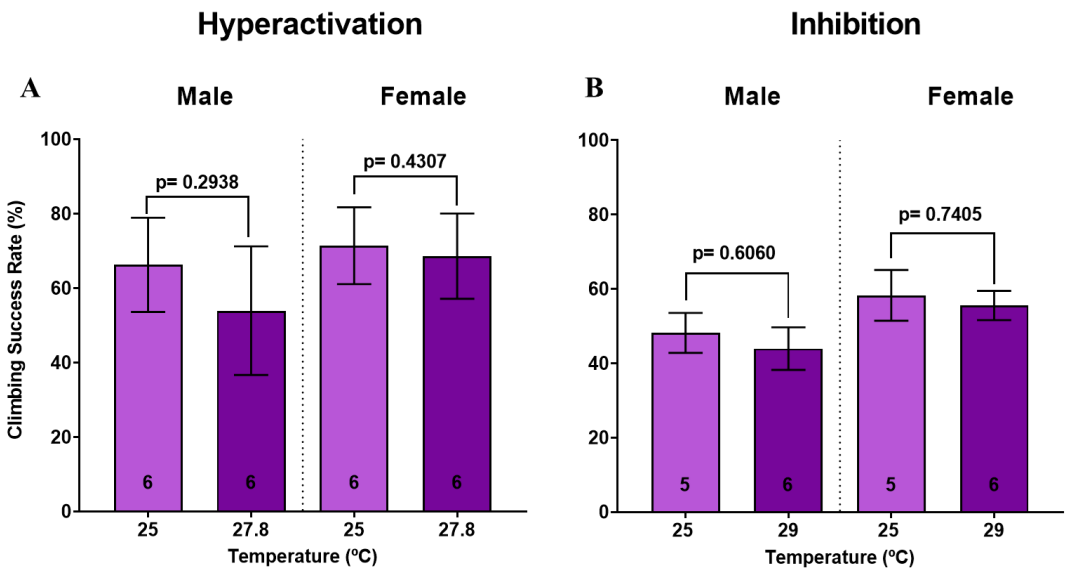

Figure 25. Climbing ability is not affected by altering neuron transmission in the PB.

Bars represent the percentage of flies that climbed successfully within a 15 second timespan at 25 ºC versus activation temperature for *PB>TrpA1* (A) and *PB>shi^{ts}* (B) flies, separated by sex. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 20-60 flies per genotype for each sex).

3.1.9 Manipulating neuron transmission in *nlg3*-neurons affects social space in a sexually dimorphic manner

Once I determined that the MB and PB are involved in social spacing, I decided to investigate whether *nlg3* neurons are involved in this behaviour, due to where *nlg3* is expressed. The *nlg3* neurons are present in the MB, PB, and optic lobes of the fly brain (Robinson & Bechard, in preparation). Thus, these results provide direction for determining which other structures may affect social spacing if differing behaviour is observed using this driver. I used the *nlg3-Trojan* driver to express *TrpA1* and *shi^{ts}* in *nlg3* neurons (*nlg3-Trojan>TrpA1* and *nlg3-Trojan>shi^{ts}*) and conducted social spacing assays on these flies at control and activation temperatures.

Using *nlg3-Trojan>TrpA1* flies, I found that hyperactivating *nlg3*-neurons increases social space in both sexes, but significantly only in females (Welch's two-tailed unpaired t-test; males p= 0.2252, females p=0.0016). Inhibiting *nlg3*-neurons using *nlg3-Trojan>shi^{ts}* flies increased social space only in females (Welch's two-tailed unpaired t-test; males p= 0.9789, females p=0.0138) (**Figure 26**).

3.1.10 Altering neuron transmission in *nlg3* neurons affects climbing ability in males

I used *nlg3-Trojan>TrpA1* and *nlg3-Trojan>shi^{ts}* flies to test whether manipulating *nlg3* neurons affects climbing ability. When I transiently hyperactivated (*TrpA1*) or inhibited (*shi^{ts}*) *nlg3* neurons I found that there was no effect on climbing ability in females (Welch's two-tailed unpaired t-test; p= 0.4597 and p= 0.7772, respectively). The climbing ability of males was slightly reduced when *nlg3* neurons were hyperactivated (Welch's two-tailed unpaired t-test; p= 0.0552), but not when they were inhibited (Welch's two-tailed unpaired t-test; p= 0.6837) (**Figure 27**).

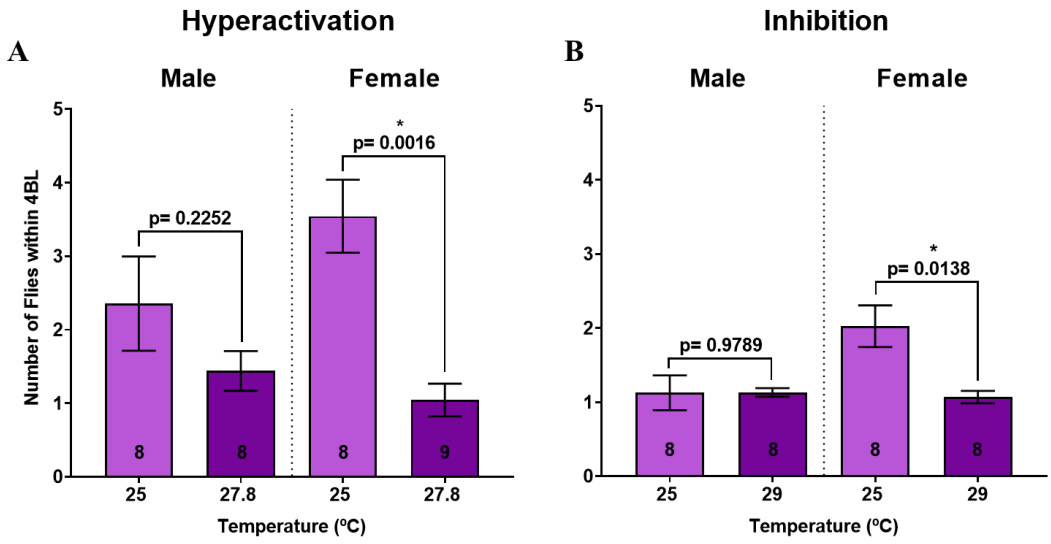

Figure 26. Manipulation of *nlg3*-neurons affects social space differently in males versus females.

