Recovery from social isolation in Drosophila: The role of dopamine and the autism-related gene nlg3.

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology
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Abstract

Within a group, individuals establish their preferred distance from each other, or social space, a form of social behaviour. The resulting distance depends on the exchange of social cues from others that needs to be perceived and integrated within the organism’s neural circuitry. In humans, social spacing can be impaired in neuropsychiatric disorders such as autism and schizophrenia. When organisms are subject to social isolation, profound changes in social behaviour are observed in a variety of species from insects to mammals, including social space. However, the genetic and molecular mechanisms modulating a behavioural response to isolation and possible recovery remain to be elucidated. I first investigated the effects of social isolation and recovery on social space of Drosophila melanogaster. Then I studied the role of two potential modulators of social space in response to isolation: neuroligin3, an ortholog of the autism-related neuroligin genes, and dopamine, a neuromodulator. Manipulations of both neuroligin3 and dopamine affect social behaviour in many organisms, making them prime candidates to study their involvement in isolation and the recovery from isolation. Using a loss of function mutant, I determined that the neuroligin3 gene is required for a typical response to isolation, but protein levels remained unchanged after isolation. Using the fly Gal4-UAS system, I expressed RNAi against tyrosine hydroxylase in dopaminergic neurons to reduce dopamine levels. I found that dopamine was required for a response to isolation in a sex-specific manner and that dopamine levels decrease in males, but not females after isolation. To determine if neuroligin3 and dopamine are working together in modulating social space, I first assessed dopamine levels and found that they are reduced in the neuroligin3 mutant and without neuroligin3, dopamine levels did not change in response to social experience. Lastly, I conducted a small targeted genetic screen using RNAi against post-synaptic proteins at the synapse to begin identifying other candidates required for a response to isolation. This research identified neuroligin3 and dopamine in the modulation of Drosophila social space after isolation and recovery, and that role could potentially be conserved, as other basic molecular mechanisms first discovered in flies.
Keywords

*Drosophila melanogaster*

Social behaviour

Social space

Isolation

Recovery

Dopamine

Neuroligin
Summary for Lay Audience

Social behaviour is fundamental to the lives of humans and other organisms. For social behaviour to occur, organisms must interact with others which relies on the functioning of neural connections, or circuitry. Social behaviour can be affected when neural circuitry is unable to properly transmit information throughout the brain, like in individuals with neuropsychiatric disorders. One social behaviour shared between humans and other organisms is the determination of a personal bubble, referred to as social space. It is unknown how the functioning of neural circuitry affects social space. Social isolation causes changes in social behaviour in many animals and has detrimental psychological and health consequences in humans. How social isolation or the recovery from isolation affects the neural circuitry and alters social behaviour is unknown. Using the powerful genetics of the vinegar fly (commonly called a fruit fly), *Drosophila melanogaster*, I examined how social space is affected by isolation and looked for a potential recovery after resocialization. Flies were further apart from each other after isolation, but social space was recovered when flies had time to interact with others. I also examined two components of neurons that are important for social behaviour in many organisms: *neuroligin*, that in humans is found to be mutated in people with autism spectrum disorders, and a chemical in the brain called dopamine that both humans and flies share. I discovered that changes in social space in flies didn’t happen after isolation when the two components are either absent or reduced in males (but not females), indicating they may be involved in the functioning of the neural circuitry regulating social space. Dopamine levels in males decreased in the brain after isolation but were recovered. This research can give us insight into the components of neurons that drive social behaviour that are potentially shared between flies and other organisms including humans.
Co-Authorship Statement

The background knowledge and research presented in this thesis are part of three manuscripts, on which I am the primary author, and one review with myself as second author.

Parts of the Introduction (Chapter 1) have been published in a review in *Genes, Brain, and Behaviour* (Brenman-Suttner *et al.*, 2019), with myself as second author. Dova Brenman-Suttner performed the original literature review and wrote the first draft. I wrote sections on the range of sociality (including a table), neurotransmitter levels in aging, and edited the overall drafts and final manuscript. The writing of sections and final manuscript editing was also done by Ariel K. Frame, J. Wesley Robinson, Amanda J. Moehring and Anne F. Simon.

Background information written in the introduction was also published in the *Journal of Chemical Ecology* (Yost *et al.*, 2021), of which I wrote and for which I am the first author. This background information is also included in the pre-print version (Yost *et al.*, 2019). The final manuscript was also edited by the following co-authors: Tim McDowell, Jeremy N. McNeil, and Anne F. Simon.

This thesis contains modified data (for presentation, format or colour scheme), from manuscripts either published or under revision (Chapter 3), which I am the first author. The first manuscript is published in the *International Journal of Molecular Sciences* (Yost *et al.*, 2020). This publication is co-first authored with JWR. Data collection, analysis and writing of the manuscript was shared, but only data collected by me is presented in this thesis. Data from Chapter 3 is also modified and written in a second manuscript that is under revision in *Frontiers in Neural Circuits*, on which I am the first author.
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First and foremost, I would like to express my deepest gratitude and appreciation for my supervisor, Dr. Anne Simon. When you approached me in my undergrad and asked about my interest in research, I had no idea we would be working together for the next seven years. Your support has been the most important part of my degree. Support in research, professional development, and everyday tasks have made my time in your lab an experience I will never forget. We have moved labs twice, travelled to international conferences, and mentored students together. You have taught me what it means to be an excellent researcher and mentor. I would not be where I am today without you.

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<th>Description</th>
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<tr>
<td>3-IT</td>
<td>3-iodo-L-tyrosine</td>
</tr>
<tr>
<td>4BL</td>
<td>4 body lengths</td>
</tr>
<tr>
<td>ASDs</td>
<td>Autism spectrum disorders</td>
</tr>
<tr>
<td>BDSC</td>
<td>Bloomington Drosophila Stock Center</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>Cs</td>
<td>Canton-Special</td>
</tr>
<tr>
<td>Cyp6a20</td>
<td>Cytochrome P450 6a20</td>
</tr>
<tr>
<td>cVA</td>
<td>Cis-vaccenyl acetate</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine reuptake transporter</td>
</tr>
<tr>
<td>dILP5</td>
<td>Drosophila insulin-like peptide 5</td>
</tr>
<tr>
<td>DopR1/2</td>
<td>Dopamine receptor ½</td>
</tr>
<tr>
<td>DopEcR</td>
<td>Dopamine/ecdysone receptor</td>
</tr>
<tr>
<td>elav</td>
<td>embryonic lethal abnormal vision</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>Gal4</td>
<td>Gal4 protein</td>
</tr>
<tr>
<td>GH</td>
<td>Group housed</td>
</tr>
<tr>
<td>GluR1A</td>
<td>Glutamate receptor 1A</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>3,4-Dihydroxy-L-phenylalanine</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>nlg</td>
<td>neurologin</td>
</tr>
<tr>
<td>Nlg3-FL</td>
<td>Neuroligin3-Full Length</td>
</tr>
<tr>
<td>Nlg3-S</td>
<td>Neuroligin3-Short</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PPM</td>
<td>protocerebral posterior medial</td>
</tr>
<tr>
<td>PTPy</td>
<td>Protein tyrosine phosphatase y</td>
</tr>
<tr>
<td>REC</td>
<td>Recovery</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SH</td>
<td>Single Housed</td>
</tr>
<tr>
<td>SINs</td>
<td>Social interaction networks</td>
</tr>
<tr>
<td>sxp</td>
<td><em>sex pistol</em></td>
</tr>
<tr>
<td>th</td>
<td><em>tyrosine hydroxylase</em></td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activating sequence</td>
</tr>
<tr>
<td>VDSC</td>
<td>Vienna Drosophila Stock Center</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
</tbody>
</table>
Nomenclature

This thesis will include nomenclature for species, genes, protein, and genotypes of Drosophila and other invertebrates based on Carbon et al. (2019):

Drosophila (alone in text): not italicized

*Drosophila melanogaster* (full species name): italicized

*nlg3, neuroligin3*: general, gene name, or referring to the transcript

Nlg3, Neuroligin3: refers to the protein; first letter capitalized

*w; th-Gal4*: a designation representing genotypes that differ from wildtype; semicolons separate chromosomes

*th>RNAi*: represents a fly line in which the driver (*th*) is expressing a RNAi in the expression pattern and location of the driver itself

All vertebrate genes and proteins in text will follow nomenclature based on Bruford et al. (2020):

*NLG3, Neuroligin3*: gene name, or referring to the transcript

NLG3, Neuroligin3: refers to the protein
Chapter 1

1 Introduction & Literature Review

In this chapter, I will outline the current knowledge on topics of social behaviour and the regulation of social behaviour through the functioning of neural circuitry, and at its most basic unit, the synapse. I will also introduce how components of the synapse are altered by genetics and the environment to modulate social behaviour.

1.1 Social Behaviour

1.1.1 What is social behaviour?

Behaviour is defined as the response of an individual or group to internal or external stimuli not attributed to the developmental stages of life (Levitis et al., 2009). Thus, social behaviour is a response of an individual or group to other individuals that influences immediate or future behaviour (Levitis et al., 2009; Robinson et al., 2008; Sokolowski, 2010). For social behaviour to occur, organisms must exchange information via social cues, which are visual, auditory, olfactory and/or tactile and are critical for the successful development, survival, and reproduction of many organisms (Dahanukar and Ray, 2011; Sokolowski, 2010; Yost et al., 2021). Social behaviour is observed in simple organisms, such as microbes and bacteria, up to complex species including humans (Crespi, 2001; Ebstein et al., 2010). The spectrum of social behaviours depends on the environment and context of social cues, which can occur between conspecifics or non-conspecifics.

1.1.2 The social spectrum

Not all organisms are considered to be social, yet they are able to interact with others and reproduce. Some organisms are completely solitary and only interact for the purpose of reproduction, whereas others can be completely eusocial (Costa, 2018; Gadagkar, 1987). For example, the puma (Puma concolor) only interacts with another individual for the sole purpose of mating and is solitary when not engaged in courtship (Elbroch and Quigley, 2017). Many organisms fall somewhere between solitary and eusocial, and those who alternate between solitary and social are termed facultatively social (Seguret et al., 2016).
On this spectrum, organisms can belong to different social classes including subsocial, communal, quasisocial, and semisocial. Each step from solitary to eusocial introduces a new social trait that is observed in the species, while maintaining the social trait from the previous class (Gadagkar, 1987). Table 1.1 summarizes the social classes and traits acquired in the following class, with examples of organisms that display the trait.

While the puma (*Puma concolor*) only interacts to reproduce, eusocial organisms such as the naked mole-rat (*Heterocephalus glaber*) and honeybee (*Apis mellifera*) engage in reproduction, parental care, have overlapping generations, cooperative brood care, reproductive castes, and division of labour (Jarvis, 1981; Libbrecht and Keller, 2015). In between solitary and eusocial species are humans and the vinegar fly (*Drosophila melanogaster*). Humans lack a reproductive caste and division of labour, which means that humans are also not considered a eusocial species (Gintis, 2012). Like humans, Drosophila also lack a reproductive caste and division of labour, but in addition, they do not participate in cooperative brood care. This places Drosophila within the parasocial division of sociality (Gadagkar, 1987). The parasocial division includes communal, quasisocial and semisocial categories, but excludes subsocial, while presocial includes all categories beyond solitary but excludes eusocial (Gadagkar, 1987).
Table 1.1 Range of social classes with associated behavioural traits and examples from different species.

<table>
<thead>
<tr>
<th>Social Class</th>
<th>Social Trait Acquired</th>
<th>Puma Puma concolor</th>
<th>Vinegar Fly Drosophila melanogaster</th>
<th>Human Homo sapiens</th>
<th>Mole Rat Heterocephalus glaber</th>
<th>Honeybee Apis mellifera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presocial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solitary</td>
<td>Reproduction</td>
<td>✓</td>
<td>✓</td>
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Each social class has its own unique behavioural trait. When sociality increases, a new behaviour is observed and includes the traits found in lower classes. Checkmark indicates presence of social traits. Adapted from Brenman-Suttner et al., 2019.
1.1.3 Evolution of social behaviour

All living organisms can be organized into hierarchal levels (Bourke, 2011). Through evolution, independent self-replicating molecules have grouped into genomes, separate cells have joined to become symbiotic unicells (i.e., mitochondria in eukaryotic cells), unicellular organisms grouped into multicellular organisms, and multicellular organisms (or individuals) to societies. These grouping events have occurred over time and are termed the major transitions in social evolution (Bourke, 2011; Okasha, 2022). Through evolutionary time, the major transitions are only possible if natural selection favours a joining of two lower levels to form new hierarchy. Each hierarchal level requires selfish traits to be overcome for the better of the group.

Proposed by Hamilton (1964), kin selection theory has become the best-known and most studied theory of how organisms became social. Kin selection theory describes that social behaviour evolves when the actions of individuals increase the fitness of the individual. As such, social behaviour should correlate with the degree of genetic relatedness (Birch and Okasha, 2015; Boomsma and Franks, 2006; Hamilton, 1964). Investing resources in the survival of progeny and siblings’ progeny would indirectly increase the fitness of an individual because that individual is aiding in shared genes being transmitted to the next generation. Familial social behaviour networks are evidence of this theory and can explain why individuals perform acts that do not have a direct benefit to them.

However, social behaviour doesn’t just occur between relatives, so an additional explanation is necessary to also explain why individuals engage with others. Inclusive fitness theory also proposes that, even among non-relatives, social behaviour evolves if there is cooperation between individuals as collectively they could increase the fitness for all individuals of the group (Hamilton, 1964). Robert Trivers (1971) proposed a similar idea in which reciprocal altruism drives evolution of social behaviour because, although individuals are not relatives or even the same species, everyone mutually benefits from the interaction (Rankin and Taborsky, 2009; Taborsky, 2013; Trivers, 1971). For example, food gathering and grooming is shared among primates and is likely beneficial as less
energy is invested into finding resources and maintaining hygiene (Gomes et al., 2009). Selection has allowed these mutually social beneficial behaviours to persist in populations.

Animals have evolved social and cognitive frameworks in order to respond to social cues in the environment. The complexity of the nervous system has allowed for the evolution of the social brain. Certain behaviour may exist to allow for novel brain patterns and behaviours to be included in a social framework. The brain is considered to be highly evolvable allowing the nervous system and therefore behaviour to adapt to the environment. Clearly, the nervous system is fundamentally important to driving social behaviour.

1.1.4 Neural regulation of social behaviour

How organisms interact with others and their environment is dependent on the functioning of their neural circuitry and how social cues from others are perceived, integrated, and responded to. When organisms interact, they receive sensory cues from another individual that must be processed in the brain before subsequently leading to a behavioural response. However, interactions are occurring between two decision-making individuals, so the response to others is dynamic, and in turn, alters the response of the receiving individual to create a feedback loop (Rubenstein and Hofmann, 2015; Figure 1.1A). For example, when a male is courting a female, the female must also be receptive to this behaviour for copulation to occur, and in turn her response affects both the immediate and future behaviour of both individuals (Chen and Hong, 2018). During the decision-making stage, the integration of social cues from other individuals and environmental cues, may alter and simultaneously depend on the internal state of the individual (Rubenstein and Hofmann, 2015). The internal state is a set of cellular and metabolic activities that shape how sensory information is transformed into a behavioural response (Kanwal et al., 2021). Memory is a good example of how past experiences can shape the internal state and alter behaviour (Figure 1.1A).

What drives the integration of social cues is the communication through the neural circuitry of an organism. At the cellular level, the communication between neurons occurs mostly through the synapse (Figure 1.1B). The abnormal communication between neurons, and
inability to integrate cues with the neural circuitry are characteristic of individuals with neuropsychiatric disorders (or synaptopathies), such as autism spectrum disorders (ASD) and schizophrenia (Allocca et al., 2018; Grant, 2013; Miles, 2011).
Figure 1.1 Schematic of the interaction occurring between individuals and representation of the synapse.

A. Diagram of the sensory information exchanged between two individuals. Sensory inputs are processed within the brain and depend on the individuals' internal state of the individual,
leading to a behavioural response. Adapted from Chen & Wong, 2018. B. Representative synapse connecting two neurons. Neurotransmitters or neuromodulators are synthesized in the pre-synaptic neuron, loaded into vesicles, and released in the synapse where they bind to receptors and ion channels to communicate with the post-synaptic neuron. Cell adhesion proteins are also present within the synapse. Created with BioRender.
1.1.5 Studying social behaviour

Since social behaviour is fundamental to the survival and reproduction of many organisms, importance has been placed on studying social behaviour and how genes, environment, and neurobiological processes affect individual behaviour and how this potentially translates into group dynamics (Coria-Avila et al., 2014; Ebstein et al., 2010; Rubenstein and Hofmann, 2015; Sokolowski, 2010). Social behaviour can be studied in “simpler” organisms to examine the underlying social interactions that contribute to both basic and complex behaviours (Sokolowski, 2010). While behaviour may be species-specific, the underlying biological processes driving behaviour is shared among species. For example, the genetic and molecular mechanisms regulating circadian rhythm were first discovered in Drosophila and later found to have homologues in mammals, including humans (NobelPrize.org, 2023). Other commonly studied behaviours in less complex organisms include aggression (Anholt and Mackay, 2012), courtship (Dahanukar and Ray, 2011), learning and memory (Androschuk et al., 2015), foraging (Leech et al., 2017), parental care (Lee et al., 2023), and sleep (Ganguly-Fitzgerald et al., 2006). The use of simpler organisms allows for more control over environmental and social settings. Underlying mechanisms regulating social behaviour in many of these organisms may be conserved, thus combining the knowledge from multiple organisms can give insight into the regulation of social behaviour and the basic underlying mechanisms driving behaviour (Sokolowski, 2001).

1.1.6 Drosophila as a model for social behaviour

*Drosophila melanogaster* is commonly known as a model organism due its fast generation time and relative affordability to maintain. Additionally, *D. melanogaster*’s genome size is ~143 Mb pairs and encodes ~14,000 genes (R6.52; http://flybase.org/static_pages/docs/release_notes.html), with almost 66% of genes associated with disease in humans having a homolog in Drosophila (Lee et al., 2015; Okray and Hassan, 2013). As a result, these flies have been extensively utilized in genetic research. Drosophila has been the model involved in the winning of six Nobel prizes (Jennings, 2011; Roberts, 2006). The first was to Thomas H. Morgan for his work on role
of the chromosome and heredity in 1933, followed by Hermann J. Muller in 1946 for work involving X-ray induced mutations. In 1995, the third Nobel prize was awarded to Edward B. Lewis, Christiane Nüsslein-Volhard and Eric F. Wieschaus for identifying conserved mechanisms controlling embryonic development and in 2004, Richard Axel and Linda B. Buck were awarded the Nobel prize for work identifying odorant receptors. The fifth prize was awarded to Bruce A. Beutler, Jules A. Hoffmann, and Ralph M. Steinman for their work on adaptive and innate immunity in 2011. Lastly, in 2017, Jeffrey C. Hall, Michael Rosbash and Michael W. Young won the final Nobel prize for identifying the molecular mechanisms regulating circadian rhythms.

