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Identifying the neural basis of female receptivity within and between Drosophila species

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

Abstract

The neural mechanisms that underlie a female's willingness to mate remain largely unknown. To identify the neural basis of female receptivity, I used a combination of genetic tools to induce temporary hyperactivation or suppression of particular neural regions and receptors, then scored their effect on *Drosophila melanogaster* female receptivity towards conspecific or heterospecific males. I found that silencing the antennal lobe reduced female receptivity while silencing the mushroom body increased receptivity towards conspecific males. Hyperactivation of Odorant receptor 47b neurons, the Johnston's organ or the mushroom body increased female receptivity to conspecific males. In contrast, silencing or hyperactivation of target regions had no effect on female receptivity between species. Identifying the neural basis of female receptivity within a species can illuminate how neuronal circuits integrate multiple sources of information from various modalities to subsequently produce behavior. Further, identifying the regions that allow for between-species discrimination can also contribute to our understanding of the neural origin of speciation.

Keywords

Drosophila, female receptivity, speciation, genetics, Gal4-UAS, *shibire*, *dTrpA1*, behavior, neurobiology

Acknowledgments

I acknowledge my supervisor, Amanda Moehring for her boundless compassion, superior intellect and unfailing support. Without her, I would not have been able to complete this degree. I acknowledge my wonderful advisors, Anne Simon and Beth MacDougall-Shackleton who were always constructive and offered meaningful input. In addition, I acknowledge my examiners, Marc-André Lachance, Jamie Kramer and Bryan Neff. I also acknowledge my fellow lab mates, both past and present, Tabashir Chowdhury, Heather Ward, Jalina Beilaska-Da Silva, Josh Isaacson, Rachelle Kanippayoor, Trinh Nguyen and the undergraduate thesis students. You all made a significant contribution to my experience, research and growth as a person. Thanks to all other departmental colleagues and friends, who made this degree enjoyable and manageable. I also thank all of the individuals who helped share the workload, particularly, Andrea Bevan, Shilpa Kumar, Bennet Jaworski and Vyshnave Jeyabalan. In addition, I thank a few professors and friends whom I met during the course of my degree, for their advice, insight and kindness. I also thank Simon Bonner, Alexandru Draghicu and Siobhan Schenk for their statistical assistance. Lastly, I would like to thank my family and guides who have provided me with not only the foundation to make this possible but for always believing in me.

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List of Abbreviations and Symbols

AL- Antennal Lobe

AMMC- Antennal Mechano-sensory and Motor center

dsx- doublesex

FC- Florida City

GFP- Green Fluorescence Protein

HU- Hydroxyurea

JOS- Johnston's Organ

MB- Mushroom Body

Or47b- Odorant Receptor 47a

Or88a- Odorant Receptor 88a

PBS- Phosphate Buffered Saline

SP- Sex Peptide

SOG- Suboesophageal Ganglion

UAS- Upstream Activating Sequence

Chapter 1

1 Introduction

1.1 Background

Charles Darwin and Alfred Wallace described the theory of evolution as "descent with modification" (Darwin & Wallace 1859). Natural selection is the primary mechanism that governs the prevalence of adaptations between successive generations. Variation exists within most populations. Individuals with variants that confer a survival benefit within a particular environment are more likely to have successful offspring, and over time those variants can come to dominate the gene pool. However, some features pose an obvious threat to individual survival. For example; the elaborate plumage exhibited in peacock tails (Dimijian 2005), the spectacular ornaments seen in the male birds of paradise (Irestedt 2009) and the conspicuous and costly courtship call displayed by the male Túngara frog (Bulbert et al. 2015) increase the visibility of these males to predators. Despite the obvious importance of natural selection, it fails to address non-adaptive exaggerated sexual traits seen within species (Darwin 1871).

Sexual selection may be responsible for the observed amplification of sexual dimorphism of particular secondary sexual characteristics (Jones & Ratterman 2009). This is primarily achieved through the members of one sex selecting mates based on the relative quality of these secondary traits. In most sexually-reproducing species, females determine whether mating occurs. For instance, strong female choice can be seen for male tail length in the lekking Jackson's widowbird, *Euplectes jacksoni* (Andersson 1989), and for high male roaring rate in red deer, *Cervus elaphus* (McComb 1991). These examples illustrate the importance of female mate choice in evolution (Workman & Reader 2004).

1.2 Sexual selection and speciation

According to the biological species concept (BSC), species are defined as reproductively isolated groups (Mayr 1942). Reproductive isolation can also be an

evolutionary mechanism that is sufficient for the establishment and maintenance of new species (Andresson & Simmons 2006; Büker et al. 2013; Coyne & Orr 1998; Dobzhansky 1935). Reproductive isolation can be achieved through various barriers, such as: temporal isolation, ecological isolation, mechanical isolation, geographic isolation and behavioral isolation (Gregorius 1992). Behavioral isolation is a reproductively isolating mechanism by which two species do not mate due to differences in courtship behavior. For example, in the Congo basin, African weakly electric fish (*Mormyrinae*) male mating behavior includes exuding a species-specific electrical discharge that attracts conspecific females, but not heterospecific females (Feulner et al. 2009). Behavioral isolation can also be seen between the closely related species pair *Drosophila melanogaster* and *Drosophila simulans* (Coyne & Orr 1998; Figure 1.1). These species currently exist in sympatry (Capy et al. 1993), which means that they co-exist within the same geographical region and yet remain distinct species (Rabosky 2016). This evolutionarily established relationship, coupled with a wide variety of genetic and neural tools in *D. melanogaster*, qualifies this species pair as a model system for the study of sexual selection and behavioral isolation (Beckingham et al. 2005).

1.3 *Drosophila* as a model system for behavior

Drosophila melanogaster has been used as a model organism for the study of behavior, disease and genetics for over a century (reviewed in: Hales 2015). This organism is well suited for scientific investigation due to its relatively short life cycle, high conservation of neuronal gene function across related taxa, relatively small genome, ubiquitous availability of biological tools, and stereotypical courtship displays (Beckingham et al. 2005; Griffith & Ejima 2009; Jennings 2011). Further, the widespread and longstanding use of *Drosophila* as a model system (Hales et al. 2015) has provided researchers with the justification to continue to develop more complex tools (Spradling et al. 2011). It is important to note, however, that the simplicity of this species offers certain limitations. For example, the inability to identify equivalents to complex human behaviors, such as emotions, makes the species untenable for pathophysiological studies (Flanagan-Cato 2011).

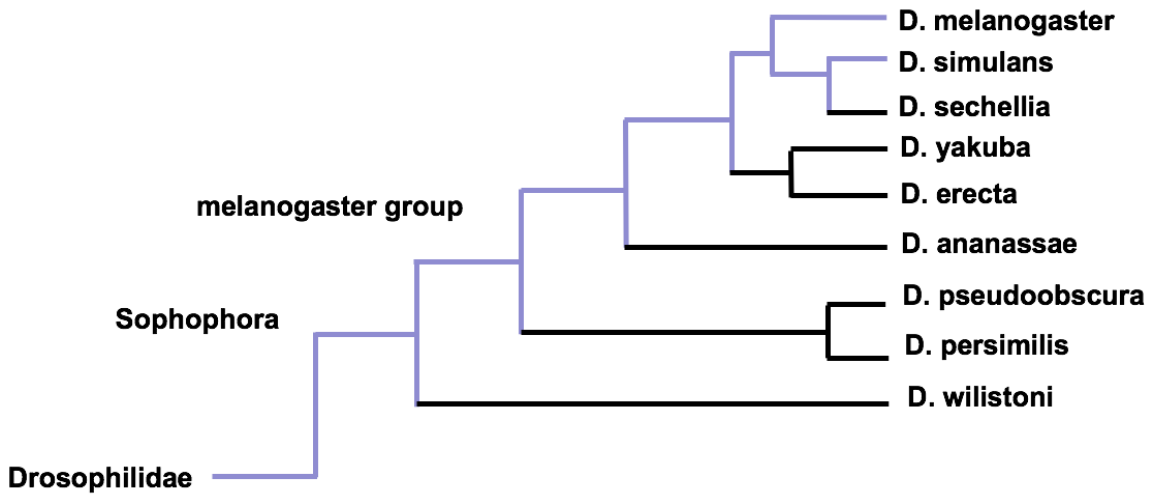

Figure 1.1: Phylogenetic relationship between *Drosophila melanogaster* and *Drosophila simulans*, highlighted in lavender to emphasize species relatedness. Branch lengths indicate approximate relative divergence times. Figure adapted from Flybase (2017).

The number of genetic tools available in *Drosophila* is one of the primary reasons it is widely used as a genetic model system. For example, transposable elements, which are sections of genes that have the ability to move around in the genome (Spradling et al. 2011), have been used for gene disruption and insertion of transgenic elements in *Drosophila*. *P*-elements, one type of transposon, have been paired with sequences of interest and used to insert those sequences into the genome (Hales et al. 2015). One commonly used tool that was generated using this method is the Gal4/UAS system (discussed in detail in Section 1.6.1; Figure 1.2). The Gal4 protein binds to the UAS (upstream activation sequence) and induces expression of the gene adjacent to the UAS. Transposable elements have been used to insert the *Gal4* gene into various locations in the genome. The expression of Gal4 can be in the pattern of nearby enhancer elements, creating tissue- or temporally-specific expression lines of Gal4, and thus tissue- and temporally-specific expression of the locus of interest that is transgenically placed adjacent to the UAS (Hales et al. 2015). The generation and continual refinement of these "enhancer–trap" lines have allowed for unparalleled specificity of expression (Hales et al. 2015).

Multiple factors and several sensory cues contribute to the stereotyped mating behavior of *D. melanogaster*, the most widely-studied *Drosophila* species (reviewed in: Sokolowski 2001). Males of many *Drosophila* species can court females indiscriminately (Dukas at al. 2006), but even in those cases, male courtship displays serve as an important precursor to copulation. Males engage in a multimodal courtship display: in *D. melanogaster*, the male orients himself towards the female, then taps her abdomen with his front tarsi, followed by a species-specific song generated by wing pulses, concluding with genital licking followed by attempted copulation (Hall 1994; Thoma et al. 2016). During this process, the male is both sampling the female and revealing important information to the female about his species and quality.

Female receptivity has been invoked as the driving force behind both mating occurrence within species and isolation between species. Sexual receptivity is defined as female behaviors that allows or helps a male to fertilize her eggs (Ringo 1996). In *D. melanogaster*, the female detects cues from the male through a variety of senses:

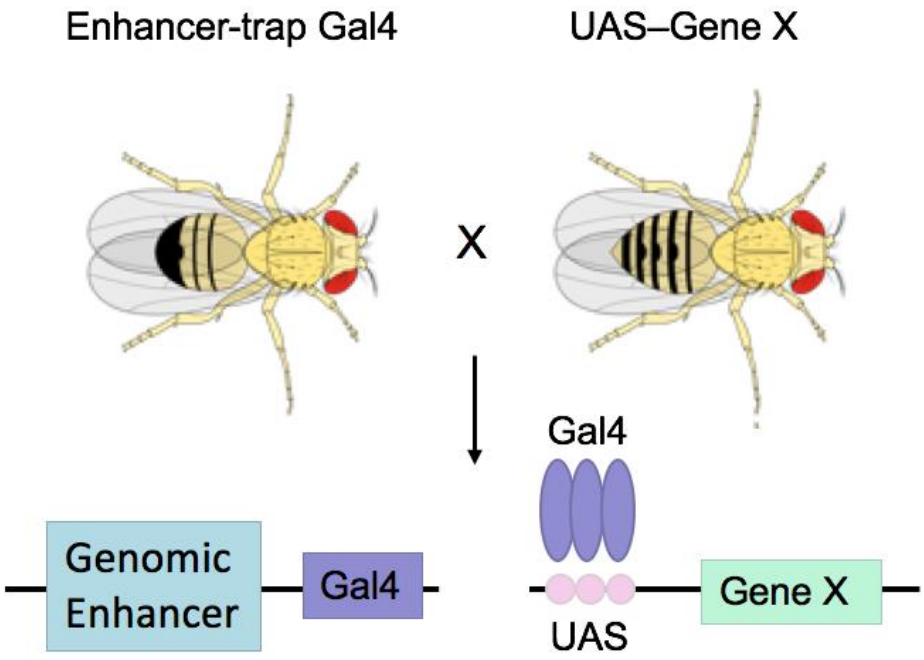

Figure 1.2: The Gal4/UAS system. The fly on the left carries the *Gal4* transcription factor and the fly on the right fly carries the cis-regulatory upstream activating sequence (UAS) that is bound to "gene x" (gene of interest). Crossing these flies results in F1 offspring that contain both the *Gal4* and UAS sequences. Wherever the Gal4 protein is expressed, it binds to the UAS, which then induces expression of the gene adjacent to the UAS. Figure adapted from Johnston (2002).

auditory, olfactory, tactile, and to a lesser extent visual (Hall 1994). Then the female must parse both her internal and external environment before sexually rejecting or receiving a male (Bussell et al. 2014). A female can display receptivity by reducing motion, pausing, and partially extruding her ovipositor (Lasbleiz et al. 2006). Conversely, a non-receptive female may show avoidance behaviors which include, but are not limited to, increased motion, kicking, and general decamping activity (Bontonou & Wicker-Thomas 2014).

