Title: Regulation of Behavioral Plasticity: Mechanism Underlying Olfaction-Dependent Changes in Male Specific Courtship Behaviors in *Drosophila melanogaster*

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Animal behaviors consists of both innate and plastic components. Hardwired neural circuits are established during development and confer animals the innate potential to solve the survival challenges. However, in an ever-changing environment, animals need to modify their behaviors constantly and accurately to increase their fitness. Sensory experience modulates animal behaviors via changes in gene expression and circuits neurophysiology. Olfaction, one of the most ancient modalities, regulates feeding and social behaviors such as courtship. It is intriguing how olfactory experience modifies animal behaviors. To study this question, we use the olfaction-driven male courtship in the fruit fly *Drosophila melanogaster*, which consists of stereotypical and quantifiable rituals wired by a well-defined, sexually dimorphic circuits.

We found that social experience, conveyed by olfactory detection of fly body pheromone, converges with internal hormone signaling, exerts chromatin-based regulation on the important behavioral regulator, $fruitless^{M}$. fru^{M} is a transcription factor that regulates male-specific behaviors in flies, and it is both necessary and sufficient for male courtship behaviors. We found that juvenile hormone signaling via its receptors Methoprene-tolerant (Met) and Germ-cell expressed (Gce), works coincidentally with calcium signaling and the histone acetyl transferase p300 downstream of Or47b pheromone signaling, to exert transcriptional regulation on fru^{M}, and subsequently

modifies neurophysiology and behaviors. As result, group-reared males gain courtship advantage in Or47b- and fru^M- dependent manner.

Next, we investigated if Or47b is involved in other aspects of behavioral plasticity. fru^M mutant males do not court other flies when held isolation, but they acquire courtship learning with grouping experience in olfaction-dependent manner. We found that Or47b is involved in male-female courtship learning and male-male chaining learning in fru^M mutant males.

Our work elucidated the roles of Or47b in regulating courtship behavioral plasticity in males reared in different social environments. Pheromone signaling via Or47b enhances courtship success in group-reared males with or without the innate fru^M -dependent programs. Our findings validated olfaction-driven male courtship behavior as a good model in studying sensory experience dependent behavioral plasticity, and lays ground for future studies on pheromone-driven modifications on genes and circuits driving male courtship.

Dedication

Dedicated to my parents and my fiancé, with love.

Contents

List of Figures

Acknowledgements

I would like to acknowledge my PI, Pelin Volkan for giving me tremendous support and help throughout my years at Duke. Without her it would be impossible for me to get to where I am. I would want to thank and acknowledge all the current and previous lab members, who gave me valuable suggestions and help in my experiments, especially Bryson Deanhardt, Qichen Duan, Paulina Guerra-Schleske, George Thomas Barlow, Scott Barish, Aishani Saha, Chengcheng Du, and Khanh Vien. I would also like to acknowledge my committee members Rebecca Yang, Mohamed Noor, Anne Yoder, and members of the Duke Neurogenetics community who gave me valuable criticism and suggestions to my experiments.

1. Introduction

"Survival the fittest", as Darwin puts. Organisms evolved strikingly diverse morphological, physiological, and behavioral patterns to adapt to the environment. To strive through the day-to-day challenges, animals must maintain balance between the innate and adaptable components of behaviors. Innate behaviors root from hard wired programs, as they are vital for the survival of the organism. From the earliest moment of life, hard-wired programs prepare the experience-novice organism for growth, development, competition, and reproduction. Innate behaviors such as sucking reflex and startle reaction in newborn human babies, which do not require previous experience or learning, prepare the newborn for life challenges. the delicate pattern of song waves produced by male songbird can be attributed to its sexually dimorphic brain circuitry(Brain et al., 1976). However, this is not enough. In an ever-changing environment, organisms need to maintain the ability to adapt. For example, human child failed to learn language if not exposed to learning within the critical period(Kuhl, 2004). depriving one eye of visual input in the newborn cat leads to the change in ocular dominance column innervation(Wong-Riley, 1979), Those learned behaviors are vital for adaptation to the environment. Despite ample information on the molecular programs for structure and function of neural circuits for innate behaviors, the molecular mechanism underlying behavioral plasticity remain less well-studied.

Courtship behavior of *Drosophila melanogaster* is an ideal model to study this question as courtship behaviors have both innate and plastic components and is heavily regulated by multi-sensory cues.

1.1 The courtship ritual

In Drosophila, courtship consists of a series of stereotypical, and quantifiable rituals. These hard-wired steps require multimodal inputs, integrating olfactory, visual, gustatory, auditory, mechanical, and proprioceptive signals, assessing the quality of potential mate to ensure reproductive success. After flies are brought to a communal substrate by food odors and aggregation pheromones, the ritual starts with a male orienting toward a target female, chasing after and circling the female, tapping the female's pheromone-coated abdomen, raising its two wings alternatively to produce species-specific courtship song, licking of the female genitalia, mounting, bending of abdomen, and copulation(Hall, 1994; Pavlou & Goodwin, 2013) (Figure 1A). A receptive female would slow down in response to a conspecific song, raise her wings and opens the vaginal plate to accept the male for copulation. Unreceptive or mated females reject a male by walking away, kicking with hind legs, wing flicking and ovipositor extension(Ferveur, 2010; Rezával et al., 2012). These steps are robust controlled by hard-wired programs.

Courtship and copulation success is ensured by the selection of appropriate mating target, that is, a sexually mature, receptive, conspecific adult of the opposite sex.

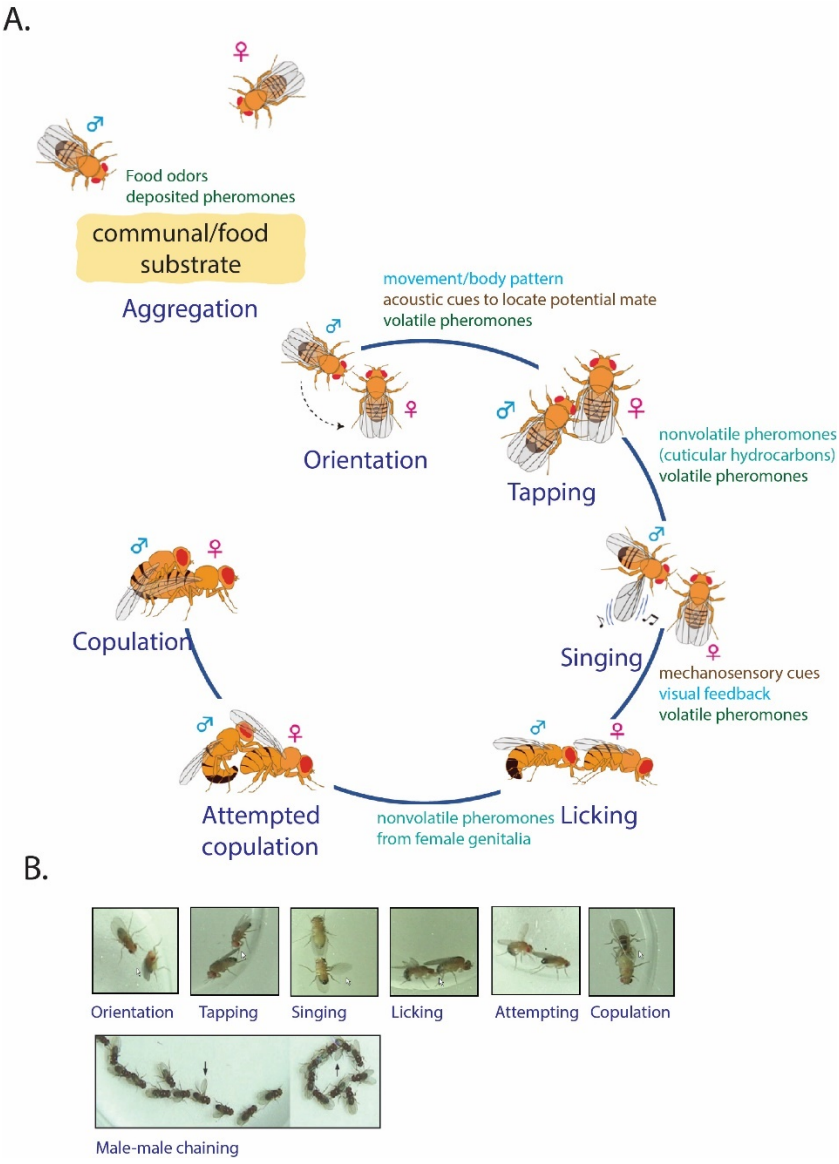

Figure 1: Male specific behaviors in the courtship ritual

Flies are brought to a communal substrate by food odors and deposited pheromones, where they feed, court, and lay eggs. The courtship ritual consists of orienting, tapping, singing, licking, attempted copulation (abdomen bending), and copulation (A and B). Males lacking fru^{M} exhibits male-male chaining. The cartoon graphics and video screenshots are taken from (Yamamoto et al., 2014), (Dai et al., 2008), and (Kitamoto, 2002) with permissions from the publisher.

From the male fly's perspective, it needs to find and pursue the female fly, compete, and win over other males, ensuring its reproductive success. These interactive steps require the males to responds to multi-modal sensory cues, execute the innately wired courtship behaviors, and modify the behaviors in response to changes in sensory cues.

In order to understand how sensory experience modulates behavioral plasticity in *Drosophila* male courtship, we will first start by reviewing the genetic and molecular circuitry underlying male innate and learned courtship behaviors.

1.2 The master regulator of male-specific behaviors: Fru^M

In Drosophila, sex-specific behaviors are encoded by sex-specific circuits, that consists of all-or-none, sex-specific cells. This is achieved by a hierarchy of sex-determination genes (SDGs). Unlike in mammals, where sex is determined by hormones, In Drosophila, sex is determined in a cell-by-cell manner by a series of alternative splicing on SDGs: *sex-lethal* (*sxl*), *transformer* (*tra*), and the bottom of hierarchy lies *doublesex* (*dsx*) and *fruitless* (*fru*) (Figure 2A). *Sxl*, *tra*, and *dsx^F* are made in the XX females, where X chromosome-to-autosome ratio exceeds 1. In the XY males, *dsx^M* and *fru^M* are made(Clough et al., 2014) (Figure 2A-B, Figure 3A). The stories of innate and adapted sex-specific behaviors unwrap around these important behavioral regulators, *dsx^M* and *fru^M*.

Fru^M is a transcription factor that regulates the structure and function of the sex specific behavioral circuits(Figure 2B) (K. I. Kimura et al., 2005; Manoli et al., 2005;

Yamamoto & Koganezawa, 2013b; Yu et al., 2010). Fru^M is both necessary and sufficient for male-specific behaviors. The loss of fru^M in males leads to reduced male-female courtship and elevated male-male courtship and chaining(Stockinger et al., 2005). Ectopic activation of the Fru^M circuitry in intact or even headless solitary male elicits courtship song production, abdomen bending, and ejaculation, components of the courtship rituals(Pan et al., 2011). Furthermore, ectopically expressing the male form of *fruitless* in female masculinizes the female, where she starts to chase after other females and try to court with them(Demir & Dickson, 2005; Manoli et al., 2005; Stockinger et al., 2005).

Given its importance as a master regulator for male-specific behaviors, fru^M is expressed in approximately 2000 neurons in the nervous system, regulating downstream genes involved in a board array of neural developmental activities such as axon guidance and synaptogenesis(Manoli et al., 2005; Neville et al., 2014). In the peripheral nervous system, Fru^M is expressed in a small fraction of olfactory and gustatory neurons on the mouth, leg, genitalia, and antenna and work as detectors of pheromones. It is also expressed in motorneurons mediating song production in the wing joint(Figure 2C)(G. Lee et al., 2000; Stockinger et al., 2005). In the central nervous system, fru^M is expressed in ~2% of the neurons that are organized into 21 distinct clusters in the brain and ventral nerve cord(Figure 2D) (G. Lee et al., 2000).The interesting aspect of fru^M positive neurons is that they are interconnected to one another. For example, in the olfactory system, fru^M

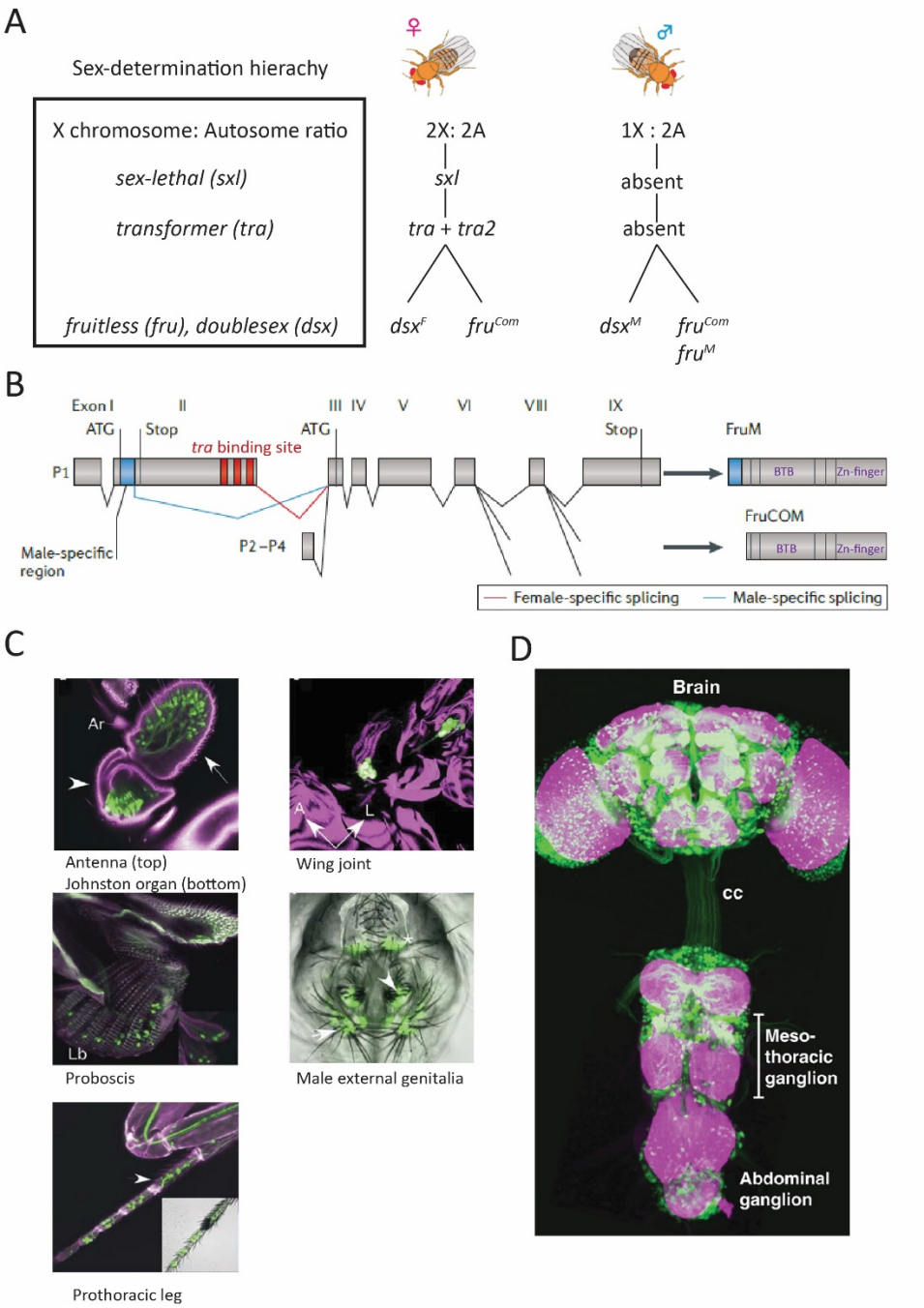

Figure 2: *fru*^{M} is the master regulator of male-specific courtship behaviors

(A). In Drosophila, sex is determined in a cell-by-cell manner by a cascade of sex-specific splicing of the sex-determination genes. In females, X chromosome to autosome ratio exceeds 1, leading to the production of *sex lethal* (*sxl*), *transformer* (*tra*) and the female isoform of *double sex* (*dsx*^{F}). In males, in the absence of *sxl* and *tra*, *dsx*^{M} and

fru^{M} are produced. **(B).** In the absence of *tra* binding to the *tra*-binding sites, *fru* undergoes the default splicing from the P1 promoter, resulting in the production of male-specific *fru* (*fru*^{M}) with the male-specific 101 amino acid residues on the N-terminal, that is absent in the non sex-specific *fru* common isoforms (*fru*^{Com}) generated from P2-P4 promoters. The Fru proteins are BTB-Zinc finger transcription factors that regulates broad arrays of genes involved in neurodevelopment and axongenesis. Exons are shown in boxes, introns are shown as lines. B is modified from Yamamoto & Koganezawa, 2013a with permission from the publisher. (C). In the peripheral nervous system, *fru*^{M} is expressed in the antenna, proboscis, prothoracic legs as pheromone detectors. *fru*^{M} is also expressed in proprioceptive and mechanosensory cells in the wing joint, Johnston's organ, and male external genitalia. Ar, arista; Lb, labellum; arrow heads indicate sex comb of prothoracic leg, lateral plates (top) and claspers (bottom) of the male external genitalia. Asterisk indicates the ventral-most part of the analia. C is adapted from Manoli et al., 2005 with permission from the publisher. (D). In the central nervous system, *fru*^{M} is expressed in ~2000 neurons in the brain and ventral nerve cord. D is adapted from Yu et al., 2010 with permission from the publisher.

positive olfactory receptor neurons connect with *fru*^{M} positive second order neurons, all the way to the motorneurons. This makes *fru*^{M} an excellent molecular marker for neural circuits driving sex-specific behaviors(Cachero et al., 2010).

Temporally, *fru*^{M} expression onset at the wandering third-instar stage, peaks in ~30hr pupae, corresponding to the development and remodeling of the imaginal tissues during metamorphic transition, allowing *fru*^{M} to exerts its regulatory roles in establishing sexual dimorphic circuitry. *fru*^{M} expression then gradually declines to a low level into adult(Lee et al., 2000).

In addition to the sex-specific *fru*^{M}, there are also common isoforms of *fru*, the *fru*^{Com} are detected in the beginning of embryogenesis, regulating neuronal differentiation and development and vital for life(Song et al., 2002). The Fru proteins are BTB zinc-finger proteins, containing a common protein-protein interaction N-terminal

domain, and distinct zinc-finger DNA-binding C-terminal domain, making them suitable modulators for the transcription state in Fru-positive cells(Figure 2B)(Ito et al., 1996; Neville et al., 2014; Sato & Yamamoto, 2020). Fru^M complexes with chromatin regulatory proteins and other transcription factors in the sexual development of the nervous system(Yamamoto et al., 2014; Yamamoto & Koganezawa, 2013b),and regulate genes such as *Notch*, *Abl*, *fra*, *fz*, *spen*, *mam*, and *unc-5*, which are important for neurogenesis and axonogenesis during development(Neville et al., 2014). The TF activity and broad spectrum of genes regulated downstream of fru^M makes it a powerful effector in modulating neurophysiology, neurotransmission, and neural connectivity.

1.3 Dsx

Doublesex and *mab-3*-related transcription factors (DMRTs) is a conserved regulator for sex determination across many species(Zarkower, 2013). In *Drosophila*, Dsx is responsible for sexual dimorphism in morphology, physiology and behavior, the loss of dsx or simultaneous expression of both male and female dsx isoforms leads to intersexual phenotype, such that the male and female genitalia are present simultaneously, rendering its name(Clough et al., 2014; Nagoshi & Baker, 1990).

Dsx^M is neither necessary nor sufficient for male-specific courtship. However, it is important in modulating the plastic aspects of courtship. For example, dsx^M is

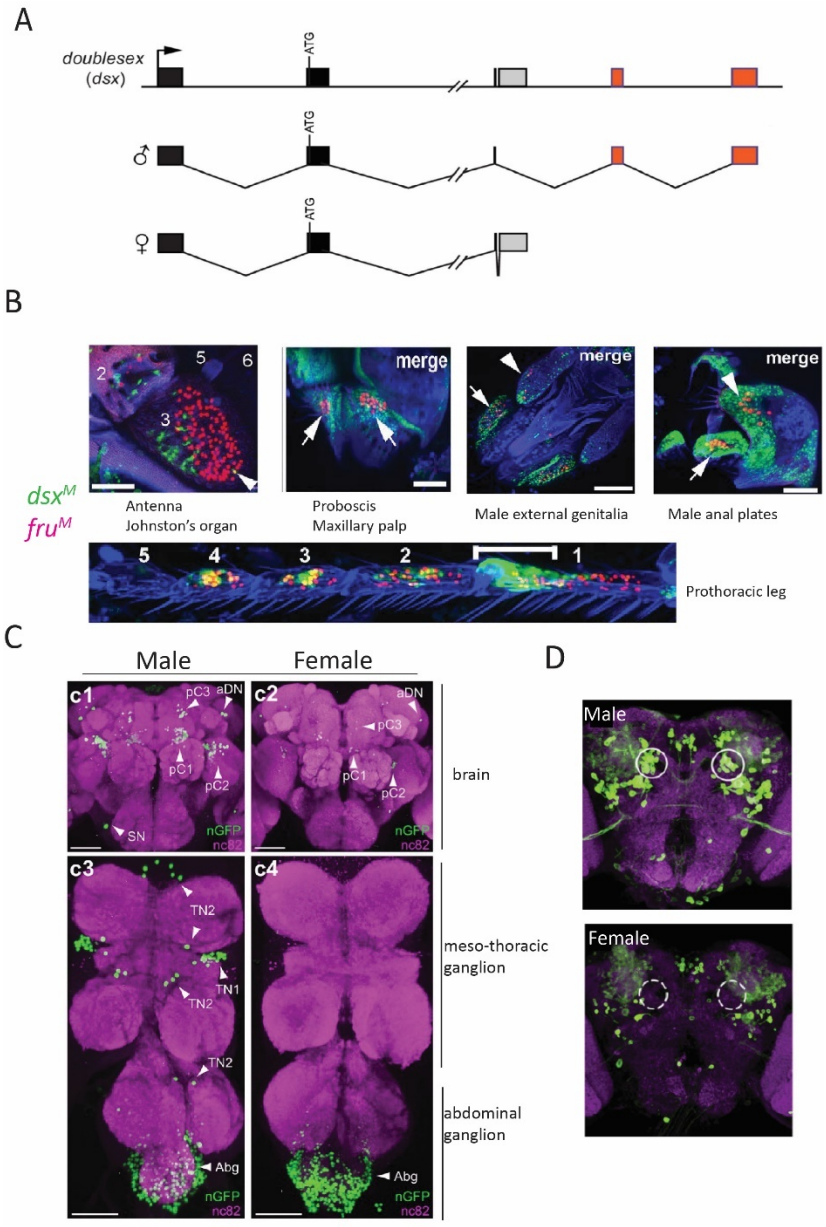

Figure 3: *Doublesex (dsx)* regulates formation of sexual dimorphic circuits

(A). *dsx* is alternatively spliced in males and females, generating the sex-specific male isoform dsx^M and female isoform dsx^F. Exons shown in boxes, red: male exons, generating 152 amino acid male-specific regions; grey: female-specific exons, generating 30 amino acid female-specific regions; black: the common region containing the DNA binding domain. Introns are shown in lines. B. In the peripheral nervous system, dsx^M is expressed in non-overlapping cells with fru^M in the antenna, Johnston's organ, proboscis, and maxillary palps. In the prothoracic leg, male external genitalia, and anal plates, dsx^M

is co-expressed in a small subset of cells with fru^M. C. *dsx* shows sexual dimorphic expression in male and female central nervous system. dsx^{Gal4} driving *UAS-nuclear GFP* in adult male (c1, c3) and female (c2, c4) central nervous system. D. The P1 cluster is present in males (circle) but absent in females (circle with broken line) as result of Dsx^F-driven cell death in female brains. A and C are adapted from Pavlou et al., 2016 , free of copyright available under available under the Creative Commons CC0 public domain dedication. B is adapted from Robinett et al., 2010 free of copyright available under the Creative Commons (CC BY) license. D is adapted from Kimura et al., 2008 with permission from the publisher.

required for courtship-learning in otherwise non-courting fru^M mutant males(Pan & Baker, 2014). On the other side, Dsx^F is required for normal female locomotion, ovipositor extrusion and wing spreading(Rideout et al., 2010). It is important not only in the formation of female-specific circuit, but also important for shaping the male-specific circuit (Figure 3B). For example, a sexually dimorphic cluster of ~20 neurons in the dorsal posterior brain, that is present in male but absent in females, is resulted from female-specific death driven by Dsx^F(Figure 3D). This cluster of cells, later named P1 neurons, sending extensive dendrites in the bilateral protocerebrum, and descending their axons to motor centers in the brain and ventral nerve cord, serve as important integration and decision center in males(G. Lee et al., 2002; Rideout et al., 2010; Sanders & Arbeitman, 2008). P1 is sufficient but not necessary for initiating male courtship behaviors. Activating the male P1 cluster or masculinizing the female's P1 cells is capable of eliciting the early steps of male courtship behaviors, such as chasing and wing vibration(Kimura et al., 2008; Pan et al., 2012; Stockinger et al., 2005).

Dsx is expressed in cells derived from the primary germ layers(Foronda et al., 2012; Tanaka et al., 2011). In the CNS, there are ~700 *Dsx*-positive neurons, that ~300 in the brain, 60 in the thoracic ganglia, and majority in the abdominal ganglion(Figure 3C)(Lee et al., 2002; Pan et al., 2011). The sex-specific isoforms are important in sex-dimorphic neurophysiology and behaviors.

The Dsx^F and Dsx^M proteins have the same DNA binding and dimerization domains, binding to thousands of same targets in diverse tissues, yet modulating sex-specific functions in these tissues via the sex-specific C-termini, a site for different cofactors to modulate gene expression(Clough et al., 2014)(Figure 3A). Dsx regulates sex- and tissue-specific morphologies via genes of the disruptor of telomeric silencing-1 (DOT1) complexes in an evolutionarily conserved manner(Murphy et al., 2010). In addition, Dsx regulates genes involved in short-range (WNT, EGF, DPP) and long-range (insulin and ecdysone) signaling, transcription factors which are known to have sex-specific expression patterns, epigenetic transcription regulators, members of the sex-determination hierarchy (including Sxl, Fru and Dsx itself), and genes known to regulate itself(Clough et al., 2014).

1.4 Decoding the message: olfactory detection of volatile signals

The first step in courtship is finding and orienting toward the female, for an animal of such tiny size in an avian world, how is it achieved? At long distance, olfactory signals such as food odors bring flies from distance into an appropriate

substrate, where they eat, mate, and lay eggs. Flies use aggregation pheromones, that consists of species-specific blends of chemicals to facilitate social encounters. Within this medium-range, flies could use visual and auditory cues to locate the appropriate target(Nojima et al., 2018). Males usually start chasing a moving object and start courting, regardless of the quality of such potential mate. However, these attempts discontinue soon, as the male learns the properties of the target. The onset or initiation of courtship is low threshold, probably as a way of enhancing courtship and reproductive success. However, the continuation or maintenance of courtship attempt, where several rituals happen multiple times, allow the male to explore the chemical profiles of the target, requires appropriate signal feedback. Chemosensory feedback is vital in regulating Drosophila male courtship. Amongst which are volatile chemicals, detected at variable distance using the olfactory organs, and nonvolatile chemicals that are detected by gustatory receptors via direct contact.