Over the past few decades, Drosophila has been used as a model to gain insight into the role of genes and neural processes in behaviour. While the human brain consists of approximately 100 billion neurons, the Drosophila brain contains around 200,000 neurons (Herculano-Houzel, 2009; Raji and Potter, 2021). Although the neuronal network is less complex in Drosophila than in human, both share the basic building blocks of the brain, including neurons and glial cells (O'Kane, 2011). Neurons in the fly have most of the functional and molecular components of mammalian neurons, which includes the ability to produce action potentials, the molecular machinery for synaptic vesicle release, as well as post-synaptic receptor active zones. Drosophila also share similar classes of neurotransmitters and neuromodulators and have conserved underlying brain organization (O'Kane, 2011). In fact, the common ancestor of humans and Drosophila share similar subdivisions of the brain. Using ontology and localized gene expression, the developing fly brain can be divided into the protocerebrum, deutocerebrum, and tritocerebrum, which are thought to be evolutionarily homologous to the forebrain, midbrain, and hindbrain of vertebrates, respectively (Reichert, 2005). While the specific neurons driving behaviour are not conserved, the functioning of the neurons, and at the cellular level, the synapse, display similar neurobiological processes. Drosophila has contributed to the understanding of circadian rhythms, learning, memory, aging, neurodegenerative diseases, and drug responses (Baier et al., 2002; Camiletti and Thompson, 2016; Greenspan and Dierick, 2004; Hales et al., 2015; Lee et al., 2015; Martin and Krantz, 2014; Narayanan and Rothenfluh, 2016; Okray and Hassan, 2013; Pandey and Nichols, 2011; Pfeiffer et al., 2008; Sokolowski, 2001).
1.2 Social behaviour in Drosophila

Drosophila, while not considered a eusocial species, do engage in behaviours that rely on the interactions with others and have a repertoire of behaviours affected by genetics and social experiences. Commonly studied Drosophila behaviours include courtship and aggression. Other social behaviours studied in Drosophila include courtship conditioning, the response to stressed flies, social learning, sociability, the structure of social interactions networks, and the now popular social space. Outlined here are some Drosophila social behaviours and what is known about the genetic or environmental modulators of those behaviours.

Studying courtship has revealed a very precise, coordinated, and elaborate behaviour that relies on multiple social cues (visual, auditory, olfactory and tactile) to influence both male and female behaviour, immediately or in the future (Yamamoto and Koganezawa, 2013). Multiple genes (eagle, pale, yellow), neural modulators (dopamine), and the environment (past mating experience and age of courting partner) have shown to regulate courtship behaviour (Baxter et al., 2015; Drapeau et al., 2003; Hu et al., 2014; Kuo et al., 2015; Liu et al., 2009; Moehring and Mackay, 2004; Neckameyer et al., 2000).

When male flies are rejected or exposed to an unreceptive mated female, they learn to reduce courtship behaviour (Siegel and Hall, 1979). This is known as courtship conditioning and can be used to study learning and memory (Montague and Baker, 2016). Courtship conditioning is modulated by pheromone perception, neuromodulators, and the circadian rhythm gene per (McBride et al., 1999; Montague and Baker, 2016; Sakai et al., 2012; Zhao et al., 2018).

Aggression involves attacks from one individual towards another and often in the form of male-male aggression, and sometimes female-female, or rarely male-female aggression. Aggression occurs over competition for food, mates, or territory (Zwarts et al., 2011). Some of the neural pathways driving aggressive behaviour have been identified and are involved in pheromone sensing, food presence, and visual cues (Alekseyenko et al., 2013; Alekseyenko et al., 2010; Hoyer et al., 2008; Zhou et al., 2008). Some genes involved in the sex determination pathway, fruitless and transformer, as well as the neurotransmitter
serotonin also affects aggression (Alekseyenko et al., 2013; Fernandez et al., 2010; Kravitz and Fernandez, 2015).

Stressing flies by electric shock or mechanical agitation causes the release of the *Drosophila stress odorant* (dSO) which acts as repulsive stimulus and is considered a general alarm signal (Suh et al., 2004; Yost et al., 2021). The first identified component in dSO is CO$_2$, however CO$_2$ is a general non-specific avoidance cue (Dahanukar and Ray, 2011; Suh et al., 2004). Flies process CO$_2$ via specific neural pathways indicating CO$_2$ is a robust but context-dependent social cue and depends on the internal state of the neural circuitry and environment the flies have been exposed (Siju et al., 2014). We recently identified approximately 32 volatiles in dSO, mostly smaller alkanes, but more work is needed to understand the specific role of each of those volatiles (Yost et al., 2021).

**Social learning** has been identified in Drosophila and involves an individual making choices based on the social cues from another individual (Durisko and Dukas, 2013). Social learning of egg laying is an example, where a naïve fly perceives cues from a demonstrator fly to determine the best location to lay eggs (Battesti et al., 2015). Egg laying affects lifetime reproductive success because eggs may not survive if laid in an area of high predation or lack of resources. Social learning requires the neuromodulators dopamine and serotonin (Monier et al., 2018).

**Sociability** is the tendency of flies to engage in non-aggressive interactions with other individuals (Scott et al., 2018). When placed in an arena, flies choose between different compartments of the arena and whether they share that compartment with other flies or be alone. Sociability is genetically inherited and can be sex-specific, depending on the genetic background of flies (Scott et al., 2018).

A central component of social behaviour, particularly flies within a group, are the non-random interactions occurring using social cues (Ramdya et al., 2017). Focus has turned to investigating individual and group interactions by analyzing **social interaction networks** (SINs) (Pasquaretta et al., 2016; Schneider and Levine, 2014). SINs allow the quantification of multiple measures of interactions occurring, which have been shown to be influenced by previous social experience (Liu et al., 2018).
Finally, a well-established measure of social interactions is the distance between flies within a group, or **social space**.

### 1.3 Introduction to social space

Motile organisms, from bacteria to humans, display a preferred inter-individual distance, or social space, which is influenced by genetics and the environment (Brenman-Suttner *et al.*, 2018; Kennedy *et al.*, 2009; Lough *et al.*, 2015; Vieira and Marsh, 2014). Aggregation around resources has set the stage for the study of social space. Social space occurs between organisms already in a group, and is environmental resource independent, unlike aggregation (Burg *et al.*, 2013). Social space often occurs before other more complex behaviours, as organisms must be within close proximity to exchange social cues (Simon *et al.*, 2012). Social space is also independent of the nature of the interaction. For example, organisms may have a close social space and engage in courtship behaviour, while others must be close to engage in aggression or attacks (Saltz and Foley, 2011).

Social space can be observed and quantified in many organisms: humans, herds of sheep, tribes of goats, flocks of birds, schools of fish, and swarms of insects (Parrish and Edelstein-Keshet, 1999). More specifically, sheep will maintain certain distances from each other while foraging on grass patches and avoid others due to competition (Sibbald and Hooper, 2003). Goats will move and eat less when reared in a higher density with less social space between individuals (Vas and Andersen, 2015). Birds maintain social space in flocks as these flocks are often a reward of a positive interaction and mitigates the negative aspects of isolation (Emlen, 1952). Fish, on the other hand, maintain smaller social space for the exchange of social cues related to finding a mate, migration success, and avoiding predation (Larkin and Walton, 1969) and insects including Drosophila also engage in social spacing.

#### 1.3.1 Social space in Drosophila

*Drosophila melanogaster* have a preferred social space that is affected by many factors including genetic and environmental conditions (Brenman-Suttner *et al.*, 2019; Simon *et al.*, 2012). Although most research on Drosophila occurs in a lab setting, one study has focused on the behaviour of Drosophila in the wild. Soto-Yeber *et al.* (2018) studied the
behaviour of wild flies in different orchards. Flies of the same species (e.g. Drosophila melanogaster or simulans) can recognize conspecifics and settle on resources at specific distances from others, forming groups of different species (Soto-Yeber et al., 2018). Stationary flies only deterred from this set distance for the purpose of courtship. Dukas (2020) also demonstrated flies showed sociability around resources in the wild such as grapes.

Studying social space in Drosophila has become a popular tool for analyzing interactions occurring between individuals in a group. Using genetic and molecular tools along with behavioural assays, we are only beginning to understand how flies perceive and integrate social cues within their neural circuitry to determine their preferred social space. Sensory modalities, including pheromone perception has been shown to influence aggregation. For example, cis-vaccenyl acetate (cVA) is known as an aggregation pheromone but does not affect social space, nor does classical olfaction (Bartelt et al., 1985; Simon et al., 2012). Vision plays a role in social interaction networks (Schneider and Levine, 2014), but only has a limited effect on social space (Simon et al., 2012), indicating the final decision of social space may not require some sensory modalities.

1.3.2 Modulators of social space in Drosophila

A significant amount of work has gone into investigating extrinsic (e.g., environmental) and intrinsic (e.g., genetic) influences on social space. Genetic and environmental modulators of social space have been of particular interest. Some of the environmental factors that affect social space include exposure to toxins and prior social experience. Flies exposed to the chemical Bisphenol A, which is linked to neurodevelopmental disorders (Nguyen et al., 2021; Wolstenholme et al., 2013) and other health effects, have a progeny with decreased social space (Kaur et al., 2015). Social space also depends on previous social experiences including mating status and social isolation. Virgin flies have increased social space compared to mated flies whereas flies reared single housed have increased social space compared to flies reared in a group (Simon et al., 2012).

Given the wealth of behavioural studies, neural and genetic mechanisms regulating social space are starting to be elucidated. Neurotransmitters have been implicated in modulating
social space. Reduction of acetylcholine in the mushroom bodies — the sensory integration, learning, and memory center in the fly brain — caused increased social space (Burg et al., 2013). Altering dopamine signaling in the fly brain also changes social space. Mutations in tyrosine hydroxylase (rate limiting enzyme in dopamine biosynthesis), Catsup (a negative regulator of tyrosine hydroxylase), and the vesicular monoamine transporter results in altered social space in a sex-specific manner (Fernandez et al., 2017; Xie et al., 2018). Genes that encode post-synaptic proteins are also important for social space and some have been implicated in neuropsychiatric disorders. The large scaffold protein, rugose (homolog of Neurobeachin), and forkhead box P are important for social space (Castells-Nobau et al., 2019; Wise et al., 2015). The cell adhesion proteins, neuroligins, are also important for social space (Yost & Robinson et al., 2020). Neurobeachin and some of the neuroligins are candidate genes for autism spectrum disorders (Castermans et al., 2003; Jamain et al., 2003).

Finally, aging influences social space. When flies are young, they have a closer social space then at an increased age of 30 to 50 days old (Brenman-Suttner et al., 2018). When fathers are aged, only their sons had altered social space (Brenman-Suttner et al., 2018), suggesting a sex-specific transgenerational effect of age on social space. The reasons for age related changes to social space, and other social behaviours are reviewed in Brenman-Suttner et al. (2020). Many of the modulators of social space mentioned above are altered with age potentially driving the change in social space with aging. Brain structures, like the mushrooms bodies, have a reduced number of neurons with age (Technau, 1984) and decreases to neurotransmitters, dopamine in particular, occur with age and can affect social space (Neckameyer et al., 2000).

It is evident that many factors affect social space, including previous social experience (mating status and social enrichment), neural circuitry, and genes. However, we are only beginning to understand how flies integrate social cues within the brain and process those cues to subsequently respond. Using social space as a quantifiable behavioural output, the cellular and genetic mechanisms driving social space can continue to be investigated.
1.3.3 Quantifying social space

In *Drosophila melanogaster*, social space can be quantified using the social space assay (McNeil *et al.*, 2015; Simon *et al.*, 2012). Flies are forced into a group but allowed to explore the chamber freely before deciding how close or far to settle from others. The distance between flies can be analyzed in different ways, but the most common measure is to calculate the distance of a fly to its closest neighbour (Anderson *et al.*, 2016; Brenman-Suttner *et al.*, 2018; Burg *et al.*, 2013; Corthals *et al.*, 2017; Fernandez *et al.*, 2017; Hahn *et al.*, 2013; Kaur *et al.*, 2015; McNeil *et al.*, 2015; Schneider *et al.*, 2012; Schneider and Levine, 2014; Wise *et al.*, 2015). However, this method only describes the distance between two flies and does not account for other flies in the group. To gain more information about the group of flies, a more recently used calculation involves averaging the number of flies within a certain radius of each fly in the chamber. The radius size around each fly can be described as the number of body lengths. A radius size of four body lengths (~1cm) has been used in previous studies since it is reproducible, takes the size difference of males and females into account, and is a more ecologically relevant measurement (Woodward *et al.*, 2005; Xie *et al.*, 2018; Yost & Robinson *et al.*, 2020). Since *D. melanogaster* settles at a preferred distance of two body lengths from other flies it allows increases or decreases in social space to be quantified.

1.4 Social isolation

Social isolation is the complete or near-complete lack of interactions with others. Current research highlights that human social relationships are declining in quantity and/or quality (Holt-Lunstad *et al.*, 2010). Trends in lifestyle have revealed reduced intergenerational living, higher social mobility between groups of people, marriage at later ages, multiple-career families, and higher single-residence households. These findings reveal that people are becoming more socially isolated, highlighting the importance of studying the mechanisms affected by isolation (Holt-Lunstad *et al.*, 2010). A prime example of social isolation was imposed by the recent COVID-19 pandemic.

Humans subject to social isolation report increases to loneliness, depression or suicidal thoughts, anxiety, and increased sleep disturbances (Arzate-Mejia *et al.*, 2020; Killgore *et
Isolation also increases the risk for mortality, a phenomenon first reported over three decades ago. In fact, the risk of mortality in isolated people is as strong as the risk of mortality in people who smoke, are obese, have a sedentary lifestyle, or have high blood pressure (Arzate-Mejia et al., 2020). Also, humans with neuropsychiatric disorders like autism spectrum disorders or schizophrenia are often self-isolating because of inabilities to integrate and appropriately respond to others or forced into isolation as others reduce their interaction with the individual (Meyer-Lindenberg and Tost, 2012). Longitudinal studies have begun to identify potential behavioural, hormonal, neuronal, cellular, and genetic effects of isolation (Arzate-Mejia et al., 2020), however this requires further investigation.

1.4.1 Animal studies on social isolation

Animal studies have begun to shed light on the mechanisms underlying the negative aspects of social isolation. Behavioural, neural, endocrine, and altered food intake have been observed in organisms from mammals to insects. Macaque monkeys have decreased social and cooperative behaviour potentially resulting from changes in brain structure and function (Harlow et al., 1965; Harlow and Suomi, 1971; Mitchell and Clark, 1968). Also, macaques have reduced grey matter and less activity in the frontal and temporal cortices of their brain (Sallet et al., 2011). Piglets display increased locomotion and vocalization, as well as increased cortisol levels from higher stress or anxiety (Kanitz et al., 2009). Cichlid fish have decreased social and cooperative behaviour (Hesse et al., 2015) and prairie voles have an altered gut-brain-axis, decreased social affiliation with others and neural activity (Donovan et al., 2020). A significant amount of research has utilized mice for studying the effects of isolation. Behavioural changes include increased aggression and anxiety (Matsumoto et al., 2005; Wongwitdecha and Marsden, 1996; Zhang et al., 2012), lower frequency of ultrasonic vocal communication (Keesom et al., 2017), impaired memory (Benfato et al., 2022; Okada et al., 2015), and changes in social interactions (Endo et al., 2018; Okada et al., 2015). Mice consume more food to the point of being obese and display symptoms of type II diabetes (Nonogaki et al., 2007). More details of the effects of isolation in rats and mice are reviewed in Arzate-Mejia et al., 2020.
Insects also have altered behaviour after social isolation. Honeybees reduce nestmate affiliation and interactions with others, reduced sleep, and reduced performance on discrimination learning (Eban-Rothschild and Bloch, 2015; Hewlett et al., 2018b; Tsvetkov et al., 2019). Bees have altered expression of nlg genes (Biswas et al., 2010), and also reduced dopaminergic signaling, possibly due to a reduction in reward pathway signaling as a consequence of social isolation (Tsvetkov et al., 2019). Isolated ants have decreased longevity and impaired brain development, particularly in the mushroom bodies (Ahronberg and Scharf, 2021). Cockroaches eat less and have altered oocyte production (Holbrook et al., 2000). Finally, crickets have increased aggression after only 3 hours of isolation (Stevenson and Rillich, 2013).

1.4.2 Social isolation in Drosophila

Drosophila are no exception to the detrimental effects of social isolation (Chen and Sokolowski, 2022; Vora et al., 2022). Some of the studied behavioural changes and genetic or molecular underpinnings of social isolation will be outlined in this section (Figure 1.2).
Figure 1.2. Schematic representing the behaviours and development affected by isolation.

Isolation affects behaviours including courtship, aggression, social space, feeding, social interaction networks, sleep, as well as, learning and lifespan. Adapted from Vora et al. (2022). Made with BioRender.com.
1.4.2.1 Aggression

Aggression is a well-studied behavioural effect of isolation in Drosophila. Both male and female flies display increased aggression after isolation (Hoffman, 1990; Ueda and Kidokoro, 2002; Ueda and Wu, 2009). This increase in aggression is such that isolating flies prior to aggression studies is used to raise the baseline aggression of flies, which is typically low (Baxter et al., 2019). As a result, some of the genetic and molecular underpinnings driving isolation-induced aggression have been identified. One study identified that blocking the function of olfactory pheromone-responsive neurons expressing the odorant receptor Or65a eliminates the modulation of aggressive behaviour (Liu et al., 2011). Additionally, the gene encoding a cytochrome P450 (cyp6a20), is upregulated after social interactions and is known to suppress aggressive behaviours, indicating that without social interactions, cyp6a20, could be contributing to isolation-induced aggression (Wang et al., 2008). Drosulfakinin, a neuropeptide, was identified through protein knockdown to increase aggression in isolated flies and has been shown to decrease in expression after isolation (Agrawal et al., 2020). In females specifically, isolation induced has caused changes to nerve and muscle excitability. In addition, two mutant genes (hyperkinetic and glutathione S-transferase-S1) involved in reactive oxygen species (ROS) metabolism increased female aggression in isolated flies indicating ROS metabolism could be involved in aggression caused by isolation (Ueda and Wu, 2009). In addition to the genes and proteins involved in aggression, some of the neural circuitry has been identified as well. Sexually dimorphic neurons regulating aggressive behaviour have increased dimorphic neural activity after isolation (Chiu et al., 2021). These neurons include the common aggression promoting neurons, male aggression-promoting neurons and female fpC1 neurons (Chiu et al., 2021). Neurons expressing the male specific fruitless transcription factor release the neuropeptide tachykinin that affects aggression in males (Asahina et al., 2014).