Mutations in genes such as *spinster* (Sakurai et al. 2013), *icebox* (Sakai et al. 2010), and *apterous* (Aranha et al. 2017) result in lowered sexual receptivity in *D. melanogaster* females; however, the genetic basis of female receptivity remains largely unknown (reviewed in: Laturney & Moehring 2012). Despite the critical role that female choice plays in reproductive success, genetic propagation and even the creation of distinct species, most investigation to date on the genetic and neural basis of mating behavior has been conducted in males. While the genetic and neural underpinnings of female receptivity are inherently interesting, they also provide a tractable framework for understanding how complex behavioral decisions are made.

1.4 Neurobiology of *Drosophila* female receptivity

Across taxa, the nervous system plays a fundamental role in enabling organisms to process sensory information and form proper behavioral responses (Beatty 1995). Neuroscience seeks to understand how the brain, perhaps the most complex electrochemical machine on earth, works, in terms of molecules, membranes, cells, neuronal substrates, development, plasticity, learning, memory, cognition, and behavior (Strumwasser 1994). *Drosophila melanogaster* offers researchers a remarkably tractable model to gain insight into the neuronal basis of complex animal behaviors (Auer & Benton 2016). In the last several years, *Drosophila* sexual behavior has become a favored model for researchers interested the "innate" behaviors of the nervous system (Griffith & Ejima 2009). The first time a sexually naïve and mature male fly is exposed to a female fly, or even a stimulus resembling a female, courtship behavior is triggered. This indicates that this behavior is innate because it does not have to be learned and is the

result of genetic hardwiring (Pan 2014). Similarly, a sexually naïve female is usually receptive towards a male of her own species (Moehring lab, unpublished data).

Female receptivity has primarily been explored in the context of neurons formed by genes that are involved in sexual dimorphism, such as *fruitless* or *doublesex* (Feng et al. 2014, Bussell et al. 2014, Zhou et al. 2014, Rezaval et al. 2013). One such study investigated the role the gene *doublesex* (*dsx*) played in virgin female receptivity towards conspecific males. They found that activation of the *dsx*-expressing neurons in specific neural clusters (located in the dorsolateral protocerebrum; Figure 1.3), called the pCd and pC1, promotes receptivity, while silencing these neural clusters renders females unreceptive (Zhou et al. 2014). The role of these specific *dsx* neurons in female receptivity was further explored in the Moehring lab (Andrea Bevan Honors Thesis 2017). Specific neurons have also been identified that mediate the behavioral changes induced in females by male sex peptide (SP) in the ejaculate (Feng et al. 2014; Heifetz & Wolfner 2004). A special class of neurons called ascending SAG neurons, which are found in the abdominal ganglion within the body of the fly, obtain input from SP and then synapse in the protocerebrum of the *Drosophila* brain (Figure 1.3). The protocerebrum is considered part of the central complex of the *Drosophila* nervous system (Wolff et al. 2015). Silencing these neurons triggers rejection behaviors, whereas activating them enhances the receptivity of sexually experienced females. This effect was recapitulated by an experiment which showed that silencing *Abdominal-B* neurons in the abdominal ganglion of adult virgin females significantly decreased female receptivity (Bussell et al. 2014).

An analysis of the distinct components of courtship processing found a critical pathway for auditory processing in female flies, which allows females to detect conspecific wing song (Vaughan et al. 2014). As previously mentioned, during courtship, male *Drosophila* produce a species-specific courtship song. This song is detected by the Johnston's organ in females, a mechanosensitive organ found in the antennae (Eberl & Boekhoff-Falk 2007; Liu & Yang 2014; Figure 1.3). The emitted pulses activate highly sensitive stretch receptor neurons in the Johnston's organ (Dickson 2008). This organ plays a critical role in conjunction with odorant receptor neurons to input initial sensory

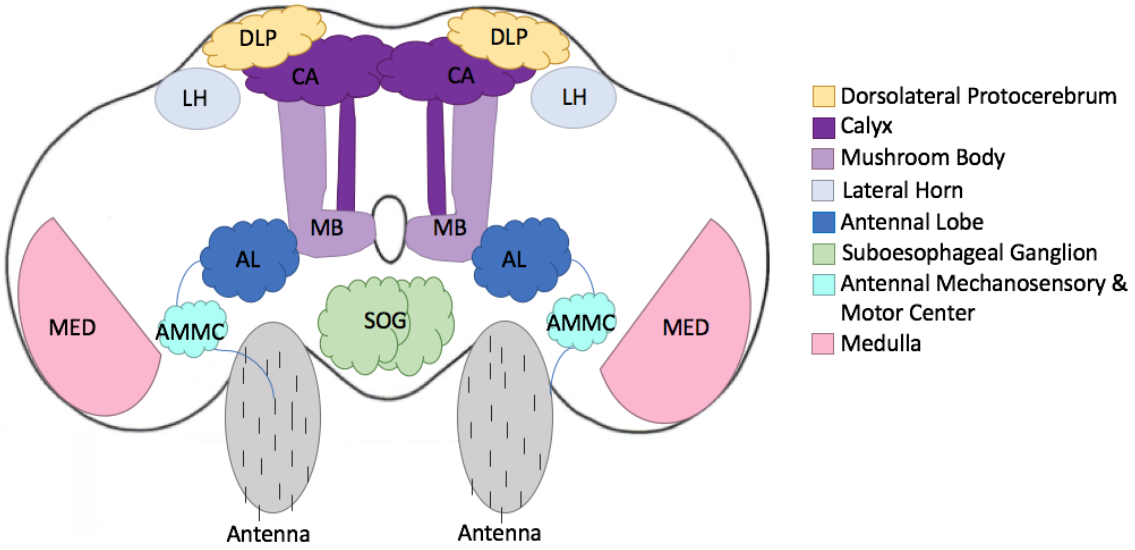

Figure 1.3: Anatomy of the *Drosophila* Brain oriented in the anterior coronal plane. This figure illustrates the antenna and a simplified brain. The approximate locations of neural regions of interest are indicated. The mushroom Body (lavender) is situated inferior to the lateral horn (periwinkle) and superior to the antennal lobe (blue). The suboesophageal ganglion (green) is medial to the antennal mechano-sensory and motor enter (cyan) and superior to the antenna (grey). All other anatomical regions are included for spatial reference.

information that the female receives during courtship (Boekhoff-Falk & Eberl 2014). The courtship song is perceived in a subset of mechano-sensory neurons that converge onto the Antennal Mechano-sensory and Motor center (AMMC; Aranha et al. 2017). Since information from the Johnston's organ is transmitted to the AMMC where an acoustic representation of the information is created, auditory projection neurons that span from the Johnston's organ to the AMMC may also be a candidate for regulating female receptivity in *D. melanogaster* (Boekhoff-Falk & Eberl 2014).

The role of auditory processing in female receptivity is supported, in part, by research on the *painless* (*pain*) gene. While most loci identified for female receptivity cause reductions in receptivity when mutated (Sakai et al. 2010; Sakurai et al. 2013; Aranha et al. 2017), mutations in the *pain* gene induce higher female sexual receptivity, as *pain* mutant females mate more readily than wild-type females (Sakai et al. 2010). The *Drosophila pain* gene is a homolog of the mammalian TRPA1/ANKTM1 gene, which is necessary for regulating avoidance behavior of noxious heat or mechanically generated pain (Sakai et al. 2010). The *pain* gene is expressed in the mushroom body, the central complex and the Johnston's organ, and these may be the neural regions through which the gene exerts its effect on female receptivity (Sakai et al. 2010). This is consistent with the notion that the females' ability to detect, process and respond to the males' species-specific courtship song is a crucial feature of reproduction.

The suboesophageal ganglion (SOG) region of the *Drosophila* brain (Figure 1.3) is part of the arthropod central nervous system and is primarily responsible for gustatory processing, but also plays a central role in pheromone perception (Yamamoto et al. 2010). Gustatory neurons from the proboscis, mouth and legs project to the SOG of the fly brain (Stocker 1994). Unlike the primary olfactory relay, the SOG does not exclusively process taste information. Instead, there are thousands of neurons associated with the SOG, which serves as a general relay center between the brain and the ventral nerve cord (Brody 1999; Kwon et al. 2014). Work on the *spinster* locus suggests that the SOG may be involved in female receptivity: mutations in this gene significantly reduce female receptivity in response to the advances of conspecific males (Sakurai et al. 2013; Yamamoto et al. 2010). The gene *spinster* expresses in two neuronal clusters, Spin-A and

Spin-D. Spin-A neurons are found in the SOG. The SOG therefore seems to play a role in both the mating response, via Spin-A neurons, and post-mating response, via regulating behavior changes induced by sex peptide, making the SOG a candidate region involved in female receptivity. Spin-D can be found in a specific glomerulus of the antennal lobe and also responds to male-produced chemical cues (Sakurai et al. 2013), making the antennal lobe a potential contributor to female receptivity behaviors (discussed further below).

The neural processing of olfactory cues can play a significant role in mate discrimination. Many organisms, including *Drosophila*, have evolved olfactory systems of remarkable sensitivity and discriminatory power to process chemical information gleaned from their environments, including potential mates. Afferent olfactory information is first detected by specific receptors housed in the antenna, which serves as the primary odorant detecting unit in *Drosophila* (Laissue & Vosshall 2008). Odorant receptor axonal projections bundle together in the antennal nerve, and then odorant information is transferred to the first odor relay station in the fly brain, the antennal lobe (Berry et al. 2008; Figure 1.3). The antennal lobe is a large bilaterally paired neuropil found in the *Drosophila* brain, where odorant receptor neurons synapse onto either local interneurons or projection neurons. At the site of the antennal lobe there is a high level of plasticity; for example, allowing for habituation to continuous odorant stimuli (Sudhakaran et al. 2012). These sexually dimorphic structures have been conserved across a variety of insects (Vosshall 2008) and are functionally analogous to the olfactory bulb in vertebrates (Bhandawat et al. 2007). The role of olfaction in female receptivity has been explored in a handful of studies. For example, the pheromone 11-cis-vaccenyl acetate activates specific olfactory receptors in the antenna that promote the sexual receptivity of females (Davis 2007).

Olfactory receptors Or47a and Or88a are expressed in the trichoid sensilla in the antennae and respond to attractant pheromones in *Drosophila* species (Dweck et al. 2015). Or47b exclusively detects methyl laurate which is a general attractant molecule for males and females across *Drosophila* species. While Or88a-expressing olfactory sensory neurons detect three different attractant molecules: methyl laurate, methyl

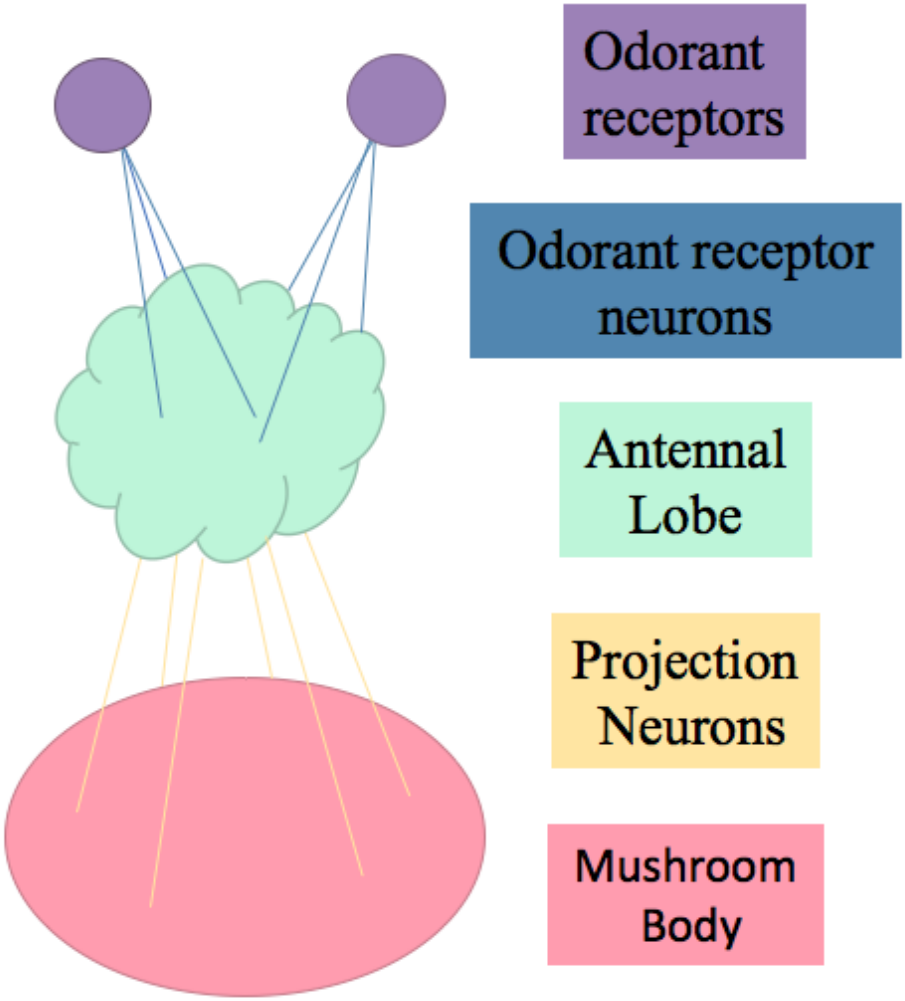

Figure 1.4: Simplified overview of the olfactory pathway in the *Drosophila* brain. Neural regions that communicate to process olfactory information are indicated. Odorants are detected by odorant receptors (purple); information is relayed via odorant receptor neurons (blue) to the antennal lobe (green); the stimulus is transduced and feed to the mushroom body (pink) via projection neurons (yellow) for sensory integration and associative processing.