Bars represent the average number of flies within 4 BL of each fly at 25 ºC versus activation temperature for *nlg3-Trojan>TrpA1* (A) and *nlg3-Trojan>shi^{ts}* (B) flies, separated by sex. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 12-18 flies per genotype for each sex).

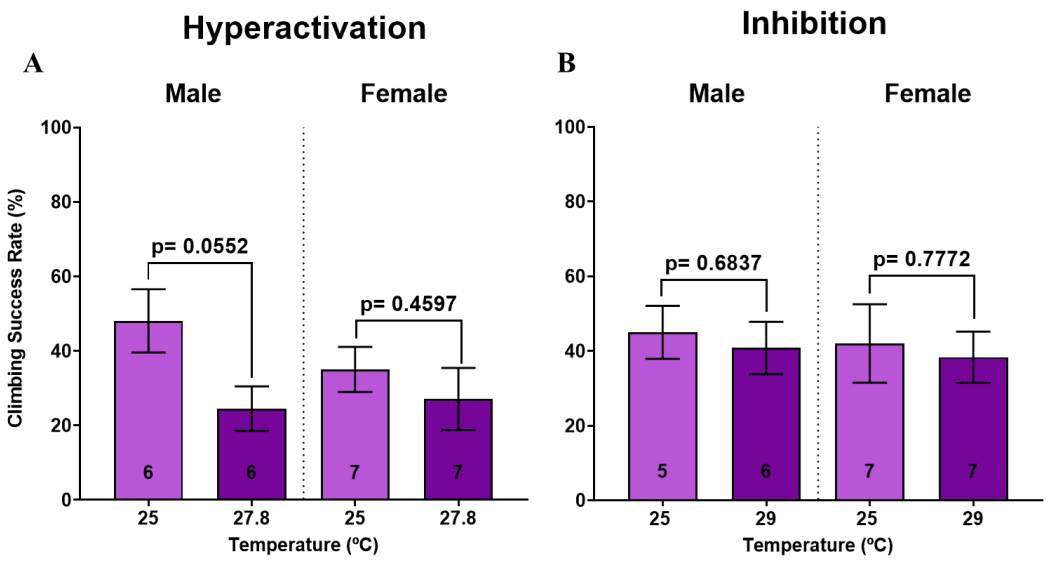

Figure 27. Altering neuron transmission in *nlg3*-neurons may affect climbing ability in males.

Bars represent the percentage of flies that climbed successfully within a 15 second timespan at 25 ºC versus activation temperature for *nlg3-Trojan>TrpA1* (A) and *nlg3-Trojan>shi^{ts}* (B) flies, separated by sex. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 20-60 flies per genotype for each sex).

3.2 Exploring the role of *nlg3* gene expression in social spacing and climbing

3.2.1 RT-PCR confirmation of *nlg3-RNAi* efficacy

After observing a sexually dimorphic effect on social spacing when manipulating *nlg3*-neuron transmission, I then wanted to determine if knocking down *nlg3* expression also affects social spacing. Although the efficacy of the *nlg3-RNAi* flies was previously confirmed through protein analysis, I wanted to conduct reverse-transcription polymerase chain reaction experiments as well to determine if the mRNA was reduced. To assess gene expression, I conducted RT-PCR and gel electrophoresis using the brains of *CS, nlg3-Trojan>nlg3-RNAi, nlg3-Trojan/+,* and *elav>nlg3-RNAi* flies. To quantify *nlg3*

expression for each genotype, I conducted a multiplex PCR and normalized its expression to an internal control gene, *rpl32*. However, the results of the RT-PCR were not interpretable; there may have been binding between primer sets or other technical difficulties which resulted in off target DNA binding in the PCR. I continued with my research despite this because of the previously established functionality of the *nlg3-RNAi* effector via western blot analysis by lab mate Ryley Yost.

3.2.2 Reducing *nlg3* expression has a slight sexually dimorphic effect on social space

I conducted social space assays using *nlg3-Trojan>nlg3-RNAi* and *nlg3-Trojan/+* flies to assess whether social space is affected by reduced *nlg3* expression. I found that reduced *nlg3* expression increases social space in both sexes- but to a greater degree in females (Welch's two-tailed unpaired t-test; males p=0.0258; females p=0.0131) (**Figure 28**). I chose to compare *nlg3-Trojan>nlg3-RNAi* and *nlg3-Trojan/+* flies to assess social spacing because these two sets of progenies share the largest amount of genetic background, therefore making observed differences more attributable to the treatments I imposed.

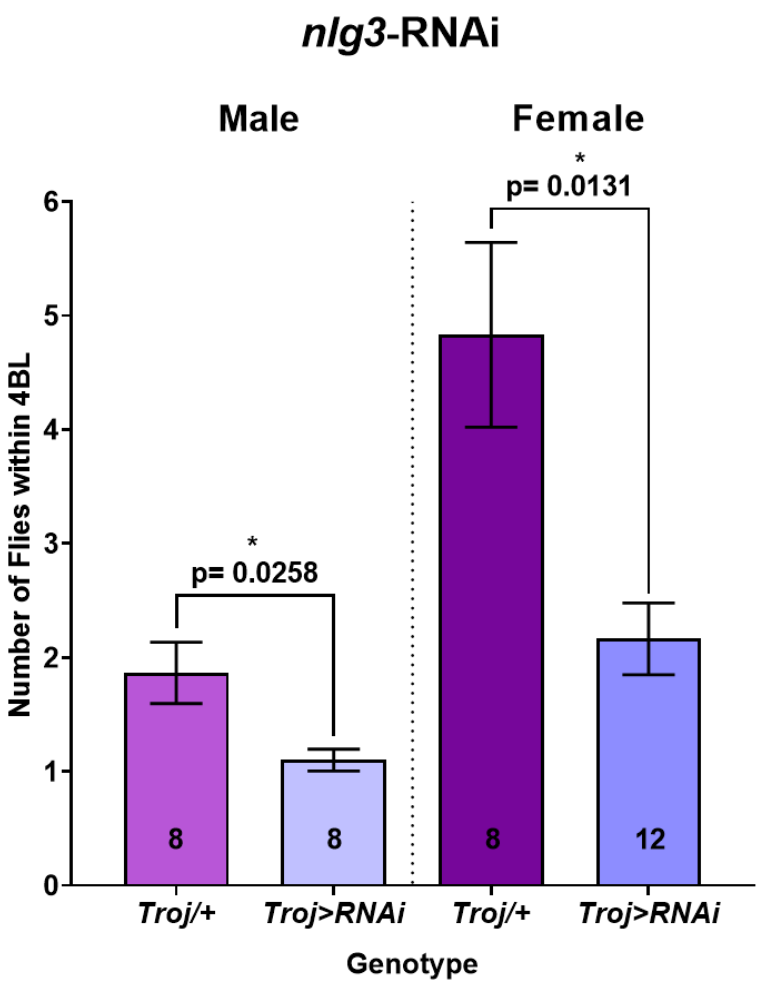

Figure 28. Knocking down *nlg3* expression in *nlg3*-neurons affects social space in both sexes.

