UNIVERSITY OF CALIFORNIA SAN DIEGO

Integration of state and sense: modulation of pheromone perception by hormones and social environment

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

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The Dissertation of Sachin Sethi is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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DEDICATION

To my parents, who worked hard and made sacrifices so that I could have the privilege of choosing my path.

EPIGRAPH

The midday sun slips behind mountains
the Yellow River turns for the sea
trying to see for a thousand miles
I climb one more story

On the Stork Tower by Wang Zhihuan
(translated by Red Pine)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE DISSERTATION

Integration of state and sense: modulation of pheromone perception by hormones and social environment

by

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Animals modify their behaviors in response to changes in their external social environment and internal physiological state to maximize their chances of survival and reproduction. This behavioral flexibility allows animals to have different responses to the same sensory stimulus depending on the needs of the animals and their environment. To understand how such flexibility arises in the nervous system, we studied how social

environment can modulate mating behavior in the vinegar fly, *Drosophila melanogaster.* We found that when mature male flies are raised in an environment of high population density, they enhance their courtship display towards females. A similar enhancement is not observed in immature males, suggesting that there is an interaction between signals arising from the internal state (reproductive maturity) and the external environment (population density) of the fly. We identified the neural substrate of this behavioral modulation as plasticity in a single type of aphrodisiac pheromone sensing neuron that enhances its pheromone sensitivity only in mature males raised in dense housing conditions. Neuronal sensitization is induced by a synergistic interaction between juvenile hormone, a signal for reproductive maturity, and activity-dependent signaling pathways. Further, the adaptive responses of pheromone sensing neurons are sexually dimorphic in nature - they are observed in males but not in females. Sex differences in neuronal plasticity are regulated by Fruitless^{M}, a male-specific transcription factor that regulates pheromone sensitivity in these neurons. Our findings indicate that Fruitless^{M} likely functions as a genomic coincidence detector—integrating internal reproductive maturity and external population density—to modulate mating behavior in a sexually dimorphic manner.

Mechanistic studies into cellular and molecular basis of behavioral flexibility are largely possible due to the availability of genetic tools that allow precise manipulations of neural circuits. In a parallel effort, we expanded the fly toolkit by developing a new method to alter gene expression in a spatially and temporally controlled manner, and demonstrated its utility in mapping and manipulating neuronal circuits underlying odor preference behavior. We anticipate that the development of these tools will further

advance our understanding of how internal state and environmental context modulate behaviors in an integrated fashion.

Chapter I.
Introduction

“Change is the only constant in life.”

— Heraclitus

1.1. Context-dependent modulation of social behaviors

Animals constantly monitor their external environment and their internal physiological state to choose appropriate behaviors for their survival. Thirst induces water seeking, starvation induces foraging, and extreme heat or cold causes animals to seek shelter. Behavioral flexibility—the ability to prioritize particular behaviors in the face of fluctuating internal and external environments—is critical to the reproductive and evolutionary fitness of the individual (Oliveira, 2009). In addition to activating behaviors that promote homeostasis, external and internal factors also heavily influence social behaviors like mating, aggression, parenting and play. The display of social behaviors subjects individuals to various fitness costs, such as the risk of predation, reduction in lifespan, and energetic costs associated with courtship or parenting. It is therefore beneficial that the timing and intensity of social behaviors be tuned to the availability of resources and opportunity in the environment to minimize the cost-to-benefit ratio associated with social interactions.

How does behavioral flexibility manifest itself in the display of social behaviors? Depending on the context, social behaviors can be modulated in three major ways, illustrated here with examples. First, animals may alter the choice of behavior such that the same sensory stimulus elicits different behaviors under differing conditions. For example, on encountering juvenile pups, adult naive male mice usually kill the pups to eliminate the offspring of potential rivals. However after mating, male mice perform parenting behaviors rather than committing infanticide, thereby promoting the survival of their own offspring (Chen and Hong, 2018). Thus,