myristate, and methyl palmitate (Dweck et al. 2015). Olfactory information picked up by olfactory receptor neurons is integrated and reformatted within the antennal lobes and then projected to the mushroom body for further consolidation and interpretation (Berry et al. 2008; Perisse et al. 2013; Figure 1.4). The mushroom body is a region of the brain that is known to play a crucial role in associative olfactory learning and memory, including learning associated with courtship (Aso et al. 2008; Figure 1.3). Ablating the mushroom body results in the complete elimination of both short-term and long-term memory (Griffith & Ejima 2009). The mushroom body is a bilaterally paired structure found in the brain of most arthropods and some annelids (Heuer et al. 2010).

The mushroom body, lateral horn, and central complex are considered to be the "higher" brain regions (Akalal et al. 2006; Heuer et al. 2010). The central complex is a segregated set of neuropils that play a crucial role in the integration of sensory information, locomotion and memory (Chang et al. 2017). The lateral horn primarily processes olfactory information; it is considered part of the olfactory relay system and is connected to the mushroom body (Schultzhaus et al. 2017). The mushroom body in particular, however, may play a role in higher-order processing of sensory information related to female receptivity.

Among other things, the mushroom body receives and processes olfactory information from the antennal lobe via dendrites located in the calyx (Hu et al. 2010). The calyx is a synapse-dense region characterized by its shape that is functionally responsible for the integration of sensory and olfactory information, making it a site of high convergence (Gramates et al. 2017; Perisse et al. 2013). Both the calyx and the lateral horn receive olfactory information from collateral projection neurons (Heisenberg 1998). Females that are mutant for the *icebox* gene have defects in central brain structures, including the mushroom body, and show reduced sexual receptivity (Sakai et al. 2010). Expression of the *rutabaga* gene is highly enriched in the mushroom body (Quinn et al. 1974). *Rutabaga* plays a role in learning and memory; it acts as a coincidence detector which enables an organism to recognize associative sensory information which may be either spatially or temporally separated in the brain (Han et al. 1992). Thus, there are multiple lines of evidence that the mushroom body play a critical

role in processing sensory information and may affect female receptivity. Surprisingly, however, female sexual receptivity is unaffected by the complete ablation of mushroom body (Neckameyer 1998). Incongruent and contradictory information surrounding the role of the mushroom body in female receptivity provides justification for further exploration.

1.5 Female receptivity and species isolation

While female receptivity is a key variable underlying copulation success within a species, it also can serve as a barrier between species. A model system for between-species behavioral isolation is the closely-related sympatric species pair of *D. simulans* and *D. melanogaster* (Gramates et al. 2017; Figure 1.1). In the wild, these two species engage in intermittent interspecific mating (Sturtevant 1920). While males of both species will court females of either species, and *D. melanogaster* females will mate with *D. simulans* males (albeit at reduced frequency), *D. simulans* females strongly and consistently reject the advances of *D. melanogaster* males (Carracedo et al. 2000). Therefore, this species pair represents a model of behavioral isolation strongly underpinned by female choice. Further, the array of tools available for *D. melanogaster* make this species pair a powerful genetic and neural model for understanding behavioral isolation.

1.6 Manipulating neurons

1.6.1 Targeting neural regions: The Gal4/UAS System

In 1993, Brand and Perrimon developed the Gal4/UAS system. Since then, the Gal4/UAS system has been widely applied in *Drosophila* for the study of targeted gene expression (Brand & Perrimon 1993). This bi-partite biochemical tool was developed based on the properties of the yeast *Saccharomyces cerevisiae* Gal4 transcription factor, and is one of the most powerful techniques currently available in the study of gene expression (Duffy 2002). As discussed previously, the *Gal4* gene has been inserted into various locations within the *D. melanogaster* genome using *P*-element transformation. Gal4 is an exogenous transcription factor whose expression can be determined by nearby enhancer elements. When it is inserted in the genome, its expression is determined by the

enhancers affecting that region of the genome ("enhancer trap"); different insertion sites can generate different Gal4 expression patterns. The Gal4 protein binds to the UAS (Upstream Activating Sequence) which activates transcription of the locus adjacent to the UAS, which is usually transgenically generated to be a gene of interest. Wherever Gal4 is expressed, the UAS is bound, and the gene of interest is expressed (Johnston 2002; Figure1.2). Therefore, in enhancer-traps, the location of the *Gal4* insertion determines the locality of gene expression and the flexibility of choosing whichever gene of interest allows the investigator to dictate the effect exerted on pre-defined locations.

1.6.2 Manipulating neural activity: Silencing and hyperactivating

To identify neural regions that affect female receptivity when suppressed, Gal4/UAS-*shibire* can be used to drive expression of the neural silencer *shibire* in candidate brain regions. The *Drosophila* gene *shibire* (*shi*) encodes a motor protein, dynamin (Gonzalez-Bellido et al. 2010). Dynamin is an enzyme that plays a critical role in the regulation of vesicle endocytosis and therefore recycling (Mettlen et al. 2012). When expression is induced with Gal4, UAS-*shi* blocks vesicle endocytosis, preventing synaptic vesicle recycling, which prohibits neurotransmitter transmission (Kitamoto 2001). Once activity in the Gal4-positive neurons is halted, behavioral consequences of spatial and temporal suppression of neurotransmission can be observed. A temperature-sensitive version of *shibire* (*shi*^{ts}) allows for temporal refinement – activation of the transgene, and thus suppression of neural activity, occurs only at high temperatures (Kitamoto 2001). The UAS-*shi*^{ts} product regains its activity and synaptic vesicles are restored immediately after the animals are returned to the permissive temperature (Gonzalez-Bellido et al. 2010).

To hyperactivate neurons, *Drosophila Transient receptor potential cation channel A1* (*dTrpA1*) can be used. As with UAS-*shi*^{ts}, the temperature-sensitive UAS-*dTrpA1*^{ts} can allow for both spatial and temporal control of neural activity. *TrpA1*, an orthologue of the mammalian TRPA1 channel, is a warmth-gated cation channel that regulates thermotactic behavior in *Drosophila* (Sakai et al. 2009). When activated, calcium ions (Ca^{2+}) rush into the neural cell which causes the cell to depolarize, triggering an action

potential (Berni et al. 2010). Activation of *dTrpA1* therefore artificially stimulates neurons. By ectopically expressing *dTrpA1^{ts}* (using the Gal4/UAS system), and then switching the temperature from permissive to restrictive, neural activity can be hyperactivated in a discrete and non-invasive manner and resultant behaviors can be observed in freely moving animals.

1.7 Objectives

1.7.1 The neural basis of conspecific female receptivity

Manipulating neural activity in candidate constituents of the brain can allow for the identification of the neural basis of female receptivity within *D. melanogaster*. Subjecting treated females to behavioral assays and observing deviations from normal sexual behavior can provide information into which neural components function to regulate these behaviors. In the present study, I employed the Gal4/UAS system to investigate how the brain controls and coordinates virgin female receptivity.

The first objective was to modulate activity in a suite of the brain regions that are involved in the sensory processing of the conspecific male courtship display, as they are likely involved in regulating female receptivity. As previously mentioned, there are a variety of systems involved in this process, including auditory, olfactory, gustatory and integration networks. The battery of selected Gal4 lines to accomplish this included those expressed in the suboesophageal ganglion, specific odorant receptors (47b and 88a), Johnston's organ, the antennal and mechano-sensory motor center, the antennal lobe, the mushroom body and *pain* gene neurons. Each Gal4 is paired with both a UAS-*shi^{ts}* line to silence neural activity, and a UAS-*dTrpA1^{ts}* to hyperactivate neural activity. Specific brain regions were either suppressed or hyperactivated during the assay and female receptivity was quantified.

Based on the literature, I predicted that suppressing the suboesophageal ganglion would increase receptivity, while hyperactivating it may decrease receptivity. Contrarily, suppressing expression of the neurons encoded by the *pain* gene should decrease receptivity and the opposite effect is expected when expression of this gene is hyperactivated. In addition, suppressing the antennal lobe, odorant receptors 47b and 88a

and the mushroom body should elicit decreased mating while hyperactivating these regions in olfactory processing should enhance receptivity. The same prediction holds regarding modulating activity in the auditory system. I anticipated that suppressing activity in the Johnston's organ and antennal mechano-sensory and motor center will interfere with a female's willingness to mate with males of the same species, while hyperactivating these regions may cause females to mate more frequently with conspecific males. Since olfaction and audition are the primary mechanisms by which females perceive and process courtship cues, suppressing underlying sensory networks may dampen the females' ability to recognize components of the courtship display. Likewise, hyperactivating these sites could serve to heighten sensitivity to courtship cues and therefore enhance a females' ability to detect particular aspects of male courtship.

1.7.2 The neural basis of behavioral isolation between species

As previously mentioned, *D. melanogaster* females mate with *D. simulans* males in the laboratory at reduced frequency compared to conspecific pairings (Coyne & Orr 1998). One way to investigate the neural mechanisms that serve to maintain the integrity of separate species is to take advantage of this pre-established relationship. This can be done by repeating the processes described in the previous objective, but rather than paring off transgenic females with conspecific males, pairing them off with heterospecific, *D. simulans* males. This objective aimed to answer a different question, namely whether the same regions that regulate female receptivity within a species are also responsible for maintaining behavioral isolation between species.

The same set of Gal4 and UAS lines used for the previous objective were used to screen for regions that may be involved in mediating female discrimination. This was done to unveil the neural mechanisms that govern and maintain behavioral isolation. Since these regions have not been previously investigated regarding their role in maintaining behavioral isolation, predictions were made based on functionality rather than previous research. Based on what is already understood about the suboesophageal ganglion, suppressing *spinster*-expressing neurons acts to decrease female receptivity within species (Sakurai et al. 2013). Depending on how these neural clusters work, which

still remains poorly understood, the same effect may be seen between species. Hyperactivating these neurons may enhance female receptivity to heterospecific males. Similarly, silencing neurons expressing the *pain* gene should trigger increased mating between *D. melanogaster* females and *D. simulans* males and hyperactivating it should have the opposite effect. Furthermore, since sexual pheromones are essential for species recognition (Cobb & Jallon 1990), suppressing units responsible for detecting and discriminating odorants may impair the females' ability to recognize whether the courting male is of a different species. Therefore, suppressing the antennal lobe and mushroom body may cause enhanced female receptivity to *D. simulans* males compared to females that have all neural networks intact (Ferveur et al. 1995), while hyperactivating these regions may enable *D. melanogaster* female to have a heightened ability to discriminate against heterospecific males. Since odorant receptors 47b and 88a detect general attractant molecules, hyperactivating them may artificially trigger the receptors in the brain and deceive the transgenic female into mistaking the courting *D. simulans* male as one of her own males, resulting in increased copulation levels. Lastly, courtship songs serve as an important indicator to females about whether the courting male is of the same species. The regions that are involved in processing this song such as the Johnston's organ and the antennal mechano-sensory and motor center may serve are likely candidate regions for behavioral isolation. Therefore, suppressing these regions could allow for higher heterospecific copulation levels if *D. melanogaster* females are less competent at discriminating and recognizing that the courting male is not of the same species. However, as mentioned in objective one, interfering with the females' ability to process sound may cause her to mate less regardless of the species of the male due to her inability to perceive the song if it acts as a gateway stimulus to copulation.