Bars represent the average number of flies within 4BL of each fly for *nlg3-Trojan>nlg3-RNAi* flies vs. genotypic controls. Error bars represent S.E.M; Welch's two-tailed unpaired t-test; p-values reported; n values indicated inside bars (n= trials of 12-18 flies per genotype for each sex).

4 Discussion

4.1 Summary of results

In this study I determined that MB, PB, and *nlg3*-neurons are indeed involved in social spacing neural circuitry, in a sex-specific manner. I demonstrated that either hyperactivation or inhibition of MB neurons increases social space in both sexes, whereas neuron manipulation in the PB affects social space only in females. Interestingly, hyperactivation of the PB caused female flies to settle closer to each other and inhibiting the structure caused them to settle further apart. The hyperactivation of *nlg3*-neurons caused both sexes to display increased social space, although to a lesser degree in males. Inhibiting *nlg3*-neurons increased social space in females and did not affect social spacing in males (**Table 5**). I also demonstrated that *nlg3* must be knocked down in the MB and PB simultaneously (as well as in the OL) to affect social space. Knocking down the expression of *nlg3* in the *nlg3*-neurons increased social space in both sexes (**Table 5)**.

When the *nlg3*-neurons are hyperactivated, as well as when *nlg3* is knocked down in these neurons, the effect on social space is the same as when the MB are manipulated (both hyperactivated and inhibited); this may mean that the MB plays a large role in social space determination in both sexes (**Table 5**). However, the results of both hyperactivating *and* inhibiting neuron transmission in *nlg3*-neurons did not match what was seen in the MB or PB results; this indicates that there may be other structures mediating neuron transmission during social spacing. Also, the *nlg3* knockdown did not correspond with the results seen when changing *nlg3*-neuron transmission; this alludes to the possibility of another regulator at these synapses that affects whether it is inhibitory or excitatory (**Table 5**).

Table 5. Summary of results.

Green cells indicate the treatment was hyperactivation, red cells indicate inhibition, and blue cells *nlg3* downregulation via RNAi. Observations of interest written in the "Notes" column. (MB: mushroom bodies, PB: protocerebral bridge, SS: social space, hyp: hyperactivation, inhib: inhibition, *nlg3*: *nlg3*-neurons).

Brain Region	**Treatment**	**Sex**	**Effect on social space**	**Effect on climbing**	**Notes**
MB	Hyperactivation	M	Increase	None	
MB	Hyperactivation	F	Increase	None	
MB	Inhibition	M	Increase	None	
MB	Inhibition	F	Increase	None	
PB	Hyperactivation	M	None	None	
PB	Hyperactivation	F	Decrease	None	Only females affected.
PB	Inhibition	M	None	None	
PB	Inhibition	F	Increase	None	Only females affected.
nlg3-neurons	Hyperactivation	M	Increase (small)	Slight decrease	Same effect on SS as MB hyp & inhib.
nlg3-neurons	Hyperactivation	F	Increase	None	Same effect on SS as MB hyp & inhib.
nlg3-neurons	Inhibition	M	None	None	
nlg3-neurons	Inhibition	F	Increase	None	Only females affected.
nlg3-neurons	*nlg3* downregulation	M	Increase	Not completed	Same effect on SS as MB hyp & inhib.
nlg3-neurons	*nlg3* downregulation	F	Increase	Not completed	Same effect on SS as MB hyp & inhib.

4.2 Interpretations

4.2.1 The mushroom bodies, protocerebral bridge, and *nlg3*-neurons are involved in social spacing

In my research I found that all the sets of neurons I tested are involved in determining social space, albeit in different ways. There was a discrepancy in the effect on social space when manipulating neuron transmission in *nlg3*-neurons compared to downregulating *nlg3* in the same neuron set (**Table 5**). The *nlg3*-neurons are located in various structures in the *D. melanogaster* brain, including the MB, PB, and OL (Robinson & Bechard, in preparation.). Interestingly, manipulating neuron transmission in the MB alone (both hyperactivation and inhibition) produced the same effect on social space as knocking down *nlg3* expression in *nlg3*-neurons. However, manipulating neuron transmission in the *nlg3*-neurons did not produce the same results; inhibiting these neurons had no effect on social space in male flies (**Table 5**).

The MB are known as a centre for integrating olfactory sensory stimuli, learning and memory, (Baltruschat et al., 2021; Keleman et al., 2012; Li et al., 2020; Lin, 2023; Menzel, 2014) and receive inputs from over 2000 neurons, including from the antennal lobes, and some neurons involved in vision (Kind et al., 2021; Li et al., 2020; Scheffer et al., 2020). Based on the distribution of *nlg3*-neurons and the MB's role as an integration centre for sensory input, it is likely that signals transmitted within *nlg3*-neurons are regulated by inputs from the sensory structures they connect to (such as the OL or antennal lobes) before they contact the MB. This means that the MB may receive and integrate sensory signals it receives from *nlg3*-neurons and send out appropriate signals based on that information supplied by *nlg3*-neurons. Perhaps the sexually dimorphic effect on social space is due to a different base level of excitation in *nlg3*-neurons that innervate the MB; this may explain why direct MB manipulation affects social space similarly to *nlg3* knockdown, whereas *nlg3*-neuron manipulation does not. For example, if the male *nlg3*-neurons typically have a low level of excitation, then inhibiting those neurons would either not affect social space or produce a small effect. But, as stated previously, the signals the MB sends out are based on the input of a multitude of neurons; inhibiting neurons that supply input to the MB does not mean the MB output will also

become inhibited (**Figure 29**). And so, directly inhibiting a large portion of MB neurons could produce a different effect compared to manipulating the transmission of its inputs. This hypothesis suggests that, in males, *nlg3*-neurons typically have a low level of excitation, and that the outputs from the MB are tightly regulated such that inhibiting or hyperactivating MB neurons will affect social space (**Figure 29**). It is hypothesized that *nlg3* plays a role in determining whether a synapse (the connection between neurons) is excitatory or inhibitory (Xing et al., 2014; Yost et al., 2020). Therefore, reducing *nlg3* expression may affect whether its inputs to the MB are excitatory or inhibitory; based on my hypothesis, in male flies, the inputs would become excitatory, thus affecting social space (**Figure 29**). I also hypothesize that social space is more strongly regulated by *nlg3*-neurons in female flies, which is why both inhibition and hyperactivation of those neurons, as well as the MB and PB neurons, affects social space. A second hypothesis to explain these results is that there is sexual dimorphism in the distribution of *nlg3*-neurons in the *D. melanogaster* brain.