Olfaction is important in male courtship. Flies with impaired olfaction or gustation, such as the *smellblind* (*smb*) and *olf D* mutants, displayed reduced courtship(Tompkins et al., 1980). In this chapter, we will review the roles of olfactory sensing in regulating male courtship behaviors.

1.4.1 Structural and functional organization of the olfactory System

Drosophila has 50 classes of olfactory receptor neurons (ORNs) housed in two olfactory organs, the antenna, and the maxillary palps, which are covered by ~60 and

~410 fine sensory hairs sensilla, respectively(Figure 4A)(Couto et al., 2005; Goldman et al., 2005). Sensilla are characterized into three morphologically distinct types, the basiconic, coeloconic, and trichoic sensilla, each house between 2 to 4 different ORNs(Bruyne et al., 2001). Each ORN expresses a single receptor from the odorant or ionotropic receptor gene family. ORNs of the same class are distributed throughout the antennal surface in a spatially defined pattern(van der Goes van Naters & Carlson, 2007), but converge their axons to the same glomerulus in the antennal lobe (AL), they connect to second order projection neurons(Figure 4B)(Fishilevich & Vosshall, 2005; Gao et al., 2000; Vosshall et al., 2000).

The AL is the primary olfactory association center, a site of synaptic integration between the olfactory afferents and the dendritic arborizations of their target interneurons. There are two classes olfactory interneurons, the GABAergic local interneurons, which connect within the AL, and the cholinergic projections neurons (PNs), which connects with higher brain centers(Kaupp, 2010; P P Laissue et al., 1999; Vosshall et al., 1999; Wong et al., 2002). AL is potentially site of reciprocal interactions between the ORNs, local interneurons, and PNs(Figure 4B)(Wilson, 2013).

PNs from different glomeruli exhibits distinct but often interdigitate axonal projection patterns in the lateral horn of the protocerebrum, providing integration of input from multiple glomeruli(Wong et al., 2002). The PN also makes en passant

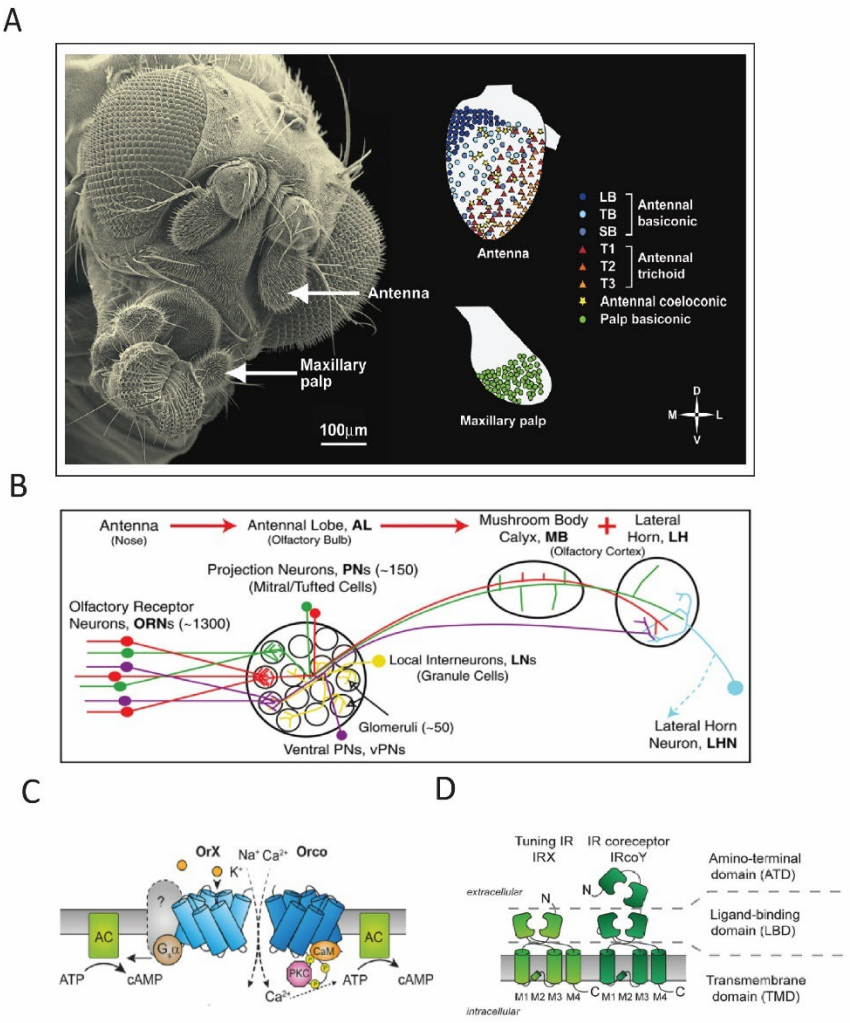

Figure 4: The organization of *Drosophila* olfactory system

(A). In *Drosophila*, the olfactory organs antenna and maxillary palps are covered by olfactory receptor neuron (ORN) housing-sensilla (shown by scanning electron micrograph of the Drosophila head on the left). Sensilla are divided into three functional groups: the basiconic, trichoid, and coeloconic sensilla. (B) Olfactory receptors (ORs) of the same class are distributed throughout the antenna but converges to the same glomerulus in the antennal lobe (AL). The AL is a synaptic integration sites between the OR afferents and the target interneurons- the local interneurons and the projection neurons (PN). PNs project to the lateral horn (LH) of the protocerebrum, making en passant synapses in the calyx of the mushroom body. A small group of PN (vPNs) project directly to the LH. Each color represents ORNs expressing a given OR or their postsynaptic PN partner. (C). Olfactory receptors are nonselective cation channels that conduct Na^+, K^+ and Ca^{2+}. A given OR pair with the olfactory co-receptor Orco to form a functional dimer. Activation of the OR leads to increase of intracellular Ca^{2+} and cAMP.

cAMP activates adenylyl cyclase (AC). cAMP and PKC phosphorylation can lead to increase in OR sensitivity. (D). Functional unit of IRs is made of heterotetramer of two tuning receptor (IRX) and two coreceptor subunits (IRcoY). A is adapted from Laissue & Vosshall, 2008 and Couto et al., 2005 with permissions from the publishers. B is adapted from Jefferis et al., 2007 free of copyright available under the Creative Commons (CC BY) license. C and D are adapted from Wicher & Miazzi, 2021 free of copyright available under the Creative Commons Attribution 4.0 International License .

synapse with the mushroom body, where the passing axon branches and form identity-stereotyped terminal boutons in the mushroom body(Figure 4B)(Wilson, 2013).

Interestingly, PN patterning in the LH shows topographic segregation of food odors and pheromone cues into the posterior-dorsal and anterior-ventral regions of LH, respectively(Jefferis et al., 2007). In addition, glomeruli that respond primarily to aromatic odors cluster in the ventral central region, and ones respond preferentially to aliphatic odors in the medial region(Laissue et al., 1999). Furthermore, the representation of aliphatic compounds is arranged according to carbon chain length(Couto et al., 2005). The fly cuticular pheromones are hydrocarbon with varying carbon chain lengths, thus this topographic segregation is important in pheromone-odor perception and processing in the higher brain regions.

1.4.2 The olfactory receptors and Ionotropic receptors

In Drosophila, and winged insects in general, ORs are odor-gated seven transmembrane non-selective cation channels that conduct Na^+, K^+, and $Ca2^+$. The odor-specific OR form heteromeric complexes with the co-receptor Or83b (Orco), which is required for the normal functioning in all ORs (Figure 4C). Though Orco is not directly

involved in odor recognition, it chaperones the trafficking of ORs, escorts the OR-Orco complex to the cilia, where it acts as cognate co-receptor(Wicher & Miazzi, 2021).

Odor stimulation evokes a two-component response that differs in size, latency, and duration. The initial small, rapid and transient response is accounted by direct ion-binding, whereas the prolonged, larger component is attributed to a GPCR-based metabotropic mechanism including calcium signaling(Kaupp, 2010). Stimulation of the OR leads to Ca^{2+} influx from the 10,000-fold higher extracellular environment. Intracellular Ca^{2+} signaling modulates signal amplification and OR response profile(Mukunda et al., 2016). Calcium dynamics is important for OR responses, and regulators for Ca^{2+} homeostasis are important in regulating OR sensitivity(Halty-Deleon et al., 2018; Lucke et al., 2020). In addition, Gq downstream signaling components are important in regulating Odor responses(Figure 4C)(Wicher & Miazzi, 2021).

Whereas Or responsible for volatile chemical detections, IRs also involved in taste sensation, hygrosensation, and cool temperature sensation(Bruyne et al., 2001; Philippe P Laissue & Vosshall, 2008; Shanbhag et al., 1999). The *Drosophila* ionotropic receptors (IR) are ligand-gated ion channels of the ionotropic glutamate receptor (iGluR) family. It functions in heterodimer or heterotrimers, consisting of one or two broadly expressed co-receptors, Ir8a and Ir25a, and an odor-specific receptor. IRs are directly gated by odors, conduct monovalent cations Na^{+}, K^{+}, as well as low Ca^{2+} (Figure

4D)(Abuin et al., 2011). At least 14 antennal IR are conserved across insects, and 45 divergent IRs are specific to Drosophilids(Abuin et al., 2011).

1.4.3 Olfactory detection of fly pheromones

Olfaction is required for pheromone-mediated courtship in both males and females. Antenna-less females show reduction in courtship and receptivity(Grillet et al., 2006). So, what are the pheromones and olfactory receptors mediating courtship?

Fly extracts elicit responses from the trichoid sensilla, which houses 11 different ORs(Kurtovic et al., 2007; van der Goes van Naters & Carlson, 2007). Amongst which it had been characterized that Or47b and Or88a responds to both male and female extracts, whereas Or67d and Or65a responds to male extracts only(Grosjean et al., 2011; Tal & Smith, 2006; Xu et al., 2005).

The male pheromone *cis*-vaccenyl acetate, previously known for its roles in aggregation(Bartelt et al., 1985), was identified as the ligand for Or67d and Or65a, where the activation is most robust on Or67d and to a lesser degree on Or65a(Amina Kurtovic et al., 2007; van der Goes van Naters & Carlson, 2007). cVA has sex-specific roles in male and female flies. cVA is aphrodisiac for females, promoting male-female courtship. However, such attraction alters shortly after mating, and females are no longer attracted to cVA. This postmating behavioral switch depends on activation of Or65a, which suppresses the cVA's activation on Or67d PN neurons(Sébastien Lebreton et al., 2014). cVA is anti-aphrodisiac to males, suppressing male-male courtship and mediates

intermale aggression, acting via Or67d signaling(Datta et al., 2008; Amina Kurtovic et al., 2007; Tal & Smith, 2006; L Wang et al., 2011).

Recently, it was reported that an oenycyte-independent pheromone produced in both male and females, methly laurate (ML), activates both Or47b and Or88a(Dweck et al., 2015). Or88a also detects methyl myristate (MM) and methyl palmitate (MP), which, in addition to ML, elicit short-range, but not long-range attraction to flies of both sexes(Dweck et al., 2015). The attraction to ML, MM, and MP is conserved in Drosophilid species, among the sibling species of *D. simulans*, *D. mauritiana*, *D. yakuba*, and *D. erecta*(Dweck et al., 2015). Though being activated by the same ligand, Or88a and Or47b are responsible for different aspects of behavioral responses to ML. ML promotes attraction via Or88a and promotes courtship via Or47b(Dweck et al., 2015). In addition, Or47b responds to fatty acids (FAs) pheromones myristoleic acid, myristic acid, palmitoleic acid, palmitic acid, and fatty acids methyl esters (FAMEs) pheromones methyl myristoleate, as well as the previously identified ML and MM. Amongst those FAs and FAMEs, palmitoleic acid elicited the strongest response and activates Or47b specifically, and is important in promoting male courtship success(Lin et al., 2016).

Recently, it was found that Or7a, which was previously identified as a generalist odorant receptor, is responsible for the detection of 9-tricosene, a volatile male-specific CHC and requires olfactory but not gustatory detection. Or7a detects many alcohols and aldehydes, such as the green leaf volatile E2-

hexenal, which has high volatility and can act as a long-distance cue. Upon activation of Or7a by food odors, male deposit 9-T on food substrate, that acts as an aggregation pheromone for flies of both sexes. Unlike other OR pheromone detectors, which are specifically tuned to respond only to the identified pheromones, Or7a detect both food odor and pheromones, enables 9-Tricosene to link food odor perception with aggregation and egg-laying(Lin et al., 2015).

1.4.4 The *fru*-positive, sexually dimorphic ORN circuits

Out of the 50 classes of ORNs, only three (~15% of the ORN population) classes are fru^M positive- the Or67d, Or47b, and Ir84a ORNs(Kurtovic et al., 2007; Manoli et al., 2005). These *fru*-positive ORNs send afferent projections to the sexually dimorphic DA1, VA1lm, and VL2a glomeruli in the antennal lobe, respectively, with PNs project to the specialized pheromone processing center on the lateral horn of the protocerebrum(Cachero et al., 2010; Grosjean et al., 2011; Jefferis et al., 2007; Shanbhag et al., 1999; Yu et al., 2010). Synaptic silencing the fru^M positive ORNs leads to impaired male courtship, suggesting that these ORNs are important in male courtship(Grosjean et al., 2011; Jefferis et al., 2007). Indeed, these ORNs detect odors associated with courtship and modulate courtship behaviors.

Or67d and Or47b are pheromone detectors. Or67d detection of the male pheromone cVA is responsible for attraction in females, repulsion and aggression in males. The dimorphic connections in the interneurons of the lateral horn, and DA1 PNs

to higher order neurons might accounts for the sex-specific behavioral responses elicited by cVA(Datta et al., 2008; Amina Kurtovic et al., 2007; Ruta et al., 2010; Tal & Smith, 2006; Liming Wang & Anderson, 2010). Or47b and Ir84a have roles on regulating more plastic aspects of courtship. Or47b detects pheromones the methyl ester and fatty acid pheromones such as methyl laurate and palmitoleic acid(Dweck et al., 2015; H.-H. Lin et al., 2016). It is responsible for age-dependent increase in courtship and copulation success in males(H.-H. Lin et al., 2016). On the other hand, Ir84a, an ionotropic receptor housed in the ac4 sensilla, is required to detect food availability by recognizing the odors phenylacetic acid and phenylacetate in both male and females, to modulate the onset of courtship(Grosjean et al., 2011). Phenylacetic acid and phenylacetate are found in fruits (for example, overripe bananas and the actus *Opuntia ficus-indica*), plant tissues, as well as their fermentation products, that had been found as food sources and oviposition sites for fruit flies(Grosjean et al., 2011). Ir84a mutant males show reduced male-female as well as male-male courtship, but the copulation success, latency, and duration remain unaffected. In addition, in the presence of phenylacetic acid, male courtship increases in Ir84a-dependent manner, indicating that Ir84a detection of food odors is important in mediating the onset of courtship. Interestingly, the PN of Ir84a, but no other IR-expressing PNs, are highly interdigitated with the pheromone pathways and not food pathways. Specifically, the PNs from the Or84a glomerulus VL2a intercalate with the PNs of the DA1 and VA1lm, and innervating the pheromone integration region,

rather than the food order processing center(Cachero et al., 2010; Clough et al., 2014; Grosjean et al., 2011).

However, how does the *fruitless* expression in these ORNs contribute to the physiological and behavioral changes in response to the olfactory experience remain unknown. Recently, our lab reported that Or47b and Ir84a, but not Or67d signaling is required for the maintenance of fru^M expression in the respective adult ORNs(Catherine E Hueston et al., 2016). Specifically, the histone acetyl transferase CBP/p300 and its phosphorylating agent CamKI, are required for the maintenance of fru^M expression, indicating that Or47b and Ir84a signaling modulates fru^M expression via the epigenetic mechanism of chromatin acetylation(Catherine E Hueston et al., 2016). It is intriguing why fru^M expression is tied to the pheromone detector Or47b, and how does the olfaction-mediated change in fru^M leads to change in neurophysiology and behaviors.

1.5 Gustatory detection of the nonvolatile pheromones

Flies are coated with a cloud of hydrocarbon compounds on their cuticles, which serve as pheromones mediating courtship. The long carbon chain of many fly cuticular hydrocarbon compounds (CHC) rendered them nonvolatile or semi-volatile, therefore, the flies must detect these molecules in short distance. Flies use the gustatory receptors (GRs) to detect non-volatile pheromones on potential mate's body during the tapping and licking rituals of the courtship.

GRs are seven-pass transmembrane proteins, located in the labellum, distal tarsal segments of the legs, anterior wing margin, ovipositor, and the pharyngeal organs lining in the esophagus(Freeman & Dahanukar, 2015). GRs are housed in sensilla, where they selectively activated by palatable (sweet, salty, water), noxious (high salt, bitter, low pH) tastants and pheromones(Freeman & Dahanukar, 2015). For example, the male pheromone 7-Tricosene is detected by the L2 gustatory neuron in the mouth part(Lacaille et al., 2007; Miyamoto & Amrein, 2008; Moon et al., 2009). The female pheromone 7,11-HD is detected by GRNs housed in the tarsal Tm4c sensillum(Lu et al., 2012).

There are 68 Grs, each expressed in a very small subsets (1-4%) of taste neurons(Chyb, 2004). Gustatory receptor neurons (GRNs) innervate the primary gustatory center suboesophageal ganglion (SOG), as well as neuromeres of the thoracic ganglia. In the brain, second order neurons relay information from SOG to the antennal and mechanosensory motor center (AMMC), connecting with *fru*-positive neurons of the ventrolateral protocerebrum, which relay information to higher order protocerebrum(Freeman & Dahanukar, 2015).

Gustatory signals are important in feeding, grooming, and courtship. Specifically, GRs involved in pheromone detection are important in regulating both male and female courtship behaviors. Gr32a, Gr66a, and Gr33a are involved in the detection of male-specific inhibitory signals(Billeter & Levine, 2013; Jois et al., 2018;

Laturney & Billeter, 2016; G. Lee et al., 2001; Miyamoto & Amrein, 2008), where Gr32a and Gr33a are identified as detectors the male specific *Z*-7-tricosene (7-T) that is aphrodisiac for females and anaphrodisiac for males(Fan et al., 2013; Lacaille et al., 2007). Gr68a and Gr39a had been identified as detectors for female aphrodisiacs(Billeter & Levine, 2013; Watanabe et al., 2011). In addition, a subset of dsx^{M}-positive Gr68a are required for the detection of male pheromone (3*R*, 11*Z*, 19*Z*)-3-acetoxy-11,19-octacosadien-1ol (CH503), and is responsible for courtship avoidance toward mated females, who receives CH503 from the male mate during copulation(Bray & Amrein, 2003; Shankar et al., 2015; Yew et al., 2009).

Recently, it had been shown that the Deg/ENaCs family pickpocket (*ppk*) ion channels are also involved in gustation and pheromone detection in flies. Deg/ENaCs had been implicated in the detection of water, sodium, acids, mechanosensory stimuli and peptides. Interestingly, GRNs that express *ppk23*, *ppk29* and *ppk25* are also *fru*-positive, and they had been implicated in detection of molecules involved in sex-specific behaviors(Lu et al., 2012; Thistle et al., 2012). Specifically, *ppk25*-mediated detection of the female pheromone 7,11,-HD is required for normal courtship initiation(H. Lin et al., 2005; Starostina et al., 2012). *ppk29* detection of its unidentified ligand is important for normal male-female courtship(Toda et al., 2012). Most interestingly, *ppk23* neurons responds to both male and female pheromones(Thistle et al., 2012). The *fru*-positive tarsal chemosensory bristles are innervated by two ppk23 cells of opposing roles: one

responds to the female specific 7,11-HD and 7,11-ND, whereas the other cell responds to 7P, 7T, and cVA(Thistle et al., 2012). These *fru*^{M}/*ppk23* cells are sexually dimorphic, showing doubling in numbers in male, and are specifically tuned as female sensing or male sensing cells to regulates courtship behaviors. Defects in *ppk23* leads to increased male-male courtship and decreased male-female courtship(Lu et al., 2012; Thistle et al., 2012).

1.6 Using the olfactory and gustatory cues to find best mate

Olfactory cues bring flies from distance together, allowing social encounters that are prerequisites to courtship. Once the male initiate chasing the moving object, chemosensory cues determine how long the male pursue the object(Agrawal et al., 2014). The species- and sex-specific, mating status-indicative fly pheromone profiles are important in maintenance of the courtship rituals, where males rely on olfactory and gustatory detection of these pheromones to proceed into later steps of the courtship ritual.

1.6.1 "CV" written in chemical words: the fly's cuticular hydrocarbon compounds.

To ensure reproductive success, the male fly needs to accurately distinguish target that is conspecific, opposite sex, sexually mature, and not mated recently. The target fly's pheromone profile tells everything.

Insect cuticles are coated with a cloud of hydrocarbons, which protects them from desiccation, confer them water- and disease-resistance. These cuticular

hydrocarbons (CHCs) had been evolved to serve as pheromones, regulating behaviors such as aggregation, foraging, aggression, and courtship(Jallon, 1984; Joseph et al., 2018; Shorey & Bartell, 1970; Svetec & Ferveur, 2005; Symonds & Wertheim, 2005). The CHCs profiles are sex and species-specific, differing in the identity of the compound (determined by carbon chain length, position and number of double bonds), relative ratio and absolute quantity of each compounds(Cobb & Ferveur, 1996; Jallon, 1984; Svetec & Ferveur, 2005). The CHCs changes with age, mating status and social experience, serving as a chemical readout of the fly's social identity and life history(Everaerts et al., 2010; Farine et al., 2012). CHCs acts as pheromones in three forms: the waxy cloth on fly's body, "deposits" on food/substrate surface, and volatile chemicals. Deposited CHC represents 1% of the amount of body CHCs and had roles in promoting aggregation and egg-laying(Farine et al., 2012). The nonvolatile and volatile body CHCs mediate important aspects in courtship and aggression. Since volatility decreases with carbon chain length, most fly CHCs of long carbon chain are low-volatile.

The CHC profile is non sex-specific at young age, abundant with hydrocarbons of longer chains (29-35 carbons), some of which could mimic aphrodisiac of mature female(Cobb & Ferveur, 1996; Jallon, 1984). The sexual dimorphic CHC profile is under the influence of the sex determination genes, where manipulation of the *sxl*, *tra*, *dsx*, and *fru* are capable of masculinizing or feminizing the CHC profile(Savarit et al., 1999; Wicker & Jallon, 1995). The sexual dimorphic CHC arises later as the flies mature.

Disruption with the gonads and neuroendocrine system maturation leads to defects in producing those CHCs.

CHC are produced by the fat body cells oenocytes. The synthesis of the 7,11-dienes requires a sex-specific and species-specific desaturation step carried by *desaturase F*, and occurs only in females but not males, *D. melanogaster* but not *D. simulans*, making it a sex-specific and species-specific aphrodisiac(Jallon, 1984; Joseph et al., 2018).

In *D. melanogaster*, the female CHC bouquet consists of 23-29 carbon hydrocarbons of monoenes (one double-bond) and dienes (two double-bond). Amongst which the ones with 27± 2 carbons and at least one double bond in position 7 appears to be potent aphrodisiac. 7,11- heptacosadiene (7,11-HD), appearing in abundance (~400ng/fly) in females, is the most potent aphrodisiac, capable of eliciting courtship and copulation attempt in male. 7,11-nonacosadiene (7,11-ND), which is also enriched the females (~200ng/fly), had also been suggested as an aphrodisiac to males. The female-specific 7,11-HD and 7,11-ND induce wing vibration in conspecific males.

The overall CHCs are lower in males compared than in females(Farine et al., 2012). In males cuticular extracts, dienes are not found. Monoenes of 23 carbons are most abundant. 7-tricosene is the major compound (~500ng/fly). 7-T suppresses male-male courtship, increase female receptivity(Grillet et al., 2006). 7-pentacosene (7-P) is also considered primarily a male pheromone, though present in small amount in female cuticular extracts as well (~100ng/fly)(Jallon, 1984). 7-P works together with the non sex

specific 9-pentacosene (9-P), acting stimulatory to courtship conditioning and increase the probability of copulation(Siwicki et al., 2005). In addition, there are volatile, male-specific pheromones: 7-docosne, 5-tricosene (5-T), 23-methyldocosane, and 9-tricosene (9-T)(Farine et al., 2012). 5-Tincrease courtship latency and inhibits male courtship.

1.6.2- Screening for proper mate: finding the conspecific partner

Olfactory and gustatory cues assist flies to find conspecific mates. First, flies are attracted to food odors of their ecological niche, and they prefer to court, copulate and lay eggs near food sources. For example, *S. sechellia*, a specialist feeder of the *Morinda citrofolia* fruit, is attracted to hexamoic and octanoic acid indicative of such fruit, whereas its sister species *D. simulans*, a generalist, is repelled by the same odors. *D. virilis* prefers decaying phloem, whereas *D. melanogaster* prefer fruit as main breading sites, and detection of fruit odor phenylatic acid via the ionotropic receptor 84a (Ir84a) brings the conspecific flies together. Evolutionary changes in the olfactory neuroanatomy and functioning accounts for the host specialization in different Drosophilid species(Dworkin & Jones, 2009; Wern Pan et al., 2017).

Second, flies use species-specific pheromones and behavioral patterns to further select flies of the same species. Female cuticular profiles differs between the Drosophilid species and are important in inhibiting intra-species courtship. Drosophila species differ in sexual differences, compositions, as well as the relative abundance of the CHCs. For example, *D. melanogaster* has a sexual dimorphic CHC profiles in males and females,

whereas flies of *D. serrata*, *D. takahashii*, and *S. pseudoobscura* have monomorphic CHC profile, producing same CHCs of different abundance in male and females. Also, cVA, the male-specific pheromone in *D. melanogaster*, is not produced in *D. suzukii*. The differences in CHC profile allows intra-species selection and discrimination important in promoting reproductive success(Everaerts et al., 2010). In *D. simulans*, 7-Tricosene is abundant in both sexes, as compared to primarily a male pheromone in *D. melanogaster*. 7-Tricosene is anaphrodisiac to *D. melanogaster* male but to aphrodisiac for *D. simulans* males. Food and pheromone preferences mediated by olfactory receptor differences contribute to species selection for mating.

1.6.3 – Screening for proper mate: finding receptive partner

Mating status alters the pheromonal profiles in the flies. The sex predominant CHCs acquired during mating alters the attractiveness of a fly to the opposite sex(Everaerts et al., 2010; Yew et al., 2008). Males could "read" the female's mating state by smelling at distance, or tapping and licking, and determines the next step.