1.4.2.2 Courtship

Courtship behaviour in male flies increases after isolation, along with higher territoriality (Hoffman, 1990). However, males also displayed shorter courtship song bursts, which is
thought to lower stimulation in females (Marie-Orleach et al., 2019). Group housed flies were more successful in mating than isolated flies, possibly due to a decrease in the sensitivity of Or47b expressing olfactory receptor neurons (Sethi et al., 2019). Downstream calcium signaling including the calmodulin-dependent protein kinase 1 and the CREB binding protein was affected by the reduced sensitivity after isolation (Sethi et al., 2019). A genetic mutant called sex pistol (sxp) has slightly reduced expression of calpain A and adducin isoforms, and increased insulin-like peptide dILP5. After isolation, sxp male flies had higher male-male courtship, increased resistance to ethanol sedation, and decreased activity compared to isolated control flies (Eddison, 2021).

1.4.2.3 Social network behaviours

Social isolation causes changes to the overall social interaction networks, but data are inconsistent. Jezovit et al. (2021) observed that flies interacted with others less but spent more time during each interaction, while Schneider et al. (2012) found no effect of isolation on social structures. Isolated flies had a higher clustering coefficient (the strength of the interaction) and weighted degree (tendency of flies to interact) than group housed flies (Liu et al., 2018), but Bentzur et al. (2020) found a lower social clustering value in isolated flies, forming less structured and stable groups. This later result is consistent with the fact that sociability decreases in isolated flies (Scott et al., 2018).

1.4.2.4 Social space

Social space is also altered by isolation. Males and females have increased social space after isolation compared to group housed flies (Simon et al., 2012). However, the genetic and molecular effects of this alteration have not been extensively explored. It is known that dopamine neurons are important for social space in response to isolation. Reducing tyrosine hydroxylase in the protocerebral posterior medial 2 (PPM2) dopamine neurons resulted in flies having increased social space in isolated flies and decreased social space in group housed flies (Xie et al., 2018). This is the opposite response to isolation seen in control flies. Thus, the genetic and molecular mechanisms modulating social space in response to isolation warrants further research.
1.4.2.5 Other behaviours and conditions affected by isolation in flies

Aside from the commonly studied behaviours in Drosophila, other behaviours are affected by social isolation. Isolated male flies spend less time foraging for food than grouped reared flies although their feeding increases (Leech et al., 2017; Li et al., 2021). Flies communicate less when isolated and paired with a socialized helper fly (Kacsoh et al., 2018). Locomotor activity might be increased in male flies after isolation, although this is not consistent across studies (Hoffman, 1990; Leech et al., 2017; Liu et al., 2018). Few studies have focused on sleep disturbances resulting from isolation. Isolation caused overall decreases in total sleep and more sleep fragmentation (Ganguly-Fitzgerald et al., 2006). Changes in sleep patterns are possibly affected as isolation alters the expression of multiple circadian rhythm genes including period, timeless, and clock (Kent et al., 2008; Krupp et al., 2008). Li et al. (2021) determined that P2 neuron activation is important for inducing chronic social isolation-induced sleep loss. Finally, flies have decreased neural fiber number in the mushroom bodies and overall decreased lifespan after isolation (Leech et al., 2017; Technau, 1984).

1.4.3 Recovery from social isolation

It is evident that social isolation is detrimental, not only to Drosophila but to many organisms. This leads one to wonder about the possibility of recovery from isolation. Can organisms adapt and recover from a changing environment to a behavioural state present before isolation occurred? To address this, we must investigate the genetic and molecular modulators of a response to isolation and also determine the plasticity of isolation-induced behavioural and molecular changes.

Evidence would suggest that isolation-induced changes are plastic and recovery can occur quickly. Honeybees, following 5 days of isolation, recover their sociability after a single day of being exposed to their natal colony (Hewlett et al., 2018b). Mice recover cognitive deficits, aggression, and partially rescue social proximity when reintroduced with other mice (An et al., 2017; Chen et al., 2016; Endo et al., 2018). In Drosophila, sleep and aggression were recovered after group housing (Ganguly-Fitzgerald et al., 2006; Wang et al., 2008). Together, there is evidence that recovery from isolation can occur in some
organisms, but the recovery of behaviour and molecular mechanisms driving recovery needs further investigation. Some candidate molecules are those already shown to alter social spacing in group-housed flies include synaptic proteins and dopamine, making them prime candidates to investigate their role in isolation and recovery.

1.5 Neuroligins

1.5.1 Introduction to neuroligins

*Neuroligins (nlg)* are genes encoding post-synaptic cell adhesion proteins that are important for social behaviour in many organisms (Cao and Tabuchi, 2017; Ding *et al.*, 2015; Mackowiak *et al.*, 2014). This makes the neuroligins ideal candidates to investigate their role in social behaviour and a response to and recovery from isolation.

Neuroligins have been identified in numerous organisms ranging from the nematode, *C. elegans*, to Drosophila, honeybees, mice, and humans (Biswas *et al.*, 2008; Calahorro, 2014; Knight *et al.*, 2011; Nguyen *et al.*, 2020; Tu *et al.*, 2015). Neuroligins are important for synapse development, maturation, and stability of excitatory and inhibitory synapses. Neuroligins are all type 1 transmembrane proteins that have an extracellular acetylcholinesterase domain, transmembrane domain, and cytoplasmic tails (Nguyen *et al.*, 2020). The neuroligins function in part by binding with their pre-synaptic partner neurexin to trigger presynaptic development in connecting neurons. On the other hand, neurexin binding to neuroligin can promote the differentiation of the synapse into an excitatory or inhibitory connection depending on the identity of the presynaptic neuron (Sudhof, 2018; Sudhof, 2021).

1.5.2 Human studies

Humans have five genes that encode neuroligins: *NLG1, NLG2, NLG3, NLG4X*, and *NLG4Y* (Nguyen *et al.*, 2020). *NLG4* has one gene located within the X chromosome and males have a second *NLG4* on the Y chromosome. Neuroligins differ in what types of synapses they belong to. NLG1 and NLG4X are in excitatory synapses, NLG2 in inhibitory synapses and NLG3 is the only neuroligin present in both excitatory and inhibitory synapses (Nguyen *et al.*, 2020). Alternative splicing within the acetylcholinesterase domain
of NLG1, NLG2, and NLG3 dictates which isoform of neurexin that NLG binds to, which may alter the maturation of the synapse (Michetti et al., 2022; Nguyen et al., 2020).

The neuroligin family of genes in humans has been linked to neurodevelopmental disorders. Mutations in *NLG1, NLG2, NLG3* and *NLG4X* have been discovered in humans with autism spectrum disorders (ASDs), intellectual disability, and mental retardation (Jiang et al., 2013; Laumonnier et al., 2004; Nakanishi et al., 2017; Parente et al., 2017; Quartier et al., 2019; Redin et al., 2014; Yan et al., 2005). Most identified mutations in the *NLGs* are missense mutations that alter a single amino acid, sometimes in a conserved region of the gene (Michetti et al., 2022). For example, an arginine to cysteine knock-in mutation (termed R451C) in *NLG3* (Jamain et al., 2003; Jamain et al., 2008). *In vitro* modelling provides evidence that these mutations cause issues with protein folding, trafficking to the membrane, impaired interactions with pre-synaptic partners, and accumulate in the endoplasmic reticulum, causing activation of an unfolded protein response (De Jaco et al., 2010; Maro et al., 2015; Nguyen et al., 2020; Ulbrich et al., 2016).

1.5.3 Mice neuroligins
1.5.3.1 Mice behavioural studies

Human cell line studies have been expanded to model the human mutation in mice genes encoding the neuroligins. The mice genome encodes five *NLG* genes that are homologous to human. An R451C mutation in mouse *NLG3*, like that found in a human patient with ASD, has provided some information on the molecular function and behavioural consequences of the mutation. Mutant mice had increased inhibitory neurotransmission in the brain suggesting that NLG3 alters the ratio of excitatory to inhibitory synapses (Tabuchi et al., 2007). Behavioural data reported some social deficits and cognitive impairment in adult mice, as well as, reduced vocal communication in pups (Chadman et al., 2008; Etherton et al., 2011; Tabuchi et al., 2007). Similar to a R451C mutation, *NLG3* knockout mice displayed reduced vocal communication and olfactory deficits also found in some ASD patients (Radyushkin et al., 2009). Mutations in the extracellular neurexin binding domain impaired neurexin binding and cause increased sociability (Yoshida et al., 2021). In addition to neurexin, *NLG3* has a second binding partner, protein tyrosine phosphatase
PTPy), that competes with neurexin for binding. Mice with mutations impairing PTPy binding display social defects but enhanced motor learning (Yoshida et al., 2021). This is the first evidence to suggest that canonical and non-canonical NLG3 signaling regulates social behaviour.

A general characterization has been done for other mice neuroligins. In short, NLG1 knockout mice showed social deficits, higher repetitive behaviour (grooming), and reduced spatial memory (Blundell et al., 2010), whereas the human mutation in NLG1 (P89L) modeled in mice had decreased social ability, aggression, and spatial memory (Nakanishi et al., 2017). NLG2 knockout mice are more anxious, have social and cognitive deficits and reduced vocal communication (Babaev et al., 2016; Blundell et al., 2009; Hines et al., 2008; Wohr et al., 2013). Mice with a NLG2 human mutation R21H also displayed higher anxiety and memory deficits (Chen et al., 2017). Finally, NLG4 knockout mice had deficits in a range of social behaviours including social interactions, social approach, and social memory, but did not display repetitive behaviours as seen in other NLG mutants (El-Kordi et al., 2013; Jamain et al., 2008).

1.5.3.2 Neuroligin regulation

Neuroligins are regulated at multiple levels of transcription and translation, which subsequently affects their function. Much like humans, NLGs in mice undergo alternative splicing of the extracellular acetylcholinesterase domain, which is thought to be responsible for binding different neurexin isoforms (Chih et al., 2004; Hines et al., 2008). As a result, there is more diversity of neuroligin-neurexin interactions and recruitment of protein complexes at the neuron membrane. No evidence suggests that neuroligins undergo alternative splicing in the intracellular domain of the transcripts in mice or human. Nlg3 in the honeybee is alternately spliced in the acetylcholinesterase-like domain, however this does not occur in nlg1 (Biswas et al., 2008), nor is there evidence of splicing in other honeybee nlgs. Interestingly, honeybees isolated since emergence showed decreased nlg1 and increased nlg3 transcript abundance (Biswas et al., 2010), indicating the neuroligins could be responding to the environment. In C. elegans, the only neuroligin gene (nlg1) is alternatively spliced in the extracellular and c-terminal domains. This c-terminal alternative
splicing of nlg1 in the worm is predicted to produce as many as 24 distinct nlg1 isoforms (Hunter et al., 2010).

In addition to transcript processing, most of the mice NLG transcripts undergo activity dependent local translation in the synapse that is controlled by RNA binding proteins such as fragile X mental retardation protein (Chmielewska et al., 2018). Neuron activity also drives the proteolytic cleavage of mice NLG1 to NLG3 proteins (Chmielewska et al., 2018). Some evidence suggests that the intracellular c-terminal domain of NLG1 undergoes cleavage resulting in small c-terminal fragments of the protein that affected the number of dendritic spines (Suzuki et al., 2012), but the functionality of the small protein fragments or effect of the cleavage on NLG function has not been explored.

1.5.4 Drosophila neuroligins

Drosophila contains fours genes encoding the nlgs (Calahorro, 2014). Drosophila nlgs have undergone gene duplication and independent divergence from the vertebrate NLGs, yet they still have similar functions (Knight et al., 2011). Regardless, all Drosophila nlgs share a common ancestor with human nlg1. Much like humans, the four Drosophila nlgs have distinct, yet overlapping, localization and function. Drosophila nlg1 encodes a protein that is localized within the larval neuromuscular junction and not the central nervous system (Banovic et al., 2010). Nlg2, nlg3, and nlg4 are located within the central nervous system and neuromuscular junction (Li et al., 2013; Sun et al., 2011; Xing et al., 2014). Nlg4 is localized within clock neurons (Li et al., 2013), however specific Nlg2 and Nlg3 localization has yet to be determined. Social behaviour is affected by mutations within nlg2 and nlg4. While nlg4 knockout flies have decreased social space, both nlg2 and nlg4 mutants prefer smaller and larger group sizes, respectively (Corthals et al., 2017). Little is known about the behavioural consequences of a loss of nlg3 in Drosophila.

1.5.4.1 Drosophila neuroligin3

Drosophila nlg3 is of particular interest because it is the least understood nlg, yet it is the closest ortholog to the human NLG3 (Gramates et al., 2022). This is important because mammalian NLG3 is the only NLG present is both excitatory and inhibitory synapses. To date, nlg3 in the fly has been confirmed in excitatory synapses (Xing et al., 2014), but there
is no evidence yet to support its presence in inhibitory synapses. With regards to structure and function, $nlg3$ knockout flies have reduced larval and adult locomotion (Wu et al., 2018; Xing et al., 2014). Nlg3 is cleaved in an activity-independent manner in the extracellular domain by tumor necrosis factor $\alpha$-converting enzyme (Wu et al., 2018). This cleavage occurs directly at the cell membrane and results in two protein variants, a full-length protein (termed Nlg3-FL) and a shorter variant (termed Nlg3-S) (Wu et al., 2018). Since Nlg3-S is cleaved in the extracellular domain, neurexin binding cannot occur and Nlg3-S would therefore have neurexin-independent function. The only known difference between the protein variants is that the cleavage only occurs in the central nervous system and not neuromuscular junction, and that Nlg3-S is required for proper locomotion (Wu et al., 2018). A recent publication demonstrated that Nlg3 is also cleaved in the intracellular C-terminal domain resulting in the release of a small fragment (Xie et al., 2023). This smaller fragment was trafficked to the nucleus and the resulted in increased expression of immune responsive genes (Xie et al., 2023). Lastly, in a previous microarray analysis study, $nlg3$ was the only nlg to exhibit expression changes with prior social experience. Male flies had increased $nlg3$ transcript abundance after social isolation [referred to as CG34127 in (Ellis and Carney, 2011)]. In addition, the evidence that $nlg3$ transcript increases after isolation in bees (Hewlett et al., 2018b), indicates that $nlg3$ could be responding to the environment and modulating behaviour. This makes $nlg3$ a good candidate to investigate as a modulator of social space, especially in response to the environment.

1.6 Dopamine

Dopamine is a monoamine neuromodulator highly conserved through evolution important for behaviour in many organisms (Yamamoto and Vernier, 2011). Dopamine has roles in motor coordination, motivation, reward, addiction, learning and memory. Misregulation of dopamine levels and synaptic release has been implicated in human disorders (Franco et al., 2021), highlighting the importance to understand molecular and cellular mechanisms driving dopamine signaling.
1.6.1 Dopamine and social behaviour

Animal models have been used to investigate dopaminergic modulation of social behaviour. Dopamine not only impacts social interactions between organisms but is in part responsible for the motivation and rewarding aspect of social interactions (Dai et al., 2022; Torquet et al., 2018). Reducing dopamine in the brains of mice reduced the time spent in arenas where another mouse was present, but using a dopamine agonist rescued the time spent interacting with others (Liu et al., 2017). Dopamine also regulates behaviours in bees including learning and memory and is important for the regulation of division of labour (Scheiner et al., 2006). When injected with dopamine, bees were more social and spent more time interacting with nestmates than control bees (Hewlett et al., 2018a). Sociability scores are positively correlated with dopamine levels in bees where isolated bees had reduced dopamine and lower sociability scores (Hewlett et al., 2018a).

1.6.2 Role of dopamine in Drosophila

Dopaminergic regulation of Drosophila behaviour has also been extensively studied (Yamamoto and Seto, 2014), as many behavioural paradigms have been used as tools to understand dopamine dynamics and signaling within the fly brain. Typically, dopamine levels are positively correlated with activity levels [reviewed in (Yamamoto and Seto, 2014)]. This may result from the role of dopamine in regulating circadian rhythm, sleep, and arousal (van Swinderen, 2011; Van Swinderen and Andretic, 2011). Specific dopaminergic neural networks regulating learning and memory have been identified (Aso et al., 2010; Berry et al., 2012; Liu et al., 2012) and dopamine is thought to play roles in reward and addiction. Reduced dopamine suppressed locomotion defects caused by ethanol, cocaine, or nicotine (Bainton et al., 2000). Blocking dopaminergic synaptic transmission suppressed behavioural effects of ethanol exposure (Kong et al., 2010).

Drosophila social behaviour is modulated by dopamine as well. Female receptivity required for courtship is reduced when dopamine levels are decreased (Neckameyer, 1998), and male flies increased courtship behaviour when dopamine was increased. In addition, decreased dopamine levels promoted increased male-male attraction (Chang et al., 2006; Liu et al., 2009). Aggression levels are increased when dopamine neurons are either
activated or inactivated, specifically the PPM3 dopamine neurons (Alekseyenko et al., 2013).

1.6.2.1 Dopamine and social space

Social space is another behaviour that is modulated by dopamine. Manipulating the vesicular monoamine transporter, thereby increasing, or reducing dopamine release into the synapse, alters social space in a sex-dependent manner. Male flies were closer when dopamine release was increased and further apart when dopamine release decreased (Fernandez et al., 2017). Females had increased social space regardless of an increase or decrease to dopamine release (Fernandez et al., 2017). Some of the neural circuitry regulating social space has been identified. As previously mentioned, Xie et al. (2018) found that two dopaminergic neurons (PPM2) regulate social space, with group housed flies being closer and isolated flies being further apart when dopamine levels were reduced in those neurons. In addition, dopamine levels respond to isolation in males with decreased levels after isolation (Ganguly-Fitzgerald et al., 2006). Taken together, these results indicate that dopamine is a great candidate to study the regulation of social space after isolation and could be involved in the recovery from isolation.

1.6.2.2 Dual modulation by neuroligin and dopamine

Independently, neuroligin and dopamine have a role in social behaviour, however evidence suggests that neuroligin and dopamine may be part of a similar pathway, both required to modulate behaviour. Overlapping transcript expression of nlg3 and tyrosine hydroxylase in the Panuco swordtail fish (Xiphophorus nigrensis) suggests that dopamine and nlg3 may be within the same neurons (Wong and Cummings, 2014). Similarly, mouse nlg2 was found to mediate the contact between dopaminergic presynaptic neuron and GABAergic postsynaptic neurons (Uchigashima et al., 2016). In addition, a study by Rothwell et al. (2014) found mutations in mice nlg3 impaired synaptic transmission onto D1-dopamine receptor expressing neurons, indicating dopamine and Nlg3 could potentially be localized within the same synapse. Knocking down nlg3 in dopamine neurons resulted in mice spending less time interacting with other mice and altered reinforcement of the interaction. Finally, the knockout of nlg1 in C. elegans caused defects in a locomotor response (termed
the basal slowing rate) that is a dopaminergic-mediated response (Izquierdo et al., 2013). Interestingly, this study showed locomotion was restored when $nlg1$ was replaced with the human $nlg1$ but not this did not occur when they expressed $nlg1$ mutations (Izquierdo et al., 2013). Thus, ample evidence exists to indicate neuroligins and dopamine function together within similar pathways, possibly regulating social behaviour in Drosophila.