mating status switches the choice between infanticide and parenting in male mice in response to a juvenile pup stimulus. The second manifestation of behavioral modulation involves the regulation of behavioral intensity, typically measured as bout duration or frequency of a given behavior. An example of this type of modulation can be observed in many species where the presence of a female in the vicinity enhances the level of aggression in males competing for access to the female (Doutrelant et al., 2001; Taylor, 1975; Yuan et al., 2014). The third way in which social behaviors can be altered involves modulation of the timing of behavior display. Internal factors such as circadian rhythms and external factors like temperature and photoperiod can entrain daily or seasonal cycles in mating behavior of many animals. For example, the Syrian hamster is typically a long-day breeder and exposure to short photoperiod suppresses reproductive activity in these animals (Goldman and Elliott, 1988). Thus, seasonal fluctuations in photoperiod can impose constrains on the timing of mating behavior. In summary, animals can show flexibility in their choice, intensity or timing of social behaviors. Notably, these categories are neither exclusive nor exhaustive, but rather represent a framework for the discussion of potential neuronal mechanisms underlying different types of behavioral modulation. In practice, social behaviors can probably be fine-tuned in many subtle ways across different species to suit the needs of the animal. Further, these modifications may be reversible or irreversible, and may be expressed in the same individual or distributed across individuals in a population.

Factors that influence social behaviors can be broadly classified into intrinsic-arising from within the individual, and extrinsic-arising from the environment. Here, we will discuss the nature of these factors and their impact on social behaviors. Intrinsic factors can generate inter-individual behavioral variations in response to the same stimulus even when the subjects are genetically identical and raised in same environment. Intrinsic factors can operate at many levels to modulate social behaviors: changes in hormones or neuromodulators, neuronal activity in brain circuits, subcellular distribution of ion channels and receptors, or chromatin remodeling in individual neurons. Typically, multiple intrinsic factors across different levels of organization

act in concert to promote a behavioral state that may last anywhere from a few seconds to years. This behavioral state is loosely defined as the internal state of the animal. In the realm of social behaviors, longer lasting internal states such as those evoked by changes in reproductive maturity, mating status, sexual satiety or social dominance hierarchy have a profound impact on animal behaviors. For example, in the African cichlid fish, dominant males display territorial and courtship behaviors, whereas subordinate males are reproductively suppressed (Cardoso et al., 2015; Fernald and Hirata, 1977). Additionally, internal states arising from homeostatic disruption can compete with social behaviors to prioritize appropriate behavior for the immediate needs of the individual. For example, sleep deprivation suppresses mating and aggression behavior and promotes sleep in male *Drosophila* (Chen et al., 2017; Kayser et al., 2015). Finally, internal states can also change on the timescale of seconds or minutes, such that even within the same social encounter, animals utilize different strategies as they transition between different states (Calhoun et al., 2019; Chen and Hong, 2018).

In addition to monitoring their internal physiological state, animals gather sensory information from their surroundings and use it to bias their decision to display social behaviors. Extrinsic modulators of social behavior include abiotic components such as temperature, light and food availability; and biotic components such as predators and conspecific individuals. In particular, social context has a strong influence on the behavior of animals that naturally occur in groups. Variations in parameters like group size, male-to-female ratio, territorial intruders, or dominance hierarchy can alter reproductive opportunities and rival competition among conspecific individuals. To maximize their reproductive fitness, animals must exhibit social competence—the ability to monitor social environments and adapt behaviors accordingly (Oliveira, 2009). Social competence is demonstrated in many animals, from insects to mammals. A striking example of the effect of group size is observed on aggregation behavior in the locust, *Locusta migratoria.* In response to crowding, locusts show a density-dependent switch from a solitary phase to a gregarious phase in which they swarm and migrate collectively

(Applebaum and Heifetz, 1999a). Another remarkable example of behavioral flexibility in response to a change in social context is observed in the parenting behavior of the poison frog, *Allobates femoralis*. In this species, typically only males are involved in tadpole transport, a form of parenting behavior. However, in the absence of males, female frogs take over parental duties (Ringler et al., 2015). These examples demonstrate how extrinsic factors, especially social context, can have marked effects on social behaviors.