Mated female displays decreased levels of 5-P, 9-H, 7,11-HD, 7,11-PD, 7,11-ND, 9,13-HD, n-C25, n-C27, 27-Br and 29-Br, and increased level or appearance of cVA, 7-D, 6-D, 5-D, 9-T, and 7-T.

The decrease in the aphrodisiac 5-P in mated female is likely due to the male licks the substance away from genitalia during courtship, whereas the appearance of antiaphrodisacs 7-T and cVA is due to male's transfer during copulation. The level of

cVA in mated female decrease over time, as they remove the cVA by sperm ejection, which takes place few hours within copulation, restoring attractiveness and re-mating(Laturney & Billeter, 2016; Schilcher, 1976).

1.7 Successful completion of the courtship rituals

1.7.1 The love song

Courtship is a highly interactive process, while the stereotypical steps are hard-wired, flies need to change their behaviors actively in response to the real-time feedback they receive. After the initial olfactory, visual, auditory and gustatory encounter, the male needs to integrate the multimodal sensory information, and execute the appropriate step in response to the activating or inhibiting signals.

If the male decides to further pursue the female, he proceeds to produce courtship song, in attempt to elicit the female to accept him. The male courtship song consists of a sustained sinusoidal oscillation of 120-180 Hz (sine note), short clicks of symmetrical slow pulse note of 200-250 Hz Pslow, and a newly discovered biphasic higher pitch pulse song of 250-400 Hz Pfast(de Bivort, 2018; Schilcher, 1976). The male sings in attempt to slow down female's locomotion and get into proximity with the female to execute the next steps in courtship. dsx^{M} is required for the production of sine song(Coen et al., 2014; Crossley et al., 1995; Trott et al., 2012), and fru^{M} is required for production of species-specific pulse song(Crossley et al., 1995; Marie-Orleach et al., 2019; Yoon et al., 2013).

The female determines if the male is an appropriate mate by recognizing the species-specific courtship song. If the female is receptive, she will slow down and let the male approach.

1.7.2 Female receptivity

A mature, unmated female is receptive for courtship and copulation. In response to the male's courtship song, a receptive female will slow down her locomotion and allow the male to copulate. Female receptivity is a physiological state determined by her previous sexual activity, as well as a decision to be made during the courtship rituals.

Olfactory and gustatory detection of pheromones regulate female receptivity. It had been shown that, female activity is regulated by two clusters of neurons-one conveying olfactory information, whereas the other conveying gustatory information(Sakurai et al., 2013). Furthermore, the *Spinster-rapamycin (mTOR)* signaling in Or47b circuit play key role in determining female sexual receptivity(Sakurai et al., 2013).

Male pheromones not only functions at the female ORNs and GRNs, but also on her reproductive tissues to regulate female receptivity. During copulation, seminal fluids containing sperms and male pheromones are transferred into the female's reproductive tissue, amongst which is sex peptide (SP), an important regulator for female post mating behaviors, modulating increase in egg laying and decrease in receptivity in females(Carvalho et al., 2006; Kubli, 2003).

Interestingly, subsets of SPR expressing neurons are also *fru*-, *dsx*-,and *ppk*-positive, and they are important for SP triggered-postmating behavioral changes(Häsemeyer et al., 2009; Tompkins et al., 1980; Yang et al., 2009).

1.6.3 Executing the final steps in courtship: copulation

In wild type flies, copulation usually persist for 15±2 min, fluctuating with the female's previous mating experience and pheromones(Jois et al., 2018). Successful copulation depends on the integration of sensory signals from the genitalia, signals for ejaculation at the reproductive organs, and proper coordination of the copulatory muscle for a species-specific duration(Pavlou et al., 2016). The ability for the male to bend its abdomen toward the female's genitalia is prerequisite for initiation of copulation, which requires the "maleness" of the neurons located in ventral nerve cord (VNC)(Keleman et al., 2012). The male needs to be sexually mature, under the proper endocrine control, to develop intact and functional internal and external reproductive organs with healthy sperm and seminal fluid proteins. The sperm and proteins transferred from the internal organs to the copulatory organ aedeagus and ejaculated into the female's reproductive tract, and the females begins to lay fertilized eggs an hour postmating.

It had been characterized that the *dsx*-positive neural circuits in the abdominal ganglion is required for the initiation, seminal fluids transfer, and genital uncoupling for successful copulation(Jois et al., 2018; G. Lee et al., 2001; Pavlou et al., 2016). A small

subset of these neurons is also fru^M positive. In addition, a small subset of *engrail*, fru^M positive neurons in the brain and ventral nerve cord are required for copulation initiation and maintenance(Latham et al., 2013).

1.8 Hormonal regulation of courtship

Juvenile hormone (JH) has prominent roles in insect development, regulating metamorphosis, reproductive maturation, longevity. JH regulates oogenesis, cuticular pheromone maturation, and receptivity in females. JH is synthesized in corpus alatum, and signals through the putative JH receptors *Met* and *Gce* in Drosophila.

Previously, JH had been shown to endocrine control of pheromone biosynthesis in a variety of insects of Coeloptera, Blattaria, and Lepidoptera(Wicker & Jallon, 1995). JH regulates the age-dependent change in CHC profile in females. Specifically, females decapitated after eclosion shows CHC characteristic of young adults three days later, which has very long chain hydrocarbons (31-35 carbons), decrease in sex pheromones and increase in monoens, characteristic of young flies. When JH analog methoprene was applied, the CHC profile is partially restored that decrease in very long-chain hydroarbons, but sex pheromones were not synthesized(Wicker & Jallon, 1995).Also, it had been reported that sex peptide (SP) stimulates JH synthesis, JH and 20-hydroxyecdysone (20E) regulates the yolk proteins (YPs) genes, which are taken up by oocyte membrane and important for oogenesis(Moshitzky et al., 1996).

However, the role of JH signaling in males had been less studied. JH synthesis and signaling is required in male courtship. Males with low JH shows defects in courtship, that can be rescued by the JH analog Methoprene(Wijesekera et al., 2016). Interestingly, genes regulated downstream of fru^M and ecdysone overlaps. In addition, fru^M regulates ecdysone-responsive genes such as reaper (rpr), a cell death gene, and crooked legs (crol).

1.9 Reshaping the hard-wired programs: Experience.

1.9.1 Effects of Social Experience on neurophysiology and behaviors

Functional plasticity is observed from the level of gene expression to a broad spectrum of complex behaviors. There is always room for changes, allowing the animal to adapt to the environment for better fitness. The neurophysiology and behavior patterns of a given animal are faithful reflections of past experience modifying its hardwired genetic programs.

It had been well-characterized that stimulating experience during development modulates neuronal connectivity. Social experience, conferred by group housing in a laboratory setting, allows the animals to receive chemosensory and mechanosensory stimulations upon social encounters. Animals raised in isolation receives no pheromone-based olfactory and gustatory input, and no social encounter-based visual, auditory, and tactile input. Flies raised in isolation show a broad-spectrum change ranging from reduction in mushroom fibers to changes in sleep patterns, showing the penetrating

effects of sensory experience deprivation from neurophysiology to behaviors. The effects of social experience had also been demonstrated on the other organisms, for example, nematode worm *Caenorhabditis elegans* raised in isolation or deprived of chemosensory input shows reduction in body size(Svetec & Ferveur, 2005) and reduced mechanosensory response, which can be reversed via mechanical stimulation(Fujiwara et al., 2002; Kent et al., 2008; Rose et al., 2005).

Social experience also regulates more complex aspects of physiology and behaviors, such as life span, sleep patterns, aggression, and courtship. For example, it had been shown that short-lived mutant flies benefited from co-housing with active or younger flies, that improved motor ability and stress resistance, leading to lifespan extension(Technau, 1984). Social experience also alters fly's sleep pattern, where visual and olfactory input are required for this experience-dependent change in sleep patterns (Ganguly-Fitzgerald et al., 2006).

1.9.2 Effects of Social Experience on CHCs

In social isolation, flies do not receive pheromone-based olfactory or gustatory signals from other flies, and interestingly, changes its own cuticular pheromone display as well. Social context changes the male fly's CHC display, altering the levels of the methyl-branched alkanes(Ganguly-Fitzgerald et al., 2006; Krupp et al., 2008; Liming Wang & Anderson, 2010). In addition, the volatile pheromone profile also shows changes with social experience. Interestingly, the 7 most volatile CHCs are more

abundant in socially isolated males, whereas the 6 less volatile CHCs are more abundant in socially enriched males. Socially isolated males show decrease in 9-, 7-, and 5-T and increased n-C23 compared to the group housed males. Strikingly, volatile cVA is not affected by development social experience, nor socialization in mature flies. In females, social experience, but not sexual context alters its volatile CHC profiles (Farine et al., 2012).

1.9.3 Effects of Social Experience on courtship behavior

Though courtship behavior is under robust genetic control, it also demonstrates plasticity. Experiences modifies fly behaviors, via the change in epigenetic gene regulation, which modifies the neurophysiology and reflects these reprogramming into behavioral outputs. Flies adapt to and learn from environmental factors such as social environment, mating experience. For example, male extends copulation duration after the sight of a rival male(W. J. Kim et al., 2012).

It had been well-documented that previous experience modifies the fly's courtship behaviors. For example, unsuccessful courtship leads to decease in subsequent courtship. More importantly, the decrease is not due to a general loss of interest, but the precise outcome of associative learning. If the male is rejected by a virgin female, the male shows decreased courtship toward intact or decapitated virgin females of the same age, indicating that the male does not learn from visual cues about the female's behavior. Such courtship depression is not observed in males with defective olfaction,

suggesting a volatile, age dependent female CHC cue is involved in associative learning(Ejima et al., 2005; Siwicki & Ladewski, 2003). The non sex-specific pheromone 9-P could accounts for this courtship conditioning(Gailey et al., 1986). Furthermore, if the male is rejected by a mated female, his courtship toward all females decreases, which is accounted by the Or65a dependent detection of cVA. This associative learning is accounted by the increase in cVA sensitivity upon unsuccessful courtship and is accounted by fru-positve aSP13 dopaminergic neurons and the DopR1 dopamine receptor in the mushroom body γ-lobe to which the asP13 neurons innervate(Keleman et al., 2012; Siwicki et al., 2005).

Previous experience also affects male-male courtship. For example, socially isolated males show increased homosexual behaviors, as well as more intense courtship compared to communally raised flies(Y.-K. Kim et al., 2004; Y.-K. Kim & Ehrman, 1998). Furthermore, immature males courted by older males show strong heterosexual courtship once they became mature. And they mated faster compared to naïve males (Benelli & Canale, 2012; Mcrobert & Tompkins, 1988; Vaias et al., 1993).

1.10 Conclusion

It is intriguing how sensory experience changes courtship circuits to ultimately modify behavior. Drosophila courtship is a good system where this question can be simultaneously interrogated at the level of genes, neurons, circuits and behavior. In this chapter, we reviewed the building of innate courtship behaviors by *fruitless*^{M} (Chapter

1.1), the construction of sex-specific circuits and behavioral plasticity by Dsx^M (Chapter 1.2). Then, we will review pheromone regulation on courtship, as detected by olfaction (Chapter 1.3) and gustation (chapter 1.4), in guiding and modifying male courtship behaviors (Chapter 1.5 and 1.6). We then examined the roles of internal (Chapter 1.7) and external signals (Chapter 1.8) in regulating courtship behaviors. Lastly, in this section, we will propose novel experiments to address the central questions of how olfactory experience modifies plastic behaviors in male courtship.

In chapter 2, we discuss our findings on how Or47b signaling, which detects the non-sex specific methyl ester pheromones to promote male copulation success, is required for the maintenance of fru^M in adult Or47b ORNs(Dweck et al., 2015; Catherine E Hueston et al., 2016). It is intriguing that *fru* expression is tied to pheromone receptor function. One possibility is that these pheromones indicate some social experience, which reprogram *fruitless* expression and fru^M-dependent behaviors. We show that social experience, conveyed via Or47b, could modulate the expression of courtship master regulator fru^M in the peripheral nervous system to modulate neuronal responses and copulation advantage.

In chapter 3, we extend our investigation of Or47b function in regulating other plastic aspects of courtship behavior. It is known that fru^M mutants don't court when isolated but when grouped with other flies, they can learn to court with them, regardless of their sex and species. This learning requires olfactory detection of social environment.

Using *Or47b-fru*^{M} double mutants, we show Or47b contributes to social experience dependent courtship learning in *fru*^{M} mutant males. Our results highlight Or47b ORN circuits as a plastic circuit, modulating experience dependent aspects of courtship behaviors.

2. Chromatin-based reprogramming of a courtship regulator by concurrent pheromone perception and hormone signaling

This chapter is modified from previously published work (Zhao, Deanhardt, Barlow, Schleske, et al., 2020)*Chromatin-based reprogramming of a courtship regulator by concurrent pheromone perception and hormone signaling. Science advances*. DOI: 10.1126/sciadvaba6913

2.1 Background

Animals tightly control social behaviors based on internal and external states, as inappropriate and untimely displays of such behaviors can interfere with reproductive success. Integration of signals such as age, reproductive state, and population density determines decisions regarding execution of specific social behaviors. Hormones act as critical signals for internal states such as age and reproductive state, which have long-lasting effects on the structure and behavioral outputs of neural circuits when coordinated with sensory experience (Cushing & Kramer, 2005). For example, female mice execute opposing social behaviors towards males using cyclic regulation of sensory neuron responses by female hormones (Dey et al., 2015). Recent studies also show the critical period for social reward learning in adult mice requires both neural activity and oxytocin (Nardou et al., 2019). In fact, many circuits and behaviors are constrained by critical periods where they rely on sensory experience early in life for maturation of circuit structure and behaviors (Hensch, 2004). Despite the overwhelming evidence that

hormones synergize with sensory experience to reprogram social and reproductive behaviors, how these are coordinated at the molecular level remain unknown or poorly characterized.

To address this question, we used neural circuits driving courtship behavior in *Drosophila melanogaster*, which are under the control of a master transcriptional regulator Fruitless^{M} (Fru^{M}) (Dickson, 2008; Yamamoto & Koganezawa, 2013a). Fru^{M} is necessary and sufficient for male courtship behavior and is expressed in approximately 2000 interconnected neurons forming the courtship circuitry (Sato & Yamamoto, 2020; Yamamoto & Koganezawa, 2013a). Fru^{M} regulates development, function and plasticity in circuits driving male-specific behaviors (Sato & Yamamoto, 2020). Social cues such as pheromones can modulate courtship behaviors, some of which are detected by two classes of *fru^{M}*-positive olfactory receptor neurons (ORNs) expressing Or67d and Or47b receptors (Dweck et al., 2015; A Kurtovic et al., 2007). Or67d neurons mediate male-male repulsion through detection of the male specific pheromone cis-vaccenyl acetate (cis-VA) (A Kurtovic et al., 2007). On the other hand, Or47b neurons detects fatty acid pheromones, such as palmitoleic acid (PA), present on both sexes, and function to increase the male courtship advantage with age (Dweck et al., 2015; H. H. Lin et al., 2016; Sethi et al., 2019; L Wang et al., 2011) . Recent studies showed that social context, juvenile hormone signaling, an age-related cue, and Fru^{M} co-regulate pheromone responses of Or47b neurons and age-relate increase in courtship advantage (Sethi et al.,

2019). However, the molecular mechanisms regulating these changes remain unknown. We previously showed that genetic perturbations to Or47b function, calcium signaling, and the histone acetyl transferase p300/CBP regulate maintenance, but not onset, of fru^M expression in adult Or47b neurons (Hueston et al., 2016). These results suggested the possibility that age and social environment can fine-tune neuronal sensitivity and courtship advantage through chromatin-mediated changes in Fru^M transcription and function.

2.2 Results

2.2.1 Social experience enhances fruM expression in Or47b neurons via chromatin-based mechanisms

To test whether social context regulates chromatin around fru^M promoter, we performed Chromatin immunoprecipitations from male whole-antennae samples using antibodies against actively transcribed chromatin, followed by quantitative PCR (ChIP-qPCR). Association of RNA polymerase II with promoters as well as increase in acetylation of histones such as H3K27 and H3K9 are hallmarks of actively transcribed chromatin (Venkatesh & Workman, 2015). ChIP-qPCR for $fruitless^M$ transcriptional start site (TSS), showed dynamic changes in chromatin around *fruitless* P1 promoter with age. In group housed (GH) male antennae, we found that RNA polymerase II and H3K27ac enrichment around *fru* P1 promoter TSS are initially high at 0-2 days, but decrease by day 5 (Fig. 5A). This is followed by an increase at 5-7 days, a peak time for sexual maturity for males (Fig. 5A). As opposed to the group house condition, single housed

(SH) socially isolated males showed a decrease in the enrichment of RNA Polymerase II and H3K27ac at the *fru* P1 promoter across time (Fig. 5A). The effect of social isolation on chromatin was similar between different wild-type strains Canton S and w^{1118}, ruling out variation due to genetic background (Fig. 5B). Acetylation of both H3K27 and H3K9 are mediated by histone acetyl transferase p300/CBP Nejire (nej) (Chan & La Thangue, 2001; Kalkhoven, 2004). Consistent with this, we detected a decrease in the enrichment of p300/CBP and H3K9ac at *fru* P1promoter in 5-day old SH males (Fig. 5C', C'''). As predicted, group housed *Or47b* mutant males ($Or47b^1$ and $Or47b^2$) showed a decrease in enrichment of all marks examined (Figure 5C-C'''). In contrast, group housed *Or67d* mutants ($Or67d^{[GAL4]}$), which previously were reported to have no effect on fru^M expression, showed no difference in RNA polymerase II enrichment from GH condition. However, an increase in H3K27ac and H3k9ac enrichment at *fru* P1 was observed in group housed *Or67d* mutants compared to wild type (Fig. 5C, C'') (C E Hueston et al., 2016). This result points to possible increase in *fru* P1 open chromatin state in *Or67d* mutants that has not been previously reported. Relative enrichment was not significantly altered around antennal *Or47b* and *Or82a* promoters in group housed or socially isolated male antennae (Fig. 5D-G). In addition, enrichment of active chromatin marks was minimal around gustatory receptor *Gr5a* promoter, which shows little to no expression in the antennae based on previous antennal RNAseq analysis (Barish Li, Q., Pan, J.W., Soeder, C., Jones, C.D., Volkan, P.C., 2016) (Fig. 5D-G). These results suggest

that social experience through Or47b signaling increases active chromatin marks around *fru* P1 promoter in sensory neurons.

To test whether social context-dependent changes in chromatin lead to changes in fru^{M} expression in Or47b and Or67d neurons, we quantified fru^{P1GAL4} driven *UAS-GFP* expression using *40xUAS-mCD8::GFP* in 2, 5, and 7-day old, GH and SH male antennae (Fig. 6A-C). Quantification of integrated GFP density in each ORN driven by fru^{P1GAL4} driven *UAS-GFP expression* showed that in group housed males fru^{M} expression is initially low in both Or47b and Or67d ORNs, increase by days 5 to 7 (Fig. 6A-C). Social isolation decreased the fru^{P1GAL4} driven *UAS-GFP expression* in Or47b neurons, but not in Or67d neurons at day 5 and 7 (Fig. 6A-C). qRT-PCR on antennal samples also showed similar changes in endogenous fru^{M} expression in different ages and social context (Fig. 6D). The changes in social isolation are not too drastic at the transcriptional level compared to the differences seen at the chromatin level. Even though this is surprising others have reported similar phenomena (Koike et al., 2012). Such differences might indicate other unexplored chromatin-based effects on gene regulation such as changes in alternative splicing, or transcriptional state (poised promoters versus transcriptional elongation) determined by phosphorylation state of RNA Polymerase II. Quantification of GFP and GAL4 transcripts from fru^{P1GAL4} driven *UAS-GFP* flies also showed comparable changes in GFP expression between GH and SH males, thus making it an

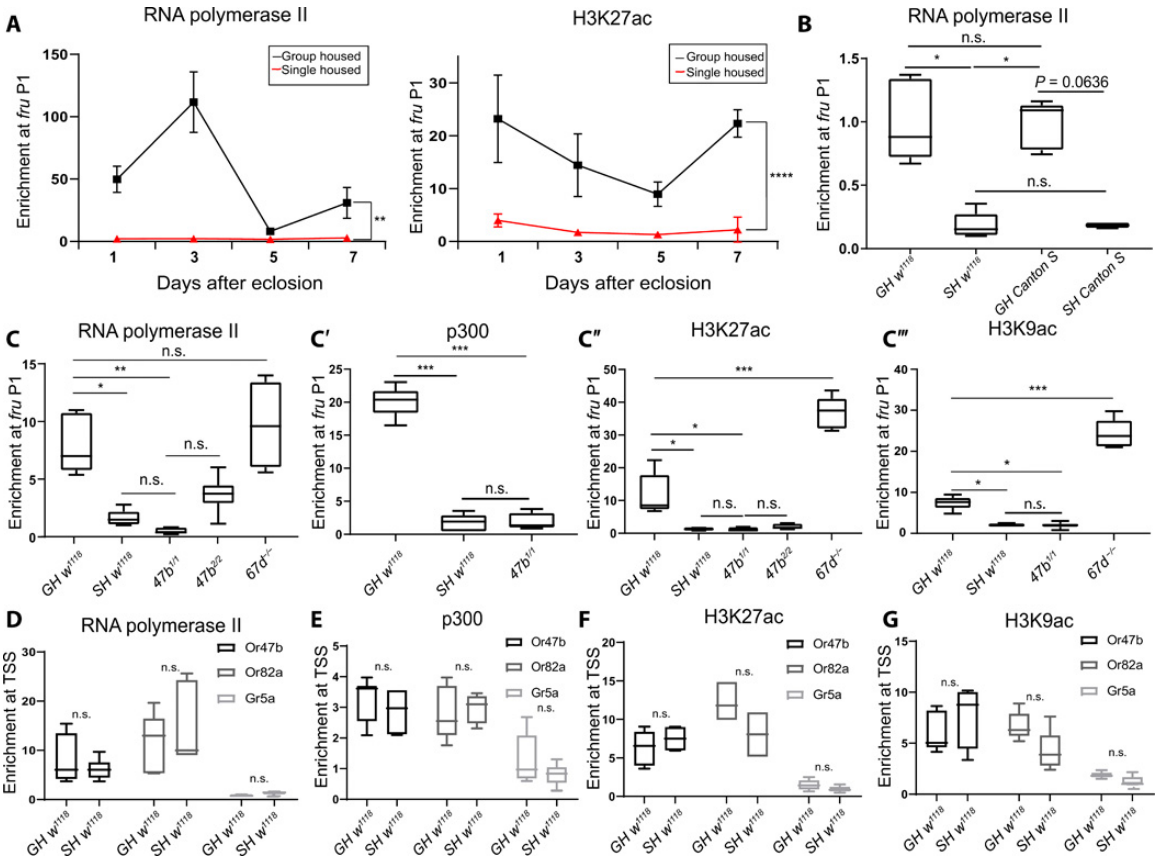

Figure 5: Social experience increases open chromatin marks around fru P1 promoter.

Antennal ChIP qPCR to measure association of open chromatin marks around fru P1 promoter using anti-RNA polymerase II, anti-H3K27ac, anti-H3K9ac, and anti-p300 antibodies from adult male antennal samples that are either GH (black) or SH (red) (A to C). y axis shows enrichment relative to no antibody control. Social isolation decreases enrichment of either mark in SH male antennae at all time points. (A) Time course of RNA polymerase II (left) and H3K27ac (right) association with fru P1 at days 1, 3, 5, and 7 after eclosion. (B) Enrichment of RNA polymerase II around fru P1 in 5-day-old GH and SH Canton S versus w1118 males. Enrichment of RNA polymerase II (C), p300 (C′), H3K27ac (C″), and H3K9ac (C‴) open chromatin marks around fru P1 are shown for GH and SH w1118, Or47b, and Or67d mutants. (D to G) Enrichment of active chromatin marks (RNA polymerase II, p300, H3K27ac, and H3K9ac) upstream of genes expressed in the antenna (Or82a and Or47b) and not expressed in the antennae (Gr5a) in different housing conditions. *P < 0.05; **P < 0.005; ***P < 0.001; n.s., not significant.

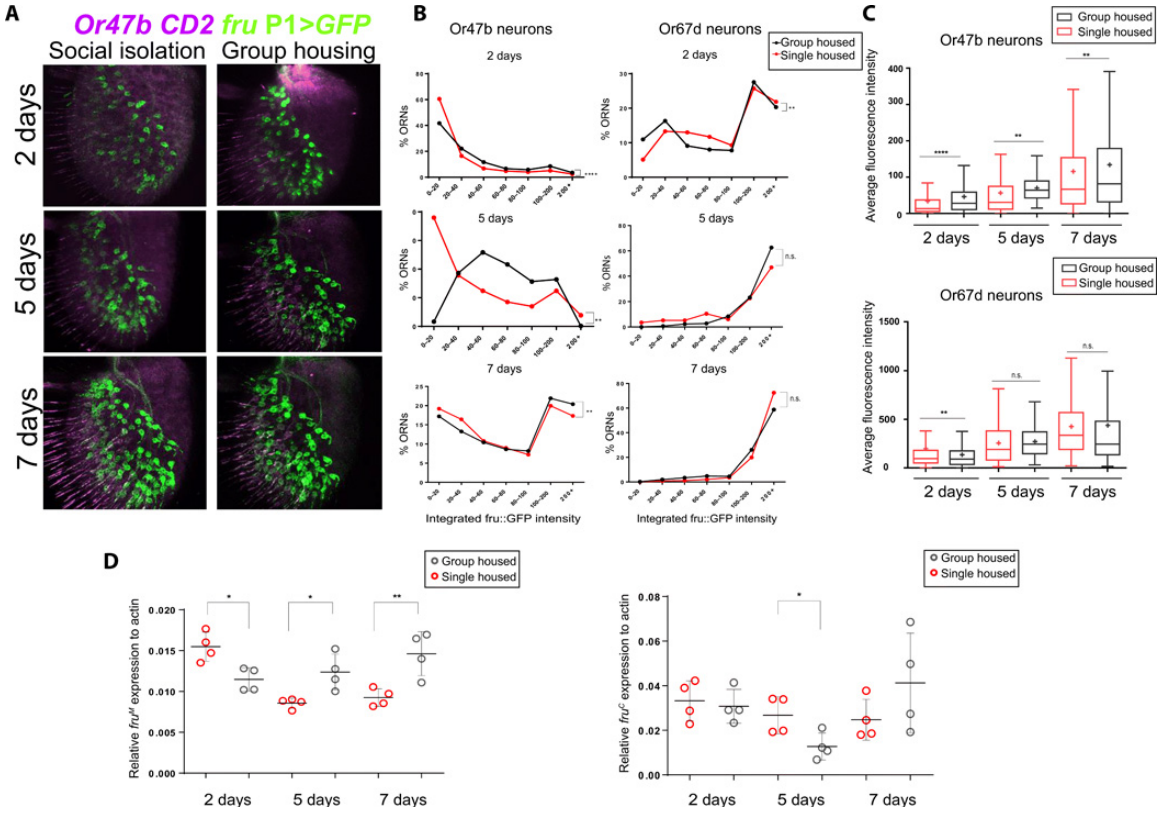

Figure 6: Effects of social experience on fru expression.