1.7 Thesis overview, hypothesis, and objectives

Understanding the genetic and molecular underpinnings of social behaviour is crucial due to its importance for survival and reproduction across many organisms. Yet, we are only beginning to understand how organisms integrate social cues within the brain and properly respond to others. Of the previously outlined genetic and molecular mechanisms regulating Drosophila social behaviour, this thesis focuses on social space since the neurocircuitry and components driving social space remains unclear. Additionally, detrimental effects of isolation are widespread and affect multiple social behaviours including social space. However, the cellular and molecular machinery altered by isolation that subsequently affects interactions with others requires more research. Post-synaptic neuroligin proteins and the neuromodulator dopamine are promising candidates for this study as both are important for social space, yet their involvement in the response to isolation has not been extensively studied independently nor together. Furthermore, even less is known about their role in the recovery from isolation. In this thesis, I assessed the role of $nlg3$ and dopamine in social space and in response to the environment.

I hypothesize that the effects of isolation on social space are plastic such that flies can recover their social space as well as that neuroligin and dopamine are required to modulate these behavioural responses.

I began with a general characterization of $nlg3$ mutants in Drosophila to determine the mutation effects on protein abundance. Then, I assessed social space in response to isolation with varying days of isolation and determined the length of group housing post-isolation required for flies to recover social space. Moving on, I tested the effects of mutant neuroligin on social space after isolation and recovery, followed by reducing dopamine levels in the fly brain and assessing the same response to isolation and recovery on social
space. I tested dopamine levels in a *neuroligin* mutant to determine if they could be operating in the same pathway. Finally, I screened for other proteins within the post-synaptic neuron that are required for social space in response to social isolation.

The objectives of my research are as follows:

1) Characterization of an isolation phenotype:
   a) Confirm the effect of social isolation on social space.
   b) Examine the effect and time required for a recovery on social space.

2) Examine the role of *nlg3* and dopamine in response to social isolation:
   a) Using mutant and transgenic lines of *nlg3* and dopamine respectively, determine the social space after isolation and determine the importance of *nlg3* and dopamine in the recovery from isolation.
   b) Determine on Western blots if *nlg3* protein abundance or extracted and quantified dopamine levels are altered after isolation and recovery.

3) Examine whether *nlg3* and dopamine were parts of a similar pathway.

4) Examine the effect of social space after isolation when knocking down transcripts of post-synaptic proteins that are known to affect social behaviour.

### 1.8 Significance

As for other fundamental behaviours in Drosophila (circadian clock, learning and memory), the underlying genetic and molecular pathways are thought to be evolutionarily conserved (Allocca *et al.*, 2018; Greenspan and Dierick, 2004; Sokolowski, 2010). While the specific neural circuitry driving social behaviour in Drosophila is likely species-specific, the molecular pathways regulating the communication between neurons and the integration of social cues within neural circuitry may be conserved in more complex organisms. Identifying these conserved mechanisms would therefore add insight into the regulation of social behaviour in other organisms.

This is the first study to investigate the role of *nlg3* in social space in response to a changing environment using male and female Drosophila. It is also the first study to examine how
dopamine levels change in females in response to isolation and the first study on the recovery of social space and the possible mechanisms regulating recovery following isolation. Finally, this study offers a list of post-synaptic proteins that are also required for a response to isolation, many of which are likely part of a molecular pathway with neuroligin and dopamine that are required to regulate social behaviour.
Chapter 2

2 Materials and Methods

All Materials and Methods presented in Chapter 2 are described in publications either published in Yost & Robinson et al., 2020 or under revision in Yost et al. *Front Neural Circuits* (Manuscript ID: 734017).

2.1 Fly stocks and husbandry

2.1.1 Rearing conditions

All flies were reared in mixed sex groups inside bottles or vials containing Jazz-Mix™ media (FisherScientific, #AS153) or handmade food using the same recipe [brown sugar (118.13 g/L), corn meal (30.24 g/L), yeast (17.48 g/L), agar (5.67 g/L), benzoic acid (1.89 g/L), methyl paraben (0.71 g/L), and propionic acid (0.71 g/L)]. Flies were reared in an incubator (Environmental Growth Chambers in the Biotron at Western University) at 25 ± 0.1 °C with 50% relative humidity (cool fog humidification) on a 12:12 h light:dark cycle with lights on at 9:00 am and off at 9:00 pm. To limit variation in behavior, parents were a maximum of 14 days old (Brenman-Suttner et al., 2018). All fly lines used in this study are reported in Appendix A and Appendix B.

2.1.2 *nlg3* fly lines

To investigate the role of *nlg3* in longevity and social behaviour, a control line [*Drosophila melanogaster* Canton-S (Cs)] and three different *nlg3* mutant fly lines were used in this study. Cs was from our laboratory stock and used to outcross the *nlg3* mutants 6 times. The three mutant *nlg3* genotypes used (see Figure 2.1) consisted of the following: a line with a P-element insertion into the fourth intron (PBac{SASTopDsRed}L04718, Kyoto stock center #140892), a line with P-element insertion into the regulatory region of the gene (P{GSV1}neurGS3205; Kyoto stock center #205074), and a deficiency line, which was a complete deletion of the *nlg3* gene. The *nlg3* deficiency line was made by Dr. Brian Mozer and described in Yost and Robinson et al. (2020)
**Figure 2.1. nlg3 gene map.**

**A.** Gene map of *nlg3* displaying insertion and deletion sites for corresponding mutants. The black arrow represents the genome. Genes within the genome are indicated by coloured bars and arrows represent the gene orientation. Grey coloured bar represents the *nlg3* gene span. Squares in the transcript represent exons and lines indicate intronic regions. Orange exons represent the coding region. Coloured arrowheads on the genome display insertion sites of *nlg3* mutants. Red line under the transcript represents the extent of the deletion of *nlg3* in the mutant *nlg3*<sup>Deff</sup>, in addition to some α-esterase genes and a long non-coding RNA (indicated under the genome). Black arrowheads above the gene span represent the P-elements used to make the *nlg3*<sup>Deff</sup>. Insertion information can be found in the Materials and Methods section. Information was obtained from FlyBase (Gramates *et al.*, 2022) using release FB2023_02. 

**B.** Protein domains of Nlg3. A signal peptide (SP) is present at the N-terminal domain (NTC). An acetylcholinesterase-like domain is extracellular and binds neurexin. Followed by a transmembrane domain (TM), and protein binding domain (PBD) that consists of a polyQ region for adaptor protein binding and the C-terminal domain (CTD). Red line represents antigen used for antibody production which is the entire intracellular region of Nlg3. Figure adapted from Xing *et al.* (2014).
2.1.3 Dopamine fly lines and crosses

To manipulate dopamine levels within the fly, the Gal4-UAS system was used to drive two RNAi (w;; UAS-THmiR-G/TM6 and w;; UAS-THmiR-C/TM6) in neurons expressing tyrosine hydroxylase (th) using w; th-Gal4. Th-Gal4 was outcrossed to Cs 6 times while the RNAi lines were not outcrossed. Appendix C depicts the crosses made for this experiment and outlines the genetic background. A RT-PCR was run to confirm the tyrosine hydroxylase (th) RNAi was effective at reducing th gene expression (Appendix D). Th-Gal4 was provided by Dr. Serge Birman and the RNAi lines were provided by Dr. Mark Wu.

2.1.4 Fly lines used in the screen

All lines used in the genetic screen were purchased from the Bloomington Drosophila Stock Center (BDSC) as part of the Transgenic RNAi Project (TRiP). A chart with the lines and stock numbers can be found in Appendix B. Each RNAi line was crossed to the pan-neuronal driver w+; elav-Gal4/CyO (BDRC, #8765) to express the RNAi in all neurons. The RNAi and driver were also crossed to a genetic control depending on the line. Genetic controls have the construct inserted at the same genomic localization, but with no RNAi for the Valium10 or attP40 vectors used to make the RNAi lines. A diagram of the crosses and the controls are shown in Appendix E.

2.2 Reverse-transcriptase PCR

To confirm the efficiency of the RNAi at reducing th gene expression, 20 fly heads, separated by sex, were used and RNA was extracted using TRIzol (Invitrogen) and phase separated with chloroform. RNA was precipitated with 100% isopropanol and washed with 70% ethanol. Genomic DNA was removed using the TURBO DNAse kit (Invitrogen) and cDNA was synthesized with the iScript cDNA Synthesis kit (BioRad). The PCR was run using primers for th gene using the following sequence: forward (5’-3’) – CCCGCAGCAAGGCAATGATTACG and reverse (5’-3’) – ACTCTGCATGGCAGCCTCGG. Each sample also included a housekeeping gene, ribosomal protein L32 (rpl32), as a technical control with the following primer sequence:
forward (5’-3’) – AAGCGGCGACGCACCTGTGTT and reverse (5’-3’) – GCCCAGCATAACGCCCCAAG. Products were run on a 1% polyacrylamide gel.

2.3 Western blot and protein analysis

Twenty male and female heads, separated by sex, were lysed, and homogenized in RIPA buffer (10 mM Tris, 140 mM NaCl, 0.1% SDS, 0.5 mM EDTA) with Halt protease and phosphatase inhibitors (ThermoFisher), followed by protein sample preparation with Laemmli sample buffer (32.9 mM Tris-HCl, 13% glycerol, 1% sodium dodecyl sulfate (SDS), 0.01% bromophenol blue; Biorad) with 1% dithiothreitol (BioRad). Protein lysates were separated on a 10% TGX FastCast Stain-Free polyacrylamide gels (BioRad) and electro-transferred to nitrocellulose membranes. Proteins were incubated with a polyclonal guinea pig anti-Nlg3 antibody (1:4000) overnight at 4 °C, followed by extensive washing and then incubated with a horseradish peroxidase conjugated secondary antibody (BioRad; 1:10,000). Total protein was visualized with the BioRad ChemiDoc using the UV illumination capability of the BioRad Stain-Free technology. A substance in the gels binds to proteins and allows for visualization of total protein which is subsequently transferred to the membrane allowing for visualization of all proteins on a membrane. Bands are normalized to total protein below ~75 kDa to avoid autofluorescence of the eye pigment. Following, anti-Nlg3 was visualized using the ClarityMax Western enhanced chemiluminescence substrate (BioRad). Western blots were analyzed with ImageLab software (BioRad). All treatments were normalized to total protein. The Nlg3 antibody was produced as described in Yost & Robinson et al. (2020).

2.4 Aging

Flies were aged as previously described (Brennan-Suttner et al., 2018). In short, flies were collected within one day of eclosion and placed in mixed sex groups of 40 flies. Flies were transferred to fresh food three times a week until being used at the desired age.

2.5 Longevity

Longevity experiments on Cs and nlg3Defi flies were conducted as previously reported (Simon et al., 2003). A new generation was started using 20 males and 20 females per
bottle to control for rearing density. Once eclosion occurred, 1-2 day old flies were collected and sexed using cold anesthesia. Flies were placed in vials with 20 same-sexed individuals and transferred to new food every 2-3 days. Dead flies remaining in the old vials were counted.

2.6 Behaviour

2.6.1 Fly handling prior to behaviour

All fly stocks, with the exception of those for isolation, were raised mixed sex in a socially rich environment (i.e., grouped-housed; see below). Newly eclosed flies were transferred to new bottles to age the flies. The day prior to a behavioral assay, 15–17 flies were collected and sexed under cold anesthesia. The morning of the experiment, all flies were transferred to new vials at least two hours prior to the assay and allowed to acclimate to the test room conditions of 25 °C and 50% relative humidity. All experiments were performed under unified light and all replicates were tested in the same room between 12:00 p.m. and 4:00 p.m., corresponding to 4–8 ZT (Zeitgeber time: time after the onset of light), to reduce behavioral variation linked to diel periodicity.

2.6.2 Social space assay and ImageJ analysis

Flies were placed into a vertical triangular chamber and allowed to explore freely (Figure 2.2A). In a vertical chamber, flies will try to escape and move to the top. As it is a triangle the flies are forced into a group, before they spread out and settle at preferred distance from other flies, as described in Simon *et al.* (2012). Once flies settled in a stable group formation, ~20–40 min after flies were placed in the chamber (i.e., time zero), photographs of each chamber were taken all at the same time point. Different ways of assessing social space have been used in previous studies, but here I used the number of flies close to each focal fly. Specifically, I chose to quantify the number of flies present within the distance of 4 body lengths of each fly, or ~1 cm, a metric also used by Xie *et al.* (2018). Images were converted to a black and white image (Figure 2.2B) and then the number of flies within 4 body lengths was calculated for each individual in the chamber and averaged to create an individual replicate (Figure 2.2C, D).
Captured images were processed using the free open access software ImageJ (Rasband, 1997-2018) and routines (i.e., code) were developed for these analyses on ImageJ and are available at: https://github.com/flugrugger/bubble (Yost & Robinson et al., 2020).

Averaging the number of flies present within four body lengths for each fly in the chamber was scored for each replicate. Chambers consist of 12–17 flies/chamber, as there is no effect of variation in density on social space within that range (McNeil et al., 2015). Data was collected by testing controls and experimental groups and each environmental treatment at the same time on each experiment day. Three replicates were tested each experiment day. To control for internal variability and effect of the day, I performed the experiments over multiple days: experiments were conducted on 3 independent days, resulting in ~9 replicates in total. Testing days were separated by at least one week to control for environmental factors beyond my control.
Figure 2.2. Social space assay and analysis.

A. Image of social space chamber. The vertical triangular shape forces flies into a group from where they can roam freely and decide how close or far to be from other individuals. Flies will then stop moving and settle at a preferred social space, which is when a photograph is taken.  

B. Images are then processed in ImageJ and converted to a black and white image.  

C. The number of flies within 4 body lengths is calculated for each individual in the chamber. For example, the bottom fly has 0 flies within 4 body lengths, while the top fly has many.  

D. The number of flies within 4 body lengths is averaged amongst the 12 to 17 flies present in the chamber resulting in 1 individual replicate, and I graphed the average of 9 trials. Flies with more individuals within 4 body lengths than the control are closer as a group (closer social space) and less flies within 4 body lengths means the group is further apart (lower social space). Cs: Canton-S control.
2.6.3 Climbing

Climbing was performed using the counter-current apparatus (Benzer, 1967), as previously described (McNeil et al., 2015). In short, 40 flies separated by sex were mechanically knocked to the bottom of the vial in the apparatus and they were allowed to climb to the top vial for 10 seconds. The number of flies reaching the top vial was counted and represented as the percent of the total flies used in the assay.

2.6.4 Social isolation and recovery

Two-day old flies were collected and sorted into individual vials under cold anesthesia to generate a single housed treatment. Flies remained single housed for 7 days, similar to that reported by Simon et al. (2012). Recovery flies were group housed following isolation for either 2 or 3 days. Group housed flies were handled in the same way as isolated or recovered flies. To perform social space flies were grouped directly before being placed in the chamber. Flies used for Western blot and LC/MS were grouped directly before being homogenized in sample buffer. A visual timeline for isolation and recovery is presented in Appendix F.

2.6.5 Sociability assay and aggregation index

The sociability assay was performed as described in Yost & Robinson et al. (2020). The sociability chamber consists of a circular arena (90 mm wide by 20 mm high) divided into 8 compartments, with a hole in the center to allow flies to enter any compartment. Each compartment contains a patch of fresh food. The chamber and performance of the assay is modified after Scott et al. (2018). Eight same-sex flies were placed in the chamber by mouth aspiration, through a hole in the lid, and they are allowed to roam freely for two hours. Every 30 minutes, a photograph of each chamber was taken and the number of flies in each compartment was counted, and an aggregation index was calculated (sample variance divided by the mean number of flies in each chamber). The variance can take values between 0 and 8. For example, the least sociable option would have 8 compartments of 1 fly each, with a variance of 0, while the most sociable situation would have 7
compartments of 0, and 1 chamber with all 8 flies. This chamber would have a variance of 8, and therefore a maximum aggregation index of 8.

### 2.7 Feeding 3-IT and L-DOPA

Flies that were group housed, isolated, or recovered from isolation were fed food with the addition of 3-iodo-L-tyrosine (3-IT; 30 mM; Sigma-Aldrich) or 3,4-Dihydroxy-L-phenylalanine (L-DOPA; 4 mM; Sigma-Aldrich) and blue food dye (Clubhouse). 3-IT was dissolved in H₂O with 0.01% acetic acid and L-DOPA was dissolved in H₂O. Food was made by melting prepared diet and adding the chemicals, or just the vehicle as a control, to produce the concentrations of 30 mM and 4 mM of 3-IT and L-DOPA, respectively, and 1 µL/mL blue food dye was added. Concentrations of 3-IT and L-DOPA were determined to be effective in previously published work (Wang et al., 2011). All flies were transferred to new food every three days. Food was prepared fresh the morning of each transfer day and the morning of each experiment. Blue dye was used to confirm all flies had eaten during the isolation, recovery and before social space experiments took place.

### 2.8 Dopamine Quantification

#### 2.8.1 Extraction

Adults were separated by sex, and dopamine extracted from their heads using the following procedure. Extraction occurred by flash freezing flies in liquid nitrogen followed by manual decapitation and homogenization of heads in 5 mM of ammonium acetate in 90% acetonitrile using microtissue grinders (Kimble Chase, USA). The supernatant was transferred and filtered through a 0.65 µm filter (Millipore) at 4°C and samples were stored at -80 °C before LC/MS detection.

#### 2.8.2 Detection and Quantification

LC/MS analysis was performed using an Agilent 1260 Infinity LC system coupled to an Agilent 6230 TOF system. A XBridge C-18 column Rapid Resolution HT was used (4.6 Å−150 mm, 3.5 µm, 600 Bar, Waters) at 25 °C and samples were eluted with a gradient of CH₃CN (Solvent B: 90% CH₃CN in H₂O, containing 0.1% formic acid) in H₂O (Solvent A: containing 0.1% formic acid). The UV lamp was set at 282 nm, the injection volume
was 10 µL. The flow rate was set to 0.4 mL/min and infused into an Agilent 6230 TOF-MS through a Dual Spray ESI source with a gas temperature of 325 °C flowing at 8 L/min, and a nebulizer pressure of 35 psi. The fragmentor voltage was set to 175 V with a capillary voltage of 3500 V and a skimmer voltage of 65 V. The instrument was set in positive ESI mode, and quantification occurred using a standard curve of known dopamine concentrations (Appendix G). Total ion count was extracted using Agilent MassHunter Qualitative Analysis software (version B.05.00).