Chapter 2

2 Materials and Methods

2.1 Fly maintenance

All *D. melanogaster* and *D. simulans* stocks were maintained over standard cornmeal medium (Bloomington *Drosophila* Stock Center recipe) in 30mL vials at 24°C with a 14:10 light: dark cycle at approximately 70% relative humidity. Fly stocks were maintained both in the controlled incubator (24°C, 14:10 light: dark cycle, approximately 70% relative humidity and on the bench (room temperature) when not within one generation of being actively tested.

2.2 Fly stocks: Wild-type and Gal4/UAS stocks

Wild-type *D. melanogaster* strain BJS was obtained courtesy of Dr. Brent Sinclair. All transgenic lines of *D. melanogaster* were obtained from the Bloomington *Drosophila* Stock Center (Table 2.1), excluding the UAS-TrpA1 line, which was a gift from Dr. Claire McKellar. Eight Gal4 lines were utilized to target expression to particular tissues. These Gal4 lines were individually paired with each of three UAS lines: UAS-GFP to visualize expression, UAS-*shibire*^{ts} to silence neural activity in a temperature-dependent manner, and UAS-*TrpA1*^{ts} to hyperactivate neural activity in a temperature-dependent manner. Wild-type *D. simulans* strain Florida City (FC) was obtained courtesy of Dr. Jerry Coyne. *D. simulans* GFP, which has GFP-tagged sperm heads (*w+; pBac{3xP3-EGFP, ProtB-EGFP}11B*) was obtained courtesy of Dr. John Belote. *D. melanogaster* GFP, which has GFP-tagged sperm heads (*P{w*$^{+mC}$*=protamineB-eGFP}2/CyO*) was obtained from Bloomington *Drosophila* Stock Center.

2.3 Genetic crosses

All crosses were performed by combining 3-5 virgin females that were aged 2-6 days of one desired genotype and pairing them in a fresh food vial with 3-5, 2-6 day old males of the other required genotype. One week later, the progenitor flies were removed to guarantee that the parents were not able to mate with offspring, ensuring that all eclosed F1 individuals were expressing the crossed genotype.

To confirm the activity of UAS-*shi* ^{ts}, UAS-*dTrpA1* ^{ts}, each UAS was crossed to the pan-neural driver Gal4-*elav* to generate an F1 that has both the Gal4 and one of the two UAS. Also, each of the test Gal4 lines were crossed with each UAS line (UAS-*shi* ^{ts}, UAS-*dTrpA1* ^{ts} and UAS-GFP) to generate an F1 that has both the Gal4 and UAS. As the Gal4 and UAS are on separate chromosomes, each is over a wild-type homolog in the F1; therefore, to generate controls for testing whether homozygous Gal4 or UAS constructs themselves affect behavior, each Gal4 and UAS line was crossed to wild-type *D. melanogaster* BJS to generate an F1 that has either UAS separately over a wild-type chromosome or Gal4 separately over a wild-type chromosome.

2.4 Behavioral assays

2.4.1 Ensuring the functionality of the thermosensitive effector lines

All assays were performed in a temperature-controlled, ~60-70% humidity incubator with a viewing window to allow for visualization. See below for temperature information. To determine proper functionality of UAS- *shi* ^{ts} and UAS-*dTrpA1*^{ts}, I followed the guidelines established by Kitamoto (2001) and Berni et al. (2010), respectively. Crossing UAS-*shi*^{ts} and UAS-*dTrpA1*^{ts} to Gal4-*elav* generated F1 females with pan-neural expression of *shibire* or *dTrpA1*, respectively. Five females with pan-neural expression aged four-six days were placed at the restrictive temperature of 30°C. However visual cues of transgene activation were not exhibited within the expected time frame of 1-2 minutes. I therefore increased the temperature to 32°C. Following this adjustment, flies were observed until activation was complete, which I empirically estimated to be between 1-2 minutes. I thus used two minutes of heat treatment at 32°C with ~60-70% humidity in the following experiments.

Silencing or hyperactivating smaller regions of the brain using the more refined Gal4 lines (Table 2.1) could potentially also affect females to an extent that they would not be able to be scored in a mating behavior assay (due to seizures or paralysis if modulating activity in the targeted regions disrupted general functions). To test this, I tested each Gal4 line paired with either UAS-*shi*^{ts} or UAS-*dTrpA1*^{ts}, as above, to ensure

that activity was not affected to such an extent that motor function was impaired (females are still able to walk and fly).

Further, in order to confirm that the temperature was not so hot that it impacted male courtship behavior, I tested approximately 10 BJS wild-type *D. melanogaster* males paired with 10 BJS wild-type *D. melanogaster* females for one hour to ensure males were still able to court. Because, courtship appeared to be dampened, with less than half of the males courting, I tested a second line of *D. melanogaster* males (GFP-sperm). During a one-hour assay, almost all of the GFP-sperm *D. melanogaster* males courted, and courtship in some cases began immediately after introduction of the female. These males were therefore used in all assays requiring *D. melanogaster* males.

2.4.2 One-hour mating assay (Pure species: *D. melanogaster*)

Virgin males and females were aged for four to six days prior to being assayed to ensure that all test subjects reached sexual maturity. Each F1 female fly was singly paired with one virgin GFP *D. melanogaster* male in a one hour, no-choice observational mating assay between 1-2 hours of 'lights on.' First, each male and female fly was placed at 32°C for five minutes to ensure sufficient acclimation and complete activation of temperature sensitive *shibire* and *dTrpA1*. Pairs were observed for 60 minutes and scored for courtship latency (time until courtship begins) and copulation latency (time until copulation begins); from these measures, latency between courtship and copulation, proportion courted, proportion copulated, and proportion copulated out of those that were courted were also quantified. The latency and proportion copulated out of those that were courted is the measure of true 'female receptivity,' as only females that are courted can exhibit receptivity. All other measures were used as confirmation that males were courting indiscriminately and to generate qualitative information for each copulation event. Every assay was conducted in the same test incubator to enhance environmental consistency. Equal numbers of Gal4/+; UAS/+ Gal4/+; + and +; UAS/+ females were tested on each assay day to control for environmental effects. As empirically determined to have higher courtship under heat stress, GFP-tagged sperm males were used in all within species assays rather than wild-type males.

2.4.3 48-hour mating assay (Interspecies: *D. melanogaster and D. simulans*)

Heterospecific assays between *D. melanogaster* females and GFP *D. simulans* males were set up the same way as the pure species assays, above. However, instead of terminating the assay after one hour of observation, males and females were kept together for a full 48-hour period. These assays were carried out over a longer period because *D. melanogaster* females take longer to copulate with a male of a different species and because *D. simulans* males are more sensitive to high temperatures than *D. melanogaster* males (Chakir et al. 2002).

GFP *D. simulans* males were used in these assays to facilitate detection of sperm after the assay. After 48 hours, each vial containing the interspecies pair was frozen, to terminate the assay and effectively kill the tested individuals in preparation for reproductive-tract dissection. The male was discarded from the frozen vial of the interspecies pair and the female's reproductive tract was dissected and imaged to detect for GFP-tagged sperm as a proxy for copulation occurrence.

2.5 Scoring for GFP-sperm

2.5.1 Reproductive tract dissection protocol

Female reproductive tracts were dissected on a dissection disc in PBS containing 0.1% Triton X-100 (PBST; pH 7.4). Dissected reproductive tracts were then mounted in PBS and a cover slip was placed on top of each droplet of PBS containing several reproductive tracts (~10/droplet). In order to keep track of the reproductive tracts once dissected, I made sure to mount them on slides segregated by genotype.

2.5.2 Imaging GFP-sperm

Reproductive tracts were imaged within 30 min of dissection, using a Nikon Eclipse Ci-L upright fluorescent microscope equipped with a DS-Fi2 colour camera. Images were acquired using Nikon Elements D software. Both the seminal receptacle (short-term sperm storage organ) and the spermathecae (long-term sperm storage organ) were visualized.

Presence of GFP-sperm was used as a proxy to indicate a copulation event while absence of GFP-sperm indicated no copulation (Figure 1.4).

2.6 Ensuring the functionality of the driver lines

A UAS crossed with green fluorescence protein (UAS-GFP) was used to confirm that each Gal4 line drove expression in the expected manner. Each Gal4 line (except Gal4-*elav*) was crossed with a UAS-GFP line to produce offspring that should show heightened fluorescence only in specific regions or receptors in the brain. Functionality of these lines was determined based on fluorescence in anticipated regions. I used an online resource (Virtual Fly Brain) to establish which regions fluorescence should be expected in and compared what I saw with these images (Milyaev et al. 2012).

2.6.1 Brain dissections

All brain dissections were performed on adult females (4-7 days old). All brains were dissected following the protocol outlined by Wu and Luo (2006). In brief, I first anaesthetized adult flies on ice or using a CO2 pad, and then the flies were placed onto a dissection dish and immersed in PBS. After being immersed in the PBS, the flies were then immersed in 75% ethyl alcohol then placed back into the PBS. After soaking for a maximum of four minutes, the head cuticle was removed from the brain using microdissection tweezers and the brain was debrided with gentle forceps manipulation under a dissecting microscope. Once the brain was in the proper orientation I collected the dissected brains using a loop without touching the brain and placed them on a slide prepared with 6-8ul 50-90% glycerol mounting medium. Glycerol is used to enhance the resolution of the images. Brains were imaged using a Nikon Eclipse Ci-L upright fluorescent microscope equipped with a DS-Fi2 colour camera. Images were acquired using Nikon Elements D software.

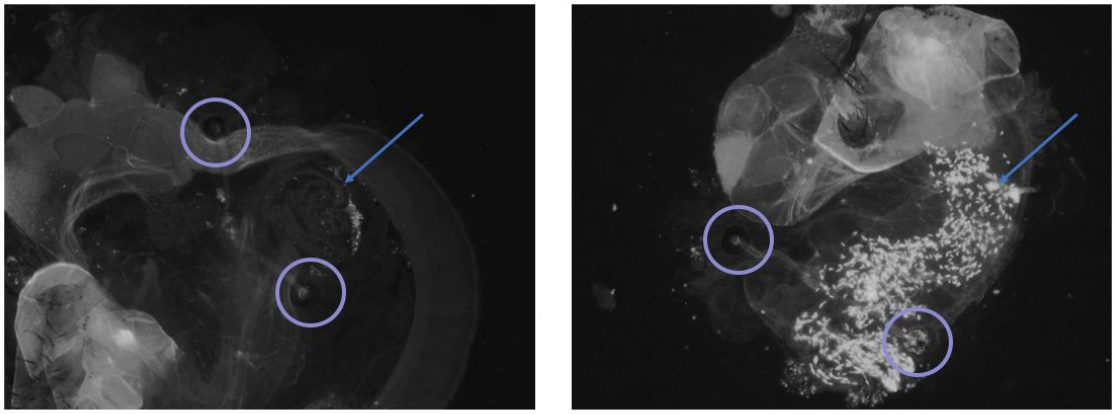

Figure 1.4: Scoring presence of sperm within the *Drosophila* female reproductive tract. The purple circles indicate the spermathecae (long-term sperm storage organs) and the blue arrows indicate the seminal receptacle (short-term sperm storage organ). It can readily be discerned if the tract is free from GFP-tagged sperm (left panel), indicating that the female did not copulate, or contains GFP-tagged sperm (right panel), indicating that copulation occurred.

2.7 Statistical analysis

To determine if specific neural region(s) were associated with female sexual receptivity or rejection behavior, the proportion of copulation out of females that were courted was analyzed using R-studio software (RStudio Team 2015), as follows. A contingency table and two-sided Fisher's Exact Test for count data was used to determine if there was a statistical difference between the expected value (mating frequency of controls) and the observed value (mating frequency of treated individuals). For tests that were statistically significant, a *post-hoc* 2×2 Fisher's exact test for each pairwise comparison was performed to determine if it was the test group that was significantly different from the two controls. This test compared the test group with each control, (Gal4-UAS/+ vs. Gal4/+ and Gal4-UAS/+ vs. UAS/+).