The distinction between intrinsic and extrinsic factors lies at the point of origin relative to the body of the animal. However, in many cases this distinction is blurred because extrinsic factors can generate long-lasting changes in the internal state of the animal. Such behavioral plasticity is evident for aggression behavior where the outcome of a prior encounter influences the results of future fights; prior winners are more likely and prior losers are less likely to win in future aggressive encounters. This behavioral plasticity—called the winner/loser effect—has been observed in mice (Oyegbile and Marler, 2005), fish (Oliveira et al., 2009), birds (Drummond and Canales, 1998), flies (Trannoy et al., 2016) and other animals. Similarly, prior social stress induced by conditions like chronic social isolation or social defeat can disrupt social behaviors in many mammals (Blanchard et al., 2001). Extrinsic and intrinsic factors can also interact to produce a combined effect on social behaviors. For example, pup retrieval behavior in response to ultrasonic distress calls is enhanced in maternal mice with repeated experience (Marlin et al., 2015). Here, internal reproductive state (mated vs virgin) and external social context (naive vs experienced) cooperate to enhance maternal behavior in a robust manner. The effect of extrinsic factors may also be gated by intrinsic factors, or vice-versa. For example, social isolation enhances aggression behavior in sexually-mature males of many species (Hashikawa et al., 2018), however, juvenile males are not similarly affected as they show low-to-no aggression in the first place. In essence, extrinsic and intrinsic factors can interact in a variety of ways to modulate social behaviors in different contexts.

1.2. Cellular and molecular mechanisms underlying modulation of social behaviors

The expression of behavioral flexibility is dependent on the ability of the brain to produce different motor programs in response to the same sensory stimulus. Flexibility in processing sensory information requires the underlying neuronal circuits to undergo some form of plasticity in their function. In this section, we will review the cellular and molecular mechanisms of neural plasticity underlying social behavioral flexibility, with emphasis on examples from insect behaviors.

Neural circuit plasticity may arise due to structural and biochemical changes (Oliveira, 2009). Depending on the timescale of induction and degree of reversibility, structural changes may arise from different mechanisms, such as morphological restructuring, synaptic plasticity, or changes in expression of surface molecules like receptors or channels. Changes in gross neuronal morphology usually occur over days and signal dramatic shifts in social behavior, such as those seen during progression of life-history stages. For example, as honeybees transition from nursing inside the hive to foraging outside the hive, there is a reduction in the volume of their primary olfactory processing center (antennal lobe) and expansion in a region associated with higher-order associative learning (mushroom bodies) (Withers et al., 1993). Morphological changes can also be observed as animals achieve reproductive maturity, such as the expansion of genital representation in the rat somatosensory cortex during puberty (Lenschow et al., 2016), or the expansion of pheromone associated glomeruli with age in male flies (Kayser et al., 2014). Studies in songbirds have shown that seasonal cycles can also affect brain structure; regions associated with social behaviors undergo expansion during the breeding cycle (De Groof et al., 2009). Although the effects of brain volume changes on functional processing have not been directly tested in most cases, it is generally assumed that the volume of a region is correlated with the ability to perform associated behaviors. Thus, morphological restructuring may be a mechanism to induce long-lasting behavioral changes.

Neuronal plasticity can also be induced due to structural changes in synaptic strength or expression of surface molecules. This form of plasticity can arise from activation of intracellular signaling molecules (like kinases) and activity-induced changes in gene expression (Cardoso et al., 2015). Synaptic plasticity is commonly observed during experience-dependent changes in neuronal circuit function and behavior. This is also true for experience-dependent modulation of social behaviors. For example, in the auditory cortex of maternal mice, coincidence of oxytocin and neural activity in response to pup calls induces a rebalancing of inhibitory and excitatory synapses (Marlin et al., 2015). As a result, experienced mice display enhanced maternal behaviors as compared to inexperienced mice. Another example of social learning is observed in male *Drosophila* where prior rejection by mated females causes males to reduce their courtship behavior in future encounters with mated females (Griffith and Ejima, 2009; Keleman et al., 2012). This behavioral plasticity is mediated by synaptic facilitation in a mushroom body circuit that enhances the sensitivity of rejected males to a mated female pheromone (Zhao et al., 2018). These examples illustrate that synaptic plasticity is a potent mechanism for modulation of social behaviors.

Biochemical changes in molecules such as monoamines, neuropeptides and hormones are known to be involved in modulating social behaviors in many animals. One way in which these chemicals alter function is by opening up critical windows during which activity dependent signaling pathways can induce structural plasticity. For example, oxytocin release after birth in maternal sheep promotes lamb-ewe bonding by inducing plasticity in the olfactory bulb of the mother (Bielsky and Young, 2004). Alternatively, synaptic release of neuromodulators like monoamines and neuropeptides can directly alter circuit function. Among monoamines, the function of dopamine in modulating synaptic strength is well understood. For example, courtship learning in *Drosophila* (described above) is mediated by dopamine-dependent synaptic facilitation in the mushroom body (Keleman et al., 2012; Zhao et al., 2018). Similarly, sexual satiety in male *Drosophila*, the process by which male flies reduce their courtship motivation

after repeated mating, is mediated by a reduction in tonic dopamine release on central courtship promoting neurons (Zhang et al., 2016). Monoamines are also involved in experience-dependent modulation of aggression; the winner/loser effect is mediated by serotonin in lobsters (Kravitz, 2000) and octopamine in crickets (Stevenson and Schildberger, 2013). Other monoamines like tyramine modulate mating behavior in insects and may also enable behavioral flexibility under certain contexts (Huang et al., 2016).