If there are more *nlg3*-neurons in the MB of female flies, this would explain why the manipulation of the MB or *nlg3*-neurons (both hyperactivating and inhibiting) produces the same effect on social spacing. This conclusion is supported by two sets of results: first, when the PB were manipulated, an effect on social space was only seen in females, perhaps due to a female-specific presence of *nlg3*-neurons in that structure. Second, when *nlg3* was knocked down in *nlg3*-neurons, a much larger effect on social space was observed in females (**Figure 30**). A recent study on the neural circuitry of social attraction found that when flies choose to approach other flies, only specific lobes of the MB were involved in this behaviour (Sun et al., 2020). This study only tested females, but it is possible that there are specific areas of the MB involved in male social spacing that is not captured by the distribution of Nlg3; this would explain why we see an effect with whole MB manipulation, but not very much with *nlg3*-neuron manipulation.

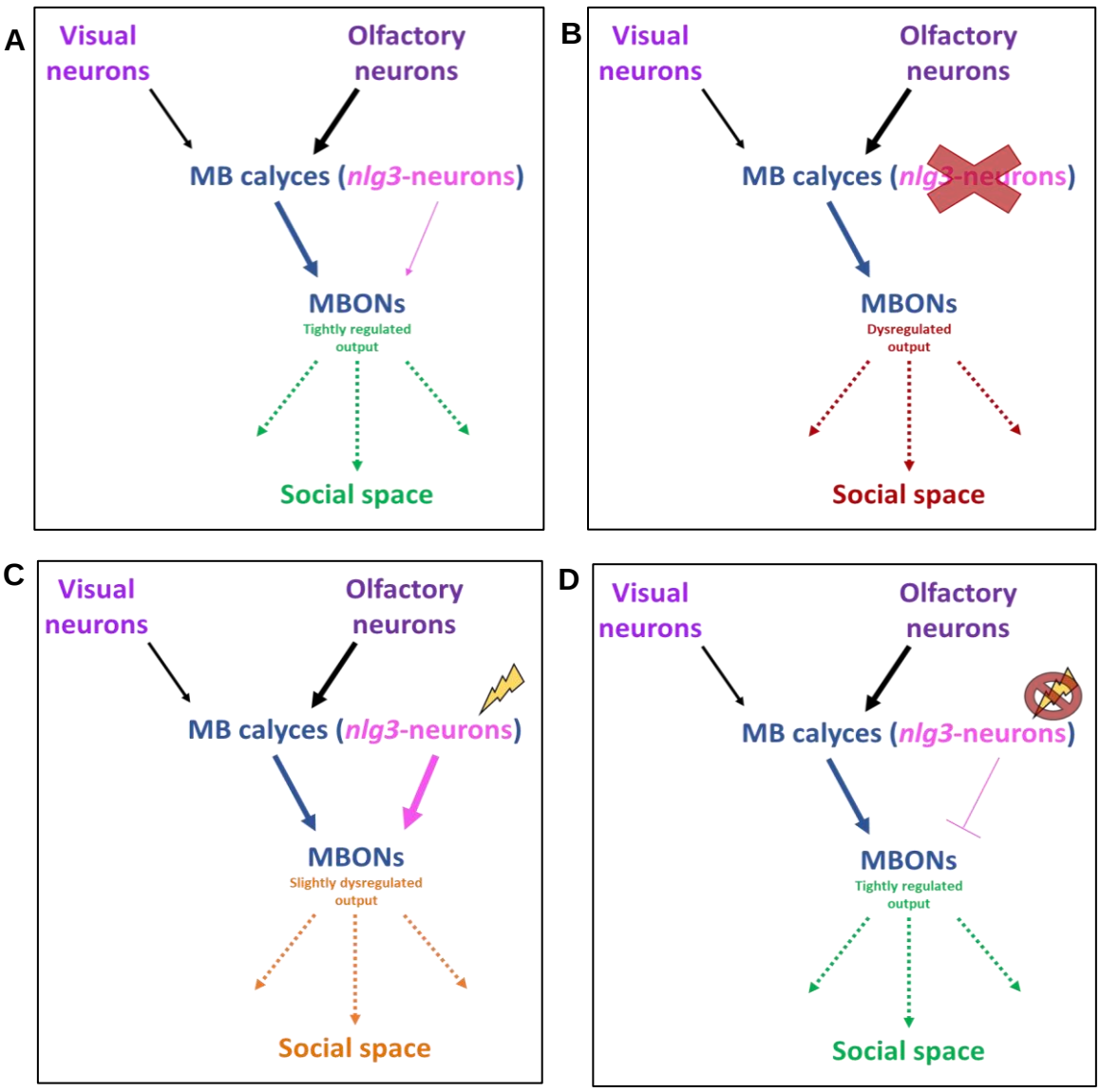

Figure 29. Hypotheses to explain the results of neuron manipulation on social spacing in male *D. melanogaster*.

Pictured above are diagrams indicating the flow of neuron transmission in (A) a typical male brain, (B) a male brain when *nlg3* expression is knocked down, (C) a male brain when *nlg3*-neurons are hyperactivated, and (D) a male brain when *nlg3*-neurons are inhibited. The thickness of arrows indicates the level of neuron excitation input, with thicker lines representing a higher level of excitation. Upside-down "T" arrow indicates inhibitory input. Green arrows indicate typical output, red and orange arrows indicate dysregulated (atypical) neuronal output, and dotted arrows represent hypothetical outputs.