In addition, to analyze courtship and copulation latency data, I used a Kaplan-Meier Survival Analysis. This analysis was conducted in consultation with Dr. Simon Bonner, using code designed by Alexandru Draghicu. Survival analysis is generally used to illustrate the expected duration of time until an event happens, and is usually used in assessing time until death in disease models. The test involves considering proportions of events (initiation of mating or courtship) of the sample size over time. Survival analysis is based on is non-parametric survival probabilities, so the statistical test is built from the data itself. Two additional statistical tests that I used to compare latency data were the Log-Rank Test (α=0.05) and the Wilcoxon Test (α=0.05). The first test compares the 'hazard function' of two groups at each observed event time and can only be used when the data has proportional effects on the predicted hazard. In cases where the data were not proportional and therefore the assumptions of the Log-Rank Test were violated, I employed the use of the weighted Wilcoxon test. Both of these tests compare between means and indicate which group means are significantly different by reporting a *P*-value. A value of 60:01 minutes: seconds was assigned in each case where males did not initiate courtship for the duration of the 60:00-minute assay. Additionally, all instances where males did not initiate courtship were removed from the courtship to copulation latency data set, as female receptivity cannot be measured without the initiation of courtship.

Table 2.1. Transgenic Fly Strains from Bloomington *Drosophila* Stock Center

Stock #	Chromosome(s) affected	Genotype	Description
5137	1;2	*y^1 w^*; P{w^{+mC}=UAS-mCD8::GFP.L}LL5, P{UAS-mCD8::GFP.L}2*	UAS-GFP
44222	1;3	*w^*; P{w^{+mC}=UAS-shi^{ts1}.K}3*	UAS-*shibire* ^{ts}
26263	1;2	*w^*; P{$y^{+t7.7}$ w^{+mC}=UAS-TrpA1(B).K}attP16*	UAS-*dTrpA1* ^{ts}
8765	2	*w^*; P{w w^{+mC}=GAL4-elav.L}2/CyO*	Gal4-*elav*
23138	1	*C(1)DX, y^1 w^1 f^1/P{w^{+mC}=Or88a-GAL4.F}51.2, w^**	Odorant Receptor (*Or88a*)- Gal4
9983	1;2	*w^*; P{w^{+mC}=Or47b-GAL4.7.467}15.5A*	Odorant Receptor (*Or47b*)- Gal4
49294	1;3	*w^{1118}; P{$y^{+t7.7}$ w^{+mC}=GMR21B03-GAL4}attP2*	*pain*- Gal4
6753	1;3	*w^*; P{w^{+mC}=J21.17-GAL4}JO15/TM3, Sb^1*	Johnston's Organ (JOS)- Gal4
49265	1;3	*w^{1118}; P{$y^{+t7.7}$ w^{+mC}=GMR15E01-GAL4}attP2*	*rutabaga*- Mushroom Body (MB)- Gal4

39159	1;3	*w^{1118};P{$y^{+t7.7}w^{+mC}$=GMR56F06-GAL4}attP2*	Suboesophageal Ganglion (SOG)-Gal4
50284	1;3	*w^{1118}; P{$y^{+t7.7}w^{+mC}$=GMR46H11-GAL4}attP2*	Antennal Mechano-sensory and Motor Center (AMMC)- Gal4
49794	1;3	*w^{1118}; P{$^{[+t7.7}w^{+mC}$=GMR34F03-GAL4}attP2*	Antennal Lobe (AL)- Gal4

Chapter 3

3 Results

3.1 Assays to establish behavior protocol

In assays testing for efficiency of neural suppression or hyperactivation, I observed that within 1-2 minutes of heat-treatment, neural activity was affected. For the Gal4-*elav*/+; UAS-*shi*^{ts}/+ F1 progeny, in which all neurons should be suppressed upon heat treatment, all flies (approximately 10) were immobilized within the first two minutes of heat treatment and remained paralyzed until removed from the restrictive temperature of 32°C. All flies regained normal functioning within seconds of shifting back to the permissive temperature of 24°C. When the Gal4-*elav*/+; UAS-*dTrpA1*^{ts}/+ females were placed at the restrictive temperature of 32°C, which should hyperactivate all neurons, motor functions became erratic and uncontrolled until all flies (approximately 10) experienced what looked like a seizure. Most (90%) lost all motor function and were unable to have coordinated movement due to motor spasms within two minutes of heat treatment; the effects took slightly longer to occur in one fly. As above, flies regained normal functioning within seconds of shifting back to the permissive temperature of 24°C.

I also ran a preliminary assay for each Gal4 line (Table 2.1) paired with each UAS line to ensure that female activity was not affected to such an extent that motor function was visibly impaired at the restrictive temperature. I found that in all protocol assays (N=~10 each), flies did not display obvious locomotor disability: there was no apparent paralysis or seizure-like activity. This suggested that the assay females likely retained their basic ability to avoid or reject males. Deviation of the level of female receptivity relative to the expected phenotype could then be interpreted as a result of modulating neural activity in regions pertinent to regulating sexual receptivity. It is still possible that the silencing or hyperactivation of specific brain regions could cause shifts in female receptivity due to a small focused effect on motor function relevant to female receptivity. However, identifying this brain region due to its effect on receptivity, if present, would still fall within the goals of this research project.

To confirm that males were able to court females at such a high temperature, I tested approximately ten BJS wild-type *D. melanogaster* virgin males paired with 10 BJS wild-type *D. melanogaster* virgin females at 32°C. Only four of 10 males courted females under these conditions. Because courtship was obviously affected by the heat, GFP-sperm *D. melanogaster* males were used rather than wild-type males, upon reference from a colleague. GFP-sperm *D. melanogaster* males proved to be much better courters at the restrictive temperature than wild-type males (nine of 10 males courted, when tested as above), suggesting that these males either court more vigorously or are more heat tolerant than wild-type males. GFP-sperm males were therefore used for all subsequent assays.

3.2 Confirming expression of driver lines

To confirm that the test Gal4 lines were expressing where previously reported, I crossed each Gal4 line (Table 2.1) to UAS-GFP and collected the F1 progeny. I dissected a minimum of three female brains per each F1 genotype and visualized them under fluorescent microscopy. I found that all Gal4 lines were driving GFP expression in the expected region(s) of the brain.

3.3 Silencing the antennal lobe and mushroom body affects female receptivity to conspecific males

A total of 90 (30 Gal4/+; UAS-s*hi*^{ts}/+, 30 Gal4/+ and 30 UAS-s*hi*^{ts}/+) transgenic females were tested for conspecific female receptivity for each candidate brain region. In females containing both the Gal4 and the UAS-*shi*^{ts}, neural activity was silenced for the entire duration of the one-hour assay. Females that had brain regions silenced were courted as rapidly as the control females, with the exception of the mushroom body, where the test group had significantly slower initiation of courtship compared to the UAS/+ control (Table 3.1). This did not have an effect on the experimental groups' overall willingness to mate, as they copulated at significantly higher levels compared to both control groups. This indicates that reductions or increases in overall copulation levels are not due to the time until the initiation of male courtship.

Table 3.1. Latency to courtship when silencing candidate brain regions.

Gal4 Drivers[1]	Group 1[2] Gal4/+; UAS/+	Group 2[2] Gal4/+	Group 3[2] UAS/+	*P*-value[3] (Group 1 vs 2)	*P*-value[3] (Group 1 vs 3)	N
SOG	1:42 ± 2:32	2:23 ± 2:41	4:14 ± 5:17	0.850	0.766	31
Or47b	4:48 ± 6:19	5:21 ± 5:19	5:57 ± 6:33	0.323	0.383	60
Or88a	3:24 ± 3:09	4:26 ± 03:25	6:40 ± 04:59	0.819	0.614	30
AMMC	4:07 ± 4:55	4:36 ± 07:43	5:12 ± 05:37	0.891	0.193	60
JOS	2:14 ± 3:11	2:20 ± 02:38	3:02 ± 02:47	0.960	0.834	30
AL	3:52 ± 3:20	4:06 ± 3:32	4:30 ± 3:38	0.967	0.899	30
MB (*rut*)	2:35 ± 2:39	3:43 ± 5:40	4:40 ± 5:02	0.168	0.003*	60
Pain	7:40 ± 6:36	5:42 ± 4:56	5:56 ± 8:21	0.843	0.791	30

[1] Gal4 Drivers abbreviations as in Table 2.1; induced by the corresponding Gal4 driver.
[2] Average latency (minutes: seconds) ± SD. Replicates are shown pooled, where applicable (see Table 3.1).
[3] Log-Rank Test or Wilcoxon Test (depending on whether data is proportional; see Methods): CI 95%, α=0.05. *P*-values indicate the probability of getting a result that is equal to or more extreme than the control groups.
* Indicates statistical significance of test group

The proportion of females who copulated out of those that were courted was used as a proxy for female receptivity when *D. melanogaster* females were paired with *D. melanogaster* males. Silencing six out of eight brain regions showed no statistical significance when mating levels were compared between treatment and control groups (Table 3.2). This indicates that silencing the neurons in the suboesophageal ganglion (SOG), odorant receptors 47b (Or47b) or 88a (Or88a), antennal mechano-sensory and motor center, Johnston's organ, and the neurons expressing the *pain* gene all do not appear to significantly increase or decrease female receptivity.

However, silencing two out of eight brain regions significantly affected mating patterns when compared to control groups. When neurons are silenced in the antennal lobe, females show significantly lower copulation levels compared to controls when neurons are silenced in the antennal lobe ($P = 0.043$) and the mushroom body ($P = 0.042$; Table 3.2). However, the significant effect of silencing the mushroom body was not observed for one of the replicates, and thus this finding may require additional confirmation. When the average latency between courtship and copulation is compared as an average value (Table 3.3), or as a cumulative survival analysis (Figure 3.1 and 3.2), none of the brain regions showed statistical significance between the test females and both controls. Although no significant difference was seen between groups for latency to copulation, the graphs for the two brain regions in which overall proportions of copulation were significant are included.

Table 3.2. Percent conspecific copulation out of those that were courted when silencing candidate brain regions.

Gal4 Drivers[1]	N	Gal4/+; UAS-*shi*^{ts}/+ (%)[2]	Gal4/+ (%)[2]	UAS/+ (%)[2]	*P*-value[3]
SOG	31	70	77	50	0.080
Or47b	30	40	40	33	0.891
Or47b (2)	30	60	77	83	0.155
Or47b (1+2)	60	48	57	58	0.512
Or88a	30	33	43	43	0.691
AMMC	30	40	50	27	0.201
AMMC (2)	30	47	60	43	0.337
AMMC (1+2)	60	43	57	35	0.092
JOS	30	43	60	50	0.469
AL	30	37	70	53	0.043*
MB (*rut*)	30	74	70	70	1.000
MB (*rut*) (2)	30	77	43	40	0.007*
MB (1+2)	60	75	57	55	0.042*
Pain	30	66	53	77	0.184

[1] Gal4 Drivers abbreviations as in Table 2.1; targeted and manipulated by the corresponding Gal4 driver. (2) indicates the second replicate of the same Gal4 driver. (1+2) means that replicates were pooled and re-analyzed.

[2] Percentages are out of n=26-30
[3] Fisher's Exact test (two-tailed): CI 95%, α=0.05. *P*-values are included to indicate statistical significance between groups. *Post-hoc* paired z-tests were used to confirm that the significantly different group is the test group compared to controls.
[*] indicates statistical significance of test group after *post hoc* pairwise comparison

Table 3.3. Latency to copulation when silencing candidate brain regions.