Neuropeptides can alter circuit function by exerting their effects locally (as neuromodulators) or systemically (as peptide hormones). Several neuropeptides have been recognized in vertebrates for their role in contextual modulation of social behaviors. Some examples include oxytocin, in maternal behaviors (Bielsky and Young, 2004); vasopressin, in pair bonding (Bielsky and Young, 2004); and tachykinin, in aggression (Zelikowsky et al., 2018). Similarly, neuropeptides play diverse roles in modulating social behaviors in insects (Nässel and Zandawala, 2019). For example, female post-mating suppression in receptivity is mediated by sex-peptide (SP) in *Drosophila* (Chapman et al., 2003) and head-peptide (HPI) in *Aedes* mosquito (Duvall et al., 2017). In the ponerine ant, *Harpegnathos saltator,* the aggression-induced establishment of dominance hierarchy and eventual transition to pseudo-queen identity is mediated by the downregulation of the corazonin peptide (Gospocic et al., 2017). Neuropeptide F (NPF) is required for modulation of male mating in *Drosophila* in response to exposure with other rival males (Kim et al., 2013).

Among non-peptide hormones, gonadal steroid hormones like testosterone and estrogen are well known for their effects on social behaviors in vertebrates. The effect of steroid hormones on social behaviors is best characterized by the organizational-activational hypothesis (Yang and Shah, 2014). During early life stages, steroid hormones play an organizational role in establishing enduring patterns in gene expression and neural circuit function. During puberty and adulthood, hormones leverage the pre-established patterns to activate behaviors. In light of this hypothesis, the role of steroid hormones in mediating

behavioral flexibility is best captured by their activational effects. For example, testosterone plays an pivotal role in the onset of behaviors like mating and aggression as males of many species transition from juvenile stage to adulthood (Sisk and Foster, 2004). Further, testosterone levels are also elevated following a win in aggressive bouts in fish and mice, suggesting that it may be involved in experience-dependent modulation of social behaviors (Oliveira, 2009). In female mice, cyclical changes in the progesterone hormone regulate attraction to a male pheromone in accordance with the ovulation state (Dey et al., 2015). Similar to vertebrates, non-peptide hormones play a major role in modulating insect social behaviors. In particular, juvenile hormone (JH), a sesquiterpenoid lipid-like hormone secreted by a structure called the corpora allata, is well known for its role in activating reproductive behaviors in many insects (Elekonich and Robinson, 2000; Flatt et al., 2005). JH is involved in the onset of mating behavior in *Drosophila,* caste-identity transitions in ants and bees, and transitions in aggregation state in locusts, among others functions (Elekonich and Robinson, 2000). Although JH receptors seem to be expressed widely in the adult brain (Baumann et al., 2017), the neural mechanisms for hormone induced behavioral plasticity in insects had remained elusive until recently. In a recent study, we found that male *Drosophila* exhibit an age-associated enhancement in their mating drive (Lin et al., 2016). This enhancement is mediated by the sensitization of a class of male pheromone sensing neurons by juvenile hormone. Thus, fluctuations in the level of JH in different contexts can alter social behaviors by inducing neuronal plasticity. However, it is worth noting that even though most studies have focused on changes in circulating hormone levels, hormone-dependent behavioral plasticity can also be induced by other mechanisms (Ball and Balthazart, 2008). In theory, behavioral changes can arise from differences in hormone receptor density, intracellular enzymes that metabolize hormones, hormone binding proteins in circulation, or transcriptional co-regulators of hormone receptor function (Ball and Balthazart, 2008). However, most of these hypotheses are derived from correlative observations and it

remains to be seen if changes in such factors can induce behavioral plasticity in the absence of variation in circulating hormone levels.