2.9 Statistical Analysis

The distributions of the data analyzed were confirmed to follow a Gaussian distribution prior to applying parametric tests. An alpha level of 0.05 for all statistical tests and analysis was completed using GraphPad Prism 9 (Prism version 9.02 for Mac, GraphPad Software, La Jolla California, USA, www.graphpad.com). Social space, climbing, and dopamine quantification data used Welch’s t-tests, One-way or Two-way ANOVAs. Western blot analysis utilized a Three-way ANOVA to test effects of genotype, age, and protein isoform or a Two-way ANOVA to test the effects of isolation and protein isoform. Two-way ANOVAs and Three-way ANOVAs test the effects of multiple factors (2 and 3, respectively) on an independent variable, as well as possible interactions between the variables. The effects of each factor and the interaction are only reported when there is a statistical significance. Holm-Sidak post hoc tests were performed to correct for multiple comparisons where indicated on the figure. Longevity data utilized a Mantel-Cox to compare the survival distributions and a Chi-square test was used to compare rates of mated flies. P-values are shown in text and significant statistics are reported in figure legends.
Chapter 3

3 Results

This chapter contains data and figures with adaptations that are either published or under revision in the following papers:


Yost, R. T., Scott, A. M., Kurbaj, J., Walshe-Rousell, B., Dukas, R. & Simon AF. Recovery from social isolation requires dopamine in males, but not the autism-related gene *nlg3* in either sex. *Front Neural Circuits* (Manuscript ID: 734017).

Unpublished data is noted in the figure legends.

3.1 Characterization of the social isolation phenotype

3.1.1 Confirming the rate of mated flies after 2 days of group housing

Since mating status has an effect on social space, and virgin flies are further apart than mated flies (Simon *et al.*, 2012), I first needed to assess how long flies should be group housed post-emergence to avoid the confounding effect of virginity on social space. I found that in Cs flies, 101 out of 112 (90.2%) of females were mated and 55 out of 72 (76.4%) of males were able to mate after spending 4-5 days mixed with females (Appendix H). In comparison, 94/103 (91.2%) of females were mated 2 days after adult emergence spent with males, and 69/81 (85.2%) of males of the same age were able to mate. The ages at which the flies were tested had no significant effect on their mating capabilities ($\chi^2(1) = 0.0213, p=0.8841$ for females, and $\chi^2(1) = 1.92, p=0.1659$ for males). Thus, the decision was to expose flies to two days of group housing post-emergence, before any social isolating took place, as the data showed that the same percentage of flies have mated by that age, as when they spend 4-5 days group housed.
3.1.2 Seven days of isolation leads to increased social space in males and females

As previously reported, seven days of social isolation leads to increased social space (Simon et al., 2012). I wanted to determine if this time could be reduced and what the minimum time of isolation was required to observe an increase in social space. Cs males had fewer flies within 4 body lengths after social isolation compared to group housed after 2, 4 and 7 days of isolation (Figure 3.1A; Effect of isolation: $p < 0.0001$). There was no effect of 2 days of social isolation in Cs females. Only after 4 and 7 days of isolation did Cs females have fewer flies within 4 body lengths and the increase in social space was larger with increasing number of days flies were isolated (Figure 3.1B; Effect of isolation: $p = 0.0131$; Effect of days of isolation: $p = 0.0327$). The effect of isolation after 7 days was similar to the effects found in earlier studies (Simon et al., 2012). Moving forward, I used seven days of isolation for subsequent experiments as this led to the largest increase in social space in both males and females.
Figure 3.1. Seven days of isolation leads to increased social space in both males and females.

A-B: Average number of flies within 4 body lengths (± s.e.m.) in males (A) and females (B) after 2, 4 or 7 days of isolation. A. Male flies had fewer flies within 4 body lengths after 2, 4 and 7 days of isolation (Two-way ANOVA and Sidak post hoc – Effect of isolation: $F_{1,46} = 22.01, p < 0.0001$; Effect of days of isolation: $F_{2,46} = 2.412, p = 0.0109$). B. Females had fewer flies within 4 body lengths after isolation and decreased further with increasing days of isolation (Two-way ANOVA - Effect of Isolation: $F_{1,47} = 6.653, p = 0.0131$; Effect of days of isolation: $F_{2,47} = 3.687, p = 0.0367$). n=7-12 chambers of 12-17 flies each. GH: Group Housed. SH: Single Housed. Adapted from Yost et al., under revision.
3.1.3 Recovery from social isolation occurs in 3 days of group housing

As recovery from isolation has been noted in various organisms, including *D. melanogaster* (see Introduction), I tested whether social space could also be recovered in flies that were group housed following isolation. Starting with 2 days of group housing following isolation and I found that males were not significantly different in the number of flies within 4 body lengths between group housed and recovery treatments, indicating they had recovered their social space. However, the data from females still showed a lower number of flies within 4 body lengths in the recovery compared to group housed treatments (Figure 3.2A, *p* = 0.0189). Females had not fully recovered after two days of group housing post isolation, so I tested social space in flies that had three days of group housing after seven days of isolation. Again, the data showed that males were not significantly different in their number of flies within 4 body lengths (Figure 3.2B). However, females now showed no difference between the recovery and group housed treatments, indicating that they too can recover from isolation, but it takes longer than males (Figure 3.2B). Thus, a period of three days of group housing following seven days of isolation was used for all other experiments investigating recovery.
Figure 3.2. Flies recover from isolation after 3 days of group housing.

**A-B:** Average number of flies within 4 body lengths (± s.e.m.) in males and females after 2 (A) and 3 (B) days of group housing (recovery) following 7 days of isolation. **A.** Males showed no difference in number of flies within 4 body lengths after 2 days of group housing. However, females had fewer flies within 4 body lengths comparing group housed to recovery treatments (Welch’s t-test: $t_{15.13} = 2.626, p = 0.0189$). **B.** Data from males and females were not significantly different in the number of flies within 4 body lengths comparing group housed and recovery flies (One-way ANOVA: $F_{3,32} = 2.649, p = 0.0656$). n=7-12 chambers of 12-17 flies each. GH: Group Housed. REC: Recovery. Adapted from Yost *et al.*, under revision.
3.2 Neuroligin mutant characterization

3.2.1 Nlg3 protein abundance is not altered in mutants or with age

To begin to understand the modulation of social space by nlg3, I needed to characterize Nlg3 protein abundance in the mutants. Western blot analysis revealed Nlg3 protein abundance in three nlg3 mutants: a deficiency line (thereafter nlg3\textsuperscript{Def1}), a line with a P-element insertion into the fourth intron with stop codons in the sense and anti-sense direction (nlg3\textsuperscript{L04}) and a line with a P-element insertion into the regulatory region of the gene (nlg3\textsuperscript{GS32}; Figure 2.1). As previously reported (Xing et al., 2014), I also detected two bands, representing full-length protein (Nlg3-FL) at ~130 kDa and a short isoform of the protein (Nlg3-S; Figure 3.3A,B) at ~90 kDa. Nlg3-S arises from cleavage of the full-length protein after translation (Wu et al., 2018). Nlg3 protein levels were assessed in mutants at two different ages (3–4 days old compared to 7–10 days old) and in both sexes. As expected, the loss-of-function nlg3\textsuperscript{Def1} line displayed no detectable Nlg3 protein (Figure 3.3A, B).

In males, there was significantly less Nlg3-S than Nlg3-FL in the control line Cs (Figure 3.3C; effect of protein isoform: $p = 0.0072$). However, no effects of moderate aging were detected on either Nlg3 isoforms in Cs flies. In contrast, the opposite effect was observed for the male mutants nlg3\textsuperscript{L04} and nlg3\textsuperscript{GS32}. Nlg3-FL and Nlg3-S both had significantly reduced protein levels at 7-10 days old compared to 3-4 days old (effect of age: $p = 0.0344$). In Cs females, there was no significant difference in protein levels between Nlg3-FL and Nlg3-S ($p = 0.7403$), no significant effect of age ($p = 0.0727$), and finally, no expression differences within the mutants (Figure 3.3D; $p = 0.3587$). Complete Western blots with molecular weight markers can be found in Appendix I.

Finally, I determined protein abundance in Cs males and females with aging (up to 50 days old) to determine if Nlg3 protein levels change with time. Data showed reduced Nlg3 levels with increasing age (Appendix J) in both males ($p = 0.0071$) and females ($p = 0.0334$). Nlg3-S levels were lower than Nlg-FL in males ($p < 0.0001$) and females ($p = 0.0003$).
3.2.2 Social space in mutants of \textit{nlg3}

J. W. Robinson performed social space assays on each \textit{nlg3} mutant at 3-4 and 7-10 days old, and results are published in Yost & Robinson \textit{et al.} (2020). The data revealed that in male \textit{nlg3}^{Def1} flies, social space was increased at both ages, while females had increased social space only at 7-10 days old (Yost & Robinson \textit{et al.}, 2020). These results indicate that Nlg3 affects social space in a sexually dimorphic manner. In contrast, regardless of age or sex, there was no significant effect of \textit{nlg3}^{L04} or \textit{nlg3}^{GS32} on social space (Yost & Robinson \textit{et al.}, 2020). Since there was no effect on social space, the subsequent data collection and analysis will focus only on \textit{nlg3}^{Def1} flies.
Figure 3.3. Protein abundance in mutants and with age.

Representative Western blots for males (A) and females (B) showing anti-Nlg3 immunoreactivity in Cs flies and mutants at 3–4 and 7–10 days old for both protein isoforms. C & D. Densitometric analysis showing mean protein abundance (± s.e.m.) in males (C) and females (D) for Cs, nlg3L04, and nlg3GS32 flies. C. No significant differences were seen in protein abundance for Cs or nlg3 mutants in males. Nlg3-FL was more abundant than Nlg3-S in Cs flies (Three-way ANOVA—effect of protein isoform: $F_{1,18} = 9.196$, $p = 0.0072$) and older flies had less Nlg3 protein than younger flies in the nlg3 mutants (Three-way ANOVA—effect of age: $F_{1,18}=5.236$, $p = 0.0344$). D. In females, there was no effect of genotype or protein isoform, although age was close but not quite a significant decrease in Nlg3 protein levels ($p = 0.0727$). All treatments are displayed as relative abundance to Nlg3-FL in Cs at 3–4 days old. Open boxes represent 3–4 day old flies and horizontal lines represents 7–10 day old flies. n=4 blots replicated independently.

*p < 0.05, **p < 0.01. Adapted from Yost and Robinson et al. (2020).
3.2.3 Longevity is decreased in \textit{nlg}^{3\text{Def1}} flies

Next, I investigated if \textit{nlg}^{3\text{Def1}} flies had altered lifespan. Mutant males showed a decrease in overall survival compared to Cs (Figure 3.4A; \( p < 0.0001 \)) and a decrease in mean survival (Figure 3.4B; \( p = 0.0035 \)). Similar results were obtained for females, showing a decreased lifespan (Figure 3.4C; \( p < 0.0001 \)) and decreased mean survival (Figure 3.4D; \( p = 0.0369 \)). An independent repeat of this experiment is presented in Appendix K and shows similar results of decreased overall survival.
Figure 3.4. Lifespan is decreased in \textit{nlg3}^{Def1} flies.

\textbf{A,C}: Survival curves; \textbf{B,D}: Mean survival, or average age for which 50\% of the population is still alive. \textbf{A-B}. Males showed a decrease in the percent of flies alive (A) compared to those in the Cs line (Mantel-Cox: $\chi^2(2) = 222.7; p < 0.0001$) and a decrease in mean survival (B; Welsh’s t-test: $t_{5,132} = 5.101, p = 0.0035$). \textbf{C-D}. Females also showed a decrease in the percent of flies alive (C) compared to the Cs line (Mantel-Cox: $\chi^2(2) = 208.0; p < 0.0001$) and a decrease in mean survival (D; Welsh’s t-test: $t_{4,139} = 5.101, p = 0.0369$). \textit{n}=4 trials of 20 flies for all genotypes and sexes.
3.2.4 *nlg3* alters startle-induced locomotor activity

The loss of function of *nlg3* was reported to reduce locomotor activity (Wu *et al*., 2018; Xing *et al*., 2014) and I wanted to confirm these findings and validate our loss-of-function mutant. To address this, a climbing assay was used to investigate startle-induced activity (a form of escape response). Results showed age-related reductions in climbing in Cs flies, as previously reported (Brenman-Suttner *et al*., 2019; Simon *et al*., 2006). This reduction occurred in both males and females (Figure 3.5A, B; $p = 0.0056$ for males; $p = 0.0014$ for females). In contrast, male *nlg3*Def1 flies at 3-4 days old had reduced climbing compared to Cs flies of the same age (Figure 3.5A; effect of genotype: $p = 0.0428$). However, no difference in climbing ability between Cs and *nlg3*Def1 male flies was observed at 7–10 days old (effect of age: $p = 0.0045$; interaction between age and genotype: $p = 0.0365$). Reduced climbing was seen in female *nlg3*Def1 flies, at both ages (Figure 3.5B; effect of genotype: $p = 0.0075$; effect of age: $p < 0.0001$).
Figure 3.5. Locomotor activity is altered in nlg3Defl flies.

A-B. Startle-induced climbing represented by the mean percent (± s.e.m.) of flies reaching the top vial. A. nlg3Defl males compared to those of the Cs line had fewer flies reaching the top vial at 3–4 days old (Two-way ANOVA – effect of genotype: $F_{1,31} = 4.464$, $p = 0.0428$). However, this was not the case at 7–10 days old, where there was no significant difference between Cs and nlg3Defl. Cs males also had fewer flies reaching the top vial at 7–10 days old compared to 3–4 days old, but this difference was not observed in nlg3Defl flies (two-way ANOVA – effect of age $F_{1,31} = 9.391$, $p = 0.0045$; interaction between age and genotype $F_{1,31} = 4.780$, $p = 0.0365$). B. nlg3Defl females had fewer flies than Cs reaching the top vial at 3-4 days old (Two-way ANOVA – effect of genotype: $F_{1,30} = 8.238$, $p = 0.0075$); however, flies at 7–10 days old had a lower percentage reaching the top vial compared to 3–4 day-old flies (Two-way ANOVA – effect of age: $F_{1,30} = 23.38$, $p < 0.0001$). n=9 trials of 40 flies per trial. Open boxes represent 3–4 day-old flies and horizontal lines represents 7–10 day-old flies. Data published in Yost and Robinson et al., 2020.
3.3 Modulation of social space by nlg3

3.3.1 nlg3 is required for a typical response to the social environment

The social environment affects Drosophila social behaviour, including social space, and in addition, nlg3 transcript expression increased after social experience (Ellis and Carney, 2011). Could nlg3 be part of a pathway responsible for the environmental modulation of social space? When group housed, male nlg3^{Defl} flies showed fewer flies within 4 body lengths compared to Cs flies (Figure 3.6A; \( p = 0.0437 \)). Following single housing of Cs and nlg3^{Defl} male flies, results showed Cs flies (as expected) had fewer flies within 4 body lengths (\( p < 0.0001 \)). This decrease, however, was not as pronounced for nlg3^{Defl} flies (effect of isolation: \( p < 0.0001 \); interaction between genotype and isolation: \( p = 0.0331 \)) since there was no significant difference between group and single housed nlg3^{Defl} flies (\( p = 0.1213 \)). A similar result was observed in females (Figure 3.6B; isolation effect: \( p = 0.0006 \); interaction: \( p = 0.0309 \)).

Western blot analysis was used to determine whether Nlg3 proteins levels varied in response to the environment. Results show that Cs flies display no significant differences in Nlg3 protein abundance after isolation (Figure 3.7) in males (\( p = 0.9880 \)) or females (\( p = 0.3415 \)). Thus, while the response to social isolation is altered by the absence of nlg3 expression, Nlg3 protein levels in control flies did not vary in response to social isolation. Furthermore, group housed Cs males and females showed there was a sex difference in Nlg3 abundance with females having lower levels of Nlg3 than males (Appendix L; \( p = 0.001 \)).


Figure 3.6. Reduced response to social isolation in nlg3Def1 males and females.

A-B. Average number of flies within 4 body lengths (± s.e.m.) in Cs and nlg3Def1 lines. A. Single housed Cs and nlg3Def1 had a lower number of flies within 4 body lengths after being single housed compared to group housed (Two-way ANOVA and Holm-Sidak post hoc – effect of isolation: $F_{1,32} = 24.56, p < 0.0001$). However, SH nlg3Def1 compared to Cs flies did not show a dramatic decrease compared to Cs (Two-way ANOVA single housed compared to group housed – interaction between isolation and genotype: $F_{1,32} = 4.96, p = 0.0331$). B. Similar results were seen with nlg3Def1 females (Two-way ANOVA – effect of isolation: $F_{1,28} = 15.10, p = 0.0006$; Two-way ANOVA—interaction between isolation and genotype: $F_{1,28} = 5.166, p = 0.0309$). n=9 trials with 12–17 flies per trial. Data published in Yost & Robinson et al. (2020).
Figure 3.7. No significant changes were seen in Nlg3 protein levels after isolation.

A. Western blot for Cs males and females that were GH or SH. The protein levels for males are displayed relative to GH Nlg3-FL and the protein levels in females are relative to GH Nlg3-FL females. B. Mean Nlg3 protein levels (± s.e.m.) in male and female Cs flies. Nlg3 protein levels of either isoform did not change after isolation in the single housed flies compared to GH. GH: group housed, SH: single housed. All samples are normalized to total protein. n=4. Data published in Yost & Robinson et al. (2020).
3.3.2 Nlg3 is not required for recovery from isolation

Since *nlg3* is important for a typical social space response to isolation, I investigated whether it was required for the recovery from social isolation. Recovery was tested and male Cs and *nlg3*\textsuperscript{Def1} flies showed a similar number of flies within 4 body lengths regardless of whether they were group housed or recovered. These results would indicate that recovery from isolation had occurred and *nlg3* is not required for recovery to occur. Although *nlg3*\textsuperscript{Def1} flies have a slightly fewer number of flies within 4 body lengths than Cs as previously shown (Figure 3.8A; effect of genotype: $p = 0.0342$). Females, showed similar results to males in that Cs and *nlg3*\textsuperscript{Def1} flies did not differ in the number of flies within 4 body lengths (Figure 3.8B; effect of recovery: $p = 0.1996$).
Figure 3.8. *nlg3*<sup>Def1</sup> flies recover after 3 days of group housing following isolation.

A-B: Average number of male and female flies within 4 body lengths (± s.e.m) in Cs and *nlg3*<sup>Def1</sup> flies that were group housed or recovery flies. A. Cs and *nlg3*<sup>Def1</sup> males were not significantly different in number of flies within 4 body lengths in group housed and recovery treatments, but *nlg3*<sup>Def1</sup> males had fewer flies within 4 body lengths compared to Cs in group housed and recovered treatments (Two-way ANOVA – Effect of Genotype: \(F_{1,25} = 5.019, p = 0.0342\)). B. Cs and *nlg3*<sup>Def1</sup> females also showed no differences in the number of flies within 4 body lengths whether they were group housed or recovery flies (Two-way ANOVA – Effect of Recovery: \(F_{1,29} = 1.723, p = 0.1996\)). n=7-9 with 12–17 flies per trial. GH: Group Housed, REC: Recovery from isolation. Under revision in Yost *et al.* Front Neural Circuits.
3.4 Dopaminergic modulation of social space

3.4.1 Dopamine is required for a response to isolation and decreases after isolation in a sex-specific manner

Dopamine is important not only for social space in both sexes (Fernandez et al., 2017) but also for the response to social isolation in males (Xie et al., 2018). To recapitulate these results an RNAi approach was used to knock down tyrosine hydroxylase (th), the rate limiting enzyme for dopamine biosynthesis. This gene, referred to as UAS-THmiR-G in Xie et al. (2018), was co-expressed with a th-Gal4 driver and the fly line expressing the th-RNAi in th cells is denoted as th>ThmiR-G.