Gal4 Drivers[1]	Group 1[2] Gal4/+; UAS/+	Group 2[2] Gal4/+	Group 3[2] UAS/+	*P*-value[3] (Group 1 vs 2)	*P*-value[3] (Group 1 vs 3)
SOG	7:29 ± 4:24 (21)	6:21 ± 4:50 (23)	7:44 ± 3:23 (15)	0.613	0.089
Or47b	8:37 ± 7:14 (29)	9:07 ± 11:33 (34)	11:30 ± 6:32 (35)	0.727	0.644
Or88a	10:18 ± 5:49 (10)	5:51 ± 4:51 (13)	5:51 ± 3:01 (13)	0.466	0.514
AMMC	5:55 ± 5:12 (26)	6:38 ± 5:57 (33)	6:31 ± 6:42 (21)	0.182	0.477
JOS	5:28 ± 2:51 (13)	4:23 ± 2:44 (18)	4:44 ± 2:38 (15)	0.126	0.658
AL	8:00 ± 4:10 (11)	5:37 ± 2:59 (21)	7:30 ± 5:04 (16)	0.004*	0.228
MB (*rut*)	7:19 ± 7:25 (45)	6:48 ± 5:27 (34)	9:25 ± 7:53 (33)	0.052	0.014*
Pain	9:57 ± 7:36 (20)	6:26 ± 6:43 (16)	8:05 ± 7:08 (23)	0.519	0.568

[1] Gal4 Drivers abbreviations as in Table 2.1; induced by the corresponding Gal4 driver.
[2] Average latency (minutes: seconds) ± SD (sample size listed in parentheses). Replicates are shown pooled, where applicable (see Table 3.1).
[3] Log-Rank Test or Wilcoxon Test (depending on whether data is proportional; see Methods): CI 95%, α=0.05. *P*-values indicate probability that the test group is different from the listed control group.
* Indicates statistical significance of test

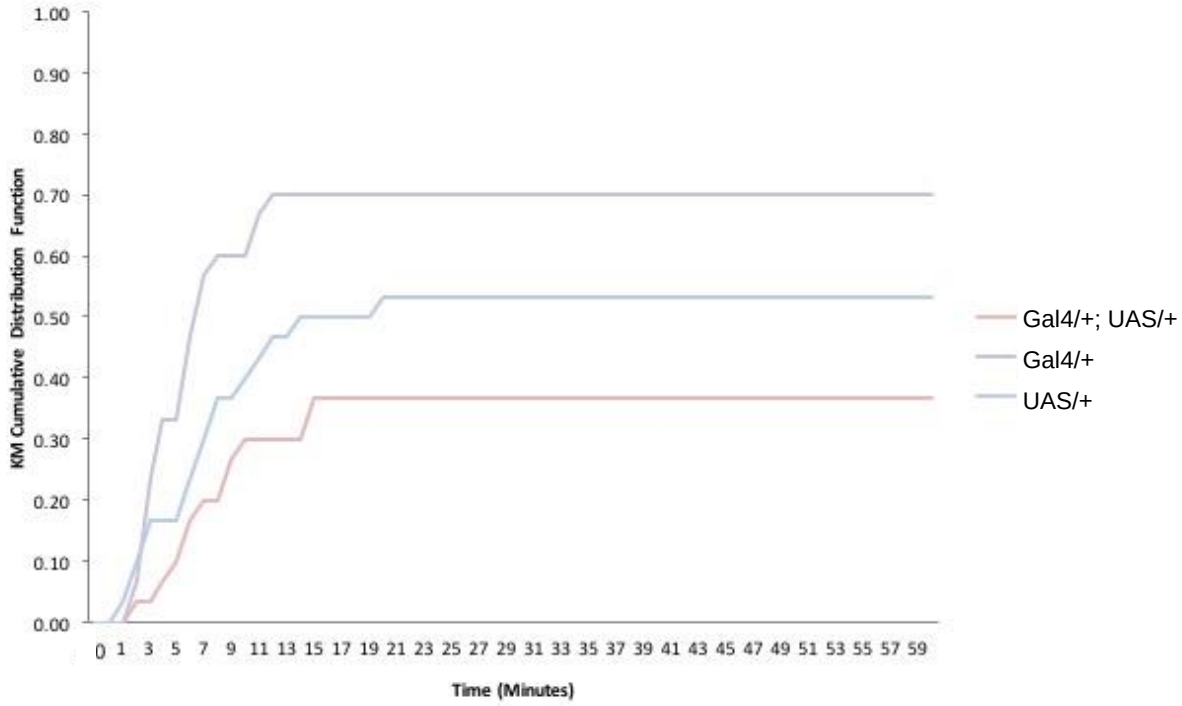

Figure 3.1: Antennal lobe silencing assay- latency to copulation. Kaplan–Meier (KM) Survival analysis: Cumulative Distribution function. Silencing the antennal lobe caused females to copulate significantly slower than the Gal4/+ control (P=0.004) but not the UAS/+ control (P=0.228).

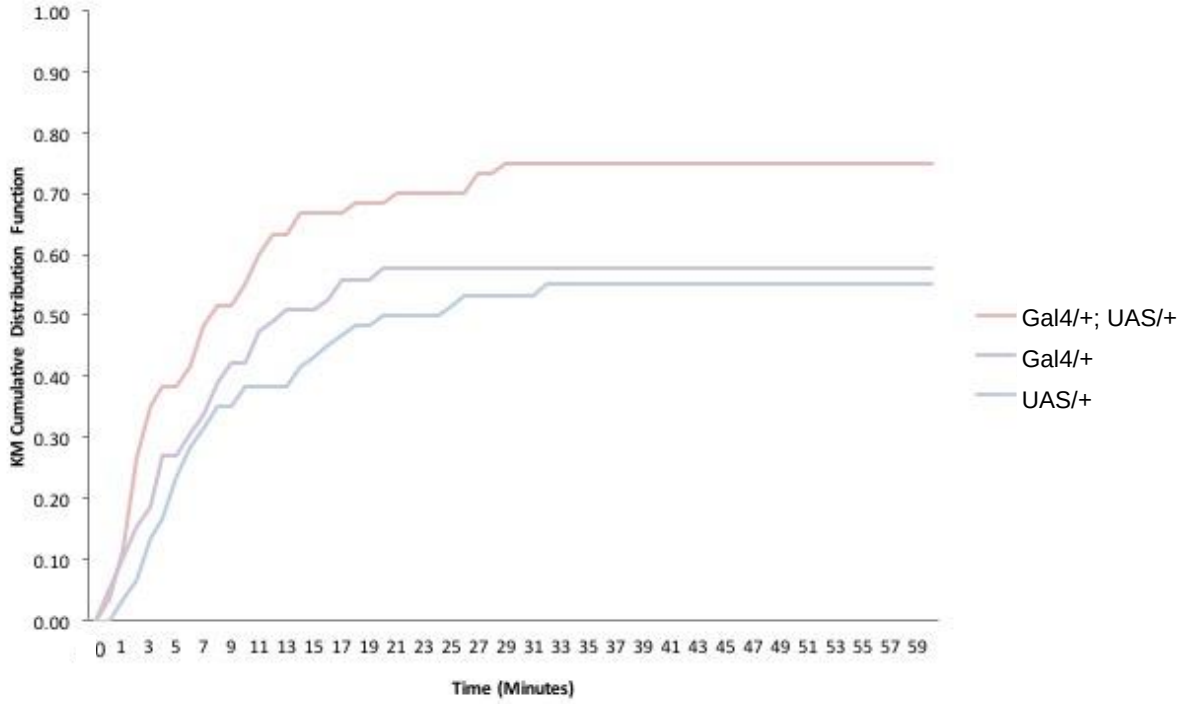

Figure 3.2: Mushroom body silencing assay- latency to copulation. Kaplan–Meier (KM) Survival analysis: Cumulative Distribution function. Silencing the mushroom body caused females to copulate significantly faster than the UAS/+ control (P=0.014) but not the Gal4/+ control (P=0.052).

3.4 Hyperactivating odorant receptor 47b, the Johnston's organ and the mushroom body affect female receptivity to conspecific males

As above, females that had brain regions hyperactivated were courted as rapidly as the control females (Table 3.4). There were no statistically significant differences between Gal4/+; UAS-*dTrpA1*^{ts}/+ females and the control groups. This indicates that reductions or increases in overall copulation levels are not due to differences in the latency until the male begins courtship.

A total of eight candidate brain regions were tested for within-species (*D. melanogaster* female paired with *D. melanogaster* male) female receptivity. Five out of eight brain regions showed no statistical significance when mating levels, out of those females that were courted, were compared between treatment and control groups (Table 3.5; Table 3.6). Manipulating activity in three out of eight brain regions significantly affected the proportion of females that copulated with conspecific males. Cumulative survival graphs for latency to copulation were included for all instances where the experiential group copulated significantly faster or slower than control groups (Table 3.6; Figure 3.3; 3.4; 3.5).

Females with hyperactivation of the Or47b neurons showed significantly higher levels of copulation when compared to controls in one of the replicates and when replicates were pooled ($P = 0.022$; $P = 0.018$, respectively), while the other replicate approached significance ($P = 0.056$). The average latency between courtship and copulation was not significantly different from controls when Or47b neurons were hyperactivated (Table 3.6).

Hyperactivation of the Johnston's organ increased the proportion of females that mated compared to both control groups ($P = 0.014$; Table 3.5). The average latency between courtship and copulation was also significantly reduced (faster copulation) compared to the two controls ($P = 0.001$; 0.014; Table 3.6), and the survival curve of the time to copulation was significantly different between the hyperactivated females and the two controls (Figure 3.3).

Table 3.4. Latency to court when hyperactivating candidate brain regions.

Gal4 Drivers[1]	Group 1[2] Gal4/+; UAS/+	Group 2[2] Gal4/+	Group 3[2] UAS/+	*P*-value[3] (Group 1 vs 2)	*P*-value[3] (Group 1 vs 3)	N
SOG	2:40 ± 3:01	3:20 ± 3:01	3:38 ± 2:33	0.869	0.783	31
Or47b	5:09 ± 7:39	4:18 ± 5:39	6:00 ± 4:55	0.658	0.702	60
Or88a	5:42 ± 5:46	6:22 ± 6:52	3:52 ± 3:29	0.975	0.995	30
AMMC	4:26 ± 4:49	4:32 ± 5:43	4:28 ± 4:45	0.590	0.733	60
JOS	1:42 ± 2:26	2:55 ± 2:50	4:58 ± 4:59	0.530	0.313	40
AL	4:20 ± 4:39	4:41 ± 4:02	6:37 ± 5:09	0.787	0.394	45
MB (*rut*)	3:58 ± 4:22	3:53 ± 5:30	4:43 ± 4:49	0.747	0.136	60
Pain	4:04 ± 4:59	3:12 ± 4:10	3:12 ± 3:01	0.902	0.978	30

[1] Gal4 Drivers abbreviations as in Table 2.1; induced by the corresponding Gal4 driver.
[2] Average latency (minutes: seconds) ± SD. Replicates are shown pooled, where applicable (see Table 3.1).
[3] Log-Rank Test or Wilcoxon Test (depending on whether data is proportional; see Methods): CI 95%, α=0.05. *P*-values indicate the probability of getting a result that is equal to or more extreme than the control groups.

Table 3.5. Percent conspecific copulation out of those that were courted when hyperactivating candidate brain regions.

Gal4 Drivers[1]	N	Gal4/+; UAS-*dTrpA1^{ts}*/+ (%)[2]	Gal4/+(%)[2]	UAS/+ (%)[2]	*P*-value[3]
SOG	30	47	33	33	0.775
Or47b	30	60	50	30	0.056
Or47b (2)	30	87	57	60	0.022*
Or47b (1+2)	60	73	50	53	0.018*
Or88a	30	47	33	40	0.938
AMMC	37	76	51	68	0.095
AMMC (2)	30	53	53	30	0.246
AMMC (1+2)	67	60	45	45	0.141
JOS	40	78	48	53	0.014*
Antennal Lobe	45	60	40	38	0.105
MB (*rut*)	30	93	73	60	0.007*
MB (*rut*) (2)	30	77	33	27	0.001*
MB (1+2)	60	85	53	35	6.236e-8*
Pain	30	73	47	43	0.061

[1]Gal4 Drivers abbreviations as in Table 2.1; induced by the corresponding Gal4 driver. (2) indicates the second replicate of the same Gal4 driver. (1+2) means that replicates were pooled and re-analyzed.

[2] Percentages are out of n=30-60 and numbers in parentheses indicate sample (in some cases, not all individuals were courted, however the difference was minimal (>4) and did not impact statistical inferences)

[3] Fisher's Exact test (two-tailed): CI 95%, α=0.05. *P*-values are included to indicate statistical significance between groups. *Post-hoc* paired z-tests were used to confirm that the significantly different group is the test group compared to controls.

[*] indicates statistical significance of test group after *post hoc* pairwise comparison

Table 3.6. Latency to copulation when hyperactivating candidate brain regions.