Most long-lasting changes in neuronal function are accompanied by changes in gene expression induced by activity-dependent signaling pathways, activation of G-protein coupled receptors or intracellular hormone receptors. Neuronal plasticity is also accompanied by epigenetic changes such as DNA methylation and chromatin modifications. Several studies have shown that genetic and epigenetic changes are involved in inducing flexibility in social behaviors (Cardoso et al., 2015). For example, differential feeding of royal jelly induces development of otherwise genetically identical larvae into queen honeybees (Kucharski et al., 2008). This effect can be artificially induced by suppressing DNA methylation, suggesting that nutrition induced epigenetic changes can alter reproductive behavior in honeybees by silencing and activating relevant genes (Kucharski et al., 2008). Although behavior state transitions are accompanied by brain-wide changes in gene expression, it is often hard to determine which changes are required and which are an outcome of the transition. Further, it has traditionally been challenging to pinpoint how changes in hormones or neuromodulators are integrated with sensory information to induce gene expression and neuronal plasticity in specific neural circuits. These challenges may arise due to the complexity associated with larger brains that makes it difficult to interpret the effect of single cell types on behavior; another limitation may be unavailability of genetic tools to manipulate neural activity and gene expression in spatially and temporally refined manner. Recent advancements in the development of neurogenetic tools in the vinegar fly, *Drosophila melanogaster,* have enabled mechanistic studies in circuit function and behavior (Guo et al., 2019). Compared to vertebrate models, the *Drosophila* brain is simpler numerically and in redundancy. Further, flies display several forms of flexibility in their social behaviors which makes *Drosophila* an ideal model to study the underlying cellular and molecular mechanisms in unprecedented detail.

1.3. Sex differences in social behavioral flexibility

Males and females of many species display qualitative, quantitative, and latent differences in social behaviors (Asahina, 2018; Gegenhuber and Tollkuhn, 2019). Copulatory behavior is completely distinct in the two sexes, thus the difference is qualitative. Qualitatively different behavior is referred to as sexually dimorphic behavior. The two sexes often exhibit different levels of aggression, which constitutes as a quantitative difference. Latent sex difference, such as difference in post-mating behavior, appears only in specific contexts. How do sex differences arise in brain structure and behavior? Based on studies in mice and flies, mammals and insects have highly divergent mechanisms for establishing sex differences in the brain (Yang and Shah, 2014). In male mice, the Y-chromosome associated locus *Sry* induces differentiation of the gonads into testes. In the absence of *Sry,* the gonads differentiate into ovaries by default. The release of gonadal testosterone during development has an organizational effect that generates male-specific patterns in gene expression and neuronal circuitry (Gegenhuber and Tollkuhn, 2019; Yang and Shah, 2014). During adulthood, the gonads release sex-specific hormones such as testosterone and estrogen that activate sex-specific behaviors. Administration of testosterone to neonatal female mice induces male-typical behaviors during adulthood (Yang and Shah, 2014). This and other experiments suggest that gonadal hormones have a deterministic role in establishing sex differences in behavior, while neuronal sex chromosome-linked genetic differences can only modulate behavior to a limited extent in mice. In comparison, the role of sex-specific hormones in insects is unclear (Negri and Pellecchi, 2012). Rather, sex differences in insect brains are thought to be established from genetic differences in sex chromosomes in a cell-autonomous manner (Yang and Shah, 2014). In female flies, presence of two X chromosomes induces expression of Sex-lethal *(Sxl). Sxl* is a splicing factor that targets Transformer (*Tra*), which itself is a splicing factor that generates sex-specific differences in gene expression and neuronal morphology in the fly brain (see section

1.4, (Yang and Shah, 2014)). Thus, compared to mammals, sex-differences in insect behavior are thought to be established at the level of individual neurons.

Differences in susceptibility to contextual modulation in males and females can give rise to latent sex differences in social behaviors. Latent differences may be linked to the ethological needs of males and females (Asahina, 2018). For example, mating increases the sensitivity of several chemosensory pathways that mediate egg-laying preference in female flies (Asahina, 2018). Where do sex differences in susceptibility arise? One possibility is that neuronal plasticity is initiated in neurons that are found only in male or female brains. Notably however, most differences in mammalian and insect brains are quantitative rather than qualitative in nature (Gegenhuber and Tollkuhn, 2019; Yu et al., 2010). Another possibility is that sex-related differences in gene expression may alter the physiological properties of neuronal circuits. Finally, differences can arise from fluctuations in sex-specific hormones like those seen during ovulation cycles in females, or testosterone levels during aggressive encounters among males (Oliveira, 2009). Even though insects do not have sex-specific hormones, juvenile hormone can have sex-specific effects on reproductive physiology and behaviors. For example, juvenile hormone induces an expansion in gut after mating in females but not in males (Reiff et al., 2015). Such sex-specific effects may arise due to an interaction between juvenile hormone signaling and sex-determination pathways in neurons. However, in the absence of a neural substrate for the activational effect of juvenile hormone, it was not possible to test this hypothesis. The identification of *Drosophila* pheromone sensing neurons as a target of juvenile hormone function enables us to test if its effect is different in males and females (Lin et al., 2016).