In th>ThmiR-G males, there was no effect of social experience, but there was a significant reduction in the number of flies within 4 body lengths after isolation in th-Gal4/+ and UAS-ThmiR-G/+ flies (Figure 3.9A; Effect of isolation: \( p < 0.0001 \); Genotype and isolation interaction: \( p = 0.0009 \)). No differences were seen in any genotype between group housed and recovery treatments (Figure 3.9B).

In females, there was a decrease in the number of flies within 4 body lengths for all three genotypes after isolation and a decrease in th>ThmiR-G flies compared to their genetic controls (Figure 3.9C; Effect of genotype: \( p = 0.0265 \); Effect of isolation: \( p < 0.0001 \)). Female th>ThmiR-G recovery flies also showed this decreased number; however, genotypes were not different when comparing group housed to recovery treatments (Figure 3.9D; Effect of genotype: \( p = 0.0112 \)). An independent repeat of this experiment and similar results using a second RNAi against th (UAS-ThmiR-C) is shown in Appendix M.
Figure 3.9. Dopamine is important for a response to social isolation in males but not females.

A-B. Average number of flies within 4 body lengths (± s.e.m.) in isolated (A) and recovered (B) male flies. A. Male th>ThmiR-G flies were not significantly different in isolated vs group housed flies. However, th-Gal4/+ flies and UAS-th-miR-G/+ males had fewer flies within 4 body lengths if isolated (Two-way ANOVA – Effect of isolation: F_{1,43} = 19.13, p < 0.0001; Interaction between isolation and genotype: F_{2,43} = 8.254, p = 0.0009). B. Male recovery flies of all genotypes were not different in the number of flies within 4 body lengths.
lengths compared to group housed males. **C-D.** Average number of flies within 4 body lengths (± s.e.m.) in isolated (C) and recovered (D) female flies. **C.** All three genotypes showed a decrease in the number of flies within 4 body lengths after isolation, and *th>*ThmiR-G had the lowest number of flies within 4 body lengths compared to their genetic controls (Two-way ANOVA – Effect of isolation: F_{1,37} = 21.91, *p* < 0.0001; Effect of genotype: F_{2,37} = 4.009, *p* = 0.0265). **D.** Despite all three genotypes showing similar results in the number of flies within 4 body lengths between group housed vs. recovery treatments, the *th>*ThmiR-G females had the fewest number of flies within 4 body lengths compared to the controls (Two-way ANOVA – Effect of genotype: F_{2,35} = 5.124, *p* = 0.0112). n=5-9 with 12–17 flies per trial. GH: Group Housed. SH: Single Housed. REC: Recovery. Data submitted in Yost *et al.*, under revision.
3.4.2 Dopamine levels decrease in Cs males but not females

Liquid chromatography/mass spectrometry (LC/MS) was used to quantify changes in dopamine levels after isolation and recovery. After isolation, Cs males had reduced dopamine levels compared to group housed flies \((p = 0.0019; \text{Figure 3.10})\) but returned to a similar level as group housed flies after recovery. Dopamine levels in females were not different in flies isolated compared to group housed (Figure 3.10). Taken together, these results indicate that dopamine is important for social space in response to isolation and recovery in males but not females, and that dopamine levels are influenced by previous social experience in a sex-specific manner.

3.4.3 Feeding 3-IT and L-DOPA

Since using RNAi was constitutively knocking down \(th\), an alternative method was to feed Cs and \(nlg^{3\text{Def}}\) flies chemical inhibitors/activators of the dopamine pathway. 3-IT, an inhibitor of \(th\), is expected to reduce dopamine levels, while feeding L-DOPA increases dopamine levels. There was no effect of feeding males or females 3-IT or L-DOPA on group and single housed flies. In addition, \(nlg^{3\text{Def}}\) males and females behaved differently from what I have reported in previous figures (Figure 3.11A,B). In this thesis, male and female \(nlg^{3\text{Def}}\) flies had more flies within 4 body lengths as opposed to less flies within 4 body lengths. This was also true in male and females that were recovered after isolation (Figure 3.12A,B). Due to this strange phenotype and variability in the data, no conclusions can be made about the effects of feeding on the \(nlg3\) mutant or response to isolation and recovery.
Figure 3.10. Dopamine levels in Cs males and females after isolation and recovery.

Dopamine levels (average pg/head ± s.e.m.) in Cs males decreased in isolated compared to group housed flies (Welch’s t-test: t_{6.271} = 5.119, p = 0.0019) but were not different between group housed and recovery flies (Welch’s t-test: t_{4.394} = 1.29, p = 0.2607). Dopamine levels in Cs females did not decrease after isolation (Welch’s t-test: t_{3.7} = 0.8022, p = 0.4704), or in recovery flies compared to group housed (Welch’s t-test: t_{5.929} = 0.3521, p = 0.7369). n=4. GH: Group Housed. SH: Single Housed. REC: Recovery. Submitted in Yost et al., Front Neural Circuits.
Isolation
Males

# of flies within 4 body lengths

Cs (control)  nlg3
Def1
GH  SH

L-DOPA 4mM  L-DOPA 0mM  3-IT 0mM  3-IT 30mM

B

Isolation
Females

# of flies within 4 body lengths

Cs (control)  nlg3
Def1
GH  SH

L-DOPA 4mM  L-DOPA 0mM  3-IT 0mM  3-IT 30mM
Figure 3.11. Cs and nlg3Def1 flies group housed or isolated and fed 3-IT and L-DOPA. Average number of flies within 4 body lengths (± s.e.m.) in males (A) and females (B) group or single housed showing social space in either sex was not significantly affected by feeding. However, nlg3Def1 flies had more flies within 4 body lengths than Cs when group housed. n=5-6 with 12–17 flies per trial. GH: Group Housed. SH: Single Housed.
Recovery

A

Males

# of flies within 4 body lengths

Cs (control)  nlg3^Def1

L-DOPA 4mM  L-DOPA 0mM  3-IT 0mM  3-IT 30mM

GH  Rec

GH  Rec

B

Females

# of flies within 4 body lengths

Cs (control)  nlg3^Def1

L-DOPA 4mM  L-DOPA 0mM  3-IT 0mM  3-IT 30mM

GH  Rec

GH  Rec
Figure 3.12. Cs and \textit{nlg3}\textsuperscript{Dcf1} flies group housed and recovery fed 3-IT and L-DOPA. Average number of flies within 4 body lengths (± s.e.m.) in males (A) and females (B) group housed or recovered showing an effect of feeding on social space, but \textit{nlg3}\textsuperscript{Dcf1} flies had more flies within 4 body lengths than Cs when group housed and recovered. n=5-6 with 12–17 flies per trial. GH: Group Housed. REC: Recovery.
3.4.4  *nlg3*<sup>Def1</sup> flies have less dopamine, but its levels are unchanged after isolation.

Previous work with other organisms has demonstrated that Nlg3 and dopamine are part of similar pathway and to determine if this interaction was occurring between dopamine and *nlg3* in Drosophila too, I tested dopamine levels in group housed Cs and *nlg3*<sup>Def1</sup> flies. Dopamine levels in males and females were decreased in *nlg3*<sup>Def1</sup> compared to Cs flies and females had lower dopamine levels compared to males (Figure 3.13A; Effect of sex: *p* = 0.0428; Effect of genotype: *p* = 0.0043). However, dopamine levels were not different in *nlg3*<sup>Def1</sup> group housed, isolated or recovery males and females (Figure 3.13B; Effect of social experience: *p* = 0.2922), indicating that dopamine levels are not responding to isolation or recovery in the absence of Nlg3.
Figure 3.13. \textit{nlg3\textsuperscript{Def1}} flies have less dopamine, and those levels are not altered by isolation or recovery.

A. Dopamine levels (average pg/head ± s.e.m.) in group housed Cs and \textit{nlg3\textsuperscript{Def1}} males (left) and females (right) show \textit{nlg3\textsuperscript{Def1}} flies had less dopamine compared to Cs, with females showing a lower amount of dopamine overall (Two-way ANOVA, Effect of genotype: $F_{1,7} = 17.21$, $p = 0.0043$ and Effect of sex: $F_{1,7} = 6.107$, $p = 0.0428$). B. Dopamine levels (pg/head) in male and female group housed, isolated and recovery \textit{nlg3\textsuperscript{Def1}} flies are unchanged after isolation or recovery (Two-way ANOVA – Effect of social experience: $p = 0.2922$). n=3-6. GH: Group Housed. SH: Single Housed. REC: Recovery from social isolation. Submitted in Yost \textit{et al.}, \textit{Front Neural Circuits}.
3.5 Screening for post-synaptic components required for social space and response to isolation

3.5.1 Designing the screen and picking candidates

I have identified that dopamine shares a link with Nlg3, however, other proteins that are part of the post-synaptic neuron are likely involved in social space and part of a pathway downstream of Nlg3. Specifically, I wanted to determine whether other post-synaptic proteins were required for a response to social isolation and if they interact or share a pathway with Nlg3. The goal is to identify other players, adding to my model for the cellular mechanisms downstream of Nlg3.

I conducted a small-scale genetic screen using RNAi for different candidates. The candidates were selected based on one the following criteria: (1) they have been published as being important for social behaviour in animal models and are expressed in the central nervous system, (2) they are candidate/risk genes for neuropsychiatric disorders in human or (3) the encoded proteins have physical connections or genetic interactions with proteins known to affect social behaviour.

3.5.2 Setting up the sociability assay

It was decided to set up and test candidates using a new assay in the lab. The sociability assay is a behavioural test that quantifies the tendency for flies to engage in non-aggressive group activities (Scott et al., 2018). The assay was created by Dr. Reuven Dukas, McMaster University (Scott et al., 2018) and our collaboration suggests that social space and sociability are correlated (Yost & Robinson et al., 2020).

The sociability assay is more amenable for this type of screen because more transgenic lines can be tested each day and a faster data analysis can take place. A first step was to purchase the materials and make the sociability chambers (pictured in Appendix NA). Making the chambers was accomplished with the help of the Department of Earth Sciences materials lab.
Next, Cs and \textit{nlg3}^{Defl} flies were tested in the sociability assay, where flies were allowed to roam for up to two hours and photographs were taken every 30 minutes for comparison. The data was very variable within genotypes and treatments over the two-hour testing time (Appendix NB, C) and because of this variability and time constraints for data collection, it was decided to use the well-established social space assay for the screen. Toward this end, one RNAi line per candidate gene was tested in males and females, and as this is a quick screen, only 1-3 replicates for each RNAi line was tested.

3.5.3 Summary of screen results

Each candidate had RNAi expressed pan-neuronally using \textit{elav-Gal4}. I tested 28 candidates including different receptors, scaffold and adaptor proteins, and other candidates known to affect social behaviour were tested. Figure 3.14 is a summary of each category of candidate genes, whether those candidates are important for social space in a group housed environment, and whether there was a presence of a response to social isolation. A typical response to social isolation (\textit{i.e.} increased social space) would indicate the candidate may not be required for a response to the social environment. Of the candidates tested, some are important for modulating social space in a group housed environment, while fewer candidates are important for a response to isolation. Interestingly, a few candidates did not affect social space nor are they required for a response to isolation, mainly the \textit{ndmds} and \textit{yellow} genes. Each candidate will need to be tested with more independent repeats and additional genetic controls to avoid false positives from the screen.
Figure 3.14. Summary of screen data.

Candidates outlined in red are important for social space in a group housed environment and those written in green indicates they are important for a response to social isolation. This summary includes data from both males and females. Created with BioRender.com.
3.5.4 Example data using RNAi against GluR1A

As an example, Figure 3.15 shows the results for one candidate, an RNAi against the gene *gluR1a*, which is an excitatory glutamate receptor. Males had fewer flies within 4 body lengths in single housed compared to group housed *elav-Gal4/+* (Figure 3.15A; \( p = 0.0435 \)) and \( elav>UAS-gluR1a \) RNAi flies (\( p = 0.0165 \)). Males had more flies within 4 body lengths in group housed \( elav>UAS-gluR1a \) RNAi flies compared to GH *elav-Gal4/+* flies (\( p = 0.0382 \)). These results would suggest that *gluR1a* is required for social space in males, but it is not necessary for a response to social isolation. In females, there were more flies within 4 body lengths in group housed \( elav>UAS-gluR1a \) RNAi flies compared to group housed *elav-Gal4/+* flies (Figure 3.15B; \( p < 0.0001 \)) but had no difference between group and single housed \( elav>UAS-gluR1a \) RNAi flies (\( p = 0.8761 \)). These results with females would suggest *gluR1a* is required for both social space in a grouped environment and a response to social isolation.

A summary of each candidate tested, number of replicates and results can be found in Table 3.1 and a graph of each candidate tested is found in Appendix O to Appendix T.
Figure 3.15. GluR1A is required for social space in males and females but is only required for a response to isolation in females.

Average number of flies within 4 body lengths (± s.e.m.) for males (A) and females (B) in flies expressing RNAi against gluR1a. A. Males flies had less flies within 4 body lengths in SH compared to GH *elav-Gal4/+* (Two-way ANOVA and Tukey post hoc: *p* = 0.0435) and *elav-Gal4>UAS-gluR1A RNAi* (Two-way ANOVA and Tukey post hoc: *p* = 0.0165). Similarly, males had more flies within 4 body lengths in GH *elav-Gal4>UAS-gluR1A RNAi* compared to GH *elav-Gal4/+* (Two-way ANOVA and Tukey post hoc: *p* = 0.0382).

B. With females, there were more flies within 4 body lengths in GH *elav-Gal4>UAS-gluR1A RNAi* compared to GH *elav-Gal4/+* (Two-way ANOVA and Tukey post hoc: *p* < 0.0001), but had no differences were seen between GH and SH *elav-Gal4>UAS-gluR1A RNAi* (Two-way ANOVA and Tukey post hoc: *p* = 0.8761). n=2 with 12–17 flies per trial for all treatments. GH: Group Housed. SH: Single Housed.
Table 3.1. Screen results for all candidates tested.

Results warranting further testing in bold. Arrows represent an increase or decrease in social space. The increase in the number of flies within 4 body lengths corresponds to a decrease in social space.

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<td>Group Housed (RNAi vs. Control)</td>
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Chapter 4

4 Discussion

In this discussion I will summarize some of the key findings of this work and my contributions to the field. I have proposed a possible model for the interaction of dopamine and Nlg3 contributing to social space.

4.1 Summary of key findings

Here I report that social isolation alters one measure of Drosophila social behaviour, social space. Social space was increased after seven days of isolation in males and females but could be recovered after three days of group housing.

To better understand the contribution of a homolog of an autism-related gene nlg3 on social space, I completed a characterization of the mutant by examining Nlg3 protein levels in the brain, longevity, and startle-induced climbing, and social space. Protein levels were absent in the deficiency line, and both longevity and climbing were reduced. Next, I provided evidence that nlg3 is required for social spacing and a typical response to social isolation, but not its recovery. While nlg3 is required for a response to isolation, proteins levels did not change after isolation.

I have also provided evidence that the neuromodulator dopamine is required for social space in response to isolation and is required for recovery in males, but not females. Dopamine levels were reduced after isolation in males but recovered after group housing. Dopamine levels were also reduced in the nlg3 loss of function mutant and dopamine levels did not respond to isolation in the absence of nlg3. This data suggests a link between Nlg3 and dopamine in modulating social space.

4.2 Effects of isolation on social space and other social behaviour

My first objective was to determine the effect of isolation on social space and determine if social space could be recovered after group housing following isolation. How long do flies need to be isolated to see an effect of isolation on social space? Previous studies have used
seven days, representing long-term isolation (Li et al., 2021; Simon et al., 2012). In males, increased social space was observed after only two days of isolation, while females appeared to be more resilient and were less effected in the early days of isolation. Seven days of isolation was required for females to display increased social space. Social space in both sexes appears to be correlated with sociability as isolation also resulted in decreased sociability (Scott et al., 2018; Yost et al., under revision). Together, this work was the first evidence for the recovery of isolation on social space in Drosophila.

Tested two days of group housing after isolation revealed that males recovered their social space, but females did not recover until three days of group housing. Thus, the effects of isolation on social space seem to be more plastic in males than females because females take longer to be affected by isolation and also take longer to recover. While seven days of isolation is a commonly studied length of time (Bentzur et al., 2020; Simon et al., 2012; Xie et al., 2018), the effects of isolation on other behaviours varies. For example, three days of isolation is sufficient to increase aggressive behaviour (Ueda and Kidokoro, 2002), while sleep disturbances are not observed until 5 to 7 days of isolation (Li et al., 2021). Differences in the molecular mechanisms driving different behaviours that are altered with isolation could be a cause for the difference in time required for the effects of isolation to be observed (Arzate-Mejia et al., 2020).

4.3 Characterization of nlg3 mutants

4.3.1 Protein abundance in nlg3 mutants is not decreased as expected

To better understand the role of nlg3 in social space, I first needed to determine the protein abundance of Nlg3 in the mutant flies before conducting any other experiments. The Western blot analysis revealed that the nlg3Def1 flies had no Nlg3 and acted as a confirmation of the deficiency. However, mutants with P-element insertions (nlg3LO4 and nlg3GS32), had similar protein levels to the control. Nlg3LO4 incorporates a 3-4 kb insertion within the fourth intron of the gene. The insertion contains stop codons along with splice acceptors in both the sense and antisense directions. Therefore, I expected the protein to be truncated, which does not seem to be the case. Nlg3GS32 contains a 5.2 kb insertion within the regulatory region of the nlg3. Due to such a large insertion, disruption of gene
expression would be expected (Toba et al., 1999). Protein abundance, however, was similar to the control in this mutant as well. The effect of these mutations beyond protein abundance needs to be investigated because protein function and/or cellular localization could not be assessed in this study. Mice models with missense mutations in \textit{NLG3} have shown reduced ability for protein processing, ER protein export and protein dynamics, indicating the spatial and/or temporal expression could be altered (Comoletti et al., 2004; Etherton et al., 2011; Sudhof, 2018). In addition, a PCR should be run to confirm the presence of the insertions in the genome.