Confocal images of fru^{P1GAL4}-driven UAS-GFP in the antennae from GH and SH males of different ages. (B) Quantification of fruP1GAL4-driven UAS-GFP expression in Or47b and Or67d ORNs from (A). x axis shows integrated GFP density, and y axis represents the percent ORNs. (C) Average of the data points from (B). (D) qRT-PCR for fruM (left) and fruC (right) from antennal samples of w1118 males that are either SH or GH. *P < 0.05; **P < 0.005; ***P < 0.001.

appropriate readout of *fru*^{M} transcription (Fig. 7). Expression of olfactory receptors (*Or47b*, *Or65a*, *Or13a*) and housekeeping genes did not show a significant change in response to social isolation in males (Fig. 7). These results suggest that social experience opens chromatin around *fru* P1, whereas social isolation decreases these active chromatin marks.

2.2.2 Pheromone and social exposure of isolated males open chromatin and rescue fru^{M} expression in Or47b neurons

Given that - (1) *Fru*^{M} expression in Or47b neurons requires a functional Or47b receptor (Hueston et al., 2016) and, (2) group housing increases *fru*^{M} expression, we reasoned that pheromone exposure or re-group housing may enhance *fru*^{M} expression in Or47b neurons. To test this hypothesis, we asked whether the decrease in *fru*^{M} expression seen in socially isolated males can be rescued by re-grouping or exposure to Or47b ligand Palmitoleic acid (PA). In addition, we wanted to determine the effect of age and duration of the social exposure on *fru*^{M} expression and chromatin state. We transferred socially isolated males to vials containing 4.5 mg PA starting day 0, 2, or 3 after eclosion, and quantified *fru*^{P1GAL4} driven *UAS-GFP expression* at days 5 and 7 (Fig. 8A-F). We found PA exposure rescues the *fru*^{M} expression to group housed levels if exposed at day 0 in Or47b, but not Or67d ORNs, albeit levels of rescue depended on the length of exposure (Fig. 8A-C). For example, exposing socially isolated males to PA from 3 to 5 days does not rescue the *fru*^{M} expression to group housed level; however, 3

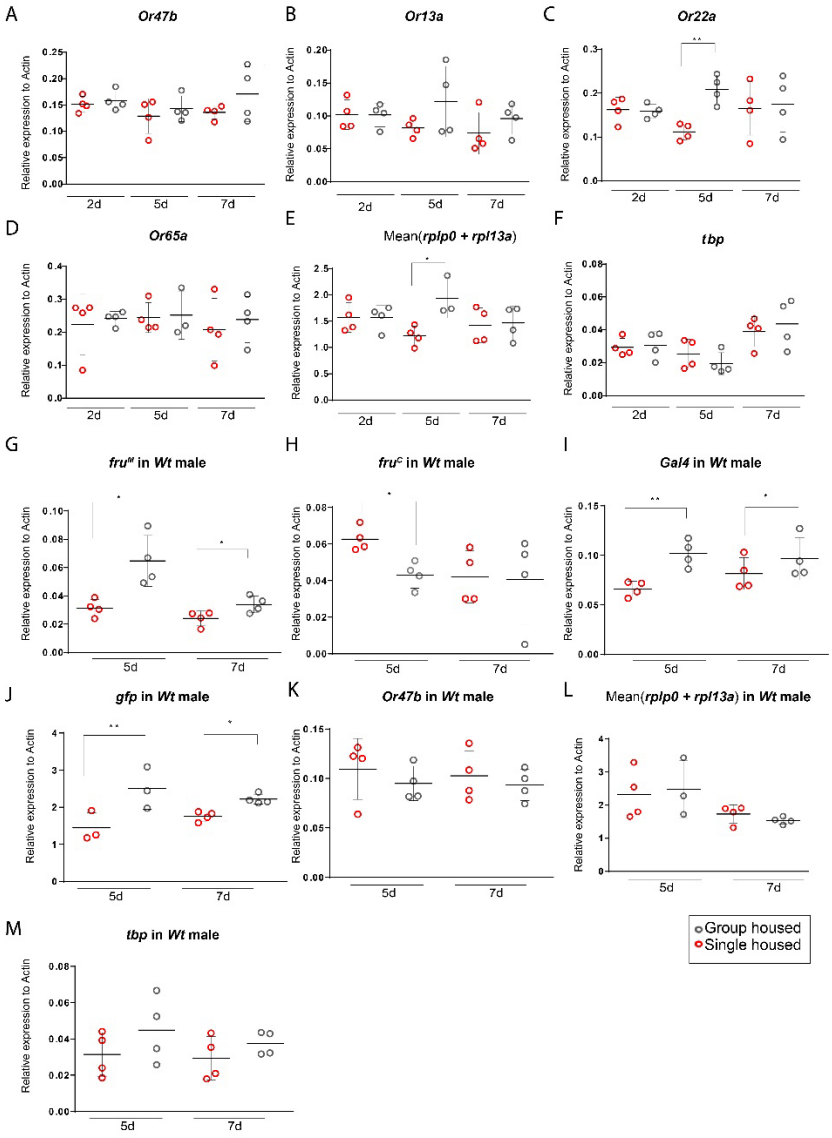

Figure 7: Effect of social experience on OR and housekeeping gene transcription.

qRT-PCR from SH and GH w1118 male antennal samples for control genes (A) Or47b, (B) Or13a, (C) Or22a, (D) Or65a, (E) Mean of rplp0 and rpl13a, and (F) tbp shows social condition does not show a significant effect on expression of these genes throughout the developmental stages, except for at 5 day, there is higher Or22a and ribosomal protein expression in group housed flies. (G-M) qRT-PCR from SH and GH males with fruP1-Gal4 driven GFP antennal samples for control genes.

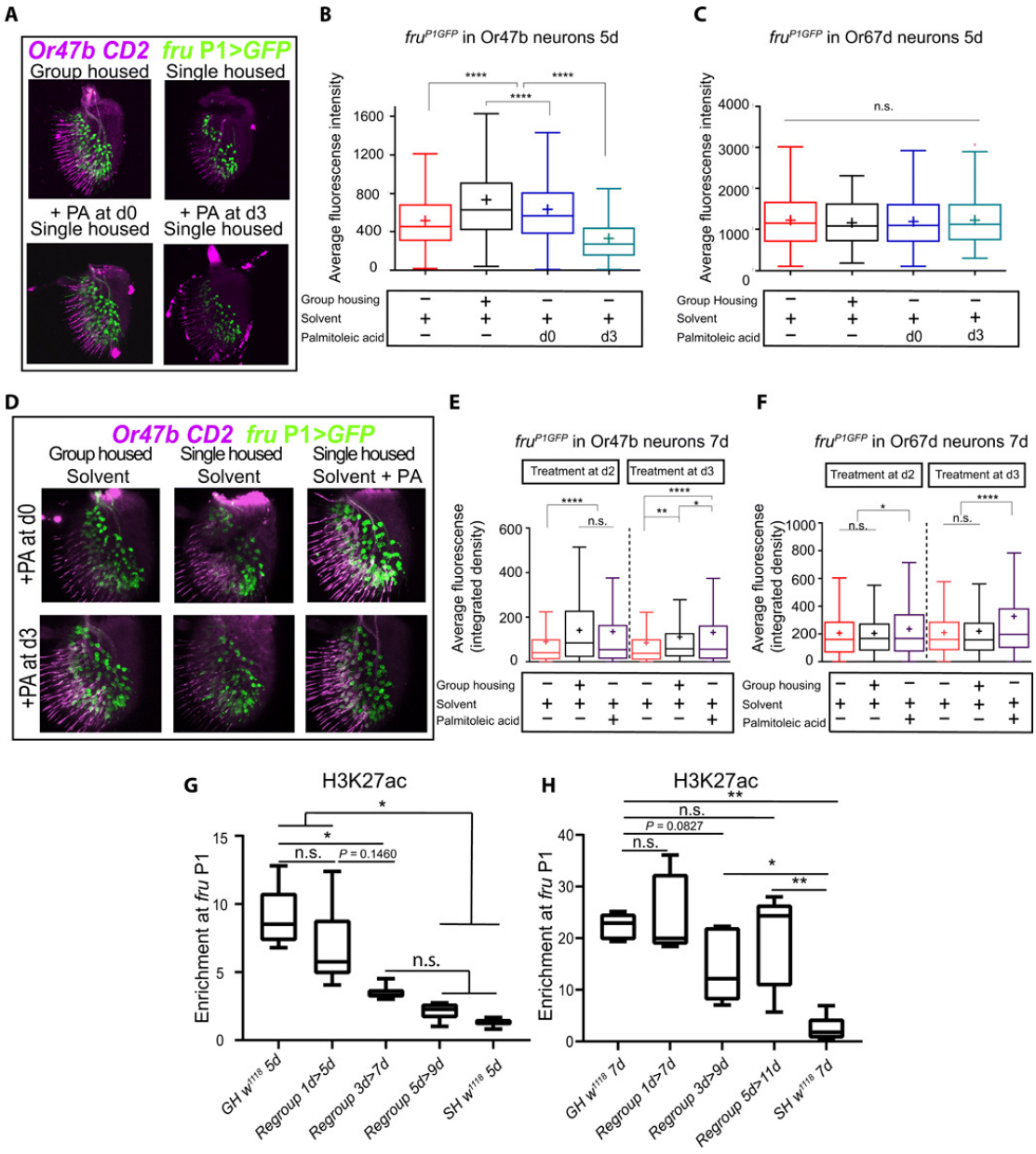

Figure 8: Pheromone exposure and regrouping rescues chromatin-based effects around fru P1.

fru^{P1GAL4}-driven UAS-GFP expression in male antennae from GH (top left) and SH (top right). Lower panels show fru^{P1GAL4}-driven UAS-GFP expression in SH males exposed to Or47b ligand PA between 0 to 5 or 3 to 5 days after eclosion (A) and 0 to 7 or 3 to 7 days after eclosion (D). Quantification of integrated GFP density from Or47b ORNs (B) and Or67d ORNs (C) in response to group housing, single housing, and 0 to 5 or 3 to 5 days of exposure of SH males to solvent or PA. Quantification of integrated GFP density from

Or47b ORNs (E) and Or67d ORNs (F) in response to group housing, single housing, and 2 to 7 or 3 to 7 days of exposure of SH males to solvent or PA. (G) H3K27ac enrichment around fru P1 in w1118 male antennae socially isolated and then regrouped for a duration of 5 days (1 to 5, 3 to 7, and 5 to 9 days after eclosion) compared to SH male antennae. (H) H3K27ac enrichment around fru P1 in w1118 male antennae socially isolated and then regrouped for a duration of 7 days (1 to 7, 3 to 9, and 5 to 11 days after eclosion) compared to SH male antennae. *P < 0.05; **P < 0.005; ***P < 0.001; ****P < 0.0001; n.s., not significant.

to 7-day exposure does (Fig. 8D-F). In Or67d neurons, PA exposure at day 3 and taken to 7 days also showed significant effects fru^{M} expression not seen in group housed flies.

Given the amount of tissue required to perform ChIP, we proceeded with a naturalistic paradigm of regrouping flies in groups of 30, rather than exposing them to PA. In agreement with an increase in fru^{P1GAL4} driven *UAS-GFP*, active chromatin marks also showed increased enrichment when socially isolated flies are regrouped starting at different ages for 7 days, yet the level of rescue was lower if flies were regrouped for a shorter period of 5 days (Fig. 8G-H). The rate of rescue was comparable among flies grouped for similar durations, suggesting absence of a critical period for social experience to modulate fru^{M} expression. Overall, these results show that duration of exposure to PA or regrouping, at any age, is sufficient to rescue chromatin-based increase in fru^{M} expression.

2.2.3 Calcium signaling induces p300 association with fru P1 promoter and facilitates open chromatin

We next sought to determine the cellular signaling mechanisms downstream of Or47b that regulate fru^{M} transcription and chromatin around *fru* P1. Drosophila

olfactory receptors (ORs) are ion channels that conduct calcium and sodium (Tiwari et al., 2019). In response to increase in calcium from membrane receptors, CaMKI is activated and phosphorylates P300/CBP (Impey et al., 2002). This results in the activation of p300/CBP and leads to its association with transcription factors at the promoters of genes (Impey et al., 2002), which is followed by acetylation of histones, such as histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27), and recruitment of RNA polymerase II to the promoters facilitating open active chromatin (Chan & La Thangue, 2001; Vicent et al., 2010). In a previous study, we showed that calcium signaling from Or47b receptor is required to maintain the expression of fru^M in Or47b neurons (Hueston et al., 2016). Knocking down CaMKI and its phosphorylation target histone acetyl-transferase p300/CBP in fru^M-positive olfactory neurons deceases fru^M expression in Or47b neurons (Hueston et al., 2016). Interestingly, these RNAi knockdowns also exhibit defects in OR47b neuron sensitivity and age- and social experience-dependent courtship competition (Sethi et al., 2019).

We predicted that in Drosophila, social experience dependent changes in the fru^M expression can be due to chromatin-based changes induced around *fru* P1 promoter. Enrichment of p300 at *fru* P1 TSS increased with group housing and decreased in response to social isolation and in *Or47b* mutants (Fig. 5C'). As with RNA PolII enrichment, p300, and its associated marks H3K9ac and H3K27ac, show no significant difference at *Or47b* or *Or82a* promoters in different social housing conditions (Fig. 5D-

G). To examine the contribution of both P300 and CaMKI to chromatin remodelling around fru^M we used Or47b ORN-specific *CaMKI* and *p300-RNAi* knockdowns. Both RNAi knockdowns led to a decrease in RNA Polymerase II, p300, H3K27ac, and H3K9ac enrichment at *fru* P1 TSS, suggesting that p300 association with the promoter requires CaMKI activity (Fig. 9A-D). Enrichment at promoters of control *Or* genes were not affected in RNAi conditions. One exception to this is the enrichment of some of the acetylation marks around *Or47b* TSS in *Or47b-GAL4* driven *p300-RNAi* knockdown, likely due to the fundamental role of p300 in transcription regulation. Even though we cannot make any direct inferences for how p300 affects the chromatin around *Or47b*, it does inform us of the cell type specificity of our knockdown (Fig. 10). Collectively, these data suggest that CaMKI signaling in response to social experience elevates fru^M transcription in Or47b neurons via p300 based chromatin modulation.

2.2.4 Juvenile hormone signaling regulates fru^M expression in Or47b neurons

Recent studies showed that juvenile hormone signaling, which relays age-related information, enhances social experience dependent increase in Or47b odor responses and courtship advantage (Usui-Aoki et al., 2000). One molecular mechanism that can account for the age-dependent differences in neuronal responses and behavior is if juvenile hormone signaling regulates fru^M expression to modulate neuronal function. To test this hypothesis, we used RNAi based knockdown of two putative JH receptors, *met*

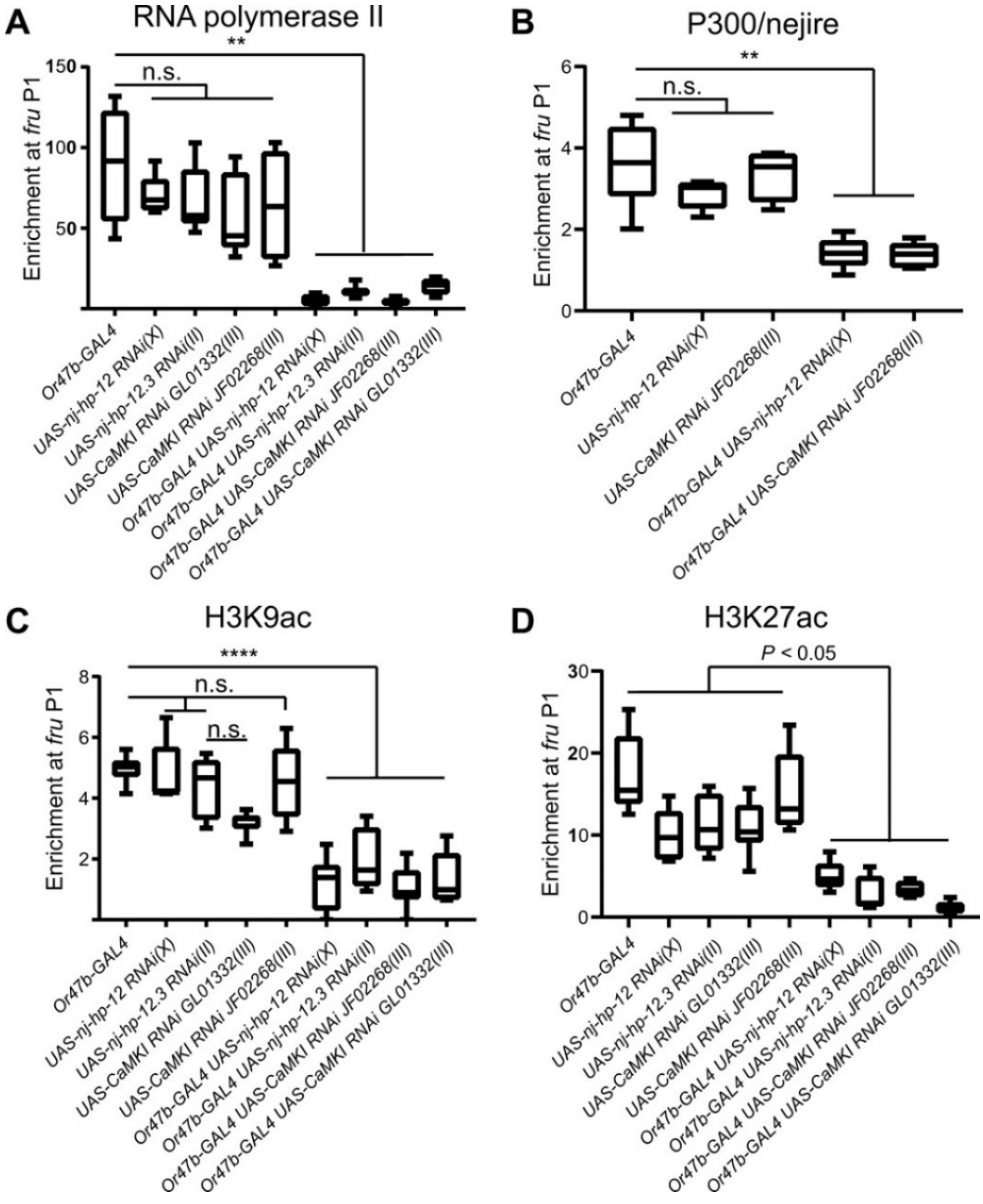

Figure 9: Calcium signaling and p300 function in Or47b neurons facilitates active chromatin around fru P1

Enrichment of RNA polymerase II (A), p300 (B), H3K27ac (C), and H3K9ac (D) around fru P1 in GH male antennae in Or47b-GAL4, UAS-CaMKI RNAi, UAS-nej (p300) RNAi, Or47b-GAL4 UAS-CaMKI RNAi, and Or47b-GAL4 UAS-nej (p300) RNAi. Multiple UAS-RNAi lines were used for each gene indicated by the chromosome and stock name. **P < 0.005; ****P < 0.0001; n.s., not significant.

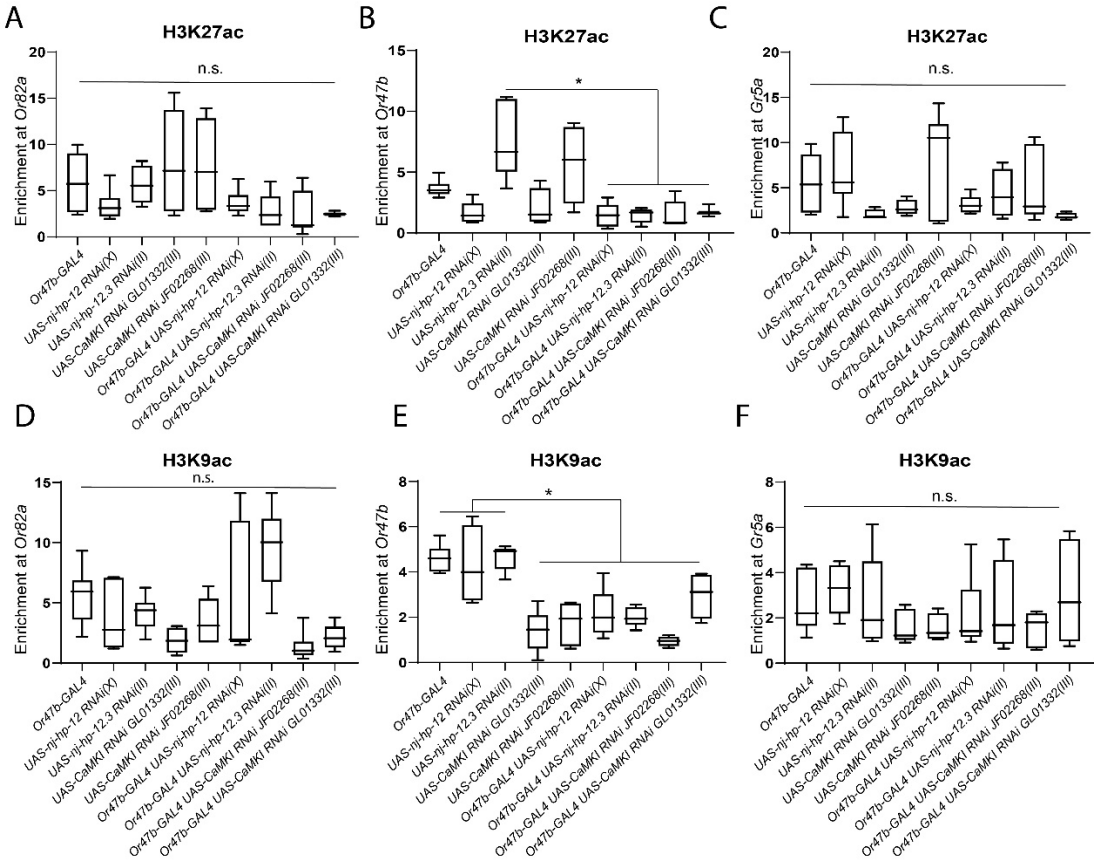

Figure 10: Enrichment of active chromatin marks H3K27ac and H3K9ac upstream of Or and Gr

Enrichment of H3K27ac (A-C) and H3K9ac (D-F) upstream of Or82a (A,D) andGr5a (C-F) in group housed 7-day old male antennae with representative genotypes are shown. Or47b-GAL4 driven UAS-p300 RNAi and UAS-CaMKI RNAi knockdown results in a slight decrease in association with Or47b promoter. No change in enrichment is detected upstream of Or82a and Gr5a

and *gce*, in either Or47b neurons or all fru^{M}-positive neurons. RNAi knockdown of both genes in fru^{M} -positive neurons resulted in a decrease in the expression of fru^{P1GAL4} driven *UAS-GFP* expression (Fig. 11A, B). Interestingly, $fru^{P1\ GAL4}$ mediated knock-down of *gce* and *met* decreased fru^{M} expression in both Or47b and Or67d neurons by day 7 (Fig. 11A, B), albeit with more dramatic effects in Or47b ORNs, which might account for differences in fru^{M} regulation in different ORN population (Fig. 11A, B).

We next analyzed open chromatin marks around *fru* P1 TSS in group housed *met* and *gce-RNAi* knockdowns. Or47b neuron specific RNAi knockdown of *met* and *gce* showed a decreased enrichment of RNA Polymerase II at *fru* P1 TSS (Fig. 11C). Enrichment of p300 and H3K27ac was also dampened in these RNAi knockdowns. These results, together with the effects of social isolation, suggest that the stabilization of active chromatin marks around *fru* P1 promoter requires both juvenile hormone signaling and social experience.

2.2.5 Time course of juvenile hormone regulation of fru^{M} expression in ORNs

Previous studies have revealed that juvenile hormone signaling in the first 3 days of life is responsible for critical period of courtship learning and maturation in Drosophila (Lee et al., 2017). This period overlaps with juvenile hormone titers, which peak at eclosion and decrease after 2-3 days into adulthood (Bownes & Rembold, 1987). Given the requirement for both group housing and juvenile hormone signaling in stabilizing active chromatin marks around *fru* P1, we predicted that the disruption of JH

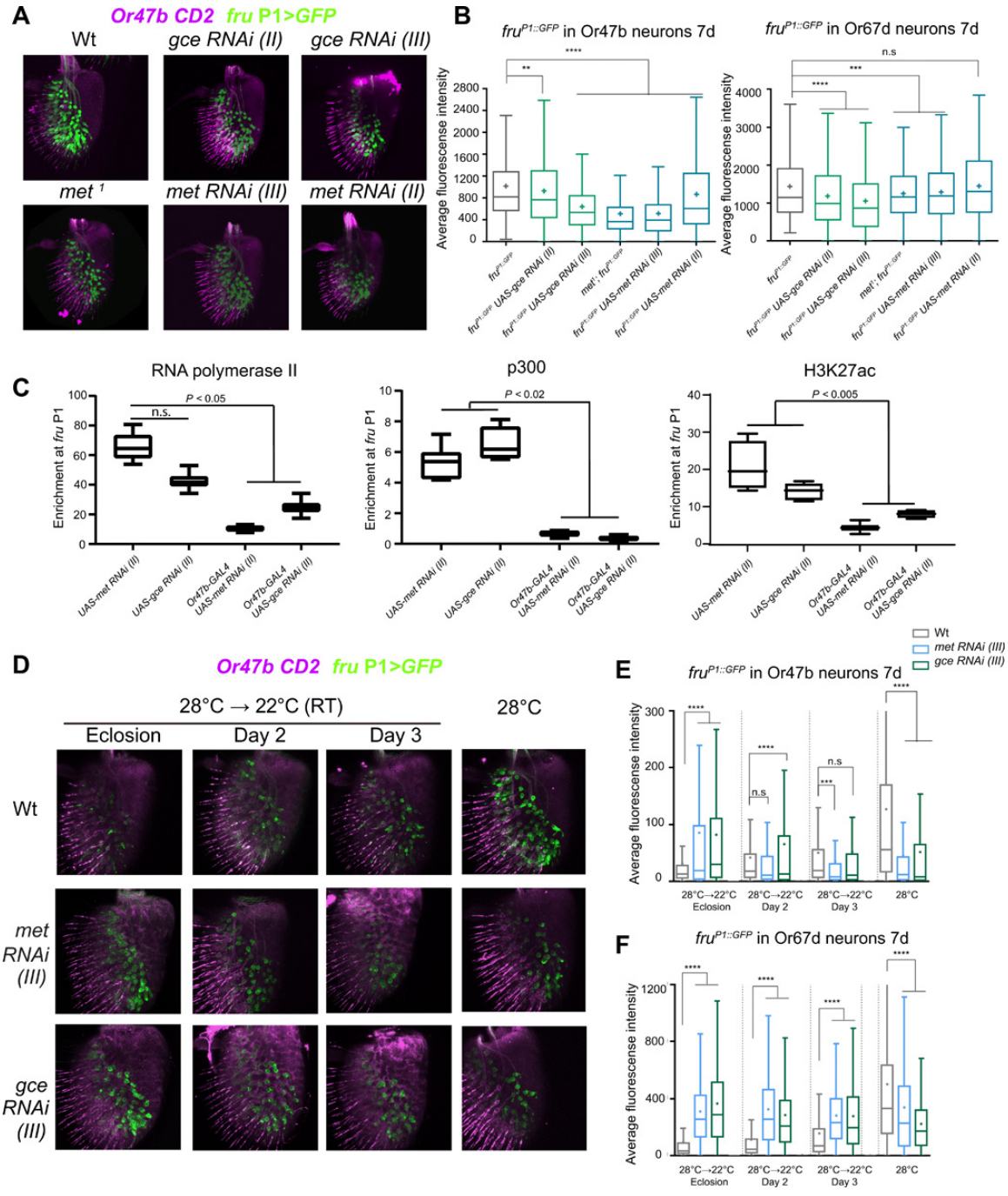

Figure 11: JH signaling recruits transcriptional machinery and facilitates open chromatin at fru P1.