1.4. Social behaviors in *Drosophila melanogaster*

To understand the cellular and molecular basis for fixed and flexible social behaviors, it is essential to manipulate the underlying genes and circuits in a spatially and temporally

controlled manner. In this regard, *Drosophila melanogaster* has emerged as a powerful model organism to study social behaviors such as mating, aggression and aggregation. Mating in *Drosophila* consists of a series of sophisticated rituals (Yamamoto and Koganezawa, 2013). On encountering female flies, male flies engage in chasing, tapping and licking like behaviors to evaluate the suitability of a potential mate using visual and chemosensory information. When the decision to mate has been made, male flies perform a courtship song to entice females. If the female flies are receptive to the advancing male, they slow down and allow the males to proceed with copulation. Alternatively, if females are unreceptive, they perform behaviors such as wing flicking, kicking or curling to signal their unwillingness to the male (Aranha and Vasconcelos, 2018). Similarly, aggression behavior in flies also consists of series of steps, starting from wing threat display and chasing, and evolving into lunging and boxing-like behaviors as the fight escalates (Chen et al., 2002). Recent advancements in high-resolution behavioral tracking have enabled the identification of neural circuits involved in discrete steps of courtship and aggression behaviors (Duistermars et al., 2018; Ribeiro et al., 2018).

The utility of *Drosophila melanogaster* as model for neurogenetics is exemplified by the discovery of the *fruitless* gene and its role in mediating many aspects of fly social behavior (Baker et al., 2001; Yamamoto and Koganezawa, 2013). *fruitless* transcripts that arise from a specific promoter (called the P1 promoter) are spliced in a sexually dimorphic manner leading to formation of a functional protein in males but not in females (Demir and Dickson, 2005; Ryner et al., 1996). The male specific isoform of the Fruitless protein (called Fru^M) is required for most aspects of male courtship behavior, and ectopic expression in females is sufficient to induce male-like behaviors (Demir and Dickson, 2005). Fru^M protein functions as a transcriptional regulator during development to promote male-fate in individual neurons (Ito et al., 2012). The functional outcome of Fru^M loss-of-function in single neurons can be observed as changes in morphological and physiological properties (Kohatsu and Yamamoto, 2015; Yamamoto and

Koganezawa, 2013). Fru^M functions during development to regulate cell death (Kimura et al., 2005), dendritic morphology (Ito et al., 2012) and synaptic connectivity (Kohl et al., 2013). In this context, Fru^M function is comparable to the organizational function of steroid hormones during mammalian development. Recent studies have proposed a role for Fru^M in the regulation of courtship behavior by social experience during adulthood (Yamamoto and Kohatsu, 2017). However, it is not clear whether this regulation explicitly requires Fru^M expression in adult neurons or is an outcome of the cellular fate specified by Fru^M during development. Thus, whether Fru^M can have an activational role in adulthood is not known.

Over the last 20 years, remarkable advances have been made in the identification of neural circuits directing social behaviors in *Drosophila.* Much of this progress is due to the generation of transgenic promoter trap lines with insertion of binary system transcription factors such as GAL4 into of the fru-P1 promoter (Manoli et al., 2005; Stockinger et al., 2005). This technology allowed the mapping and manipulation of Fru^M expressing neurons in the adult brain, many of which have since been linked to their function in executing social behaviors (Auer and Benton, 2016). For instance, this approach enabled the identification of peripheral pheromone sensing neurons that enhance male-female courtship (Thistle et al., 2012) and male-male aggression (Wang and Anderson, 2010). Fru^M expression has also been used as an entry point to identify central brain neurons that integrate multisensory input to set courtship motivation (Kohatsu et al., 2011; von Philipsborn et al., 2011), and descending neurons that trigger wing extension for courtship song production (von Philipsborn et al., 2011). It is noteworthy that while some Fru^M-expressing neurons appear sexually dimorphic in their morphology at the light microscopy level, majority of the neurons exhibit no obvious sex differences (Yu et al., 2010). This observation suggests that a large portion of neural circuitry underlying sexually dimorphic behaviors are shared by both males and females. What then is the function of Fru^M expression in these neurons? One possibility is that Fru^M expression may lead to sex-differences in physiological properties, such as differential expression of receptors

and ion-channels in males and females. However, this idea remains to the tested in the adult nervous system.