4.3.2 Longevity is reduced in \textit{nlg3}^{Def1} flies

I demonstrated that \textit{nlg3}^{Def1} flies had reduced longevity and repeating these experiments (Appendix K) gave similar results. I feel that this experiment could be repeated one more time to confirm the effects of Nlg3 in longevity, as well as testing the effect of overexpression of the cDNA in \textit{nlg3} expressing neurons. Would overexpression have the opposite effect and increase longevity? This is first study to examine the effect of \textit{nlg3} in longevity.

4.3.3 Reduced locomotion in \textit{nlg3}^{Def1} flies

Given the role of Nlg3 in locomotion (Wu et al., 2018; Xing et al., 2014), I had expected to observe some reduction in startle-induced locomotion. Climbing was assessed at two different ages (3-4 and 7-10 days old) as some behaviours begin to decline with age beginning at ~one week of age (Brenman-Suttner et al., 2019; Simon et al., 2006). At 3-4 days of age, male flies had decreased climbing ability compared to the control (Cs). Cs had reduced climbing ability at an older age, as expected, but interestingly \textit{nlg3}^{Def1} flies did not experience this decrease with age. In support, we have shown that overexpressing \textit{nlg3} protects flies from a reduction in age-related climbing decline (Yost & Robinson et al., 2020). Moreover, I found that climbing was reduced in \textit{nlg3}^{Def1} females at a younger age than controls, but it too decreased at an older age.
4.4 Response of $nlg3^{Defl}$ flies to isolation and recovery

4.4.1 Neuroligin in required for a response to isolation

Social isolation is known to affect social behaviours such as aggression and courtship (Ueda and Kidokoro, 2002; Ueda and Wu, 2009). Here, I demonstrated a role of $nlg3$ in a third social behaviour, social space. Group housed flies lacking $nlg3$ were further apart than the control in males and there was a similar result in females. This result replicates data collected by J. W. Robinson also published in Yost & Robinson et al. (2020). When flies were isolated, there was a significant increase in social space in the control line but only a slight non-significant increase in $nlg3^{Defl}$ flies. The effect of isolation was not as strong in flies with no $nlg3$. Taken together, this data demonstrates that $nlg3$ is important for social space and a gene-environment interaction is occurring on social space in isolated $nlg3^{Defl}$ flies.

4.4.2 Protein levels remained unchanged after isolation

Since $nlg3$ was required for a typical response to isolation, I wondered if Nlg3 protein levels could be responsible for changes in social space. As previously reported, $nlg3$ transcript levels are potentially upregulated after isolation (Ellis and Carney, 2011) and as shown in bees, qPCR data has demonstrated multiple $nlg$ transcript changes after isolation (Hewlett et al., 2018b). Interestingly, I found the protein levels in males and females was unchanged after isolation, thus contradicting the findings of Ellis & Carney (2011). Changes in transcript level may not have been predicative of protein levels since the Nlg3 protein seems stable with age (Appendix J). Instead of changing the relative amounts of protein, the effect of isolation could alter the subcellular localization of the protein, trafficking of synaptic components to the membrane altering plasticity, or synaptic remodeling could have occurred that will would not necessarily affect protein levels (Uchigashima et al., 2021). To address this, electron microscopy could be used to determine Nlg3 localization after isolation. Another possibility is that changes in Nlg3 protein abundance are occurring after isolation, but only in specific neurons and this change is not sensitive enough to be detected on Western blot.
4.4.3 Recovery from isolation occurs in the absence of *nlg3*

I found *nlg3* was required for a response to isolation, but it was not required for a recovery. Evidence suggests that *nlg* genes share redundant functions and may be able to compensate for each other. Furthermore, newer studies have provided insight into the complexity of Nlg protein processing and the capability of heterodimers consisting of different Nlg proteins (Uchigashima *et al.*, 2021). It is clear that more research is needed to better understand why *nlg3* in the fly was required for a response to isolation and not recovery. It is important to note that genotype effects social space, where *nlg3* 

4.5 Dopamine modulates social space in a sex-specific manner

4.5.1 Isolated males do not respond to isolation with reduced dopamine

Social space was examined in flies with reduced dopamine levels. Isolated male flies did not differ in social space compared to group housed flies which strongly suggests that dopamine is required in males for a proper response to isolation. Interestingly, Xie *et al.* (2018) reported that group housed male flies were further apart and isolated flies were closer to others when dopamine was reduced (Xie *et al.*, 2018). This is noteworthy because it is the opposite phenotype of what is normally observed with social space and response to isolation. Accordingly, the group housed flies acted as if they were isolated and the isolated acted as if group housed. My results did not show as strong of a phenotype as Xie *et al.* (2018), even when using the same fly lines. One key difference is the spacial expression of the *th* RNAi and therefore the location of reduced dopamine. I expressed the RNAi in all dopamine neurons expressing *th*, while Xie *et al.* (2018) targeted a subset of dopamine neurons. Taken together and supported by the work of Xie *et al.* (2018), my results clearly demonstrate the importance of dopamine signaling in males in the response to social environment.
4.5.2 Females do not require dopamine for a response to isolation

Here I show for the first time that female flies do not require dopamine for a response to isolation or for recovery to occur. As previously reported, males and females group housed have increased social space when dopamine levels are reduced (Fernandez et al., 2017). Therefore, in females, dopamine may not be neuromodulator involved in social space when responding to the environment in females suggesting that another neurotransmitter or neuromodulator is involved.

4.5.3 Dopamine levels are dynamic in response to isolation

Dopamine levels were measured to determine if they change in response to isolation. Results showed male, but not female flies, had reduced dopamine levels after isolation, but they recovered to levels like group housed flies. Reduced dopamine levels in males after isolation has been reported previously and were similar to my results (Ganguly-Fitzgerald et al., 2006). Also, Cs and nlg3^{Def1} flies showed a reduction in dopamine levels were in 9 and 12 day old flies regardless of social experience, consistent with age related decreases in dopamine previously reported by Neckameyer et al., (2000).

My work is the first to determine that females did not experience a change in dopamine levels in response to isolation, which is additional evidence to indicate dopamine is not required in females for this behavioural response to isolation.

4.5.4 Pharmacological manipulation of dopamine

A pharmacological approach to decrease and increase dopamine was employed to test social space. Cs and nlg3^{Def1} flies were fed 3-IT to decrease and L-DOPA to increase dopamine levels. There are some issues worth noting, but the data is inconclusive, and experiments need to be repeated. Firstly, in Cs group housed flies there was no difference in social space when flies were fed either chemical. These results are not consistent with what has been published (Fernandez et al., 2017). Secondly, results from this study with nlg3^{Def1} flies showed they were much closer to others even in group housed flies. This phenotype was opposite to what I found throughout this study. A few of theories will need to be addressed regarding this opposite phenotype.
One avenue includes atmospheric pressure differences on experiment days. Atmospheric pressure alters Drosophila behaviour (Austin et al., 2014; Pellegrino et al., 2013), however I did not find a correlation between behaviour and atmospheric pressure (Appendix U). Thus, technical limitations and the opposing phenotype displayed by our mutant prevent me from making solid conclusions about this data set.

4.5.5 Dopamine levels are reduced in \textit{nlg3}^{\text{Def1}} flies and don’t respond to changes in the social environment

Dopamine levels in \textit{nlg3}^{\text{Def1}} flies were examined to determine if \textit{nlg3} and dopamine were employed in a pathway to regulate social space and a response to the environment. Dopamine was decreased in \textit{nlg3}^{\text{Def1}} flies compared to the Cs controls, but did not change after isolation or recovery, indicating that \textit{nlg3} was necessary for dopamine to respond to the environment in male flies. This data strongly implies that \textit{nlg3} is necessary for a proper level of dopamine in the male flies, and suggests that these two players, possibly working in a similar pathway, modulate social behaviour in response to isolation in these flies. However, whether there is a common pathway and whether \textit{nlg3} and dopamine are interacting at the synapse is not known and requires further research.

4.6 Working Model

My research, coupled with previous studies, has led me to propose a model for the role of \textit{nlg3} and dopamine in social space. Since flies require \textit{nlg3} for dopamine levels to change in response to the social environment, and because the protein levels of Nlg3 do not respond to social experience, Nlg3 could be required downstream of dopamine, possibly providing feedback regulation. Nevertheless, the role of \textit{nlg} in dopamine signaling, and their common influence on behaviour, including social behaviour was seen in mice (Bariselli et al., 2018; Rothwell et al., 2014), the northern swordtail fish (Wong and Cummings, 2014), and the worm \textit{C. elegans} (Izquierdo et al., 2013). Clearly these results and my study show that \textit{nlg3} and dopamine are linked but it is unclear about the relationship between \textit{nlg3} and dopamine. Whether they are part of the same synapses and/or directly involved in post-synaptic recruitment of dopamine receptors is not known in flies. However, in mice, there is evidence that dopamine and NLG2 are part of the same synapse, and that NLG mediates
the connection between dopaminergic neurons and other neurotransmitters to modulate neuron function (Uchigashima et al., 2016). Also, NLG3 and dopamine in mice are present in the same neurons and they regulate social behaviour in mice (Bariselli et al., 2018; Hornberg et al., 2020). More research is required to determine what regulates social space after isolation in female flies.

To begin assembling potential pathways involved in the determination of social space, we must compare the location of Nlg3 and the known dopaminergic neuron projections. Nlg3 protein levels are enriched in the mushroom bodies of the fly (Robinson, 2019) (Figure 4.1A in green). The mushroom bodies are a center for sensory integration, learning and memory (Heisenberg, 1998; Menzel, 2014) and receive many inputs from other brain regions into a specialized organization of neural networks (Aso et al., 2014; Heisenberg, 2003). Furthermore, the dendrites of mushroom body intrinsic neurons are located in the calyx, the most posterior part of the mushroom bodies that connects to the different lobes. The calyx is where Nlg3 is specifically expressed (Robinson, 2019) (Figure 4.1A,B) and since Nlg3 is localized in post-synaptic neurons, its function would be in dendrites.

Dopamine neurons are known to innervate different parts of the mushroom bodies. The PPL2ab cluster of dopamine neurons have been shown to innervate the calyx of the mushroom body (Mao, 2009), the same structure where Nlg3 is expressed (Figure 4.1B). Together, I predict that dopamine neurons synapsing onto mushroom body intrinsic neurons express Nlg3 and are part of the same synapse. However, this is just one scenario and others are likely to exist since isolation is affected by reduced dopamine in PPM2 neurons (Xie et al., 2018) do not innervate the calyx of the mushroom body. Dopamine could be further upstream in a neural pathway containing interneurons that instead synapse onto Nlg3 expressing neurons. What remains to be discovered is if dopamine and Nlg3 are part of the same synapse (Figure 4.1C) and what cellular or genetic pathways are occurring within the neuron and synapse.
Figure 4.1 Working model for social space determination in response to the environment.

A. Social isolation causes changes to social space and requires Nlg3 for a proper response. Nlg3 was found to be expressed in the calyx of the mushroom body (labeled in green).

B. Dopamine neurons (PPL2ab) innervate the calyx of the mushroom body where Nlg3 is
expressed. C. Possible interactions of Nlg3 and dopamine in the synapse, with downstream cellular and genetics pathways unknown. Double arrow indicates a possible interaction between Nlg3 and dopamine receptors in the synapse. VMAT: vesicular monoamine transporter; DAT: dopamine reuptake transporter; Nlg3: neuroligin3; DopR1/2: dopamine receptor 1/2; DopEcR: dopamine/ecdysone receptor.

4.7 Identifying cellular and molecular pathways downstream of Nlg3

To complete my third objective, I conducted a small targeted genetic screen using RNAi to identify post-synaptic proteins involved in social space and a response to isolation. The purpose of this screen was to identify post-synaptic proteins involved in social space because the downstream molecular pathways involving Nlg3 are unknown.

We have previously reported a decrease in sociability correlated with a decrease in social space seen after isolation (Yost et al., 2020). The sociability assay (Scott et al., 2018), developed by a collaborator, Ruven Dukas, could be adapted to use smaller chambers and fewer flies, allowing me to set this assay more candidates in the screen. However, there was high variability of the data obtained in this assay and therefore it was deemed problematic, and the screen was not performed using the sociability assay.

The screen using the social space assay did reveal that multiple candidates were required for social space, and some of them were required for isolation. Preliminary results from using RNAi against gluR1a showed males had increased social space in group housed flies but responded typically to social isolation. Females also had increased social space in group housed flies but had similar social space after isolation, indicating that gluR1a is important for a response to isolation in females but not males.

Nevertheless, gluR1a may be a starting point for identifying genetic and molecular mechanisms regulating social space in females. Moreover, this social space assay and screen could potentially identify downstream interactions with Nlg3. Nlgs has been shown to interact with proteins of the post-synaptic density (Sheng and Hoogenraad, 2007) and
Drosophila Nlg3 regulates glutamate receptor proteins localized to larval neuromuscular junctions (Xing et al., 2014). These preliminary results have provided the lab with multiple candidate genes to investigate for their role in regulating social space.
Chapter 5

5 Limitations, Future Directions & Conclusions

In this section I will first discuss some of the limitations of this study including methodology and experimental design. I will also provide suggestions for future directions and finally, I will end by restating the key findings of this work.

5.1 Limitations

5.1.1 Technical Limitations

5.1.1.1 Generation of a new Nlg3 antibody

Our original antibody used in this study was made and provided by our collaborator, Brian Mozer. After I completed the Western blots in the characterization of Nlg3, and J. Wesley Robinson set up immunochemistry with it, the stock of antibody had all been used. Since the antibody is not commercially available it needs to be custom made, and I took the lead on the production of a new polyclonal antibody.

The first antibody was produced in guinea pigs by ThermoFisher, the same host animal as the original stock. Thermofisher designed the antigen with the amino acid data I provided from NCBI. Once we received the antibody, I tested it with my original protocol but was unable to detect Nlg3. Multiple blots were tested with variations to the original protocol recommended by ThermoFisher, including different protocol adaptations included using transfer times (1 hour or overnight), different membranes (nitrocellulose and low-florescence PVDF), changes to the time of blocking (30 min, 2 hours, overnight), primary antibody incubations (1, 2, 4 hours at room temperature, overnight and 72 hours in the fridge), and altered levels of salts in the membrane washes. Despite these modifications, I was still not able to detect Nlg3.

Moving forward, a second antigen was designed by our lab. This antigen was produced in rabbits by BioBasic, with hope that more antibodies would be obtained. Again, this antibody did not bind to Nlg3 on a blot. I repeated the above protocol variations but was unsuccessful in detecting Nlg3. Finally, a third antibody was produced by BioBasic. This
antibody targeted the entire intracellular domain of Nlg3, the same as the original working antibody. Unfortunately, the same protocol variations as with the first two antibodies, I was unable to detect Nlg3. The lab will continue to move forward with the generation of monoclonal antibody, but at present there is no Western blots or immunocytochemistry data until the antibody is delivered. Overall, this was a huge impediment to addressing the question of whether Nlg3 and dopamine are part of a similar pathway. Immunochemistry with a Nlg3 antibody and a dopamine receptor antibody would have helped.

5.1.1.2 Issue with $nlg^{3\text{Def1}}$ change in phenotype

The observation that the phenotype of the $nlg^{3\text{Def1}}$ mutant completely reversed, from flies being further apart to flies being closer, was a huge surprise. This affected my interpretation of the largest experiment I conducted. The lab will continue to investigate this new phenotype. Of note, another graduate student in the lab, Abigail Bechard, found that an RNAi against $nlg3$ expressed in $nlg3$ neurons led to the same phenotype as the original one seen in $nlg^{3\text{Def1}}$ flies.

Several reasons could explain the difference observed. Lab members will need to test different conditions including parental density, food type and a possible genetic drift. High parental density leads to progeny that are much smaller in $nlg^{3\text{Def1}}$ flies than Cs which has been noted when flies are closer together. Controlling parental density restores the size of the $nlg^{3\text{Def1}}$ flies and could alter the behavioural phenotype. Another difference between the data sets are food types. While the recipe remained the same, the lab switched from first purchased ready-to-mix food from FisherScientific to purchasing ingredients and making the food manually. Possibly, the sourcing of ingredients is different and could be affecting behaviour in the $nlg3$ mutant. Social space of the $nlg^{3\text{Def1}}$ flies using the original food sourced will be tested again. Finally, reamplifying flies from our laboratory stocks will occur to ensure that genetic drift of individual fly populations has not occurred.
5.1.2 Limitations on the experiments

5.1.2.1 Social isolation

To generate isolated conditions, flies are kept group housed for two days prior to isolation to ensure they are not virgin flies. To confirm two days was sufficient, I tested the mating status of flies after two and four days of group housing. While there was no difference in the number of mated flies between the groups, the percent of flies mated was not 100%. This means that some flies used in the study could have been virgin flies, which is known to affect social space (Simon et al., 2012). I did not determine if all flies used in the group housed condition were 100% mated after 9 days of group housing, but even though flies have had access to mates, does not mean the flies will ever mate. In the wild, and potentially lab setting, Drosophila mating, at least in females, is estimated to never be 100% (Giardina et al., 2017).

5.1.2.2 Screening post-synaptic proteins important for social space

During the screen for post-synaptic proteins involved in social space, all RNAi-expressing lines were compared to the elav-Gal4/+ where the control allele was the genetic background used for the insertions. To decrease the number of lines tested, I omitted a genetic control. Usually, a UAS-RNAi/+ is also tested (as with the th-RNAi) to determine the effect of the insertion site, or a potentially low expressing RNAi in the absence of the driver. Due to time constraints, a maximum of three replicates was conducted for each candidate and only a single RNAi line was tested. For this reason, it is not possible to make definitive conclusions about the candidates, and those of interest will be tested again with complete genetic controls and more replicates to confirm the results are not false positives.

5.1.3 Limitations of the working model

Although I have provided a proposed working model for the regulation of social space, it represents only one possible pathway regulating this behaviour. However, at present, it provides a context to generate testable hypotheses.
5.1.4 Limitations on Drosophila as a model

While Drosophila has proven to be a powerful model organism, there are some limitations to the use of Drosophila in social behaviour. For instance, some behaviours in Drosophila may not be analogous in other organisms. Therefore, it is difficult to generalize the behavioural output displayed by flies. Thus, the mechanisms and molecular components of the underlying circuits may be conserved between organisms, but behaviour is not. However, we can use Drosophila behavioural output as a tool to understand these genetic and molecular regulators and manipulations that alter behaviour.

What also needs to be considered is the division between lab settings and nature. We have shown previously that Drosophila emit a stress odor or alarm cue that causes the avoidance of other flies (Yost et al., 2021). Flies become stressed with only gentle agitation, so most manipulation of Drosophila in the lab cause flies to emit the Drosophila stress odor which may affect olfactory perception and social responses (Yost et al., 2021).

Finally, Drosophila is not a very ecologically relevant model. Few studies have focused on Drosophila in the wild, however, more ecological assays and observations are now being reported (Baxter et al., 2019; Dukas, 2020; Giardina et al., 2017; Soto-Yeber et al., 2018). More studies like these are needed about naturally occurring social behaviours that are not limited by laboratory conditions.