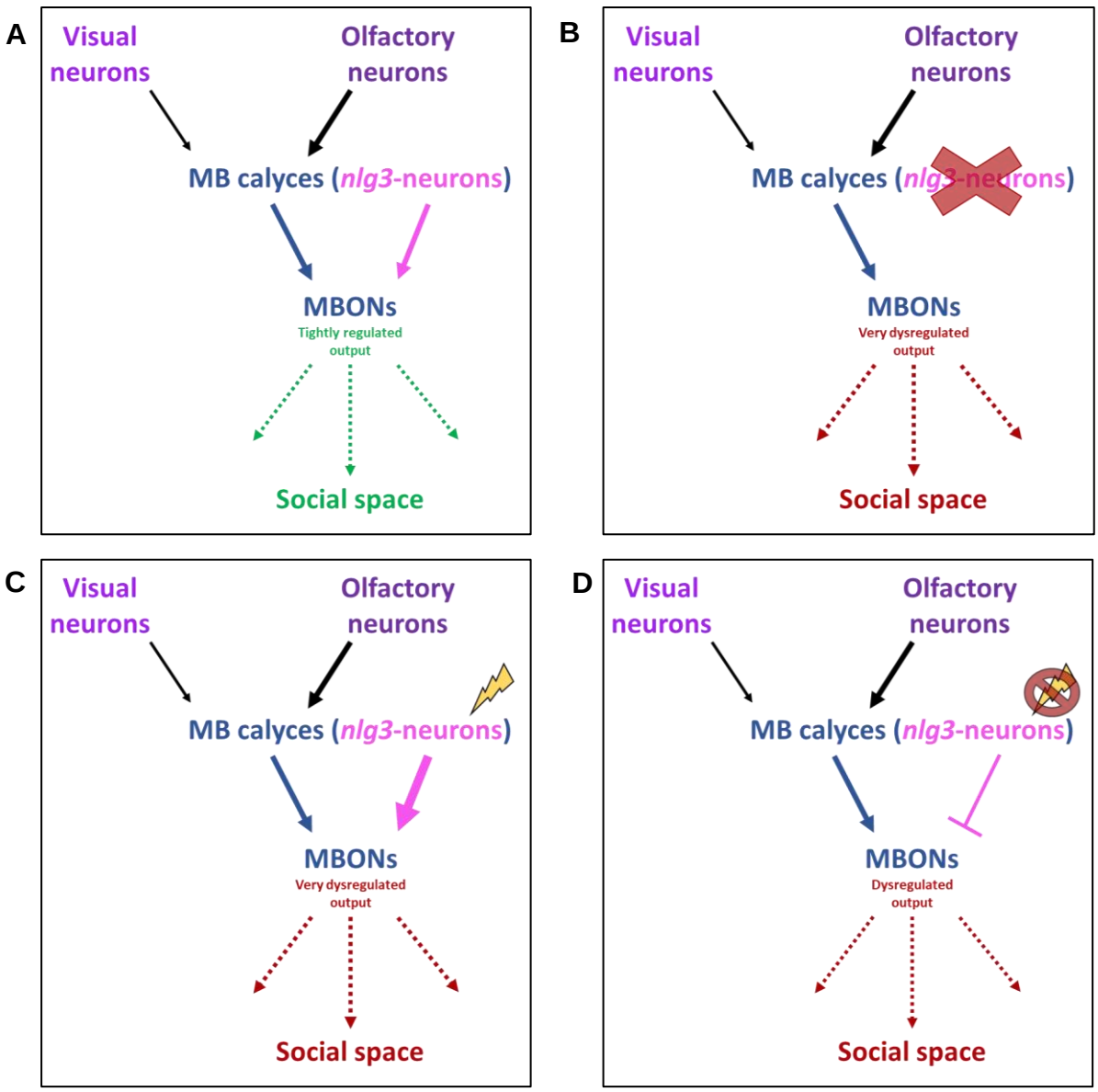

Figure 30. Hypotheses to explain the results of neuron manipulation on social spacing in female *D. melanogaster*.

Pictured above are diagrams indicating the flow of neuron transmission in (A) a typical female brain, (B) a female brain when *nlg3* expression is knocked down, (C) a female brain when *nlg3*-neurons are hyperactivated, and (D) a female brain when *nlg3*-neurons are inhibited. The thickness of arrows indicates the level of neuron excitation input, with thicker lines representing a higher level of excitation. Upside-down "T" arrow indicates inhibitory input. Green arrows indicate typical output, red and orange arrows indicate dysregulated (atypical) neuronal output, and dotted arrows represent hypothetical outputs.

4.2.2 Sexual dimorphism in the social spacing neural circuitry through differential neurotransmitter regulation of synapses

After observing that PB and *nlg3*-neurons may play more of a role in female social space neural circuitry, and the MB may play more of a role in the males', I sought to determine an explanation. A fellow graduate student (Ryley Yost) has conducted an extensive amount of work studying the role of dopamine and *nlg3* in social spacing, and much of his results, as well as work from previous lab mates, also indicate sexual dimorphism (Fernandez et al., 2017; Yost, in preparation; Yost et al., 2020). To sum up years of research in one sentence: dopamine plays a strong regulatory role in determining male social spacing.

When studying social isolation, it was found that flies that experience a period of time alone will have increased social space when tested as a group. They can "recover" after spending time with other flies and will display typical social space afterwards. Notably, only male flies had a decreased level of dopamine after social isolation, which was restored to normal once they "recovered". It was also found that a lack of dopamine prevented male flies from responding to social isolation; they no longer had increased social space afterward. A lack of dopamine in females had no effect on their response to social isolation; however, a lack of dopamine did increase the typical social space of both males and females. Finally, it was found that in *nlg3-def* flies there is significantly less dopamine in males, and also less dopamine in females. Both sexes in *nlg3-def* flies also displayed a reduced response to social isolation where they increased their social space, but to a lesser degree (Yost, in preparation).

As stated in the introduction, the neurons of the MB are strongly modulated by dopamine through the large number of dopaminergic neurons (DANs) that are innervating it (Aso et al., 2014; Li et al., 2020). The MB can also modulate themselves in response to the environment through dopamine; MB output neurons (MBONs) have connections to the DANs that innervate the MB. This means that the MB can quickly adjust the amount of signaling, or type of signaling, in response to information relayed by the DANs. This dopaminergic circuit, as well as the many others within the MB, contribute to the fly's ability to learn about the environment. This is demonstrated by the role of dopamine is

associative learning as well as the well-established role that the MB play in learning and memory (Aso et al., 2014; Baltruschat et al., 2021; Keleman et al., 2012; Lim et al., 2018; Menzel, 2014; Modi et al., 2020). The connections between dopamine, the MB, and their impacts on male social spacing provides additional evidence that the MB play a large role in the male social spacing neural circuitry. The MB likely play a role in female social space neural circuitry as well, as manipulating neuron transmission in this structure had a large effect on social space, however the neurotransmitter involved has not yet been determined.