Among the neural circuits involved in fly social behaviors, the role of chemosensory neurons is understood is great detail (Auer and Benton, 2016). Chemosensory neurons on the antenna and the foreleg detect volatile and contact pheromones respectively. Previous studies have identified the identity of the pheromones detected by these neurons and the effect of neuronal activation on fly behaviors. For example, ppk25 expressing neurons on the male foreleg detect the female specific pheromone 7,11-HD and promote courtship initiation (Thistle et al., 2012). Other gustatory neurons that express the Gr32a receptor detect the male specific pheromone 7-tricosene and suppress male-male courtship, enhance male-male aggression and suppress interspecies male-female courtship (Auer and Benton, 2016; Wang et al., 2011). Among the 50 types of olfactory receptor neurons, four types detect volatile fly pheromones (van der Goes van Naters and Carlson, 2007). Among them, two types - Or47b neurons and Or67d neurons express Fru^{M} and have enlarged glomeruli in males compared to females (Stockinger et al., 2005). Or67d neurons detect the male pheromone cVA and promote male-male aggression, enhance female receptivity, and suppress male courtship towards mated females (Auer and Benton, 2016). Or47b neurons detect fatty acid ligands such as palmitoleic acid that are present on both males and females and enhance male courtship (Dweck et al., 2015; Lin et al., 2016). Interestingly, pheromone sensory neurons appear to be hotspots of neuronal plasticity. For example, juvenile hormone mediated enhancement in Or47b neuronal sensitivity elevates courtship motivation in mature males (Lin et al., 2016). Similarly, mating induced suppression in Or67d neuronal output reduces attraction towards males in mated females (Lebreton et al., 2014). In general, plasticity in peripheral pheromone circuits may be a mechanism to modulate behaviors only in the context associated with a specific pheromone channel, thus ensuring contextual selectivity.

As expected of animals that naturally exist in groups, flies display several forms of behavioral plasticity in response intrinsic and extrinsic factors. Male courtship is modulated by reproductive maturity (Lin et al., 2016), sexual satiety (Zhang et al., 2016), circadian rhythm (Fujii et al., 2007), prior courtship experience (Keleman et al., 2012), food availability (Grosjean et al., 2011), housing density (Dankert et al., 2009) among other factors. Similarly, male aggression is modulated by housing density (Wang et al., 2008), food availability (Lim et al., 2014), prior experience (Trannoy et al., 2016) and female presence (Yuan et al., 2014). Several studies have identified the cellular and molecular mechanisms underlying such behavioral plasticity. For example, compared to virgins, mated females participate in courtship behavior at much lower levels. This mating-dependent reduction in receptivity is mediated by the release of sex-peptide during copulation from the male seminal fluid into the female reproductive tract (Chapman et al., 2003). Sex-peptide receptor activation triggers a long-lasting change in neuronal activity in ascending neurons that regulate female receptivity (Feng et al., 2014; Yapici et al., 2008). These findings, among others, illustrate the value of *Drosophila* as a model for understanding the mechanisms of behavioral plasticity. The development of new anatomical and physiological tools has now made it possible to pinpoint the molecular and cellular basis of other forms of flexibility in fly social behaviors (Guo et al., 2019). Future studies will also determine how neuronal activity and neuromodulation are coordinated with epigenomic modifications to bring about enduring changes in social behaviors.

1.5. Preview of the dissertation

In chapter II, we describe how extrinsic and intrinsic factors interact to induce neuronal plasticity and modulate male courtship behavior in *Drosophila*. Specifically, we explored how courtship behavior is differentially modulated by population density in young and mature males. Raising mature males in high density environments enhances their courtship behavior. However, population density has no effect on younger males. We show that plasticity in specific

pheromone sensing neurons is required for this behavioral flexibility in mature males. Further, using genetic tools, we identified the underlying molecular mechanism for neuronal plasticity which involves interactions between juvenile hormone signaling and activity induced gene expression. Finally, we discuss how these molecular pathways target sex-specific genomic loci, leading to induction of plasticity in males but not in females. In summary, we discovered a mechanism by which interactions between hormonal state and sensory input induce plasticity in gene expression and circuit function to facilitate behavioral flexibility in a sex-specific manner.