Gal4 Drivers[1]	Group 1[2] Gal4/+; UAS/+	Group 2[2] Gal4/+	Group 3[2] UAS/+	*P*-value[3] (Group 1 vs 2)	*P*-value[3] (Group 1 vs 3)
SOG	3:00 ± 2:42 (17)	6:09 ± 4:56 (14)	5:09 ± 5:21 (14)	0.253	0.467
Or47b	9:08 ± 9:13 (44)	6:00 ± 7:34 (30)	6:11 ± 7:08 (32)	0.050	0.189
Or88a	5:13 ± 2:56 (14)	13:18 ± 10:24 (10)	10:12 ± 8:28 (10)	0.158	0.454
AMMC	6:56 ± 14:50 (40)	6:05 ± 3:17 (30)	7:23 ± 6:49 (30)	0.188	0.224
JOS	2:56 ± 2:18 (31)	6:09 ± 5:49 (19)	5:29 ± 3:41 (21)	0.001*	0.014*
AL	5:00 ± 4:17 (27)	6:10 ± 4:17 (18)	6:00 ± 3:16 (17)	0.022*	0.064
MB (*rut*)	4:25 ± 4:53 (51)	6:37 ± 6:38 (32)	8.26 ± 7:19 (21)	6.0e-5*	2.0e-5*
Pain	2:55 ± 2:03 (22)	9:34 ± 9:02 (14)	7:14 ± 3:19 (13)	0.003*	0.016*

[1] Gal4 Drivers abbreviations as in Table 2.1; induced by the corresponding Gal4 driver.
[2] Average latency (minutes: seconds) ± SD (sample size listed in parentheses). Replicates are shown pooled, where applicable (see Table 3.1).
[3] Log-Rank Test or Wilcoxon Test (depending on whether data is proportional; see Methods): CI 95%, α=0.05. *P*-values indicate the probability of getting a result that is equal to or more extreme than the control groups.
* Indicates statistical significance of test group

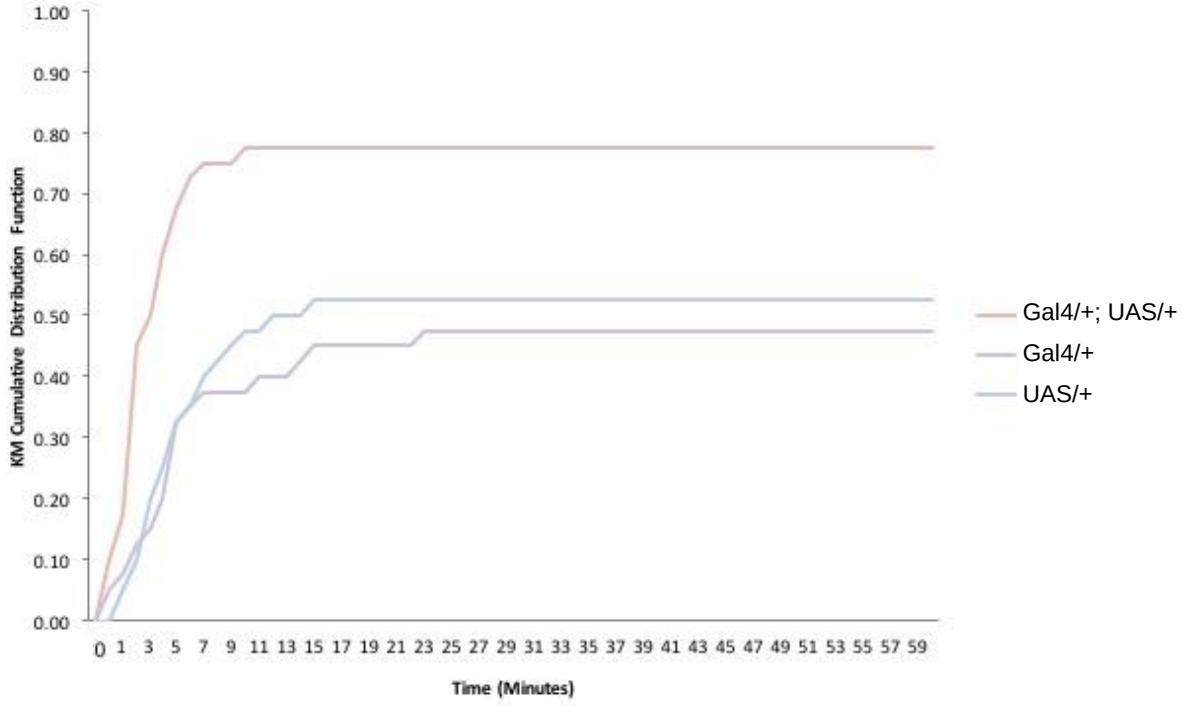

Figure 3.3: Johnston's organ hyperactivation assay- latency to copulation. Kaplan - Meier Survival analysis: Cumulative Distribution function. Hyperactivating the Johnston's organ caused females to copulate significantly faster than both control groups (*P*=0.001; 0.014).

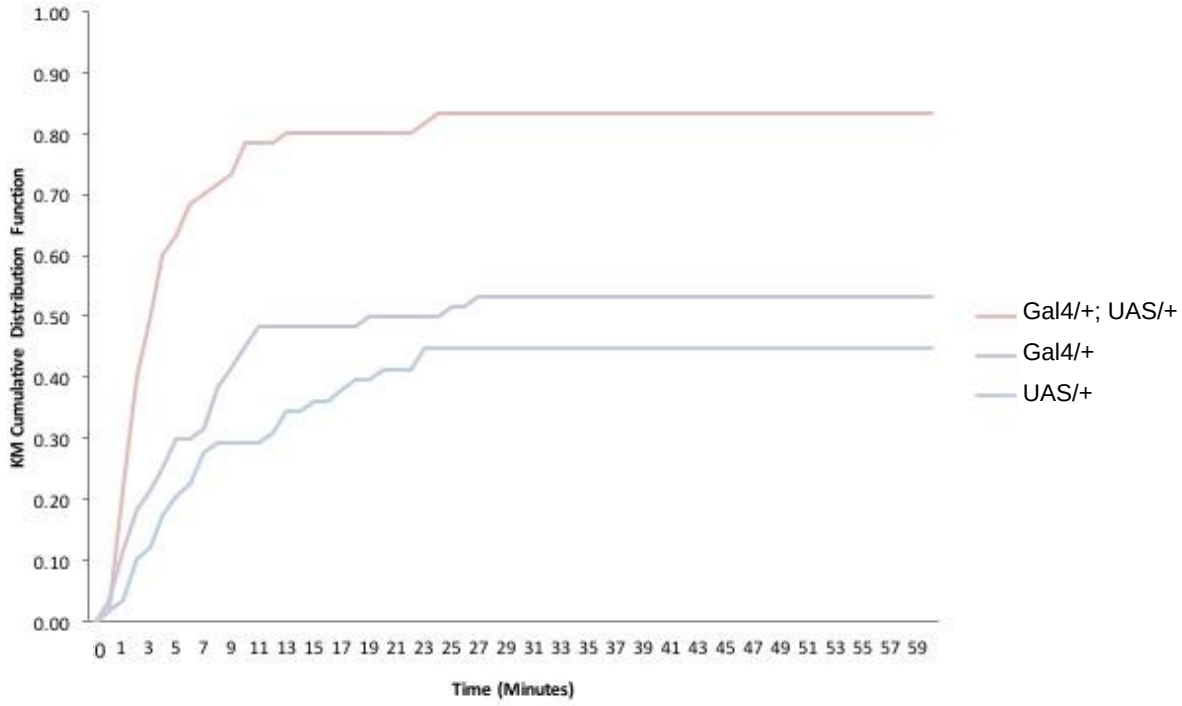

Figure 3.4 Mushroom body hyperactivation assay- latency to copulation. Kaplan - Meier Survival analysis: Cumulative Distribution function. Hyperactivating the mushroom body caused females to copulate significantly faster than both control groups (*P*=0.003; 2.0e-5).

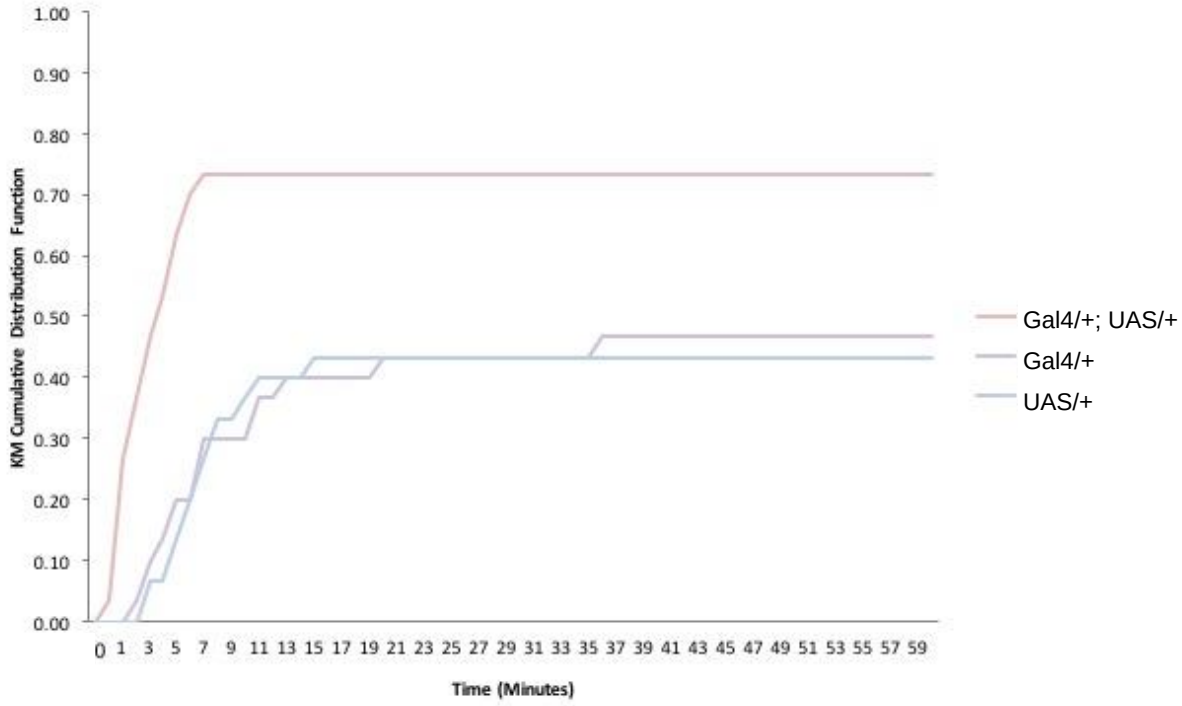

Figure 3.5: *Pain* expressing neurons hyperactivation assay- latency to copulation. Kaplan - Meier Survival analysis: Cumulative Distribution function. Hyperactivating the *pain* gene caused females to copulate significantly faster than both control groups (*P*=0.001; 0.016).

For both replicates and the pooled analysis, females with hyperactivation of the mushroom body showed significantly higher levels of copulation compared to controls (*P* = 0.007; 0.001; 6.236e-8, respectively; Table 3.5). When the mushroom body was hyperactivated, females from the experimental test group copulated faster (had reduced latency) than both controls after courtship was initiated (*P* = 6.0e-5; 2.0e-5, respectively; Table 3.6; Figure 3.3). This result is consistent with other findings that suggest that hyperactivating *rutabaga*-expressing neurons in the mushroom body may enhance female receptivity within species (Han et al. 1992).

Lastly, the when neurons expressing the *pain* gene were hyperactivated, latency to copulation was significantly reduced compared to both control groups (*P* = 0.003; 0.014, respectively; Table 3.6; Figure 3.5).

3.5 Silencing candidate brain regions does not affect female receptivity to heterospecific males

A total of 90 (30 Gal4/+; UAS-*shi*^{ts}/+, 30 Gal4/+ and 30 UAS-*shi*^{ts}/+) *D. melanogaster* transgenic females were tested for each of eight candidate brain regions, and scored for copulation occurrence with *D. simulans* males (Table 3.7). For each assay, neural activity was silenced in the candidate brain region for the entire duration of the assay (48 hours). All eight candidate brain regions showed no statistical significance when mating levels were compared between treatment and control groups (*P*>0.05; Table 3.7).

3.6 Hyperactivating candidate regions does not affect female receptivity to heterospecific males

As above, a total of 90 (30 Gal4/+; UAS-*dTrpA1*^{ts}/+, 30 Gal4/+ and 30 UAS-*dTrpA1*^{ts} /+) transgenic females were tested for each of eight candidate brain regions (Table 3.8). Percent copulation was used as a proxy for female receptivity when *D. melanogaster* females were paired with *D. simulans* males. None of the eight candidate brain regions had a statistically significant effect on female receptivity when mating levels were compared between treatment and control groups (*P*>0.05; Table 3.8).

Table 3.7. Percent heterospecific copulation when silencing candidate brain regions.

Gal4 Drivers[1]	Gal4/+; UAS-s*hi*^{ts}/+ (%)[2]	Gal4/+ (%)[2]	UAS/+ (%)[2]	***P***-value[3]
MB(rut)	7	10	10	1.000
Or47b	7	13	15	0.611
Or47b (2)	13	15	3	0.328
Or47b (1+2)	6	9	6	0.734
SOG	13	13	3	0.380
Or88a	7	0	0	0.326
AMMC	3	7	3	1.000
Antennal Lobe	15	13	10	0.925
JOS	3	3	0	1.000
Pain	7	10	13	0.905

[1] Gal4 Drivers abbreviations as in Table 2.1; induced by the corresponding Gal4 driver. (2) indicates the second replicate of the same Gal4 driver. (1+2) means that replicates were pooled and re-analyzed.

[2] Percentages are out of n=30, except in (1+2) pooled replicates, which have n=60.

[3] Fisher's Exact test (two-tailed): CI 95%, α=0.05. *P*-values indicate statistical significance between groups.

Table 3.8. Percent heterospecific copulation when hyperactivating candidate brain regions.