Next, I wanted to determine a possible route for the PB and *nlg3*-neurons in female social spacing. Thus far, *nlg3* has only been associated with glutamatergic synapses at the larval neuromuscular junction, and it has yet to be fully characterized (Xing et al., 2014). Interestingly, in an RNAi screen for abnormal social spacing conducted by Ryley Yost indicated that a glutamate receptor called GluR1A appears to be involved in social spacing, and perhaps more so in females (Yost, in preparation). When the gene expression of the GluR1A receptor is knocked down via RNAi, both males and females displayed decreased social space (Yost, in preparation). And, when these flies are subject to social isolation, the females are not able to respond to this change in social experience- they do not display the typical increase in social spacing after isolation, but males do (Yost, in preparation). When I further investigated this, I discovered that a set of neurons in the PB called Δ7 are often glutamatergic (Scott et al., 2018). The same study found that activating this set of neurons can trigger an inhibitory response in a set of neurons that connects the PB to the fan-shaped body (FSB) (Scott et al., 2018). It is possible that these connections may underly some of the female social spacing neural circuitry; in my results I found that either inhibiting or hyperactivating the PB affected female social space (**Figure 24**). This neural circuit may play a role in male social spacing as well, as knocking down GluR1A also affected male social space. And within the MB there are glutamatergic MBONs that connect back to the lobes of the MB, which offers another route for the modulation of signals underlying social spacing (Aso et al., 2014; Li et al., 2020; Scaplen et al., 2021).

Next, I wanted to understand how the MB and PB might interact to determine social spacing. The MB and the PB surprisingly do not have direct connections to each other, but both have a wealth of connections to the FSB (Kind et al., 2021; Li et al., 2020; Lin et al., 2013; Scaplen et al., 2021; Scheffer et al., 2020; Sun et al., 2020). The MB and the PB both send and receive signals from the FSB, so this structure could act as an intermediate, or even an integrator, of signals that determine social spacing. The PB has been associated with visual targeting in motor functions, proprioception, integration of light cues, and recently the ability to learn and remember self-body size in *D. melanogaster* (Krause et al., 2019; Lin et al., 2013; Manjila & Hasan, 2018; Triphan et al., 2010). The MB, as described previously, has a stronger association to learning and memory, social behaviours, as well as the integration of sensory signals (Baltruschat et al., 2021; Burg et al., 2013; Li et al., 2020; Menzel, 2014; Modi et al., 2020; Sun et al., 2020). The FSB has been connected to locomotor control, visual feature recognition, nociception, courtship maintenance, sleep, and most recently social spacing and social approach (Kato et al., 2022; Liu et al., 2006; Pan et al., 2009; Qian et al., 2017; Strauss, 2002). Perhaps the FSB integrates information from the MB related to associative learning via dopamine and olfactory cues, and information from the PB via glutamate pertaining to the visual surroundings of the fly, including its own body size and motor control based on those aspects. The integration of these signals would allow the fly to remember what it has learned about social and olfactory cues via the MB and orient its body in accordance with those cues through the PB.

4.2.3 Connecting brain structures to sensory modalities that may be involved in social space determination

After realizing that signals between the MB and PB may be relayed through the FSB, I researched sensory modalities that play a role in social spacing/social behaviour to see if these structures mediate signals from those sensory neurons. As described above, the PB is involved in integrating several cues relayed through vision and allows proper motor responses to visual targets; the FSB has also been associated with visual feature recognition (Krause et al., 2019; Lin et al., 2013; Liu et al., 2006; Manjila & Hasan, 2018; Pan et al., 2009; Triphan et al., 2010). Previous research on social spacing

determined that vision indeed plays a role in this behaviour: when flies are placed in darkness, they display decreased social space (Simon et al., 2012). A similar finding was established when these researchers assessed mutant blind flies in social spacing assays, both sexes gathered closer together (Simon et al., 2012). A recent study found that serotonergic neurons modulate vision in *D. melanogaster*, which provides a link to the study on serotonin receptors in social space in the FSB (Sampson et al., 2020). This group of researchers assessed the effect of knocking down a serotonin receptor, 5HT2B, on social space in various neuron sets within the fan-shaped body. They found that the effect on social space was the strongest when this receptor was knocked down in the dorsal side of the FSB (Cao et al., 2022). The effect on social spacing was the same in both sexes (Cao et al., 2022). A set of neurons that connect the PB to the dorsal side of the FSB have also been associated with the flow of visual information, as well as the ability of a fly to propel itself forward when walking (Lu et al., 2022; Lyu et al., 2022). This literature provides strong evidence that the FSB integrate visual signals from the PB, which may contribute to the social spacing neural circuitry.

One study investigated the possible involvement of classical olfaction in social spacing and determined that this sensory modality likely does not play a role (Simon et al., 2012). However, this study was conducted using only male *D. melanogaster*, and only a subset of olfactory sensory modalities were tested (Simon et al., 2012). The experiments used mutant lines with broad olfactory deficits, and one mutant line that is not able to perceive most odours in addition to one of the *D. melanogaster* sex pheromones, cis-Vaccenyl Acetate (Simon et al., 2012). This is likely because there is a surprising lack of knowledge pertaining to how sex pheromones are perceived (Khallaf et al., 2021). Recent studies have shed some light on this subject however, and this opens an avenue for further testing of olfactory neurons that may affect social space (Borrero-Echeverry et al., 2022; Khallaf et al., 2021; Verschut et al., 2023). For example, the sex pheromone that was tested in the initial study conducted by Simon et al. in males (cis-Vaccenyl Acetate), is likely sensed by a different set of receptor neurons in females (Borrero-Echeverry et al., 2022; Verschut et al., 2023). Also, it is important to note is that only female *nlg3-def* flies display a reduced response to an odour released by stressed flies, called *Drosophila* stress odourant (Fernandez et al., 2017). There are many olfactory signals that are

integrated in a sexually dimorphic manner in *D. melanogaster*, and this provides a route for establishing the differences in social space between males and females (Borrero-Echeverry et al., 2022; Khallaf et al., 2021). A structure called the lateral horn (LH) makes a likely candidate for mediating sex-specific olfactory signals to the FSB. The LH is associated with innate and learned olfactory cues, connects to parts of the brain involved in vision as well as olfaction, and the structure itself is sexually dimorphic- it is larger in males (Schultzhaus et al., 2017). The enlarged region of the LH in males has been specifically associated with the response to sex pheromones and receives input from sexually dimorphic antennal lobes (Schultzhaus et al., 2017). The LH has many connections to the FSB and the MB (the MB are also involved in olfactory integration) and could provide sex-specific olfactory information to these structures (Li et al., 2020; Scheffer et al., 2020; Schultzhaus et al., 2017).