(A) fru^{P1GAL4}-driven UAS-GFP expression in GH male antennae in gce-RNAi and met-RNAi knockdowns, as well as met1 mutants. UAS-RNAi expression is driven

by fru^{P1GAL4}. (B) Quantification of GFP intensity from fru^{P1GAL4}-driven 40XUAS-CD8GFP expression in Or47b and Or67d neurons from (A). (C) Enrichment of RNA polymerase II, p300, and H3K27ac at fru P1 in GH male antennae with genotypes UAS-met RNAi, UAS-gce RNAi, Or47b-GAL4 UAS-met RNAi, and Or47b-GAL4 UAS-gce RNAi. (D) fru^{P1GAL4}-driven 40XUAS-CD8GFP expression in GH male antennae in gce-RNAi and met-RNAi knockdowns raised at 28° or 22°C to vary knockdown efficiency. The flies are either raised at 28°C for 7 days or transferred to 22°C at day 1, 2, or 3 after eclosion before GFP analysis on day 7. RT, room temperature; Wt, wild type. (E and F) Quantification of integrated GFP density of antennae in (D). ****P < 0.0001; n.s., not significant.

receptor function in the first 2-3 days after eclosion could have different effects on fru^{M} expression. Previous studies have shown that performing GAL4 driven RNAi knockdown experiments at different temperatures can modify RNAi effects, where lower temperatures weaken and at times completely rescue knockdown phenotypes (Barish et al., 2018; Joo et al., 2013). Given that fru^{GAL4} mediated *met* and *gce* RNAi knockdown phenotypes are highly penetrant at 28 degrees, we probed fru^{M} expression in experiments where we restricted the knockdown to specific days by performing temperature shifts. Interestingly, restricting *met* and *gce* RNAi knockdowns to development by switching temperature the flies were raised from 28 to 22 degrees at eclosion resulted in an elevated fru^{M} expression compared to wild type (Fig. 11D-F) (see Materials and Methods). This suggests that in pupal stages JH receptors might act as repressors of *fru.* Performing the temperature shifts in successive days gradually reversed this trend in both neurons, whereby day 7 flies transferred on day 3 appeared wild type and flies kept at 28 for the entire experiment showed a significant decrease in fru^{M} expression in both ORN populations. This trend was true for fru^{M} expression in

both Or47b and Or67d neurons, with a slower rate in the latter (Fig. 11E-F). These results suggest that JH receptors possibly act as repressors of *fru*^{M} transcription in late pupal and early adult stages of life, and switch to an activator after day 3 in the presence of social experience.

2.2.6 Juvenile hormone and pheromone signaling co-regulates chromatin to enhance fru^{M} expression in Or47b neurons

Information about the social environment via pheromone sensing Or47b neurons and age-related internal states through juvenile hormone signaling can synergize to modulate *fru*^{M} expression, neuronal sensitivity, and courtship advantage. But how do hormone and pheromone signals synergize to exert these effects on *fru*^{M} regulation at the molecular level? One possible mechanism of interaction would be if juvenile hormone receptor binding upstream of *fru* establishes a permissive or poised chromatin state around *fru* P1, which is stabilized by timely social experience, through p300 activity to regulate *fru*^{M} expression and function. Indeed, nuclear hormone receptors can directly interact with p300/CBP to regulate gene expression in mammals (Mahajan & Samuels, 2005; Vicent et al., 2010), and a conserved juvenile hormone response element CACACGCGAAA (E-box) exists 7721 base-pairs upstream of *fru* P1. Thus, we hypothesized that juvenile hormone receptors can interact with *fru* P1 TSS and E-box to regulate chromatin and *fru*^{M} expression in ORNs. In order to test if juvenile hormone receptors associate with E-box and *fru* P1 promoter, we performed ChIP from group housed and single housed male antennae using Gce antibodies followed by qPCR. These

experiments showed that Gce is enriched at *fru* P1 in 5-day old group housed flies and this association is dampened in response to *gce* RNAi knockdown in Or47b ORNs, demonstrating antibody specificity (Fig. 12A). Despite the significant decrease association of RNA Pol II and active chromatin marks in response to social isolation, we observe an age-dependent increase in Gce accumulation at both *fru* P1 promoter as well as E-box upstream of *fru* P1 in single housed males (Fig. 12B, C). Disrupting Or47b signaling can recapitulate the social isolation dependent increase in Gce enrichment at both *fru* P1 and the E-box in Or47b neuron-specific knockdown of *CaMKI* and *p300-RNAi* (Fig. 12D-E). Enrichment of Gce was not significantly different around another Gce regulated gene *Kr-h1* in different social conditions and RNAi knockdowns, except for *p300* knockdown (Figure 13, 14).

The accumulation of Gce at the *fru* P1 promoter in the absence of social experience suggest that Gce complexes might function as transient repressors of transcription, and that social experience converts them to transcriptional activators by stabilizing the enrichment of active chromatin marks and recruitment of basal transcriptional machinery. To test this hypothesis we analyzed methylation state of H3K27, where H3K27me3 is associated with repressed chromatin whereas, demethylation to H3K27me2 was shown to mark chromatin that is poised for transcription (Ferrari et al., 2014). Indeed, in socially isolated males we observed a trend towards an increase in the enrichment of poised chromatin H3K27me2 marks around *fru*

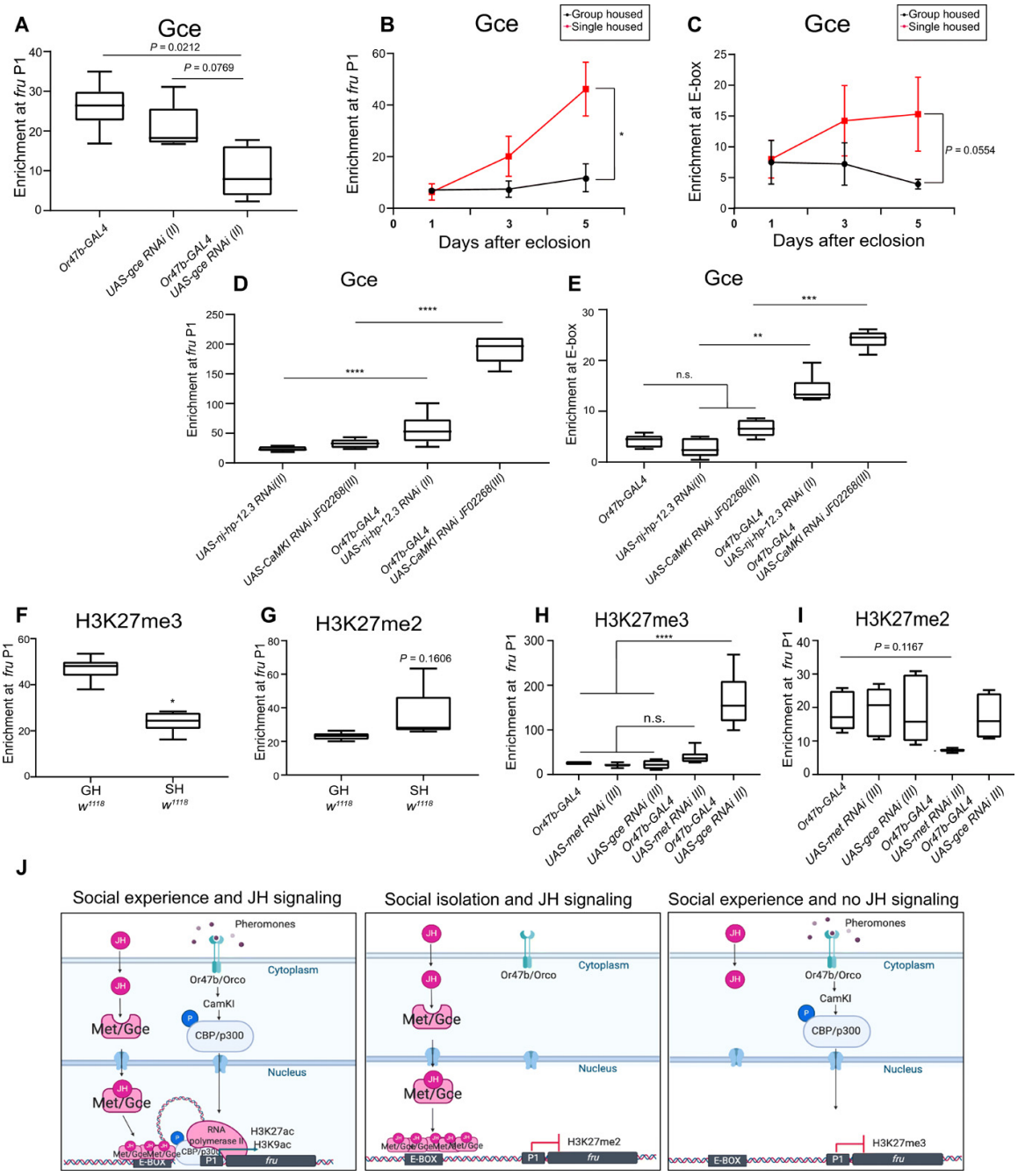

Figure 12: JH receptor binds upstream of fru P1 and primes it for chromatin-based regulation with social experience.

(A) Enrichment of Gce at fru P1 in 7-day-old GH male antennae with genotypes *Or47b-GAL4*, *UAS-gce RNAi*, and *Or47b-GAL4 UAS-gce* RNAi. The time course of Gce association with fru P1 (B) and E-box upstream of fru P1 (C) in GH (black) or socially

isolated (red) male antennae is shown. The x axis shows days after eclosion, and the y axis is enrichment relative to input. The enrichment of Gce at fru P1 (D) or E-box (E) in GH male antennae with genotypes *UAS-nj(p300)*RNAi, *UAS-CaMKI* RNAi, *Or47b-GAL4 UAS-nj(p300)*RNAi, and *Or47b-GAL4 UAS-CaMKI* RNAi is shown. (F and G) Enrichment of H3K27me3 and H3K27me2 at fru P1 in GH or socially isolated male antennae. (H and I) Enrichment of H3K27me3 and H3K27me2 at fru P1 in GH male antennae with genotypes *UAS-met RNAi*, *UAS-gce RNAi*, *Or47b-GAL4 UAS-met* RNAi, and *Or47b-GAL4 UAS-gce* RNAi. (J) A model showing coincidence detection of hormone and pheromone signals by fru promoter. The presence of both social experience through pheromones and JH signaling leads to the assembly of JH receptors, activated p300, and RNA polymerase II and increases the association of active chromatin marks H3K29ac and H3K9ac at the fru P1. In the absence of social experience, p300 activation is inhibited, and JH receptor complexes accumulate at the fru P1 promoter. This leads to an increase in the association of H3K27me2, which interferes with non–cell type–specific activation of fru P1. In the absence of JH signaling, p300 and RNA polymerase II association decreases, and this leads to an increase in repressive chromatin marks such as H3K27me3. *P < 0.05; **P < 0.005; ***P < 0.001; ****P < 0.0001; n.s., not significant.

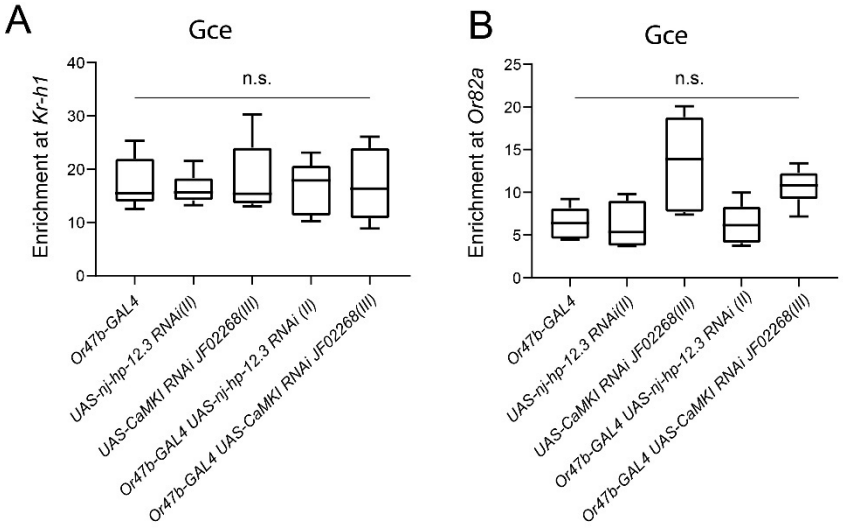

Figure 13: Gce enrichment upstream of Kr-h1 and Or82 in p300 and CaMKI knockdowns

Antennal ChIP qPCR from 7-day old w1118 males. Gce binding upstream of Or82a (B) and Kr-h1 (A), a Met/Gce regulated gene, does not change in Or47b-GAL4 driven UAS-CamKI RNAi (A and B) and UAS-p300 RNAi (C and D) knockdown

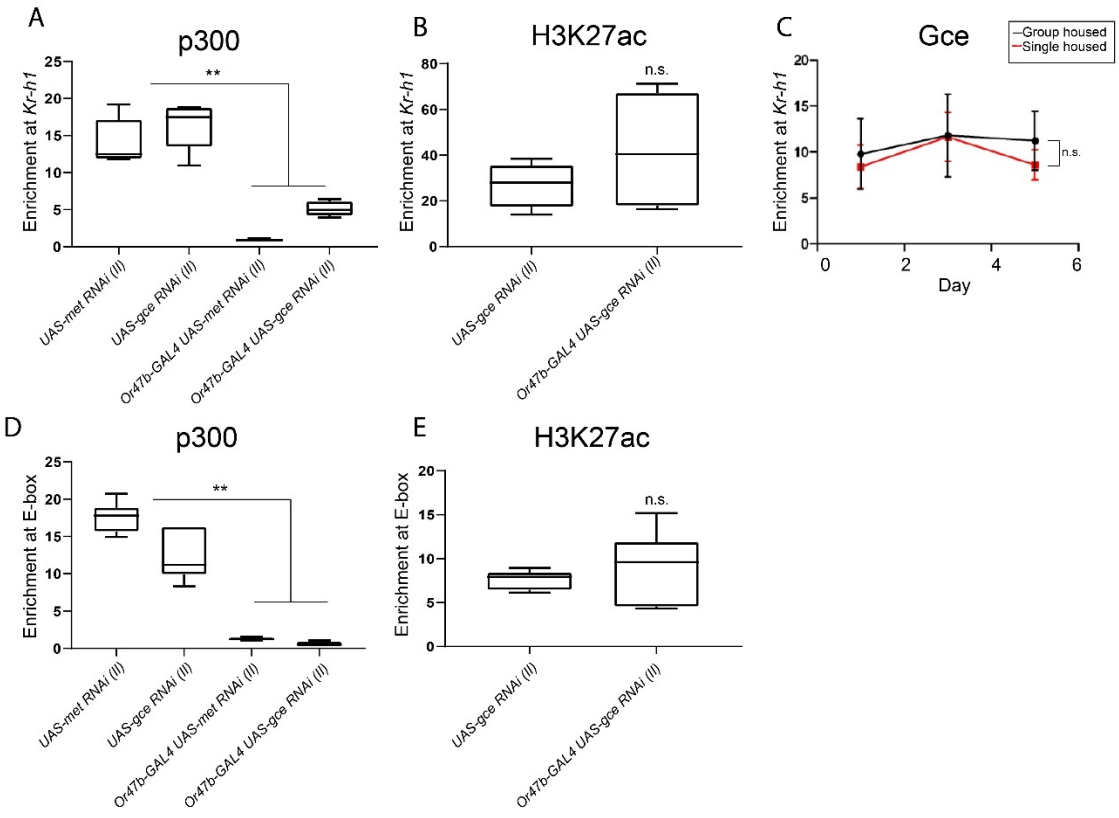

Figure 14: p300, H3k27ac, and Gce enrichment upstream of Kr-h1 and fru E-box in gce

P300 enrichment upstream of Kr-h1 and E-box in 7-day old male antennae (A and D). H3K27ac enrichment upstream of Kr-h1 and E-box in 7-day old male antennae (Band E respectively). Krh1 and the E-box show a decrease in p300 in (B and E) in Or47b-GAL4 driven met/gce RNAi, while H3K27ac does not change (B and E). Gce enrichment is not altered in single versus group housed males at Kr-h1 (C).

P1 (Fig. 12G), compared to H3K27me3, which showed a significant decrease (Fig. 12F). Furthermore, Or47b-specific *met* and *gce RNAi* knockdowns had very little to no effect on H3K27me2, except for a small trend towards a decrease in *met* RNAi knockdowns (Fig.12I). In contrast, H3K27me3, showed a significant increase in Or47b-specific *gce-RNAi* knockdowns. The differences between *gce* and *met* RNAi results might either

indicate distinct effects for the two JH receptors in regulating the chromatin state around *fru P1*, or differences in the strength of RNAi (Fig. 12H,I).

Taken together, we propose a model where activity of juvenile hormone receptors prime or poise fru^M transcription in ORNs, particularly Or47b neurons, which is stabilized by social experience via p300 dependent chromatin modulation around *fru* P1 (Fig. 12J). In the absence of JH signaling fru^M is repressed and increases its association with heterochromatin marks. In contrast, in the absence of social experience, JH receptors bind to *fru* promoter, retain a poised chromatin state, but cannot effectively induce *fru* expression because of lack of p300 necessary for histone acetylation and RNA Polymerase II binding. Only when the two signals are coincident *fru* expression is maintained in Or47b ORNs in a stable manner. In this way, the *fru* P1 promoter act as a coincidence detector for activation at an appropriate age and social context to enhance sensory neuron responses and courtship advantage.

2.3 Discussion

There is ample evidence that social experience and hormone signals are integrated by animals to modulate physiology and behavior (Dey et al., 2015; Hensch, 2004; S. S. Lee et al., 2017; Nardou et al., 2019). Interactions between hormones and social experience is vital for opening critical periods for social experience dependent reward learning, and appropriate cyclic display of mating behaviors in many organisms (Dey et al., 2015; Dölen et al., 2012; Nardou et al., 2019). Despite the known contribution

of both social and hormonal cues to physiology and behaviors, molecular mechanisms regulating these changes remains unknown or poorly described. In this study, we made use of the unique advantages of the genes and circuits regulating male reproductive behaviors in Drosophila, which are under the control of the male specific transcription factor Fru^M. Previous studies have shown that social experience and hormone signaling increases the sensitivity of Or47b neurons and courtship rigor in male flies (Dölen et al., 2012; Fryer & Archer, 1998; McGowan et al., 2009; Trollope et al., 2012; Weaver et al., 2004). Here we report coincident calcium and hormone signaling converge at *fru* P1 promoter in Or47b neurons to modify chromatin and increase fru^M expression to coordinate alterations in physiology and mating behavior. Our results provide an adaptive molecular mechanism to bridge hormone and pheromone signals at the promoters of key behavioral regulators and subsequent changes in chromatin and transcription to reprogram physiology and behavior.

Chromatin modulation can provide a relatively fast and possibly reversible effects on gene function in neural circuit modulation of behavior. For example, cyclic progesterone exposure, a cue indicating the ovulation state of the female mice, modulates the sensitivity of pheromone detecting sensory neurons, which in turn determines whether the female will accept or be "blind" to male courtship attempts. The change in the sensory neuron responses depend on sensory transduction components, PLCβ2 and PGRMC1 (Dey et al., 2015). It is possible that integration of social and

hormone signals drives behavioral decision via chromatin-based changes in the expression of genes that dial neuronal sensitivity up or down interfering with sensory circuit activation. Indeed, hormone signals were shown to cause changes in the chromatin landscape of actively expressed genes (Dölen et al., 2012; Edsinger & Dölen, 2018; Ferrari et al., 2014; Fryer & Archer, 1998; S. S. Lee et al., 2017; Vicent et al., 2010). Similarly, for behaviors with critical periods where sensory experience and hormone signals may be required, chromatin-based changes can also provide a way of cellular memory later in life once the chemical cues defining the critical period are gone. For example, increase in DNA methylation patterns around glucocorticoid receptor gene with tactile maternal behaviors can have long lasting effects on circuits and behaviors regulated by cortisol signaling (McGowan et al., 2009; Mifsud et al., 2011; Trollope et al., 2012; Weaver et al., 2004). The advantage of using Drosophila courtship behaviors is that genes (fru^M) and circuits underlying these behaviors are well characterized. Fru^M is a male-specific transcription factor that labels the interconnected neurons in the courtship circuits and typically acts as a sexual switch during the pupal stage to alter the developmental fate of individual neurons (Sato & Yamamoto, 2020; Yamamoto & Koganezawa, 2013a). Fru^M can promote male fate by inhibiting cell death, altering dendritic arborization and switching synaptic connectivity between neurons (Sato & Yamamoto, 2020). Even though Fru^M is stably expressed in the adult brain, its function in mature neurons is unknown, but likely involve regulation of Fru^M downstream genes

required for the function, sensitivity, and communication within and among neurons within the circuit (Neville et al., 2014). In this study, we uncover a novel role for Fru^M in regulating physiological properties of olfactory neurons in the adult male flies. This result is consistent with the observation that Fruitless over-expression alters the expression of several genes that are known to regulate neuronal physiology (Neville et al., 2014). Overall our study shows that despite Fru^M function to masculinize the nervous system and behaviors, pheromone and hormone signals interact at *fru* P1 to regulate Fru^M expression at the level of chromatin to fine tune neuronal properties and mating behavior. Identifying ORN-specific changes in transcription and splicing of *fru* as well as genes regulated by Fru^M in the future, combined with expanded chromatin analysis, will give insights into the genes changing neuronal sensitivity in different social conditions and age. Given the wide spread interactions between hormone and sensory signals in modulating organismal behaviors, similar molecular events converging on the regulation of key behavioral genes might also modify behavioral outputs to social signals with age and reproductive state, as well as drive critical periods of social learning in vertebrates to modulate behavior (Slattery et al., 2011).

2.4 Materials and Methods

2.4.1 Fly husbandry and Genetics

Flies were raised on standard fly food (containing yeast, cornmeal, agar and molasses) at 25°C in a 12:12 light-dark cycle in cylindrical glass vials (24 mm diameter, 94 mm height). For group housing, flies were collected within 12 hours of eclosion and separated by sex in groups of 15-30. For socially isolated flies, pupae were taken at 80-100 hours after pupae formation (apf) and placed into individual vials. Newly eclosed flies were transferred to fresh vials and aged till dissection w^{1118}, *Canton S*, *Or47b* mutant alleles (C E Hueston et al., 2016), $Or67d^{Gal4}/Or67d^{Gal4}$ mutant allele (A Kurtovic et al., 2007), and *Or47bCD2/CyO*; fru^{P1Gal4}, *40xUAS-mCD8::GFP/TM6B* were maintained at room temperature. All the flies for RNAi knock-down experiments were maintained at 28°C incubator. These following stocks were obtained from Bloomington Stock Center: *P{TRiP.JF02097}attP2* (#26323); *Mi{MIC}gceMI02742* (#60189); *P{TRiP.HMJ23341}attP40* (#61852); Met^{1} (#3472); *P{TRiP.JF02103}attP2* (#26205); *P{TRiP.HMJ23518}attP40* (#61935); *P{UAS-nej.siRNA}hp12* (#32576); *P{UAS-nej.siRNA}hp12.3* (#32579); *P{TRiP.JF02268}attP2* (#26726); *P{TRiP.GL00274}attP2* (#35362), *Or47b-GAL4* . For temperature shift experiments to define the critical period of juvenile hormone receptor function, we raised *UAS-gce RNAi* and *UAS-met RNAi* crosses at 28 degrees Celsius. The progeny was transferred to 22 degrees to decrease RNAi knockdown at eclosion, Day 2, and Day 3, and fru^{P1GAL4} driven GFP expression is analyzed at day 7.