Gal4 Drivers[1]	Gal4/+; UAS-*dTrpA1^{ts}*/+ (%)[2]	Gal4/+(%)[2]	UAS/+(%)[2]	***P*-value[3]**
MB(rut)	13	3	7	0.493
Or47b	20	13	13	0.815
Or47b (2)	15	13	7	0.592
Or47b (1+2)	11	7	6	0.472
SOG	10	7	7	1.000
Or88a	0	3	0	1.000
AMMC	3	7	3	0.318
Antennal Lobe	10	10	0	0.238
JOS	3	0	0	1.000
Pain	7	7	3	1.000

[1] Gal4 Drivers abbreviations as in Table 2.1; induced by the corresponding Gal4 driver. (2) indicates the second replicate of the same Gal4 driver. (1+2) means that replicates were pooled and re-analyzed.

[2] Percentages are out of n=30, except in (1+2) pooled replicates, which have n=60.

[3] Fisher's Exact test (two-tailed): CI 95%, α=0.05 *P*-values indicate statistical significance between groups.

Chapter 4

4 Discussion

4.1 Manipulating activity in odorant receptor 47b, the Johnston's organ, the antennal lobe and the mushroom body affects female receptivity towards conspecific males

Reproductive behaviors are essential for the survival and fitness of the species. In *Drosophila melanogaster*, as in many other species, the decision of whether or not to mate is usually under the control of the female (Bussell et al. 2014). However, we still have a limited understanding of the choosy behavior displayed by the female fly and the genes and neuronal circuits underlying it. The present study sought to bridge the existing knowledge gap by discretely and non-invasively manipulating activity in candidate regions of the female *Drosophila* brain. This was done to ascertain whether modulating brain activity in candidate regions affects female receptivity to conspecific males. I silenced and stimulated neural activity through the use of temperature-sensitive transgenes and then subjected animals to behavioral assays. My results show that modulating activity of the suboesophageal ganglion, the antennal mechano-sensory and motor center, odorant receptor 88a and the *painless* gene did not have a significant effect on female receptivity. However, manipulating activity in odorant receptor 47b, the antennal lobe, Johnston's organ and the mushroom body did have a significant effect on female receptivity towards conspecifics. I found that silencing neural activity in the antennal lobe decreased female receptivity, silencing or hyperactivating the mushroom body enhanced female receptivity, and hyperactivating odorant receptor 47b and the Johnston's organ enhanced female receptivity compared to controls when females were paired with conspecific *D. melanogaster* males. It is worthwhile to note that the majority of regions that had a significant effect on female receptivity are involved in olfactory processing. The next step in the search for the neural mechanisms of female receptivity in *Drosophila* could be to focus on the role the olfactory system plays in determining female receptivity.

In *Drosophila*, hearing is primarily mediated by the Johnston's organ, which is particularly sensitive to the subtle vibrations discharged by males during courtship (Eberl & Boekhoff-Falk 2007). Although silencing this organ did not appear to affect female receptivity, hyperactivating it was. Because hearing is a major component of courtship, perhaps heightening the females' perception of this precursor cue causes her to disregard other sensory repellents and allows her to be less discriminatory, thereby enhancing receptivity (Eberl & Boekhoff-Falk 2007).

Based on previous research, it was expected that hyperactivating Or47b, which exclusively detects the general attractant molecule methyl laurate, would enhance female receptivity (Dweck et al. 2015). As expected, I found that hyperactivating Or47b neurons significantly increased female receptivity. Hyperactivating this receptor is potentially deceiving the fly into thinking that she is detecting methyl laurate, enhancing female receptivity. In contrast, silencing activity in Or47b had no apparent effect on female receptivity, perhaps because there are several other attractant molecules that may have been detected by the female, indicating that the male was a suitable mate.

I also found that silencing activity in the antennal lobe was enough to decrease female receptivity to conspecific males. As previously mentioned, the antennal lobe is the primary processor of scent stimulus in the *Drosophila* brain (Bhandawat et al. 2007). With activity in this system turned off, the female did not have the ability to process any of the detected pheromones. In the absence of olfactory information, an important part of courtship, she will potentially not become receptive. The role of olfaction in female receptivity is further supported by studies of mutations in the *spinster* gene. Recall that *spinster* is expressed in the antennal lobe, and that a reduction in *spinster* expression reduces female sexual receptivity (Sakurai et al. 2013).

Lastly, I saw that both inhibition and hyperactivation of the *rutabaga*-expressing neurons in the mushroom body caused enhanced female receptivity. *Rutabaga* is a gene that is highly enriched in the alpha (α), beta (β) and gamma (γ) lobes of the mushroom body, and is expressed at lower levels in the calyx of the mushroom body and suboesophageal ganglion (Han et al. 1992). Perhaps normal functioning of the mushroom

body, in particular the areas where *rutabaga* is highly expressed, is essential for regulating female receptivity. As previously mentioned, the mushroom body is involved in learning and memory, associative olfactory memory and higher order cognition (Heuer et al. 2010; Aso et al. 2008). It is possible that increased receptivity in response to both hyperactivation and silencing of the mushroom body is due to altered function at a site of high convergence of sensory input, scrambling the female's ability to discriminate, leaving her in a default receptive state (Perisse et al. 2013). However, the mushroom body is more than just an integration unit and plays a role in the highest level of sensory processing. Another possible explanation for seeing enhanced female receptivity when applying antagonistic treatments to the mushroom body could be that homeostasis is being disrupted. A recent study focused on investigating the mechanisms that maintain cAMP homeostasis in the mushroom body found that two antagonistic genes, *dunce* and *rutabaga*, caused very similar defects in synaptic plasticity even though they have opposite effects on cellular cAMP levels (Lee 2015).

Identification of a role for the mushroom body in female receptivity is surprising since previous research found that the mushroom body was not necessary for female receptivity (Neckameyer 1998). In that study, hydroxyurea was used to ablate the mushroom body and there was no effect on the latency to copulation for the eight females that were assayed (Neckameyer 1998). I also did not see a significant shift in latency to copulation when the mushroom body was silenced using UAS-*shibire*, which closely resembles ablation, but rather the effect was only apparent when comparing the proportion of females that copulated. The apparent discrepancy between those findings and mine may also be due to the ablation of the mushroom body through the use of chemicals *vs.* stimulation or suppression of intact neurons. Chemical approaches can potentially be incomplete, and their effects can be more widespread than expected. A more interesting explanation for the contradictory results is that there could be important differences between an organism that has a brain region ablated compared to one that has the neurons intact, but with altered activity. The brain is an extremely plastic organ, and if an entire neural region is resected, the brain can potentially compensate for the loss either by using alternate circuitry or by recruiting other networks to maintain homeostasis (Lanet & Maurange 2014). In my study, I reversibly modulated activity within the

mushroom body rather than removing the structure entirely. Perhaps I was able to see an effect because I kept the mushroom body intact, allowing for surrounding circuitry to maintain connections that would be lost or altered if the mushroom body had been ablated. Alternatively, the mushroom body itself may actively influence receptivity only when neural activity is present; without this neural region, the default level of receptivity remains unaltered. Fundamentally, the studies asked different questions regarding female receptivity: whether copulation latency is the same in a female lacking the mushroom body *vs*. if stimulating or silencing an intact mushroom body affects copulation latency or occurrence.

4.2 Manipulating activity in candidate regions does not affect female receptivity towards heterospecific males

My results show that either silencing or hyperactivating candidate regions in the brain had no significant effect on female receptivity between species. Because candidate regions were chosen based on the sensory modalities involved in courtship recognition, the results were unexpected. These findings may mean that these particular regions are not operational in discriminating against heterospecific males. In other words, that the regions that regulate receptivity within a species are not the same as those that serve to maintain sexual isolation between species.

Alternatively, it could mean that the regions are not individually sufficient to induce a change in receptivity. If neural activity in one region is either "turned on or off" but stimulatory cues are being received and processed in other areas of the brain, the female may still maintain the same level of receptivity. Perhaps the brain regions involved in species discrimination have been evolutionarily reinforced to such a degree that they must work in concert with one another to produce species-specific sexual behavior. Therefore, shutting down or stimulating one unit at a time may not have been powerful enough to overcome the stimuli from the courting male of another species, as other normally functioning units may have been enough to maintain this isolation. However, another study (Vaughan et al. 2014) successfully used neuronal inactivation and hyperactivation to identify a critical pathway for auditory recognition during

courtship. Two neurons involved in recognizing conspecific courtship song were isolated, a projection neuron (aPN1) and a local interneuron (aLN). Thus, hyperactivation and silencing of neuronal subsets can be used to identify some of the neurons involved in species recognition.

Another explanation for the lack of change in female receptivity between species could be that each neural region that I tested had an effect on behavior, but manipulating activity in one region at a time did not precipitate a strong enough phenotype to significantly shift behavior as measured using my assay. It may be necessary to delineate which neural substrates play a role in sexual isolation through the use of larger sample sizes or different approaches entirely.

It remains unclear whether the same neural areas that are responsible species recognition are the same as those involved in mate quality assessment. There is some evidence that these brain regions may not be the same. For example, in songbirds (*Melospiza georgiana*), the neurons in the primary auditory area are selectively activated by conspecific song while neurons in the secondary auditory area are selectively activated by preferred song types (Mooney et al. 2001).

4.3 Limitations

While the Gal4/UAS system is an elegant and powerful genetic tool, with the ability to overcome many restrictions of other widely-used systems, it poses limitations of its own. Many Gal4 lines have low expression in off-target areas, which can confound interpretation of results. Further, it has been noted in previous research that the *shibire*^{ts} transgene has the potential for low levels of expression even at permissive temperatures (Margulies et al. 2005). However, this does not appear to have occurred in the present study, as I did not see significantly different amounts of female receptivity between the two control groups.

I also encountered some limitations in my methodology. In order to activate the temperature sensitive transgenes, I needed to subject the animals to temperatures above 30°C, which was essentially a heat-shock. This effect was partially alleviated by acclimating the flies to this high temperature prior to beginning the assay. All test flies and

controls were subjected to the same temperature, controlling for the effect of temperature on mating. Another limitation of the experimental design was that the experimental flies were not in the same genetic background as the controls. I could control for the effect of the genetic background by re-testing the same cohort of females at the permissive temperature as well as the restrictive temperature, then statistically comparing the same genotype at the permissive *vs.* restrictive temperature. Regions of further interest would have no difference in the controls between the permissive and restrictive temperatures, but a significant shift in receptivity for the test females when shifted to the restrictive temperature.

Lastly, *D. melanogaster* females mate very infrequently with *D. simulans* males. *D. simulans* is also more sensitive to high temperatures than *D. melanogaster* (Capy et al. 2004). These two aspects of mating behavior could account for the extremely low mating levels that I saw in my experiment. I could circumvent the effect of high temperature in the future by employing the use of a different mechanism of conditional gene activation. For example, one such system in which activation does not require changes in temperature is optogenetics, in which particular wavelengths of light induce expression of Gal4 (Inagaki et al. 2014).

4.4 Conclusions and future directions

The evolution of the brain has had a fascinating and complex history. Originating from a single common ancestor, the simple proto-brain has since evolved into a highly ordered and remarkably complex central nervous system in most animal species (Shepherd 1994). Across metazoan taxa, there is great diversity in the level of functional and structural neural complexity. Regardless of phylogeny, one of the primary purposes of the brain is to control the behavior of an animal. Because the environment is always changing, the selection pressures that act on behavior also change over time. Thus, behavior is constantly evolving. In many organisms, behavior evolves through precursory evolution of the brain, which then generates and facilitates shifts in behavior.

More research needs to be done to further investigate the role of the olfactory system in regulating female receptivity in *Drosophila*. The particular focus should be on

fine-mapping the connecting circuitry between the odorant receptor neurons, the antennal lobe projection neurons and the peripheral and core neurons of the mushroom body. Future endeavors should also focus on sensory processing units, where information converges from multiple sensory modalities that process the cues provided during courtship. Thus, it would be worthwhile to identify the discrete sub-units within the mushroom body that control female sexual behavior. This fine-scale neural analysis can be accomplished by using the split Gal4/UAS system, which has the advantage of separating the binding domain and the activating domain of the Gal4 transcription factor to allow for greater specificity (Dolan et al. 2017). To identify whether this neural region is important in regulating female receptivity more broadly, it would be useful to investigate whether manipulating the mushroom body in another species would affect female receptivity. The locust, *Schistocerca gregaria*, also has a mushroom body region of the brain. Similar to *Drosophila*, the mushroom body in the locust is responsible for olfactory learning and multimodal processing (Laurent & Naraghi 1994).

Studying the regulatory mechanisms of female reproductive behaviors in *D. melanogaster* holds the promise of revealing how the neural circuits can guide decision-making and behavior in general (Dickson 2008). Pavlou and Goodwin (2013) suggest that further investigation is required to clarify which and how neural circuits process courtship behavior. Through the study of model organisms, scientists can further identify the essential biological processes which regulate and maintain reproductive behavior and the integrity of separate species.

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