A recent study on social approach, which is a measure on how often flies choose to walk up to other flies, studied how the sensory modalities influence this behaviour. One of the main conclusions was that social approach was only affected when both vision and olfaction were impaired in the flies (Sun et al., 2020). These researchers tested how the impairment of various combinations of sensory modalities affected social attraction, and it was only when both vision and olfaction were non-functional that an effect on social approach is reduced (Sun et al., 2020). The researchers then sought to determine the neural circuitry that might be underlying this behaviour based on their sensory modality results. Through various methods of neuronal manipulation while measuring social approach they established two hypotheses to explain how vision and olfaction affect social attraction; the first is that olfactory neurons in the antenna send signals to two specific lobes of the MB (the alpha and beta lobes), which then relay those signals to a third lobe of the MB called the gamma lobe, which then affects social approach (see **Figure 12** for MB diagram). The second hypothesis is that photoreceptor neurons send signals to the FSB, which then relay those signals to the gamma lobe of the MB to affect social approach (Sun et al., 2020). It is interesting that these researchers also identified the MB and FSB as integrators for sensory signals that affect social behaviour- especially a behaviour that plays a role in social spacing. They also found that inhibiting serotonergic neurons that innervate the MB reduces social approach, which coincides

with results found by Cao et al. where they investigated the role of serotonin in the FSB on social space (Cao et al., 2022; Sun et al., 2020). The FSB and the MB both have direct connections to lateral accessory neurons (LAL) that send signals to motor neurons to control movement; the FSB also has direct connections to motor neurons (Li et al., 2020; Scheffer et al., 2020). Given the multitude of connections between the MB, PB, FSB, vision, olfaction, and social behaviour described above, I hypothesize a model for social spacing neural circuitry that involves these structures.

4.2.4 New hypotheses to describe the neural circuity underlying social spacing

I hypothesize that the FSB acts as an integration centre for signals between the MB and PB, which contribute to determining social space in *D. melanogaster*. I also hypothesize that the MB sends some signals independently of the FSB to regulate social space, and that sexual dimorphism in the connections between the FSB, PB, and LH may underlie the sex differences in social spacing behaviour (**Figure 31**).

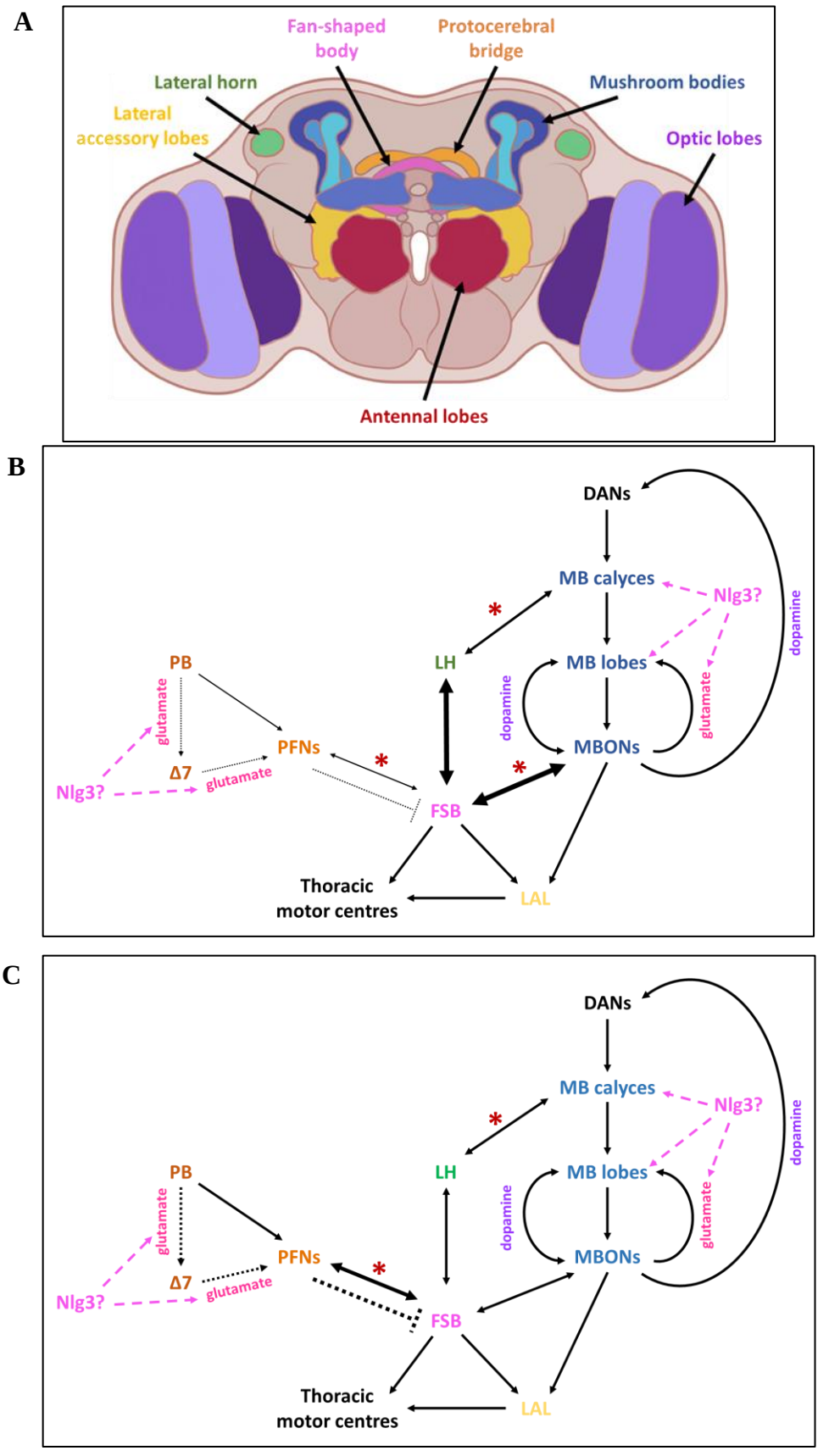
A
Fan-shaped body
Protocerebral bridge
Lateral horn
Mushroom bodies
Lateral accessory lobes
Optic lobes
Antennal lobes
B
DANs
MB calyces
Nlg3?
dopamine
LH
MB lobes
PB
glutamate
dopamine
glutamate
PFNs
MBONs
Δ7
Nlg3?
glutamate
FSB
Thoracic motor centres
LAL
C
DANs
MB calyces
Nlg3?
dopamine
LH
MB lobes
PB
glutamate
dopamine
glutamate
PFNs
MBONs
Δ7
Nlg3?
glutamate
FSB
Thoracic motor centres
LAL

Figure 31. Hypotheses to describe the neural circuitry underlying *D. melanogaster* social spacing.