2.4.2 Genotypes

2.4.2.1 Figure 5

Fig 1A *w*^{1118}

Fig 1B *Canton S* and *w*^{1118};

Fig 1C w^{1118}, Or47b^{1}/Or47b^{1}; TM2/TM6B, Or47b^{2}/Or47b^{2}; TM2/TM6B; Or67d^{Gal4}/Or67d^{Gal4}

Fig 1D, 1F, 1G Or47b-CD2/CyO; fru ^{P1Gal4}, 40xUAS-mCD8::GFP/TM6B

2.4.2.2 Figure 6

2A-2F Or47b-CD2/CyO; fru ^{P1Gal4}, 40xUAS-mCD8::GFP/TM6B

2H-I *w*^{1118}

2.4.2.3. Figure 7

A-F

w^{1118}

G-M

Or47b-CD2/CyO; fru ^{P1Gal4}, 40xUAS-mCD8::GFP/TM6B

2.4.2.4 Figure 8

3A-3F Or47b-CD2/CyO; fru ^{P1Gal4}, 40xUAS-mCD8::GFP/TM6B

3G-3H w^{1118}

2.4.2.5 Figure 9

Or47b-Gal4 UAS-mCD8::GFP/CyO; TM2/TM6B

UAS-nej hp12 RNAi(X)/+

UAS-nej hp12.3 RNAi(II)/CyO

UAS-CamKI GL01332 RNAi(III)/TM6B

UAS-CamKI JF02268 RNAi(III)/TM6B

UAS-nej hp12 RNAi(X)/+; Or47b-Gal4 UAS-mCD8::GFP/CyO; TM2/TM6B

UAS-nej hp12.3 RNAi(II)/ Or47b-Gal4 UAS-mCD8::GFP; TM2/TM6B

Or47b-Gal4 UAS-mCD8::GFP/CyO; UAS-CamKI GL01332 RNAi(III)/TM6B

Or47b-Gal4 UAS-mCD8::GFP/CyO; UAS-CamKI JF02268 RNAi(III)/TM6B

2.4.2.6 Figure 10

A-F

Or47b-Gal4, UAS-mCD8::GFP/CyO; TM2/TM6B

UAS-nej hp12 RNAi(X)/+

UAS-nej hp12.3 RNAi(II)/CyO

UAS-CamKI GL01332 RNAi(III)/TM6B

UAS-CamKI JF02268 RNAi(III)/TM6B

UAS-nej hp12 RNAi(X)/+; Or47b-Gal4, UAS-mCD8::GFP/CyO; TM2/TM6B

Or47b-Gal4, UAS-mCD8::GFP/UAS-nej hp12.3 RNAi(II); TM2/TM6B

Or47b-Gal4, UAS-mCD8::GFP/CyO; UAS-CamKI GL01332 RNAi(III)/TM6B

Or47b-Gal4, *UAS-mCD8::GFP/CyO; UAS-CamKI JF02268 RNAi(III)/TM6B*

2.4.2.7 Figure 11

A-B, D-E

Or47b-CD2/CyO; fru ^{P1Gal4}, 40xUAS-mCD8::GFP/TM6B

Or47b-CD2/UAS-gce HMJ23341 RNAi(II); fru ^{P1Gal4}, 40xUAS-mCD8::GFP/TM6B

Or47b-CD2/CyO; fru P1Gal4, 40xUAS-mCD8::GFP/UAS-gce JF02097 RNAi(III)

met^{1}/+; Or47b-CD2/CyO; fru P1Gal4, 40xUAS-mCD8::GFP/TM6B

Or47b-CD2/CyO; fru ^{P1Gal4}, 40xUAS-mCD8::GFP/UAS-met JF02103 RNAi(III)

Or47b-CD2/UAS-met HMJ23518 RNAi(II); fru ^{P1Gal4}, 40xUAS-mCD8::GFP/TM6B

C

UAS-met HMJ23518 RNAi(II)/CyO; TM2/TM6B

UAS-gce HMJ23341 RNAi(II)/CyO; TM2/TM6B

Or47b-Gal4, UAS-mCD8::GFP/ UAS-met HMJ23518 RNAi(II); TM2/TM6B

Or47b-Gal4, UAS-mCD8::GFP/ UAS-gce HMJ23341 RNAi(II); TM2/TM6B

2.4.3.8 Figure 12

A

Or47b-Gal4 UAS-mCD8::GFP/CyO; TM2/TM6B

UAS-gce HMJ23341 RNAi(II)/CyO; TM2/TM6B

Or47b-Gal4, UAS-mCD8::GFP/ UAS-gce HMJ23341 RNAi(II); TM2/TM6B

B-C, F-G

w^{1118}

D-E

UAS-nej hp12.3 RNAi(II)/CyO

UAS-CamKI JF02268 RNAi(III)/TM6B

UAS-nej hp12.3 RNAi(II)/ Or47b-Gal4, UAS-mCD8::GFP; TM2/TM6B

Or47b-Gal4, *UAS-mCD8::GFP/CyO; UAS-CamKI JF02268 RNAi(III)/TM6B*

Or47b-Gal4 UAS-mCD8::GFP/CyO; TM2/TM6B

H-I

Or47b-Gal4 UAS-mCD8::GFP/CyO; TM2/TM6B

UAS-met JF02103 RNAi(III)

UAS-gce JF02097 RNAi(III)/TM6B

Or47b-Gal4, UAS-mCD8::GFP/CyO; UAS-met JF02103 RNAi(III)/TM6B

Or47b-Gal4, UAS-mCD8::GFP/CyO; UAS-gce JF02097 RNAi(III)/TM6B

2.4.2.9 Figure 13

Or47b-Gal4, UAS-mCD8::GFP/CyO; TM2/TM6B

UAS-nej hp12.3 RNAi(II)/CyO

UAS-CamKI JF02268 RNAi(III)/TM6B

Or47b-Gal4, UAS-mCD8::GFP/UAS-nej hp12.3 RNAi(II)

Or47b-Gal4, *UAS-mCD8::GFP/CyO; UAS-CamKI JF02268 RNAi(III)/TM6B*

2.4.2.10 Figure 14

UAS-met HMJ23518 RNAi(II)/CyO; TM2/TM6B

UAS-gce HMJ23341 RNAi(II)/CyO; TM2/TM6B

Or47b-Gal4, UAS-mCD8::GFP/ UAS-met HMJ23518 RNAi(II); TM2/TM6B

Or47b-Gal4, UAS-mCD8::GFP/ UAS-gce HMJ23341 RNAi(II); TM2/TM6B

2.4.3 Social Condition

For social isolation condition, males were isolated into separate vials as pharate adults in pupal case, allowed to eclose alone and aged as single housed to different ages to deprive pheromone input from Or47b ORNs. For group housed condition, 15-30 newly eclosed males were collected and aged as group housed until dissection. Dissections were performed at 1, 2, 3, 5, 7, 9, 10, and 11 days, dependent on experiment. For Rescue experiments in the absence of PA exposure, single housed flies were re-grouped at day 1, 3, and 5 in group of 35 flies and taken out to combinations of day 5, 7, 9, and 11 to examine the effect of duration of social experience on the epigenetic modification around fru^{P1} promoter.

2.4.4 Odor and Pharmacological Treatment

To examine the effect of PA on *fru* expression in socially isolated flies, 0.45mg of Palmitoleic acid (Cayman) or 10 ul ethanol (solvent control) (E7023, Sigma-Aldrich) was applied on a piece of filter paper (1 x 1 cm) and air-dried in the hood for 60 minutes. The filter paper containing PA or solvent control was then transferred to a fresh vial with a single housed fly. Fresh filter paper was changed every other day.

2.4.5 Immunohistochemistry

Flies from the *Or47b-CD2/CyO*; *fru* ^{P1Gal4}, *40xUAS-mCD8::GFP/TM6B* stock were used for fru^{M} expression quantification. Fly heads were removed in PBT, fixed in 4% PFA for 1 hr followed by three 15- minute PBT wash. Antennae were removed from the head in PBT, fixed in 4% PFA for 0.5 hr followed by three 15-minute PBT wash. Antennae were incubated in primary antibody 1: 200 mouse mouse-α-Rat-CD2 (BIO-RAD MCA154GA) and 5% NGS at 4°C overnight, followed by three 15- minute PBT wash. Then incubated in secondary antibody 1: 1000 goat α-mouse-Cy3 and 5% NGS at 4°C overnight. Antennae were mounted using the Fluoromount-G mounting solution (SounternBiotech) and imaged. Confocal images were taken using the following confocal: Olympus Fluoview FV1000 with laser power 488 = 17%, intensity = 710, pinhole = 105 um, gain = 1, offset = -1; Zeiss 510 with Argon laser power = 35%, pinhole = 108um, detector gain = 800, amplifier gain = 1, amplifier offset = -0.185.

2.4.6 Fluorescence quantification

fru^{P1GAL4} driven *UAS-GFP* expression was quantified using Image J. Individual Or47b or Or67d ORN cell body boundaries were drawn and kept identical for each cell body. Integrated fluorescence density (for images taken using Zeiss 510) and fluorescence intensity (for images taken using Olympus Fluoview FV1000) of the cell boundary for each ORN were measured using Image J fluorescence analysis tools. Or47b ORN cell bodies were identified based on the presence of *Or47b-CD2* marker. Or67d

ORN cell bodies were identified based on the absence of *Or47b-CD2* marker and their central location on the antennae. 15-25 images per experimental condition were quantified and statistical tests were performed in Prism GraphPad.

2.4.7 Chromatin Immunoprecipitation and qPCR

ChIP protocol modified from *(38, 39).* For each genotype, roughly 180 third antennal segments were crosslinked in 1.22% formaldehyde in dissection buffer for 10 minutes at 1ul/antennae. Samples were quenched with 2.5 M glycine to a final concentration of 125 nM. Antennae were ground via electric mortar and biorupted in the Eppendorf bioruptor for 30 cycles at 30 second on/off cycles. See *(39)* for post bioruption protocol using antibodies: H3K27ac (*ab4729*), H3K9ac (*ab4441*), and RNA polymerase II (*Millipore 05-623*), H3K27me2 (*ab24684*), and H3K27me3 (*ab6002*), while control sample tubes contain no antibody. P300 and GCE antibodies were kind gifts from Mattias Mannervik(Lilja et al., 2003), and Claude Desplan, respectively. Primer pairs for qPCR DNA amplification against *fruP1*; see RT-PCR below for machine and reagent. To ensure that any differences in *fru* P1 enrichments were site specific, we examined Or82a, Or47b, and Gr5a promoters; all primers can be found in Table 1 below. Data was processed using a modified 2^(-ΔCt) calculations to determine the percent enrichment. We define fold enrichment as (1+amplification efficiency)^(-((Ct antibody)-(CtnoAntibody))), where amplification efficiency is calculated from the standard curve of the primer pair.

2.4.8 Quantitative RT-PCR

Antennae from 100 male flies were dissected on CO_2 pads and transferred into Trizol (Ambion Life Technologies). At least three biological replicates were prepared for each genotype. RNA was extracted using the RNAeasy kit (Qiagen) and reverse transcribed using the SuperScript IV First-Strand Synthesis Kit (Invitrogen). qPCR was performed using the FastStart Universal SYBR Green Master (ROX) kit (Roche) on Eppendorf Realplex. Primers used were listed in Table 1. The expression level of each gene was normalized to the expression of actin, and the relative expression level was compared across experimental conditions using the delta-delta Ct method. Statistical tests were performed in Prism GraphPad.

2.4.9 Primer list

Primer Name	Sequence
ChIP qPCR primers:	
FruM(-488)-ChIP F	CAGGAGCTGTTACCATTTCAC
FruM(-488)-ChIP R	CCTTTACAGTGCCCGTTTAC
FruM(+37)-ChIP F	AAATCAGCAGCCGACATAC
FruM(+37)-ChIP R	TTTACAGCGCTCTAGCATTT
FruM(+1224)-ChIP F	AGTTGGCTGAGCACAATTC

FruM(+1224)-ChIP R	ACACGGATTAGCCAGTTT
Or82a(+109)-ChIP F	GCTCTGACGTTGGCATAAC
Or82a(+109)-ChIP R	GCAGTTGAAACAGCCTACC
Gr5a(-109)-ChIP F	AATGCGACAGCTGAAAGG
Gr5a(-109)-ChIP R	TCATGGGATTCTAACGATTTG
Or47b(+147)-ChIP F	CTTTCTGAGGTGAATCTG
Or47b(+147)-Chip R	CTTCTTGTTGGGATACTG
Kr-h1-ChIP F (+10)	CGTGACGTTCTCCGAATTT
Kr-h1-ChIP R (+135)	ACGAGATCGATTGGTAGGT
E-box-ChIP F(-7774)	GACGCATAAACGTCTTCCA
E-box-ChIP R (-7641)	CTCAGCTGCATCTCATTCTC

qRT-PCR primers:

Or47b_qPCR F	CAAATCTCAGCCTTCTGCGG
Or47b_qPCR R	GATACTGGCACAGCAAACTCA

Gal4_qPCR F	TTATGCCCAGGGATGCTCTT
Gal4_qPCR R	CGTCGCCAAAGAACCCATTA
GFP_qPCR F	ATGGAAGCGTTCAATTAGCAGA
GFP_qPCR R	AAAGGGCAGATTGTGTGGAC
fruC_qPCR F	CAAATTTGACCGGCGTGCTAACCT
fruC_qPCR R	AGTCGGAGCGGTAGTTCAGATTGT
fruM_qPCR F	CCCGCATCCCCTAGGTACAA
fruM_qPCR R	GACTGTTTCGCCCTCGCAGG
ACT5C_qPCR F	GGCGCAGAGCAAGCGTGGTA
ACT5C_qPCR R	GGGTGCCACACGCAGCTCAT
RPL13A_qPCR F	GCGAGGAGCTGAACCTCTC
RPL13A_qPCR R	GGAAGTGGAATGGACCACGG
RpLP0_qPCR F	GTGCCCATCCTGAAGCCTG
RpLP0_qPCR R	CCTGGTTGACAATCAGACCGT
TBP_qPCR F	TAAGCCCCAACTTCTCGATTCC
TBP_qPCR R	GCCAAAGAGACCTGATCCCC

2.4.10 Statistical Analysis

15-25 antennae were used for GFP quantifications in different social and mutant conditions. For fluorescence analysis, one-way ANOVA followed by Bonferroni's multiple comparisons tests were performed to assess statistical significance between conditions. For each ChIP experiment we used 2-3 biological replicates (n), which are derived from a pool of 180 third antennal segment per biological replicate. For each of the biological replicates we performed three technical replicates of the ChIP-qPCR. Across sample preparations, fold enrichment was standardized to the wildtype sample values within each experiment, i.e. wildtype GH sample Fold enrichment values were used to normalize across different biological preparations, in order to control for batch effects. In addition, no-antibody Ct values were standardized within a plate to limit well effects. To assess significance of the effects of genotype, we then used a nested ANOVA, followed by post-hoc comparisons among genotypes and treatments, using two-stage step-up method of Benjamini, Keieger, and Yekutieli in GraphPad/Prism to correct for multiple testing by controlling the false discovery rate. For qRT-PCR experiments, we performed 3-4 biological replicates, each from a pool of 100-200 antennae. To assess effects of social condition, we used two-tailed t test. For Chip: $p>0.2$ (n.s.) p-value between 0.05 and 0.2 provide the numerical value:

All: $p<0.05$(*), $p<0.005$(**), $p<0.001$(***), $p<0.0001$(****).

2.4.10.1 Figure 5

A) For time course experiments, we ran a nested T-test on group housed versus single housed time points as whole.

PolII GH w^{1118} compared by day: SH w^{1118} 180 whole antennae (p=0.0031, n=8)

H3K27ac GH w^{1118} compared by day: SH w^{1118} 180 whole antennae (p<0.0001, n=8)

B) GH w^{1118}: SH w^{1118} 180 whole antennae (p=0.0338,n=3), GH *Canton S* 180 whole antennae (p=0.8475., n=2), SH *Canton S* (p=0.0560, n=2)

C) GH w^{1118} : SH w^{1118} 180 whole antennae (p= 0.0391, n=3), $Or47b^{1/1}$ (p=0.0246, n=3), $Or47b^{2/2}$(p=0.1120, n=2), $Or67d^{GAL4}$ /$Or67d^{GAL4}$ (p=0.7828, n=2); $Or47b^{1/1}$: SH w^{1118} 180 whole antennae (p=0.7828, n=3), $Or47b^{2/2}$(p=0.2617, n=2), $Or67d^{GAL4}$ /$Or67d^{GAL4}$ (p=0.0179, n=2);

C') SH w^{1118} 180 whole antennae (p=0.0007, n=3), $Or47b^{1/1}$ (p=0.0008, n=2)

C'') SH w^{1118} 180 whole antennae (p=0.0306, n=2), $Or47b^{1/1}$ (p=0.0295, n=2), $Or47b^{2/2}$ (p=0.0445, n=2), $Or67d^{GAL4}$ /$Or67d^{GAL4}$ (p=0.0002, n=2)

C''') SH w^{1118} 180 whole antennae (p=0.0360, n=2), $Or47b^{1/1}$ (p=0.0342, n=2), $Or67d^{GAL4}$ /$Or67d^{GAL4}$ (p=0.0002, n=2)

D) RNA PolII; *Or47b*: GH vs SH w^{1118} (p=0.6365, n=2); *Or82a*: GH vs SH w^{1118} (p=0.5283, n=2); *Gr5a*: GH vs SH w^{1118} (p=0.8865, n=2)

E) p300; *Or47b*: GH vs SH w^{1118} (p=0.5192, n=2); *Or82a*: GH vs SH w^{1118} (p=0.7744, n=2); *Gr5a*: GH vs SH w^{1118} (p=0.3698, n=2)

F) H3K9ac; *Or47b*: GH vs SH w^{1118} (p=0.4366, n=2); *Or82a*: GH vs SH w^{1118} (p=0.1420, n=2); *Gr5a*: GH vs SH w^{1118} (p=0.7075, n=2)

G) H3K27ac; *Or47b*: GH vs SH w^{1118} (p=0.4816, n=2); *Or82a*: GH vs SH w^{1118} (p=0.0899, n=2); *Gr5a*: GH vs SH w^{1118} (p=0.7918, n=2)

2.4.10.2 Figure 6

B-C) Two-way ANOVA followed by Bonferroni's multiple comparisons test

In Or47b neurons, fru^{P1GFP} expression is significantly higher in flies raised in group housing condition compared to social isolation at 2 day (p<0.0001, n=1182 for SH, n=530 for GH), 5 day (p=0.0025, n=1229 for SH, dfn=1665 for GH) and 7 day (p<0.0001, n=1602 for SH, n=1516 for GH) old males.

In Or67d neurons, fru^{P1GFP} expression is unaffected by social experience in 5 day (p=0.8511, tn=1115 for SH, n=1430 for GH) and 7 day (p>0.9999, n=1285 for SH, n=1133 for GH) old males. However, fru^{P1GFP} expression is significantly higher in socially isolated flies in 2 day old male compared to flies raised in group housing condition (p=0.0054, n=1015 for SH, n=374 for GH).

D) Two-tailed t-test

fru^{M} expression is significantly higher in group housed males at 5 day (p=0.016, t=3.321, df=6) and 7 day (p=0.01, t=3.709, df=6). However, fru^{M} expression is significantly higher in socially isolated males at 2 day (p=0.0126, t=3.514, df=6);

fru^{C} expression remain unchanged by social condition in 2d male (p=0.6901, t=0.4185, df=6) and 7d male (p=0.2203, t=1.368, df=6). *fru*^{C} expression is significantly higher in socially isolated males at 5 day (p=0.0341, t=2.733, df=6)

2.4.10.3 Figure 7

Whole antennal gene expressions in different social conditions were analyzed by two-tailed t tests

A) *Or47b* expression remain unaffected by social condition in 2 day (p=0.5896, t=0.5697, df=6), 5 day (p=0.5281, t=0.6695, df=6) and 7 day (p=0.2427, t=1.296, df=6).

B) *Or13a* expression remain unaffected by social condition in 2 day (p=0.9764, t=0.03086, df=6), 5 day (p=0.1943, t=1.461, df=6), and 7 day (p=0.3049, t=1.122, df=6).

C) *Or22a* expression remain unaffected by social condition in 2 day (p=0.8246, t=0.2315, df=6), and 7 day (p=0.8325, t=0.2209, df=6); *or22a* expression is significantly higher in group housed flies at 5 day (p=0.0021, t=5.161, df=6)

D) *Or65a* expression remain unaffected by social condition in 2 day (p=0.709, t=0.3915, df=6), 5 day (p=0.8744, t=0.1664, df=5), and 7 day (p=0.6231, t=0.178, df=6).

E) Ribosomal protein average *(rplp0* and *rpl13a)* expression remain unaffected by social condition in 2 day (p=0.986, t=0.01824, df=6), and 7 day (p=0.986, t=0.01824, df=6). Ribosomal protein average expression is significantly higher in group housed flies at 5 day (p=0.0194, t=3.392, df=5)

F) *tbp* expression remain unaffected by social condition in 2 day (p=0.8051, t=0.2578, df=6), 5 day (p=0.329, t=1.062, df=6), and 7 day (p=0.61, t=0.5379, df=6).

G) fru^M expression is significantly higher in group housed males at 5 day (p=0.012, t=3.497, df=6) and 7 day (p=0.045, t=2.515, df=6) old *Wt* males.

H) fru^C expression remain unchanged by social condition in 7d *Wt* male (p=0.9303, t=0.091, df=6). fru^C expression is significantly higher in socially isolated males at 5 day (p=0.017, t=1.733, df=6) *Wt* male.

I) *gal4* expression is significantly higher in group housed males at 5 day (p=0.0039, t=4.56, df=6) and 7 day (p=0.031, t=2.118, df=6) old *Wt* males.

J) *gfp* expression is higher in group housed males at 5 day (p=0.0564, t=2.66, df=4) and 7 day (p=0.0025, t=4.983, df=6) old *Wt* males.

K) *Or47b* expression remain unaffected by social condition in 5 day (p=0.4444, t=0.8184, df=6), and 7 day (p=0.556, t=0.6233, df=6) old *Wt* males.

L) Ribosomal protein average *(rplp0* and *rpl13a)* expression remain unaffected by social condition in 5 day (p=0.81, t=0.2534, df=5), and 7 day (p=0.2386, t=1.308, df=6) old *Wt* males.

M) *tbp* expression remain unaffected by social condition in 5 day (p=0.2666, t=1.225, df=6), and 7 day (p=0.2722, t=1.209, df=6) old *Wt* males.

2.4.10.4 Figure 8

B, C, E, F) One-way ANOVA followed by Bonferroni's multiple comparisons test

B) Compared to 5d SH + solvent, there is significantly higher fru^{P1GFP} expression in Or47b neurons in 5d GH +solvent flies (p<0.0001, n=1054). Applying palmitoleic acid at eclosion leads to significant increase in fru^{P1GFP} expression (p<0.0001, n=1218), rescuing fru^{P1GFP} expression toward the GH level (p<0.0001, n=1054), whereas applying palmitoleic acid at day 3 leads to significant decrease in fru^{P1GFP} expression (p<0.0001, n=427).

C) In Or67d neurons, the fru^{P1GFP} expression is unaffected by social experience (p=0.5152, n=540), or application of palmitoleic acid at eclosion (p>0.9999, n=769), or at day 3 (p>0.9999, n=414), compared to 5d SH + solvent.

E) In Or47b neurons, applying palmitoleic acid at day 2 (p<0.0001, n=624) or day 3 (p<0.0001, n=779) both led to significant increase in fru^{P1GFP} expression level in flies lacking social experience, bring the level to GH level when applied at day 2(p>0.9999, n=428) and day 3 (p=0.2221, n=478).

F) In Or67d neurons, applying palmitoleic acid at day 2 (p=0.2973, n=505) does not result in difference in fru^{P1GFP} expression compared to in flies raised in social isolation. However, applying palmitoleic acid at day 3 leads to significant increase in fru^{P1GFP} expression compared to both SH (p<0.0001, n=677) and GH flies (p<0.0001, n=737) applied with solvent controls.

G-I) All ChIP analyzed with ANOVA followed by post-hoc multiple comparison

G) GH w^{1118} : Regroup 1d>5d (p=0.0701, n=2), Regroup 3d>5d (p=0.0036, n=2),Regroup 3d>7d (p=0.1041,n=2),Regroup 5d>9d (p=0.0100,n=2), SH w^{1118} 5d (p=00384,n=2); Regroup 1>5d: Regroup 3d>5d (p=0.1116, n=2)Regroup 3d>7d (p=0.6642,n=2), Regroup 5d>9d (p=0.2397, n=2), SH w^{1118} 5d (p=0.1335, n=2); Regroup 3d>7d: Regroup 5d>9d (p=0.1032,n=2), SH w^{1118} 5d (p=0.*0526*,n=2); Regroup 5d>9d: SH w^{1118} 5d (p=0.7010,n=2)

H) 7 day GH w^{1118}: Regroup 1d>7d (p=0.6423, n=2),Regroup 3d>9d (p=0.0827,n=2),Regroup 5d>11d (p=0.9142,n=2), SH w^{1118} 7d (p=0.0014,n=2); Regroup 1>7d: Regroup 3d>9d (p=0.0350,n=2), Regroup 5d>11d (p=0.6743, n=2), SH w^{1118} 7d (p=0.0007, n=2); Regroup 3d>9d: Regroup 5d>11d (p=0.0446,n=2), SH w^{1118} 7d (p=0.0080,n=2); Regroup 5d>11d; SH w^{1118} 7d (p=0.0006,n=2)

I) GH w^{1118}: Regroup 1d>5d (p=0.1587, n=2), Regroup 3d>5d (p=0.2342, n=2), SH w^{1118} 5d (p=0.0109, n=2); Regroup 1d>5d: Regroup 3d>5d (p=0.0109, n=2), SH w^{1118} 5d (p=0.0125, n=2)

2.4.10.5 Figure 9

All ChIP analyzed with nested ANOVA followed by post-hoc multiple comparison controlling for false discovery.