5.2 Future Directions

5.2.1 Continuing social isolation research

Here I examined the effect of isolation on social space, however many behaviours are affected by isolation. Although, little is known about the molecular reasons for these changes, continuing to identify genes, neurotransmitters, and neural circuitry can elucidate genetic and molecular responses to isolation. Since humans with neuropsychiatric disorders are often socially withdrawn, studying homologous of candidate genes in Drosophila will undoubtedly shed new light on the molecular functions of those genes. Since females seem to be more resilient after isolation it would be interesting to determine why they are slower to respond to isolation and take longer to recover.
5.2.2 Neuroligin research

We have only begun to understand the role of neuroligins in behaviour. At the cellular level, the complexity of neuroligins in regulating synaptic function needs much more attention. What post-synaptic proteins are being trafficked by neuroligin and how this regulates the function and alters the synapse in a changing environment could offer some insight into the plasticity of behaviour in response to the social environment. More information on membrane recruitment, composition and excitability or inhibitory potential will result from using genetic approaches and molecular techniques (Xing et al., 2014). For physical interactions of Nlgs and other post-synaptic proteins, different models such as yeast can be utilized by expressing fly or human Nlgs and performing a split ubiquitin protein interactions assay (Johnsson and Varshavsky, 1994). Recently, a publication reported the cleavage of Drosophila Nlg3 in the intracellular region that alters transcript expression (Xie et al., 2023). This is the first report of intracellular cleavage of Drosophila Nlgs, a function not known to occur in mammals. This avenue needs to be explored as well.

Finally, redundancies of the neuroligins in behaviour need to be investigated. I describe here that nlg3 was required for a response to isolation but not the recovery from social isolation. Could the deletion of nlg3 be compensated for by other nlgs? Investigating transcript expression and protein levels of all neuroligins would be a good a start. Specifically, cataloguing all the nlg transcripts and proteins and determining if they are altered in nlg mutants. In addition, the overexpression of Nlgs in deficiency backgrounds could reveal which Nlgs can rescue mutant behavioural phenotypes of single mutants, including in social space.

5.2.3 Determining dopaminergic responses to the environment

Since dopamine appears to be responding to social isolation and regulating social space in males, it would be interesting to determine what specific dopaminergic neurons lead to a similar phenotype. Using the split Gal4 system where a DNA binding domain and activation domain can be expressed in specific tissues, precise dopaminergic neurons can be manipulated (Xie et al., 2018). This approach would allow investigators to further develop pathways regulating social space.
5.2.4 Research needed to advance the working model

As previously mentioned, it is still not known if Nlg3 and dopamine are part of the same synapse or if they are only part of a similar neural circuitry modulating social space. Once an antibody against Nlg3 is successfully generated, immunocytochemistry can be used to observe the co-localization of Nlg3 and dopamine receptors to establish if they are part of a similar location in the brain, and even the same synapse. Another method to visually determine if the proteins co-localize is to use GFP reconstitution across synaptic partners. With this technique, a split-GFP is fused to two proteins of choice and extracellularly expressed. Synaptic connections are revealed when GFP fragments reassemble at synaptic sites where both proteins of choice are within close proximity (Feinberg et al., 2008; Shearin et al., 2018). Together these strategies would strongly suggest that Nlg3 and the dopamine receptor are part of a similar location in the brain, and even the same synapse. Finally, a technique for transsynaptic circuit tracing called trans-Tango could be utilized to visualize the post-synaptic structures connected to dopamine neurons (Talay et al., 2017). Using a combination of these techniques would determine whether Nlg3 and dopamine are working together as my behavioural studies have shown in male flies.

5.2.5 Modulation of social space in females

Since dopamine was not important for a behavioural response to isolation in females, other mechanisms need to be investigated. Based on LC/MS data collected at the same time as dopamine (data not shown), the neuromodulator octopamine may be involved in a response to isolation in females. This modulator and candidate genes, including glur2e, dop1r1, dopEcr, and oct-tyrR, identified in my genetic screen should also be studied based on their social space in females. Some of these candidates have altered social space in females but not males.

5.3 Conclusions

In conclusion, this study examined a Drosophila social behaviour, social space, in response to social isolation. I provided evidence of the recovery from isolation in males and females. While previous, but limited, work has reported the recovery of behaviour in males, this was the first study to examine the recovery from isolation in females. My findings demonstrate
that social space is affected by isolation, but recovery can occur after a period of group housing. I then provide evidence that a homologue of a human autism candidate gene, \textit{nlg3}, is required for a typical response to isolation but is not required for recovery. Finally, I demonstrate that dopamine is required for a response to isolation in males but not females and that dopamine levels in males are decreased after isolation but are recovered. In males, I am proposing that dopamine may be modulating a Drosophila behavioural response to the environment. This was the first study to investigate dopamine changes after a recovery from isolation. In the end a model was proposed that includes Nlg3 and dopamine being part of a similar pathway modulating social space. Furthermore, this model predicts that Nlg3 and dopamine are operating within the same synapse, which are findings that contribute to the knowledge of potentially conserved mechanisms driving social behaviour.
References


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Vieira, J.B., and A.A. Marsh. 2014. Don't stand so close to me: psychopathy and the regulation of interpersonal distance. Front Hum Neurosci. 7:907.


Appendices

This appendix contains supplemental data published or under revision. It also contains additional charts and figures to support methods.
Appendix A. List of all *Drosophila melanogaster* fly lines

All *Drosophila* stocks were obtained from either the Bloomington *Drosophila* Stock Centre (BDSC) or the Vienna *Drosophila* Resource Centre (VDRC).

<table>
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<th>Stock Name</th>
<th>Stock No.</th>
<th>Source</th>
<th>Genotype</th>
<th>Description</th>
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<td></td>
<td></td>
</tr>
<tr>
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<td>N/A</td>
<td>+/+</td>
<td>Genetic Control. Laboratory strain.</td>
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<td>36303</td>
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<td>Background stock used as control for attP2 site TRiP RNAi collection with the VALIUM10 vector.</td>
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<td>36304</td>
<td>BDSC</td>
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<td>Background stock used as control for attP40 site TRiP RNAi collection.</td>
</tr>
<tr>
<td><strong>nlg3 Lines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nlg3&lt;sup&gt;3.04&lt;/sup&gt;</td>
<td>140892</td>
<td>VDRC</td>
<td>y* w*; P{w*&lt;sup&gt;W.ks=FRT(w.ks)&lt;/sup&gt;2A P{ry&lt;sup&gt;+/t7.2&lt;/sup&gt;=neoFRT}82B PBac[SAstopDsRed]LL04718 P{y&lt;sup&gt;+/t7.7&lt;/sup&gt; ry&lt;sup&gt;+/t7.2&lt;/sup&gt;=Car20y}&lt;sup&gt;96E&lt;/sup&gt;</td>
<td>P-element insertion into the fourth intron.</td>
</tr>
<tr>
<td>nlg3&lt;sup&gt;GSS2&lt;/sup&gt;</td>
<td>205074</td>
<td>VDRC</td>
<td>y[1] w[67c23]; P{w+[mC]=GSV1}neur[GS3205]</td>
<td>P-element insertion upstream of gene transcription start cite.</td>
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<tr>
<td>nlg3&lt;sup&gt;Def1&lt;/sup&gt;</td>
<td>N/A</td>
<td>Brian Mozer</td>
<td>w++; <em>Df(3R)nlg3 IID-14 (w</em>)</td>
<td>Complete deletion of nlg3.</td>
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## Appendix A. continued

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<td>Serge</td>
<td>w; th-Gal4</td>
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<td></td>
<td></td>
<td>Birman</td>
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</tr>
<tr>
<td>UAS-THmiR-G</td>
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<td>Mark</td>
<td>w; UAS-TH RNAi 3#G / TM6, Sb</td>
<td>Expresses th RNAi under UAS control.</td>
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## Appendix B. List of all RNAi Fly Lines

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<td>BDSC</td>
<td>y[1] v[1]; P{y+[t7.7] v+[t1.8]=TRiP.JF02634}attP2</td>
<td>V10</td>
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</table>
### Appendix B. continued

<table>
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<tr>
<th>RNAi Gene Target</th>
<th>Stock No.</th>
<th>Source</th>
<th>Genotype</th>
<th>Appropriate Control</th>
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<tr>
<td><em>nlg4</em></td>
<td>58119</td>
<td>BDSC</td>
<td>y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ22056}attP40</td>
<td>attP40</td>
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</table>
Appendix C. Schematic of the crosses performed to generate $TH>TH_{miR-G}$ and its genetic controls.

Created with BioRender.com.
Appendix D. RT-PCR on th>RNAi lines and genetic controls in males (A) and females (B) for THmiR-G and THmiR-C.

Rpl32 was used a technical control and no template controls were tested for each primer set.
Appendix E. Schematic of the crosses performed to generate screen lines (elav>TRiP RNAi) and their genetic controls.

Created with BioRender.com.
Appendix F. Visual timeline for isolation and recovery treatments.

Created with BioRender.com
Appendix G. Standard curves for DA quantification.

Equation displayed was used to determine DA concentration. Solid line represents line of best fit. Dotted lines represent SEM.
Appendix H. Percent of flies mated with two or four days of group housing before isolation.
Under revision in Yost *et al.*

<table>
<thead>
<tr>
<th>Days of Group Housing</th>
<th>Number of Flies Mated</th>
<th>% of Flies Mated</th>
<th>Chi-square</th>
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</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>69/81</td>
<td>85.2</td>
<td>$\chi^2(1) = 1.92$, $p = 0.1659$</td>
</tr>
<tr>
<td>4 days</td>
<td>55/72</td>
<td>76.4</td>
<td>$p = 0.1659$</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>94/103</td>
<td>91.2</td>
<td>$\chi^2(1) = 0.0213$, $p = 0.8841$</td>
</tr>
<tr>
<td>4 days</td>
<td>101/112</td>
<td>90.2</td>
<td>$p = 0.8841$</td>
</tr>
</tbody>
</table>
Appendix I. Representative whole blot images with molecular weight markers and total protein for nlg3 mutants (Figure 3.3A,B) for males (A) and females (B). Western blot of anti-Nlg3 is displayed for Cs and nlg3 mutants (top image) and total protein (bottom image). Lane 1 in each image is the molecular weight marker.
Appendix J. Protein abundance of Nlg3 from 7 to 50 days old in Cs males and females.

A. Representative whole blot images with molecular weight markers and total protein for Cs males and females. B-C. Mean protein abundance ± s.e.m. in Cs males (B) and females (C). B. Males had a slight reduction in protein with age (Two-way ANOVA – effect of age: $F_{4,29} = 4.342, p = 0.0071$) and decreased Nlg3-S compared to Nlg3-FL (Two-way ANOVA – effect of variant: $F_{1,29} = 25.82, p < 0.0001$). C. Females also had a slight reduction in protein with age (Two-way ANOVA – effect of age: $F_{4,30} = 3.014.342, p = 0.0334$) and decreased Nlg3-S compared to Nlg3-FL (Two-way ANOVA – effect of variant: $F_{1,30} = 17.11, p = 0.0003$). All male treatments are relative abundance to male Nlg3-FL at 7 days old and female treatments are relative abundance to female Nlg3-FL at 7 days old. n=3-4 for all treatments.
Appendix K. Lifespan is decreased in \textit{nlg3}^{Def1} flies.

\textbf{A,C}: Survival curves; \textbf{B,D}: mean survival, or mean age for which 50\% of the population is still alive. \textbf{A-B}. Males had a decrease in overall survival (A) compared to Cs (Mantel-Cox: $\chi^2(2) = 222.7; p < 0.0001$) and a decrease in mean survival (B; Welsh’s t-test: $t_{3.290} = 3.872, p = 0.0258$). \textbf{C-D}. Females also had a decrease in overall survival (C) compared to Cs (Mantel-Cox: $\chi^2(1) = 32.47; p < 0.0001$) and a decrease in mean survival (D; Welsh’s t-test: $t_{5.770} = 3.919, p = 0.0084$). n=4 for all genotypes and sexes.
Appendix L. Representative whole blot images with molecular weight markers and total protein (A) for group and single housed Cs including a male to female comparison in protein abundance (B).

A. Western blot of anti-Nlg3 is displayed (top image), along with total protein (bottom image). Lane 1 in each image is the molecular weight marker. B. Mean protein abundance ± s.e.m. in male and female Cs. Group housed female Cs had lower protein levels than group housed males (Two-way ANOVA: $F_{1,12}=18.81$, $P=0.001$) and both sexes had lower Nlg3-S than Nlg3-FL (Two-way ANOVA: $F_{1,12}=25.90$, $P=0.0003$). All treatments are displayed as relative abundance to male Nlg3-FL. n=4 for all treatments.
Appendix M. Dopamine is required for a response to isolation in males but not females.

A-B: Social space in isolated (A) and recovered (B) male flies. A. Male th>THmiR-G was not different in isolated vs group housed flies, nor in the th-Gal4/+ flies. However, UAS-THmiR-G/+ males had lower flies within 4 body lengths when isolation (Two-way ANOVA - Effect of isolation: F_{1,41} = 4.474, p = 0.0405). B. Male recovery flies in all genotypes were not different in the number of flies within 4 body lengths compared to group housed males (Two-way ANOVA - Effect of recovery: F_{1,41} = 0.007089, p = 0.9333).

C-D: Social space in isolated (C) and recovered (D) female flies. C. All three genotypes had a decrease in the number of flies within 4 body lengths after isolation and th>THmiR-G flies had the lowest number of flies within 4 body lengths compared to their genetic controls (Two-way ANOVA – Effect of isolation: F_{1,41} = 15.77, p = 0.0003; Effect of genotype: F_{2,41} = 7.555, p = 0.0016). D. UAS-miR-C/+ and th>THmiR-C had less flies within 4 body lengths between group housed vs. recovery treatments, however th-gal4/+ females were not different in the number of flies within 4 body lengths comparing recovery and group housed flies (Two-way ANOVA – Effect of recovery: F_{1,41} = 25.90, p < 0.0001; Effect of genotype: F_{2,38} = 4.582, p = 0.0165; Interaction: F_{2,38} = 5.570, p = 0.0076).

Effect of Isolation ($p = 0.0405$)

Effect of Genotype ($p = 0.0016$)

Interaction ($p = 0.0076$)
Appendix N. Sociability assay set up and aggregation index for Cs and $nlg3^{Def1}$ males and females.

A. Pictures of the sociability chambers. B-C. Mean aggregation index (± s.e.m.) in Cs and $nlg3^{Def1}$ males (B) and females (C) group or single housed from 0.5 to 2 hours. No conclusions can be drawn about the data due to variability within treatments and unstable aggregation indexes over time. n=5-8 for all treatment, genotype, time, and sex.
Appendix O. Average number of flies within 4 body lengths (± s.e.m.) in flies with pan-neuronal expression of RNAi against glutamate receptors in males (A) and females (B).

Each line is compared to elav-Gal4/+ where the + is the V10 genetic control.
Appendix P. Average number of flies within 4 body lengths (± s.e.m.) in flies with pan-neuronal expression of RNAi against GABA receptors in males (A) and females (B). Each line is compared to *elav-Gal4/+* where the + is the V10 genetic control.
Appendix Q. Average number of flies within 4 body lengths (± s.e.m.) in flies with pan-neuronal expression of RNAi against serotonin receptors in males (A) and females (B).

Each line is compared to elav-Gal4/+ where the + is the V10 genetic control.
Appendix R. Average number of flies within 4 body lengths (± s.e.m.) in flies with pan-neuronal expression of RNAi against dopamine receptors in males (A) and females (B).

Each line is compared to *elav*-Gal4/+ where the + is the V10 genetic control.

<table>
<thead>
<tr>
<th></th>
<th>Male 1</th>
<th>Female 1</th>
<th>Male 2</th>
<th>Female 2</th>
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<tbody>
<tr>
<td>elav-Gal4</td>
<td>1.0</td>
<td>1.5</td>
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<td>DopEcR</td>
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<td>3.5</td>
<td>4.0</td>
<td>4.5</td>
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</table>

*elav*-Gal4>UAS-RNAi

![Graph A](imageA.png)

![Graph B](imageB.png)
Appendix S. Average number of flies within 4 body lengths (± s.e.m.) in flies with pan-neuronal expression of RNAi against *octopamine-tyrosine receptor, discs large 5, homer, fmr1, tace, and foxp* in males (A) and females (B).

Each line is compared to *elav-Gal4/+* where the + is the V10 genetic control.
Appendix T. Average number of flies within 4 body lengths (± s.e.m.) in flies with pan-neuronal expression of RNAi against *yellow* and *nlg4* in males (A) and females (B).

Each line is compared to *elav-Gal4/+* where the + is the attP40 genetic control.

<table>
<thead>
<tr>
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<th>Yellow and nlg4 candidates</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>elav-Gal4&gt;UAS-RNAi</td>
</tr>
<tr>
<td>En</td>
<td>elav-Gal4/+</td>
</tr>
<tr>
<td>Y En</td>
<td>elav-Gal4/+</td>
</tr>
<tr>
<td>Y iso</td>
<td>elav-Gal4/+</td>
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<tr>
<td>Nlg4 GH</td>
<td>elav-Gal4/+</td>
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<tr>
<td>Nlg4 SH</td>
<td>elav-Gal4/+</td>
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</table>

<table>
<thead>
<tr>
<th># of flies within 4 body lengths</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
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<tbody>
<tr>
<td>elav-Gal4&gt;UAS-RNAi</td>
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</table>

<table>
<thead>
<tr>
<th>Males</th>
<th>Yellow and nlg4 candidates</th>
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<tr>
<td></td>
<td>elav-Gal4&gt;UAS-RNAi</td>
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<td>Elav</td>
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<td>Nlg4 GH</td>
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<td>Nlg4 Sh</td>
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<th># of flies within 4 body lengths</th>
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<tbody>
<tr>
<td>elav-Gal4&gt;UAS-RNAi</td>
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</table>
Appendix U. Atmospheric pressure (kPa) on days of the Cs and nlg3Deff social space experiments.

Violin plots represent atmospheric pressure throughout the day (00:00 to 16:00) and arrows indicate the direction of pressure change throughout the day. Green plots represent increased social space and red represents decreased social space on experiment days.
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May 10th, 2023
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Agreement number: KT25CLQ1VL
Journal name: Electronic Thesis and Dissertation Repository

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Curriculum Vitae

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- Queen Elizabeth II Graduate Scholarships in Science and Technology (QEII-GSST), Western University, 2018-2019, 2019-2020, 2020-2021 (Declined)
- Finalist, 3-Minute Thesis Competition, Western University, 2019
- Michael Locke Graduate Travel Bursary, Western University, 2019

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Select Conference Presentations:

Yost, R.T., Walshe-Roussel, B., & Simon, A.F. (2022). Neuroligin3 and dopamine are required for a response to social isolation, but recovery is complex and sex-specific. 63rd Annual Drosophila Research Conference, San Diego, CA, USA. [Poster]