Above are a depiction of the structures that are hypothesized to be involved in determining social space (A), diagrams depicting the potential neuronal connections in male (B) and female (C) that regulate social spacing. Structures are indicated by their acronyms as defined in the text, and the colour of the font indicates different regions of the same structure and corresponds to the structures shown in (A- created using Biorender).

4.3 Limitations of the study

4.3.1 Technical limitations

There were technical limitations that affected some aspects of my research. First, I began my MSc in 2020, so much of my first year was spent conducting literature searches at home; lab capacity was limited by Covid-19. This delayed my research progress well into 2021 so I started conducting experiments quite late. Once I started work at the bench, one limitation was that mating the *nlg3-Trojan* and *nlg3-RNAi* fly lines produced very little progeny. This may be due to genetic incompatibility between the lines, but nonetheless gathering enough flies for social spacing assays proved to be quite difficult; I was not able to gather enough flies for climbing assays in this genotype. Also, I found out quite late in my research that many of the lines I was using were not outcrossed which makes assessing the effect of genotype less reliable. This is why I chose to compare the *nlg3-Troj>nlg3-RNAi* flies with *nlg3-Troj/+* flies; the *nlg3-RNAi* line is outcrossed with *CS* so they are in the same genetic background. The *nlg3-Troj* line is not outcrossed, so it does not share a genetic background with *CS*. But, crossing *nlg3-Troj* with either *CS* or *nlg3-RNAi* produces flies with a genetic background that is approximately 50% *Troj* and 50% *CS*, making them comparable.

In the same *nlg-3 Troj* line, for reasons that we are still trying to discern, the flies have proven very difficult to dissect and thus the images of their brains were unclear. We have

begun to establish a protocol for imaging using confocal microscopy which may address this issue. Previously J. Wesley Robinson, a PhD candidate in the lab, has dissected and imaged the protein enrichment pattern of Nlg3 in adult *D. melanogaster* brains; this pattern displayed enrichment in the MB, PB, and OL. Currently, J. Wesley Robinson is dissecting the brains of flies that contain a *MiMIC* (see section 1.2.3.1 for a description of this construct), which would capture the same expression pattern dictated by the *nlg3* gene (in a way similar to that of the *Trojan* construct).

Then, I decided to conduct an RT-PCR to confirm the *nlg3-RNAi* was functional, though it was already determined to be functional via western blot analysis by Ryley Yost. I chose to conduct these experiments to expand my skillset in the lab and began them near the end of my MSc. I used previously designed primers for *nlg3* and *rpl32* (control) that functioned well in my previous RT-PCRs. However, I wanted to do a semi-quantitative assessment and thus perform a multiplex PCR with both primer sets in the same reaction. Unfortunately, they were not compatible for use in a multiplex PCR. There was likely binding between the primer sets within the PCR which prevented an accurate amplification of *nlg3* DNA. Therefore, semi-quantitate RT-PCR was not possible using those primer sets and there was not enough time to design and order new sets to test before I finished my MSc.

4.3.2 Conceptual limitations

If I were to conduct this study again, it would have been useful to find driver line that expresses within the MB and PB simultaneously in a similar fashion to the individual MB and PB drivers that I used in other experiments. This would have allowed me to draw stronger conclusions about the roles of those structures, as the *nlg3-Troj* driver expresses in areas other than the MB and PB such as the AL and OL, which likely influenced my results. Additionally, it would have been beneficial to conduct an RT-PCR to confirm the efficacy of the *nlg3-RNAi* construct early on in my research, as well as ensure that all of the lines I used were outcrossed.

4.4 Future directions

In my research I confirmed that the MB, PB, and *nlg3*-neurons are indeed involved in social spacing. I also confirmed that knocking down *nlg3* in *nlg3*-neurons produced an effect on social spacing in both sexes. Future studies to determine the expression of *nlg3* that is sufficient to produce proper social space would help us further characterize both the Nlg3 protein, as well as the neural circuitry underlying social spacing. It is possible to generate flies that are deficient in *nlg3*, but also contain an inducible *UAS-nlg3-cDNA*. This would allow us to test how the expression of *nlg3* in different brain structures affects social space, as well as discern which structures require *nlg3* expression to produce proper social spacing.

To test my current hypothesis on the neural circuitry of social spacing, I think it is important to assess whether the FSB play a role in this behaviour. Another research lab concluded that serotonergic neurons in the FSB indeed play a role in social spacing (Cao et al., 2022). I think it would be beneficial to investigate specific areas of brain structures to assess their involvement now that we have confirmed the MB and PB are involved, and the FSB likely are as well. We could again use shi^{ts} and *TrpA1* to transiently manipulate neurons in specific regions, as well as assess the effects of knocking down dopamine synthesis or *nlg3* within those areas as well. Also, the enrichment pattern of Nlg3 showed that it is present in the optic lobes which provide another avenue for investigation. It would also be interesting to investigate the co-localization of Nlg3 and various neurotransmitters to determine if Nlg3 plays an excitatory or inhibitory role in the regulation of synapses; or if it is able to play both. Finally, studying the expression of *nlg3* at a single-cell level would provide context to help discern the exact function of this protein, and therefore its role in social spacing.

4.5 Final conclusions

In my research I examined the role of MB, PB, and *nlg3*-neurons in *Drosophila melanogaster* social spacing. I provided further evidence that the neural circuitry underlying this behaviour is sexually dimorphic, and I made many new and relevant connections to other brain regions that may regulate the sex differences. I also made relevant connections between much of the previous work on sensory modalities to the brain regions under investigation, which was not previously characterized. My work contributes to understanding the role Nlg3 plays in social spacing and reveals many more routes of neural connectivity to be investigated in future studies.

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