A) All component comparisons (p>0.05); *Or47b-GAL4: Or47b-GAL4 UAS-nj-hp 12 RNAi(X)* (p=0.0013,n=2) *Or47b-GAL4 UAS-nj-hp 12.3(II) (p=.0020, n=2), O47b-GAL4 UAS-*

CamKI RNAi GL01332 (III) (*p*=0.0011, *n*=2), *Or47b-GAL4 UAS-CamKI RNAi JF02268 (III)* (*p*=0.0014,*n*=2)

B)*Or47b-GAL4: Or47b-GAL4 UAS-nj-hp 12 RNAi(X)* (*p*=0.0002,*n*=2) *Or47b-GAL4 UAS-nj-hp 12.3(II) (p<0.0001, n=2), O47b-GAL4 UAS-CamKI RNAi GL01332 (III)* (*p*<.0001, *n*=2), *Or47b-GAL4 UAS-CamKI RNAi JF02268 (III)* (*p*<.0001,*n*=2)

C) All component comparisons (*p*>0.05); *Or47b-GAL4: Or47b-GAL4 UAS-nj-hp 12 RNAi(X)* (*p=0*.0075,*n*=2), *Or47b-GAL4 UAS-CamKI RNAi JF02268 (III)* (*p*=0.0046,*n*=2)

D) All component comparisons (*p*>0.05) except *UAS-CamKI RNAi GL01332 (III)* (*p*=0.0120, *n*=2); *Or47b-GAL4: Or47b-GAL4 UAS-nj-hp 12 RNAi(X)* (*p*<0.0001,*n*=2) *Or47b-GAL4 UAS-nj-hp 12.3(II) (p=0.0001, n=2), O47b-GAL4 UAS-CamKI RNAi GL01332 (III)* (*p*<0.0001, *n*=2), *Or47b-GAL4 UAS-CamKI RNAi JF02268 (III)* (*p*<0.0001,*n*=2)

2.4.10.6 Figure 10

A) All comparison p>0.05 n=2 per condition

B) Comparisons with (*p*<0.05):, *UAS-nj-hp 12.3 RNAi (II): Or47b-GAL4 UAS-nj-hp 12 RNAi(X)* (*p*=0.0167, *n*=2), *Or47b-GAL4 UAS-nj-hp 12.3(II)* (*p*=0.0150, *n*=2), *O47b-GAL4 UAS-CamKI RNAi GL01332 (III) (p=0.0179, n=2), Or47b-GAL4 UAS-CamKI RNAi JF02268 (III)* (*p*=0.0182, *n*=2)

C) All comparisons p>0.05 n=2 per condition

D) All comparisons p>0.05 n=2 per condition

E) Comparisons with p<0.05: *Or47b-GAL4, UAS-nj-hp 12.3 RNAi (II),and UAS-nj-hp 12 RNAi(X) vs: Or47b-GAL4 UAS-CamKI RNAi JF02268 (III), O47b-GAL4 UAS-CamKI RNAi GL01332 (III), Or47b-GAL4 UAS-nj-hp 12 RNAi(X), Or47b-GAL4 UAS-nj-hp 12.3(II)*

F) All comparisons p>0.05 n=2 per condition

2.4.10.7 Figure 11

B) All genotypes analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test

Comparing to fru^{P1Gal4}, fru^{P1Gal4} *UAS-gce RNAi (III)* Or47b neurons (p<0.0001, n=513), Or67d neurons (p<0.0001, n=388)

fru^{P1Gal4} *UAS-gce RNAi (II)* Or47b neurons (p=0.0203, n=532), Or67d neurons (p<0.0001, n=534)

fru^{P1Gal4} *UAS-met RNAi (II)* Or47b neurons (p<0.0001, n=542) , Or67d neurons (p>0.9999, n=616)

met^{1}; fru^{P1Gal4} Or47b neurons (p<0.0001, n=492), Or67d neurons (p=0.0013, n=460)

fru^{P1Gal4} *UAS-met RNAi (III)* Or47b neurons (p<0.0001, n=680) , Or67d neurons (p=0.0029, n=617)

C) RNA Polymerase II. *Or47b-GAL4: Or47b-GAL4 UAS-met RNAi (II)* (p=0.0018, n=2), *Or47b-GAL4 UAS-gce RNAi (II)* (p=.0044, n=2); *UAS-met RNAi (II): Or47b-GAL4 UAS-met RNAi (II)* (p=0.0074, n=2), *UAS-gce RNAi (II): Or47b-GAL4 UAS-gce RNAi (II)* (p=0.2249, n=2)

p300. *Or47b-GAL4: Or47b-GAL4 UAS-met RNAi (II)* (p=0.0581, n=2), *Or47b-GAL4 UAS-gce RNAi (II)* (p=0.0425, n=2); *UAS-met RNAi (II): Or47b-GAL4 UAS-met RNAi (II)* (p=0.0116, n=2), , *UAS-gce RNAi (II): Or47b-GAL4 UAS-gce RNAi (II)* (p=0.0081, n=2)

H3K27ac. *Or47b-GAL4: Or47b-GAL4 UAS-met RNAi (II)* (p=0.0024, n=2), *Or47b-GAL4 UAS-gce RNAi (II)* (p=0.0095, n=2); *UAS-met RNAi (II): Or47b-GAL4 UAS-met RNAi (II)* (p=0.0012, n=2), , *UAS-gce RNAi (II): Or47b-GAL4 UAS-gce RNAi (II)* (p=0.0421, n=2)

D, E) All genotypes analyzed with two-way ANOVA followed by Bonferroni's multiple comparisons test

Comparing to WT 28 --> 22 degree Eclosion, *met RNAi* --> 22 degree Eclosion Or47b neurons (p<0.0001, n=1323), Or67d neurons (p<0.0001, n=1394); *gce RNA*i-->22 degree Eclosion Or47b neurons (p<0.0001, n=535), Or67d neurons (p<0.0001, n=762);

Comparing to WT 28 --> 22 degree day 2, *met RNAi* --> 22 degree day 2 Or47b neurons (p=0.9753, n=1449), Or67d neurons (p<0.0001, n=1252);

gce RNAi --> 22 degree day 2 Or47b neurons (p<0.0001, n=796), Or67d neurons (p<0.0001, n=1021);

Comparing to WT 28 --> 22 degree day 3, *met RNAi* -->22 degree day 3 Or47b neurons (p<0.0264, n=441), Or67d neurons (p<0.0001, n=543); *gce RNAi* --> 22 degree day 3 Or47b neurons (p>0.9999, n=600), Or67d neurons (p<0.0001, n=761);

Comparing to WT at 28 degrees, *met RNAi* at 28 degrees Or47b neurons (p<0.0001, n=1342), Or67d neurons (p<0.0001, n=999); *gce RNAi* 28 degree Or47b neurons (p<0.0001, n=443), Or67d neurons (p<0.0001, n=454);

2.4.10.8 Figure 12

All ChIP data is analyzed with nested ANOVA followed by post-hoc multiple comparison controlling for false discovery

A) *Or47b-GAL: Or47b-GAL4 UAS-gce RNAi (III)* (p=0.0212, n=2); *UAS-gce RNAi (III): Or47b-GAL4 UAS-gce RNAi (III)* (p=0.0769, n=2);

For time course experiments, we ran a nested T-test on group housed versus single housed time points as whole.

B) GH w^{1118} comparison: SH w^{1118} 180 whole antennae (p=0.0345, n=6)

C) GH w^{1118} comparison: SH w^{1118} 180 whole antennae (p=0.0554, n=6)

All experimental comparisons (p<0.0001, n=2). All control comparisons (p>0.05, n=2)

E) *Or47b-GAL4*: *Or47b-GAL4 UAS-nj-hp 12.3 RNAi (II)* (p=0.0013, n=2): *Or47b-GAL4 UAS-CamKI RNAi JF02268 (II)* (p<0.0001, n=2); *UAS-nj-hp 12.3 RNAi (II): Or47b-GAL4 UAS-nj-hp 12.3 RNAi (II)* (p=0.0007, n=2); *UAS-CamKI RNAi JF02268 (II): Or47b-GAL4 UAS-CamKI RNAi JF02268 (II)* (p<0.0001, n=2)

F) H3K27me3 GH vs SH w^{1118} (p=0.0226, n=2)

G) H3K27me2 GH vs SH w^{1118} (p=0.1606, n=2)

H) H3K27me2; Or47b-GAL: Or47b-GAL4 UAS-met RNAi (III) (p=0.1167, n=2), Or47b-GAL4 UAS-gce RNAi (III) (p=0.8249, n=2);UAS-met RNAi (III): Or47b-GAL4 UAS-met RNAi (III) (p=.4058, n=2); Or47b-GAL4 UAS-gce RNAi (III) (p<0.4901., n=2); UAS-gce RNAi (III): Or47b-GAL4 UAS-met RNAi (III) (p=0.1834, n=2); Or47b-GAL4 UAS-gce RNAi (III) (p<0.9243, n=2)

I) H3K27me3; Or47b-GAL: Or47b-GAL4 UAS-met RNAi (III) (p=0.3066, n=2), Or47b-GAL4 UAS-gce RNAi (III) (p<0.0001, n=2); UAS-met RNAi (III): Or47b-GAL4 UAS-met RNAi (III) (p=0.2842, n=2); Or47b-GAL4 UAS-gce RNAi (III) (p<.0001, n=2); UAS-gce RNAi (III): Or47b-GAL4 UAS-met RNAi (III) (p=0.31363578, n=2); Or47b-GAL4 UAS-gce RNAi (III) (p<0.0001, n=2)

2.4.10.9 Figure 13

A) Or47b-GAL4: UAS-nj-hp 12.3 RNAi (II) (p=0.4535, n=2), UAS-CamKI RNAi JF02268 (II) (p=0.1591, n=2), Or47b-GAL4 UAS-nj-hp 12.3 RNAi (II) (p=0.9570, n=2): Or47b-GAL4 UAS-CamKI RNAi JF02268 (II) (p=0.1107, n=2); UAS-nj-hp 12.3 RNAi (II): Or47b-GAL4 UAS-nj-hp 12.3 RNAi (II) (p=0.9570, n=2); UAS-CamKI RNAi JF02268 (II): Or47b-GAL4 UAS-CamKI RNAi JF02268 (II) (p<0.4840, n=2)

B) Or47b-GAL4: UAS-nj-hp 12.3 RNAi (II) (p=0.6996, n=2), UAS-CamKI RNAi JF02268 (II) (p=0.9759, n=2), Or47b-GAL4 UAS-nj-hp 12.3 RNAi (II) (p=0.7745, n=2): Or47b-GAL4 UAS-CamKI RNAi JF02268 (II) (p<0.8685, n=2); UAS-nj-hp 12.3 RNAi (II):

Or47b-GAL4 UAS-nj-hp 12.3 RNAi (II) (p=0.9173, n=2); UAS-CamKI RNAi JF02268 (II): Or47b-GAL4 UAS-CamKI RNAi JF02268 (II) (p=0.8438, n=2)

2.4.10.9 Figure 14

A) p300; UAS-met RNAi (II): Or47b-GAL4 UAS-met RNAi (II) (p=0.0016, n=2); Or47b-GAL4 UAS-gce RNAi (II) (p=0.0064, n=2); UAS-gce RNAi (II): Or47b-GAL4 UAS-met RNAi (II) (p=0.0009, n=2); Or47b-GAL4 UAS-gce RNAi (II) (p=0.0029, n=2)

B) H3K27ac; UAS-gce RNAi (II): Or47b-GAL4 UAS-gce RNAi (II) (p=0.6957, n=2)

C) GH w^{1118} comparison: SH w^{1118} 180 whole antennae (p=0.3469, n=6)

D) p300; UAS-met RNAi (II): Or47b-GAL4 UAS-met RNAi (II) (p=0.0008, n=2); Or47b-GAL4 UAS-gce RNAi (II) (p=0.0007, n=2); UAS-gce RNAi (II): Or47b-GAL4 UAS-met RNAi (II) (p=0.0036, n=2); Or47b-GAL4 UAS-gce RNAi (II) (p=0.0029, n=2)

E) H3K27ac; UAS-gce RNAi (II): Or47b-GAL4 UAS-gce RNAi (II) (p=0.6957, n=2)

3. Or47b signaling in social experience-dependent courtship learning in *fru^M* mutant males

3.1 Background

Animals need to modulate their social behaviors constantly to better adapt to the environment. They need to detect and respond to the external and internal stimuli efficiently, timely and appropriately to maximize the fitness. Olfaction plays important roles in regulating social encounters in flies, where they use food and pheromone odors to modulate their aggregation, aggression, and courtship behaviors. In the previous chapter, we described that Or47b, an olfactory pheromone receptor, is involved in the plastic aspect of courtship. Specifically, social experience, conveyed by Or47b detection of the pheromone palmitoleic acid (PA), converges with internal hormone signaling and exerts chromatin-based modulation on the important behavioral regulator *fruitless*(Zhao et al., 2020). Fru^M is a transcription factor that regulates a broad array of neurodevelopmental and axonogenesis genes and is required for the development of sex-specific circuits and male-specific behaviors(Anand et al., 2001; Hall, 1978; Stockinger et al., 2005; Villella et al., 1997). In the peripheral olfactory organ antenna, social experience, via pheromone detection, and *fru^M*, regulate the expression of genes involved in the ion transport, olfactory transmission, and hormone metabolism (unpublished results from our lab). These changes in *fru^M* expression and its downstream neuromodulatory genes are responsible for the increase in Or47b ORN

sensitivity to PA, which confers grouped males with copulation advantage over socially isolated males(Sethi et al., 2019).

Given Or47b's role in regulating increase in male courtship advantages with age and social experience, we predicted that Or47b could also contribute to other plastic aspects of courtship. Courtship conditioning and learning are classical examples of plastic modifications on the innate sex-specific programs. Interestingly, in fru^{M}-mutants, fru^{M} independent courtship learning had been characterized in males with group-housing and olfactory experience. Normally socially isolated fru^{M} mutant males do not court with females or males. However, when they are group housed they learn to court with whomever they had been group housing with, regardless of sex and species. Interestingly, when fru^{M} null males are deprived of olfactory input, this fru^{M}-independent courtship leaning is abolished(Pan & Baker, 2014). It is intriguing what olfactory input are required in regulating fru^{M}-independent courtship learning, and suggest that olfactory pheromone detecting neurons, such as Or47b, can contribute to this social experience dependent courtship learning.

To interrogate this question, we examined the courtship behaviors of fru^{M}, *Or47b* mutant males that are group housed with either virgin females or males of the same genotypes. We quantified the courtship intensity for males that are held in social isolation, and with increasing grouping experience. We reported that Or47b signaling contributes to male-female and male-male courtship learning in fru^{M} null males.

3.2 Results

3.2.1 Or47b signaling contributes to male-male and male-female courtship learning in *fru^M* males

We collected male flies at eclosion and aged in single vials for 4 days prior to the courtship assay. The tester male is then paired with either 7 virgin females (male-female assay) or 7 male flies of the same genotype (male-male assay). Socially isolated wild-type control males court with virgin females, but not males (Fig 15). In comparison, socially isolated *fru^M* null male exhibits little courtship with females or males. Similar to *fru^M* mutants, socially isolated *Or47b- fru^M* double mutant males also are defective in any courtship toward both males and females (Figure 15).

We next tested males in the subsequent days for the effect of group housing on courtship learning. In wild type males, courtship with female increases with grouping experience, and peaks at day 3, when the tester male is 7-day old and at his optimal courtship performance (Figure 16 A). Wild type male does not court or chain with other males (Figure 16 B and C). This is consistent with previous reports where immature or novice wild type males can court flies of both sex upon initial encounter, but give up the male target soon and learn to court with females only(Hall, 1994). In contrast, group housed *fru^M* null males show an increase in courtship, regardless of the target fly's gender (Fig 16 B and C). *fru^M* mutant males grouped with other males learn to court other males intensively (Figure 16 B), and engage in chaining behaviors, where more

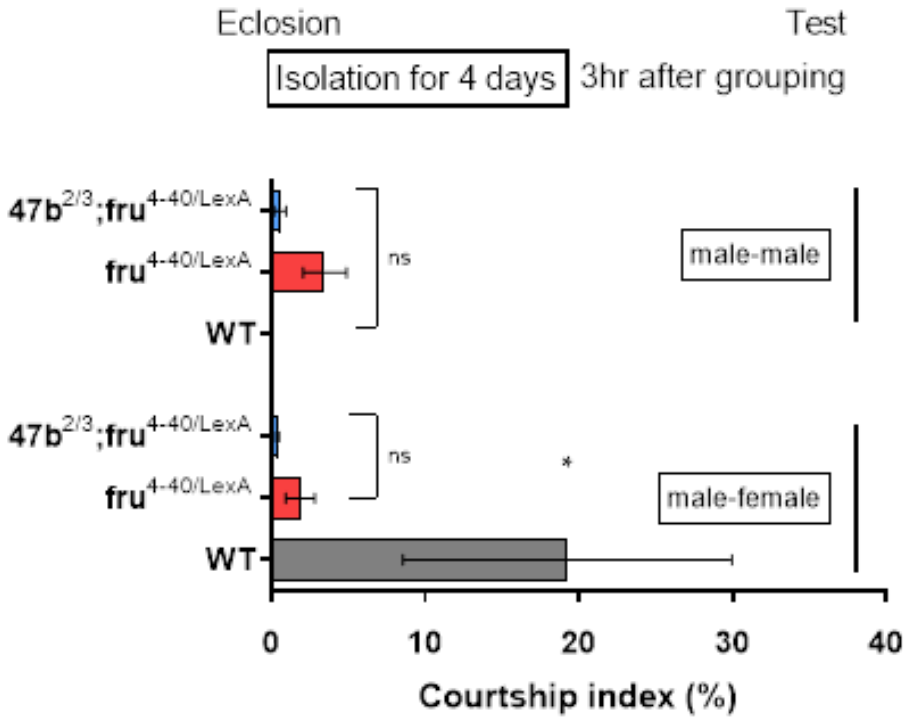

Figure 15: Courtship Index of male courtship in socially isolated males

Males were collected at eclosion, singly housed for 4 days, and transferred to the courtship chamber with 8 males of the same genotype (male-male courtship) or 7 virgin females (male-female courtship). Flies were given 3hr to rehabilitate from any aversive effects of handling prior to the assay. Courtship index (CI) is calculated as the percentage of time the male engages in any step of courtship (chasing, singing, tapping, licking, abdomen bending, attempted copulation, and copulation) during the 10 min observation time. * $p = 0.046$, $t = -2.23$, $df = 12$

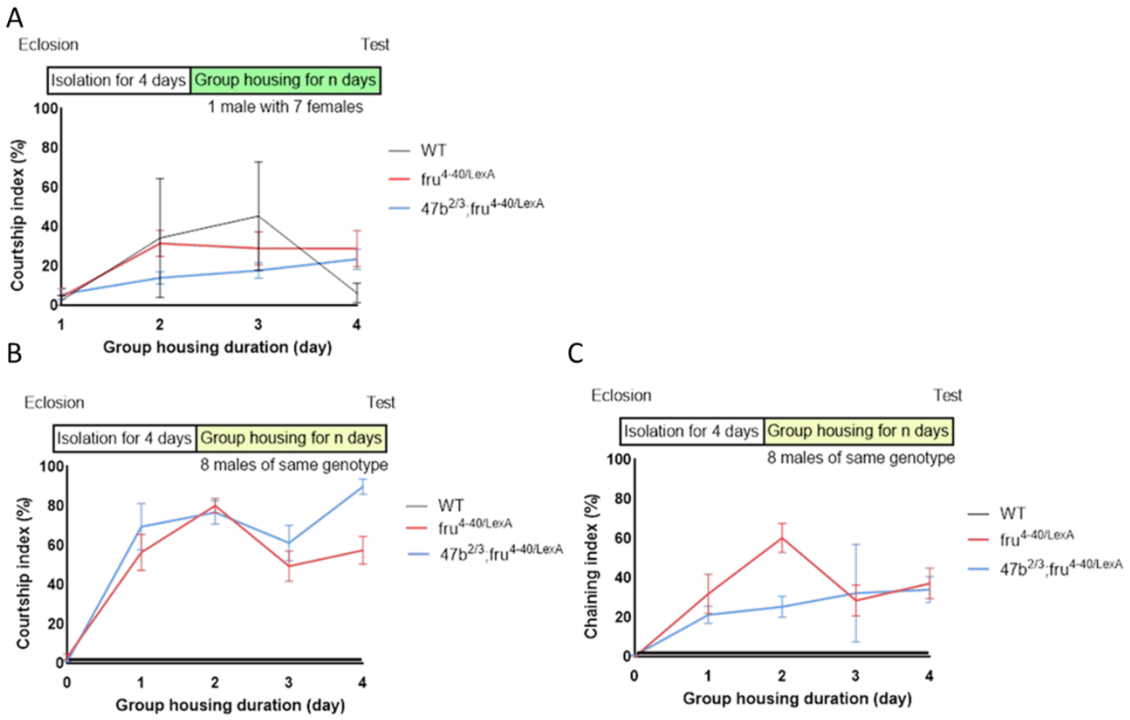

Figure 16: Social experience dependent courtship learning in *Or47b*, *fru*^{M} double mutant males

Tester males were collected at eclosion, single housed for 4 days prior to the group housing with 7 virgin females (A), or 8 males of the same genotype (B and C). The tester males were assayed daily 0, 1, 2, 3, and 4 day after group housing.

than three males are engaged in courtship with one another simultaneously (Figure 12 C).

Compared to *fru*^{M} null male, *Or47b- fru*^{M} double mutant males show impaired male-female courtship learning (Figure 16 A). However, the *Or47b- fru*^{M} double mutant males shows normal courtship learning toward males (Figure 16 B). Interestingly, the male-male chaining behavior, though initially lower compared to the *fru*^{M} null male, also

catches up with grouping experience in *Or47b- fru*^{M} double mutant males (Figure 16 C). These results suggest to us that (1) Or47b contributes to both male-female and male-male courtship learning (especially male-male chaining behavior) (2) Or47b is not the sole pheromone receptor involved in courtship learning toward females in *fru*^{M} null males, as the *fru*^{M}, *Or47b* double mutant male still exhibits increase in courtship with grouping; (3) different pheromones and olfactory signaling are involved in courtship learning toward males and females in *fru*^{M} mutant males, as the *fru*^{M}, *Or47b* double mutant male exhibits relatively normal overall courtship learning toward males, with some impairment of chaining behavior. We hypothesize that Or67d, which detects male-specific pheromone cVA, could be driving male-male courtship learning in *fru*^{M} mutant males.

3.2.2. Dampened male-male chaining in *Or47b, fru*^{M} double mutant males

When *fru*^{M} mutant males are grouped together, they exhibit high level of chaining (Figure 16 C). Specifically, each male chase after, sings toward, taps and licks the target male anterior to him, and is followed by another courting male behind. As a result, a linear, head-to-butt, moving chain of courting males is formed. Although *Or47b, fru*^{M} double mutant males exhibit chaining, it is quantitatively and qualitatively different compared to that of the *fru*^{M} double mutant males. Post-hoc analysis of the observation notes reveals that, whereas the courting chain of *fru*^{M} mutant males is long, with 6-8 males involved, and persists for a long time, the courting chain of *Or47b, fru*^{M} double

mutant males are short (3-4 males), breaks often and start again with different males. The frequent chain breaks are a result of (1) the leading fly in the front of the chain accelerates suddenly, and/or (2) the chasing fly suddenly gives up the chase, walks away with or without orienting toward another target fly. These observations suggest an increase in male rejection behaviors, and more interestingly, a shorter interest or attention span toward the target fly, that is also observed during the male-female courtship in *Or47b*, fru^{M} double mutant males. These observations suggest a possible involvement of Or47b in male-male courtship learning by decreasing the intensity by which males court one another.

In order to analyze these observations quantitatively, the locomotion track, frequency and duration of each courtship step, behavior transition pattern should be quantified in both male-male and male-female courtship assays. In addition, chain length and chaining duration, chain breakage pattern should be quantified for male-male courtship assays. These analyses are beyond accurate and valid manual scoring power, and requires computational, annotated behavior analysis programs.

3.2.3 Qualitative changes in courtship learning in fru^{M}, *Or47b* double mutant male

3.2.3.1 Non courtship-related locomotor activities

General locomotion of the male flies is unaffected in fru^{M} mutant and fru^{M}, *Or47b* double mutant males. During the observation time, the flies actively circle along and explore the edge of the grouping chamber, engage in grooming behaviors, and walk

across the plates. In general, the males are more active compared to the female in explorative behaviors. Interestingly, the overall non courtship-related, explorative locomotor activity seems to be an output of the group, rather than individuals. There is rarely an inactive male, but an inactive chamber. This suggests to us that, the flies influence one another reciprocally- in cases where the male is not active and thus excluded from analysis, all the flies in the same chamber seems low activity, regardless of sex and genotype. In the future, annotated program could be used to create a path-tracking for each individual fly and calculate the general locomotor level, to explore the possibility of the reciprocal influence on activity level.

3.2.3.2 Later steps in courtship ritual

The courtship assays were scored in a genotype-blinded fashion. Post-hoc analysis of the observation notes indicates that, among the courtship behaviors observed and quantified for courtship index (CI) for the fru^{M} and *Or47b*, fru^{M} double mutant males, chasing and courtship song occurs most frequently, constituting most of the courtship behaviors counted by the CI. In Furthermore, there are more head-circling, where the tester male circles arounds the target fly's head during the chase, observed in the *Or47b*, fru^{M} double mutant males. In the male-female courtship assays, the later steps of courtship, including licking, abdomen bending and attempted copulation, were rarely observed in the *Or47b*, fru^{M} double mutant males. Therefore, the increase in courtship with grouping days reflects increase in male potential and motivation to court with the

female target. The chase and singing reflects active pursuit and eliciting, but the male either stops abruptly during the chase and lost the target by turning away, or fails to shorten the distance with the female. As a result, the male did not get into proximity with the female where the later steps of courtship occur. The occasional observation of tapping, licking, and abdomen bending suggest that the male is physically capable of performing these later steps, but the infrequent occurrences raised the possibilities that the *Or47b*, fru^M double mutant males appear less attractive to the females, who therefore rarely slows down to let the male approach. The decrease in attraction could be due to (1). a poorly composed courtship song, and/or (2) change in the male CHC profile.

3.3 Discussion

Courtship behaviors have innate and plastic components. In flies, male-specific behaviors are hardwired in fru^M-dependent circuits, where fru^M is both necessary and sufficient for male courtship behaviors. However, the flies need to modify these hardwired behaviors in response to the changing environment, where plasticity in courtship behaviors allow them to gain better fitness. Olfaction is involved in regulating the plastic aspects of courtship behaviors. Previously, it was reported that Or47b is involved in olfactory experience-dependent increase in courtship advantage in group-reared males(Lin et al., 2016). It is intriguing if Or47b is also involved in other plastic behaviors in courtship. Interestingly, fru^M mutant males acquires courtship learning through grouping experience in olfactory-dependent manner. We hypothesized that

Or47b also regulates courtship learning in group-reared *fru*^{M} mutant males. We found that Or47b is indeed involved in male-female courtship learning and male-male chaining learning, but not required for male-male courtship learning in *fru*^{M} mutant males. This result suggests that additional ORN pathways are needed for male-male courtship learning in *fru*^{M} mutant males. Amongst the known olfactory pheromone receptors, Or67d and Or65a detect the male-specific pheromone cVA and stand out as candidates for mediating male-male courtship learning in *fru*^{M} mutant males.

In addition, we observed dampened courtship learning toward females in *Or47b, fru*^{M} double mutant males. How does the loss of Or47b signaling contribute to this dampened courtship intensity? *Or47b, fru*^{M} double mutant males rarely exhibits the later steps in the courtship ritual such as licking and abdomen bending, which happens in close distance with the female. It is possible that the *Or47b, fru*^{M} double mutant males appear less attractive to the females due to poorly composed courtship song and changes in pheromone profiles. In *fru*^{M} mutant males, the pulse song component of courtship song is missing(Villella et al., 1997; Villella & Hall, 1996). It is possible that the increase in male-female courtship in *fru*^{M} mutant male is due to experience-dependent learning in producing better quality courtship song. It had been well-characterized that visual feedback is deployed for courtship song composition, where the male actively adjusts the pulse song components in response to the change in distance with the females. The male could also use olfactory feedback as indicator for female distance and

quality during singing. Therefore, it is possible that the loss of Or47b-dependent olfactory feedback impairs the male's ability to learn to sing better, and thus unable to proceed to the later steps of courtship. Also, it is possible that the CHC profile alters in *Or47b*, fru^{M} double mutant males. Our preliminary data suggests that social experience changes the CHC profile in fru^{M} mutant male. For example, nC2, 2MeC23, C23:1(9), C23:1(7), 2MeC24 decreases with social experience in fru^{M} mutant males. The behavioral consequences for these pheromones yet need to be identified. To ask if the changes in male CHCs with social experience affects its courtship interaction with females, that determines its courtship intensity, we could group the oenocyte-depleted in fru^{M} male and probe if he could learn. It is intriguing to see if the later steps of courtship, that requires close-distance interactions, increase with grouping experience.

Furthermore, the reminiscent male-female courtship learning in *Or47b*, fru^{M} double mutant males suggest that Or47b is not the sole ORs that are required for the male-female courtship learning. It is possible that Or88a and its detection of non sex-specific methyl ester pheromones are required. In addition, a food odor detector, such as Ir84a and Or7a, might also be required to potentiate the male for the higher rate in initiating the courtship. Lastly, it is possible that an OR with unidentified pheromone ligand is required for male-female courtship learning in *Or47b*, fru^{M} double mutant males, this is discussed in further details in Chapter 4.2.4 Future directions.

And finally, what is the molecular mechanism by which Or47b pathways regulate courtship learning. In the previous chapter, we discussed how Or47b signaling regulates chromatin around *fru* modifying its transcriptional regulation. It is possible that these chromatin-based regulation of fru^M expression also cascades through the courtship circuit to the central brain. Indeed, we find both *fru* and *dsx* chromatin patterns change with social experience. Especially relevant to courtship learning in fru mutants, group housing increases active chromatin marks around dsx in *fru* mutants, which is decreased in *Or47b-*fru^M double mutants. This suggests that Or47b can affect courtship learning by modifying the regulation and function of dsx in the fru mutant male brain.

In conclusion, our current study elucidates the partial role of Or47b in male-female and male-male, courtship learning in fru^M mutant males. It also proposes possible candidate ORs in male-male courtship learning, and lays ground for studying the molecular mechanisms of social experience dependent courtship learning in *Or47b*, fru^M double mutant males.

3.4 Method

3.4.1 Fly Husbandry

Flies were raised on standard fly food (containing yeast, cornmeal, agar, and molasses) at 25°C in a 12-hour light/12-hour dark cycle in cylindrical glass vials (diameter, 24 mm and height, 94 mm). Male tester and target flies were collected within 12 hours of

eclosion and housed into individual vials for 4 days until the courtship assay. Female target flies of D. melanogaster were collected every 8 hours to ensure virginity. Target female flies were housed in 10-15/vial for 4-6 days until the assay. fru4-40 (Villella et al., 1997) and fruLexA combination was used for fruM-null male; Or472 and Or47b3 heterozygotes were used for Or47b null male; Or67d[gal4]/Or67d[gal4] (Villella et al., 1997)homozygotes were used for Or67d null male. All the tester flies were backcrossed for at least three generations with a w+ background, such as +, sp/cyo; +/+ or +, +/+, TM2/TM6B, so it carries wild-type eye color and no balancer. These following stocks were obtained from Bloomington Stock Center: w*; TI{TI}Or47b2 (# 51306), w*; TI{TI}Or47b3 (51307).

3.4.2 Preparation of the grouping chamber

Courtship assay was conducted in a round polystyrene grouping chamber (petri dish with diameter of 35mm, and height of15mm) with a commercial fly food. Fly food were heated and poured into the chamber, cooled down and solidified, allowed for water evaporation and condensation on the cover lid overnight.

3.4.3 Preparation of tester and target flies

On the day of assay, the tester and target flies were combined into a single vial by flipping, and put into sleep by putting the vial in ice for 10 min. Then, the flies were gently poured into the courtship chamber and covered with the transparent chamber lid immediately to prevent fly escape. One target male and 7 virgin females were used for

male-female assay, whereas eight target males of the same genotype were used for male-male assay. All the target males underwent 4 days of social isolation after eclosion, and are of the same age when introduced into the assay. Flies were grouped and assayed in the grouping chamber, where food was supplied for them to eat and potentially court as a communal substrate.

3.4.4 Video recording

The flies were allowed to recover for 3 hours before the first videotaped assay (3hr) and videoed for 10 min. The flies were videoed in the next subsequent days at the same time (1d, 2d, 3d, and 4d). The assays were set up in early afternoon and videotaped between 6-8 PM EST daily to ensure consistent fly locomotor and sexual activity. One to four plates were videotaped simultaneously at 720p, 30 fps. The assays were videotaped using a webcam (Lifecam Cinema # H5D-00013, Microsoft) or phone camera (iPhone 11 ProMax).

3.4.5 Courtship behavior analysis

The recorded videos were played-back to the graders in a genotype-blinded manner. The intensity of courtship behaviors was quantified using the courtship index (CI) and the chaining index (ChI). Courtship index is the percentage of observation time that a tester fly performs any courtship steps toward a target fly, as previously described(Villella et al., 1997). CI was used to score courtship intensity in both male-female assay, and the male-male assay. Chaining index measures the percentage of

observation time that at least three males are engaged in courtship together, as described previously(Villella et al., 1997).

Statistical analysis of CI and ChI were conducted using one-way ANOVA was performed in GraphPad Prism.

4. Conclusions

4.1 Conclusions

With increasing knowledge, "Nature versus Nurture" is no longer a dilemma- "Nature" is coded in the hardwired genetic programs that confers the animal with evolutionary advantages to thrive through day-to-day tasks, where "Nurture" modifies the program in response to changes in environment and experience to maximize the animal's potentials. Animal behaviors consist of both "Nature" and "Nurture" components. During development, animals need to set up the circuits correctly, where axon pathfinding, synaptogenesis and maintenance are critical in ensuring the functional properties of the circuits. These assembly instructions are genetically hardwired and evolutionarily conserved. However, once the circuits are established, animals face the challenges from the ever-changing environment that requires behavioral plasticity. It is intriguing how these circuits change with experience, via the change in gene expression, that ultimately leads to the changes in behaviors. Sensory experiences regulate behaviors, amongst which olfaction is involved in regulation of behaviors such as feeding, aggregation, aggression, and courtship. It is perplexing what is the molecular mechanisms underlying olfactory experience modulation on animal behaviors. To study this question, we use the olfaction driven courtship behaviors in Drosophila males, where the effects of genes, circuits, and behaviors can be interrogated simultaneously. We found that the olfactory receptor Or47b, via detection of the fly

pheromone palmitoleic acid (PA), conveys social presence of other flies and regulates courtship behavioral plasticity.

In Chapter 2, we reported that Or47b signaling works synergistically with hormone signaling to promote male courtship advantages via social-experience dependent regulation on the behavioral regulator the *fruitless*^{M} (*fru*^{M}). We identified chromatin-based changes at the *fruitless* sex-specific promoter in response to olfactory experience of the pheromone PA, which signals via the Or47b neurons through calcium signaling to upregulate *fru*^{M} expression. Furthermore, juvenile hormone signaling could rescue the effects of social isolation, exerting coincident control with calcium signaling to regulated *fru*^{M} expression(Zhao et al., 2020). It is well-established that *fru*^{M} exerts transcriptional regulation on neurodevelopmental and axonogenesis genes and is required for the development of sex-specific circuits and male-specific behaviors (citation, from intro). In the peripheral olfactory organ antenna, social experience, via pheromone detector signaling and *fru*^{M}, regulate the expression of genes involved in the ion transport, olfactory transmission, and neurophysiology (unpublished data from our lab). Changes in *fru*^{M} expression is responsible for the increase in Or47b ORN sensitivity to PA in socially reared males, achieved through the calcium signaling dependent activation and opening of the pickpocket 25 (ppk25) and ppk23 channels, which amplifies the signal and modulate the gain of neuronal output(Ng et al., 2019; Zhang et al., 2020). The social experience-dependent sensitization of Or47b neurons confers group

reared males with courtship advantage over socially isolated males(Sethi et al., 2019). These results suggest that social experience and pheromone dependent regulation of *fru* chromatin and transcription modulates neuronal responses and behavior by reprogramming fru target neuromodulatory gene expression. Future studies will elucidate the biological effects of these neuromodulators on neurophysiology and courtship behaviors.

In chapter 3, we extend our investigation of Or47b function in regulating other plastic aspects of courtship behavior. In Chapter 2, we described Or47b functions in fru^M-dependent increase in courtship advantage in socially reared males. In Chapter 3, we characterized Or47b function in social experience-dependent courtship learning in males lacking fru^M. fru^M is required for innate male courtship behaviors. fru^M mutant males exhibits barely any male-female courtship, lost in competition assay, elevated male-male courtship, and most strikingly, male chaining behaviors(Viiella et al., 1997). When raised in isolation, fru^M mutant males do not show courtship toward males or females. However, socially reared fru^M mutant males learn to court with whomever they were group housed with, regardless of sex and species. This social experience dependent courtship learning in fru^M mutant males is mediated by *doublesex* (*dsx*)-circuits in the central brain and requires olfaction. We found that Or47b is involved in courtship learning toward female targets in fru^M mutant males. In addition, Or47b also functions in male-male chaining learning in fru^M mutant males. In chapter 2, we characterized Or47b

signaling regulates chromatin around *fru* sex specific promoter and modifies its transcriptional regulation. It is possible that Or47b signaling exerts chromatin-based regulations on behavioral regulators, such as *dsx*, contributes to courtship learning in fru^{M} mutant males. Indeed, in the central brain, group reared *Or47b*, fru^{M} double mutant males show decrease in active chromatin marks around *Dsx* promoter compared to fru^{M} mutant males reared under the same social condition (unpublished data from our lab). This suggests that Or47b can affect courtship learning by modifying the regulation and function of *dsx* in the *fru* mutant male brain.

In conclusion, our results highlight Or47b ORN circuits as a plastic circuit, contributing to experience-dependent modulation of courtship behaviors. Social experience conveyed via Or47b detection of the pheromone PA, contributes to both fru^{M}-dependent increase in male courtship advantage, and courtship learning in males lacking fru^{M}. Our findings lay the ground for further exploring the molecular mechanism in olfactory experience-dependent behavioral plasticity.

4.2 Future Directions

4.2.1 Quantitative analysis of courtship defects in *Or47b*, fru^{M} double mutant males

In Chapter 3, we described the qualitative behavioral defects in *Or47b*, fru^{M} double mutant males, revealed by post-hoc analysis of the observation notes. The trends and patterns we observed required further quantification and validation. A

comprehensive and non-biased examination of the assays is required, where the occurrence frequency, occurrence sequence, transition pattern, and duration of each courtship steps should be quantified. However, such analysis is beyond manual scoring. To overcome this limitation, automated computer program should be used. We propose to use behavioral tracking programs for measuring the general locomotion patterns in the future, and a machine vision apparatus, as described previously(Dankert et al., 2009), to generate the behavioral ethogram to examine the changes in courtship ritual component steps in *Or47b*, fru^{M} double mutant males.

The impaired male-female courtship learning in the *Or47b*, fru^{M} double mutant males could be due to the failure to progress to later steps of courtship rituals or a nonproductive behavioral transition back to the earlier steps of courtship. We hypothesize that (1) in *Or47b*, fru^{M} double mutant males, the total courtship behaviors are consist of a higher proportion of chasing and singing, and a lower proportion of tapping, licking and abdomen bending compared to fru^{M} mutant males, (2) The behavioral transition ethogram alters in *Or47b*, fru^{M} double mutant males. It was reported that Fru^{M} positive median bundle neurons in the SOG regulates the sequential performance of the courtship rituals(Manoli et al., 2005). It is possible that the fru^{M} mutant flies learn to perform the rituals in sequential steps with experience, which is defective in flies without olfactory feedback regarding the target fly's position and distance.

We also observe impaired male-male chaining learning in the *Or47b*, *fru*^{M} double mutant males. We observed that fewer number of participants in the chain, and the chain persists for a shorter duration. It is possible that the chain breaks more frequently in *Or47b*, *fru*^{M} double mutant males because they exhibit higher level of rejective behaviors. It had been shown that, *fru*^{M} mutant males exhibits lower wing flicking, a rejective behavior, when courted by other males, compared to wild type males(Jallon, 1984; Svetec & Ferveur, 2005). It is possible that the loss of stimulatory pheromone via Or47b leads to increase in rejective behaviors in the *fru*^{M} mutant male. Therefore, we propose to quantify the length, duration, breakage pattern of the chain, and rejective behaviors of individual flies using automated machine vision, which is capable of tracking multiple flies at the same time.

4.2.2 change in courtship song in in *Or47b*, *fru*^{M} double mutant males

4.2.2.1 courtship song for male-female courtship

We hypothesize that the impaired male-female courtship learning in *Or47b*, *fru*^{M} double mutant males could be attributed to poorly composed courtship song. During courtship, the note-by-note composition of the song reflects the real-time social interaction with the female(Clemens et al., 2018; Coen et al., 2014; Trott et al., 2012). It needs to be carefully composed to contain the species-specific IPI, and adjust according to the female's response to successfully elicit the female to slow down. For example, male produces the loud P_{fast} in distance when he initiates courtship(Zhou et al., 2015),

and shift to the sine song as the females slows down(Li et al., 2018). If the females speed up, males increase pulse note(Pavlou et al., 2016). Male relies on olfactory feedback to adjust the song composition. In *Orco* mutant males with impaired olfaction, a uniform percentage of pulse song is produced regardless of female movement, disabled the males to make dynamic change of the song composition in response to female movement (Li et al., 2018). Furthermore, it had been demonstrated that social experience impacts courtship song quality. Males raised in isolation produce shorter pulse song bursts compared to socially reared males(von Philipsborn et al., 2011). It is possible that the fru^{M} mutant male, who is already defective in sine song production, learns to improve the courtship song with social experience, and such learning is impaired in *Or47b*, fru^{M} double mutant males. We hypothesize 1. fru^{M} mutant male courtship song composition changes with social experience, specifically, adjusted sine song-pulse song production in response to female distance. 2. In fru^{M} mutant *D. melanogaste*r male, the species-specific IPI is lengthened toward that of *D.simuluns* males, which made them less attractive to conspecific females(Crossley et al., 1995; Yoon et al., 2013). We hypothesize that fru^{M} mutant male adjust the IPI component with social experience. 3. The choice between pulse song types is controlled by the dsx^{M}-positive ps1 motor neurons(Ferveur, 2010; Pavković-Lučić & Kekić, 2009), and we had shown chromatin-mediated changes in *Dsx* in socially isolated *Or47b*, fru^{M} double mutant males compared to the fru^{M} mutant males. We hypothesize *dsx* expression changes in these ps1 motor

neurons with social experience, and 4. These experience-dependent changes are dampened in *Or47b*, *fru*^{M} double mutant males.

4.2.2.2 The role of courtship song in male-male chaining

In our analyses, we observed a decrease in male-male chaining in *Or47b*, *fru*^{M} double mutant males. It is intriguing why and how olfactory experience modulates this behavior. Chaining behaviors is characteristic of fru*M* mutant males, and this unique behavioral pattern is not seen in wild type males. However, chaining could be induced in the wild type *D. melanogaster* males by playing monotonous pulse-song playback, even with alterations on the species-specific IPI components(von Philipsborn et al., 2011). This robust chaining is accounted by the activation of ventrolateral protocerebrum projection Neuron 1 (vPN1) neurons and pC1 neurons(Clemens et al., 2018; Shirangi et al., 2013). It is intriguing if the courtship song produced by *fru*^{M} mutant males is capable of eliciting chaining, and if the *fru*^{M} mutant male-specific courtship song is impaired in *Or47b*, *fru*^{M} double mutant male, thus accounting for the decrease in male-male chaining observed.

4.2.3 change in pheromone profiles in *Or47b*, *fru*^{M} double mutant males

It is possible that the observed decrease in male-female courtship learning is due to a less appealing pheromone profile in *Or47b*, *fru*^{M} double mutant males. It had been reported that wild type males court *fru*^{M} mutant males(Cobb & Ferveur, 1996; Lacaille et al., 2007; Thistle et al., 2012), partially because the CHC profile of *fru*^{M} mutant males is

sexually appeal to WT males(Cobb & Ferveur, 1996). Specifically, fru^M mutant males show reduction of 7-Tricosene and increase of 7-pentacosene, making 7-P the principal CH in fru mutant males(Yew et al., 2009). Furthermore, volatile compounds chromatographic profile analysis reveals fru^M mutant males produces a compound that is characteristic of female extract(Savarit et al., 1999). It is possible that fru^M mutant males pheromone profile change with social experience, making them more appealing to the females. However, social-experience dependent change in CHC is not observed in *Or47b*, fru^M double mutant males, thus they show impaired courtship learning toward females. We propose to examine the changes in *Or47b*, fru^M double mutant male pheromone profiles, especially the evolutionarily conserved *ur*-pheromones, such as n-C25, 27-Br and 29-Br, which are known to induce non-species specific sexual excitation(Jallon, 1984).

4.2.4 Additional pheromones and olfactory receptors regulating courtship learning in fru^M mutant males.

Given that fru^M null male fails to learn when the olfactory co-receptor *Orco* is mutated, but not when they are deprived of tactile contact with others flies(Pan & Baker, 2014), we hypothesize that a volatile, fly-derived odor is responsible for the olfaction-dependent courtship learning in fru^M males. In flies, most of the identified fly-derived chemicals with behavioral consequences are found on the surface of the fly cuticle. Those cuticular compounds are mostly hydrocarbons of long carbon chains and low volatility, which reply on gustatory detections by receptors on fly forelegs and

proboscis(Amrein & Thorne, 2005; Chyb, 2004; Jallon, 1984; Svetec & Ferveur, 2005). Volatile pheromones had long been elucidated in promoting courtship(Shorey & Bartell, 1970), but their chemical identities and olfactory receptors are less studied. The only known olfactory pheromone detectors in *D. melanogasters* up to this date are: Or67d, Or65a, Or88a, Or47b, Or7a, and Or69a. As reviewed in Chapter 1.4.3, Or67d and Or65a are responsible for the detection of the male specific pheromone11-*cis*-vaccenyl acetate (cVA)(Datta et al., 2008; Amina Kurtovic et al., 2007; Sébastien Lebreton et al., 2014; Schlief & Wilson, 2007; van der Goes van Naters & Carlson, 2007). Or88a and Or47b are responsible for the detection of non sex-specific pheromones methly laurate (ML), Methyl myristate (MM), and methyl palmitate (MP)(Dweck et al., 2015). In addition, Or47b also detects various fatty acids (myristoleic acid, myristic acid, palmitoleic acid, palmitic acid) and fatty acids methyl esters (methyl myristoleate)(Lin et al., 2016). Or7a is responsible for the detection of male-specific pheromone 9-tricosene (9T) (Lin et al., 2015). Or69aB is responsible for the detection of female-specific pheromone (Z)-4-undecenal (Z4-11Al)(Lebreton et al., 2017).

We hypothesized that courtship learning is accounted by the detection of a non sex-specific, and non species-specific pheromone, that is present in all grouping conditions (conspecific *D. melanogaster* males and females, *D. yakuba* and *D. mojavensis* females) where the fru^{M} null male demonstrated courtship learning(Pan & Baker, 2014). The only known olfactory receptors detect non sex-specific, non-species specific

pheromone are Or47b and Or88. Interestingly, Or47b and Or88a are each individually responsible for one of the dual roles of the ligand ML. Or88a is required for the short-range attraction toward the pheromone, whereas Or47b is required for the pheromone-dependent increase in male courtship advantage(Dweck et al., 2015). Furthermore, Or47b also detects PA, which is also non sex-specific and known to promote courtship advantage in males(Lin et al., 2016). Thus, we first examined the role of Or47b in courtship learning in fru^{M} null males.

Our results suggest that Or47b and its ligands are not the sole olfactory inputs contributing to courtship learning toward females in fru^{M} null males. What are some other possible ligands and detectors? First, it is possible that Or88a is required in addition to Or47b for the full spectrum detection of the methyl pheromones. MM and MP elicits stronger responses in Or88a, and these pheromones might be needed in addition to ML and PA, that is detected by Or47b to convey the social presence of other flies. To examine this possibility, courtship learning in *Or47b*, *Or88a*, fru^{M} triple mutant males should be tested.

Second, it is possible that not only Or47b, but also a food detector, or detectors, are required for courtship learning in fru^{M} null males. Previously it was reported that fru^{M} null males court more actively on food substrate, and this effect is not seen in wild-type males(Viiella et al., 1997). It is possible that food odor(s) is required to potentiate the fru^{M} male for the onset of courtship and subsequent courtship learning. Flies use

food odors and deposited pheromones to attract conspecific flies of both sexes to aggregate on a communal substrate, where they can feed, court, and lay eggs. Olfactory receptors housed in the antennal and palp basiconic sensilla had been characterized for food odor detection. Amongst the food odor detectors, Ir84a and Or7a had been shown involved in aggregation and coupling the food odors to promote courtship. Ir84 signaling via the detection of fruit and plant tissue odor phenylacetic acid increases male courtship intensity(Grosjean et al., 2011). Or7a detects green leaf volatile E2-hexenal at long distance, bringing flies into its detection range of the aggregation pheromone 9T(Lin et al., 2015). It is possible that the fru^{M} null males need to be pre-aroused by food orders for the subsequent steps in courtship.

Third, it is possible that a novel pheromone, signaling via an OR of unidentified pheromone detection capability, is involved. Recently, it had been described that the Degenerin/Epithelial sodium channel (DEG/ENaC) family cation channels pickpocket 23 (ppk23) and pp25k regulate Or47b sensitivity and are required for Or47b-dependent courtship advantage in older males(Ng et al., 2019; Starostina et al., 2012; Zhang et al., 2020).However, abolishing ppk23 and ppk25 does not affect courtship learning in fru^{M} null males (Pan & Baker, 2014). This suggests to us that, the ppk23- and pp25-dependent increase in Or47b sensitivity does not account for olfaction-dependent courtship learning in the in fru^{M} null males. Therefore, it is likely that other ORs might play a more weighted roles in regulating courtship learning in the in fru^{M} null males. Previously, it is

commonly believed that only ORs housed in the trichoid sensila responds to pheromones. This presumption is rooted from the single-unit electrophysiology studies where basiconic sensilla respond to various food odors and CO2, coeloconic sensilla respond to food, water vapor, ammonia, and putresceine, and only trichoid sensilla responds to fly cuticular extracts(Bruyne et al., 2001; Philippe P Laissue & Vosshall, 2008; van der Goes van Naters & Carlson, 2007). The trichoid sensilla houses the Or2a, Or19a, Or19b, Or23a, Or43a, Or47b, Or65a, Or65c, Or67d, Or83c, and Or88a, amongst which 4 ORs had been identified later as pheromone receptors (van der Goes van Naters & Carlson, 2007). However, (1) recent characterizations of an expanded spectrum of volatile pheromone (Farine et al., 2012), as well as (2) the identification of pheromone detection roles of Or7a and Or69a, neither are housed in the trichoid sensilla (Or7a is housed in basiconic ab4A sensilla whereas Or69a is housed in basiconic ab9 sensilla), suggest to us that a more careful screen, probably by electrophysiological study from the axon terminal of different ORNs, is needed to screen for detectors of the newly characterized pheromones. Interestingly, it had been reported that males with feminized DM2, DA3, DA4 glomeruli exhibits male-male courtship (Ferveur et al., 1995). These results suggest that, olfactory information conveyed to these glomeruli are involved regulating courtship behaviors. Thus, olfactory receptor neurons innervating these glomeruli might house pheromone detecting ORs. These glomeruli are innervated by Or22a, Or23a, Or47a and Or2a (the later for DA4l and DA4m), respectively. None of

these ORs had been implicated in pheromone detection. It is intriguing (1) what is the behavioral consequences for these OR mutants, (2) if their known ligands are involved in courtship, and (3) if they have unidentified fly-derived odor ligands.

Meanwhile, a careful reexamination of the CHC compounds is needed. Previously, it is thought that CHCs, which decreases in volatility with increasing hydrocarbon chain length, requires gustatory, but not olfactory detection. For example, the male-specific pheromone 7T had been well-characterized for its detection by GRs. However, another male-specific pheromone of the same carbon chain length, 9T, was recently reported to be detected by an olfactory receptor Or7a. CHC compounds - pheromone detector mapping, especially compounds with higher volatility that could be detected in short range, should be performed. Recently, Solid Phase Micro Extraction (SPME) coupled GC-MS allows the detection of airborne 25-29 carbon long CHCs in cockroaches and termites(Bland et al., 2001; Saïd et al., 2005). This method would allow the detection of potential dispersion of fly CHCs, which escaped from the conventional fly extracts GC-MS analysis. SPME GC-MS study revealed that n-C21, cVA, 7-D, n-C22, 7,11-TD, 9-T, 7-T, 5-T, and n-C23 are detected in airborne phase (Farine et al., 2012). Up to this date, olfactory detectors for cVA and 9T had been mapped, with the remaining volatile pheromone detector identification awaits. Furthermore, recent study identified a new panel of CHC compounds of aldehyde and esters, which might have higher volatility and possible candidates for olfactory detections: Nonanal, dehydromevalonic

lactone, decanal, tridecane, butyrate ester, tetradecane, geranyl acetone, 2-tridecanone, pentadecane, hexadecane, hexadecane, octylether, monoene, heptadecane, ethanolamide ester, octadecane, nonadecane, terpene, ethanolamide ester (Dweck et al., 2015). Another study using headspace collection from fly batches revealed saturated and unsaturated aldehydes, including heptanal, octanal, nonanal, Z-4-nonenal, undecanal, Z-4-undecenal, dodecanal, tridecanal, Z-4-tridecenal, tetradecanal, pentadecanal, hexadecanal, Z-4-hexadecenal, octadecanal, Z-4-octadecenal, Z-4-eicosenal, amongst which Z-4-undecenal was identified as a long-range attractant detected by Or69aB (Lebreton et al., 2017).

In addition, the pheromone could be from non-CHC sources. Flies are attracted to conspecific frass deposits, which contains known species-specific and sex-specific pheromones such as cVA, 7,11-HD, and 7T(Keesey et al., 2016; Mercier et al., 2018). These deposits might contain uncharacterized volatile pheromones from excretory source and wait for mapping to the activating olfactory detectors. Furthermore, pheromones could be made from non-eonocyte sources, and not presented on the cuticle. For example, the female pheromone 2-MeC30 is not reduced in oenocyte-depleted females, indicating it is made elsewhere by uncharacterized pathway (Billeter et al., 2009). To distinguish this possibility, group housing with oenocyte-depleted flies, who does not produce any CHC, could be performed. If CHC is the source contributing

olfaction-dependent courtship learning in fru^M null males, grouping with CHC-free flies should elicit no courtship learning in fru^M null males.

Lastly, it is possible that courtship learning in fru^M null males is acquired based on different pheromones and receptors for male-male courtship and male-female courtship. In this case, we should examine the newly identified, female-specific pheromones for their volatility and possible detection by ORs. Those are: Tricosadiene, Tetracosadiene, (Z,Z)-7,13-pentacosadiene,(Z,Z)-7,11-pentacosadiene,(Z,Z)-7,11-Hexacosadiene, (Z,Z)-9,13-Heptacosadiene, (Z,Z)-7,11-Heptacosadiene,octacosane, (Z,Z)-7,11-octacosadiene, octacosene, and (Z,Z)-7,11-nonacosadiene) (Dweck et al., 2015). These pheromones are 23-29 carbon hydrocarbons, but it is possible that within short range of crowded flies, as in the small grouping chamber, could be detected by ORs. Meanwhile, male-specific pheromones could contribute to male-male courtship learning. The role of Or67d, Or65a, and Or7a, the known olfactory detectors for male-specific pheromones, should be tested. In addition, it had been shown that Or88a also responds to mated female genitalia extracts but not virgin female genital material(van der Goes van Naters & Carlson, 2007), suggesting that Or88a might detect an unidentified male-specific pheromone. We will start the probe by first testing the role of Or67d in courtship learning, especially for male-male courtship, in fru^M null males.

4.3 Concluding remarks

In this study, we elucidate the roles of Or47b ORN circuits in modulating behavioral plasticity in male courtship behaviors. This study provides insights into social experience dependent modulation of behaviors. The knowledge can have broader implications, as social isolation could induce aggression, depression, and changes in brain functions in human.

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