Mechanistic studies into cellular and molecular basis of behavioral flexibility are largely possible due to the availability of genetic tools that allow precise manipulations of neural circuits. In chapter III, we describe the development of new genetic tools for controlling gene expression in *Drosophila.* Conventional tools are largely based on regulation of gene expression at the transcriptional level. In this study, we expanded the fly toolkit by developing an orthogonal method for regulating gene expression at the post-translational level using a small molecule. Our approach can be combined with existing reagents to further refine spatial and temporal patterns of gene expression. We demonstrate its utility by manipulating neuronal activity and odor preference behavior. We expect the development of these and other tools will further advance our understanding of how internal state and environmental context modulate behaviors in an integrated fashion.

Chapter II.
Social context enhances hormonal modulation of pheromone detection in *Drosophila*

"Old age and treachery will always beat youth and exuberance."

— David Mamet

2.1. Abstract

Animals can dynamically regulate their social behavior in accordance with environmental cues and internal physiological states. To understand the cellular and molecular underpinnings of such behavioral flexibility, we focus on how age and social context influence courtship behavior in *Drosophila melanogaster*. We find that the courtship-promoting Or47b olfactory receptor neurons (ORNs) adapt their pheromone sensitivity to housing density in an age- and sex-dependent manner. Specifically, group housing elevates the pheromone response of Or47b ORNs in 7-day old males but not in immature 2-day old males. This change in pheromone response in turn impacts courtship behavior. Interestingly, the group housing effect can be mimicked by chronically exposing single-housed males to an Or47b ligand. Results from our experiments suggest that group housing elevates Ca^{2+} levels, which trigger CaMKI-mediated activation of the histone-acetyl transferase CBP. This in turn enhances the efficacy of juvenile hormone, an age-related regulator of reproductive maturation in flies. Downstream of juvenile hormone signaling, we find that the male-specific Fruitless isoform (Fru^M) is required for pheromone response plasticity. Therefore in Or47b ORNs, Fru^M likely functions as a genomic coincidence detector—integrating internal reproductive maturity and external population density—to modulate courtship behavior.

2.2. Introduction

Flexibility of social behavior in changing environmental and physiological states is critical to reproductive success and evolutionary fitness. Recent studies have advanced our understanding of how individual contextual inputs—such as mating status (Yapici et al., 2008), hormonal state (Dey et al., 2015), population density (Wang et al., 2008; Yang et al., 2017; Zelikowsky et al., 2018), and prior experience (Keleman et al., 2012)—modulate the display of social behaviors. The influence of environmental cues on social behavior is highly dependent on the physiological state of the animal. However, the cellular and molecular mechanisms that underlie the integration of extrinsic and intrinsic inputs remain poorly understood.

Changes in social environment, such as fluctuations in population density, have a profound impact on mating and aggression behaviors in group-living animals (Cardoso et al., 2015). Recent studies have identified key neural circuits and molecules involved in the modulation of aggression by social isolation (Liu et al., 2011; Wang et al., 2008; Yang et al., 2017; Zelikowsky et al., 2018). Social context is also known to influence many aspects of male courtship in *Drosophila* (Bretman et al., 2009; Dankert et al., 2009; Hosken et al., 2019; Kim et al., 2012; Li et al., 2018; Marie-Orleach et al., 2019; McRobert and Tompkins, 1988). For example, prior exposure to other male flies modulates the courtship display and enhances the duration of copulation (Bretman et al., 2009; Dankert et al., 2009; Marie-Orleach et al., 2019; McRobert and Tompkins, 1988). These adaptations allow male flies to adjust their courtship intensity in accordance with rival competition and reproductive opportunity.

To address the question of how external social cues are integrated with internal state of the organism, we focused on the courtship-promoting Or47b ORNs to investigate the combined effect of age and housing condition on courtship behavior in *Drosophila.* Although it is not clear where and how neuronal plasticity in the nervous system enables flexible social behavior, ORNs are often required for the contextual modulation of social behaviors (Bentzur et al., 2018;

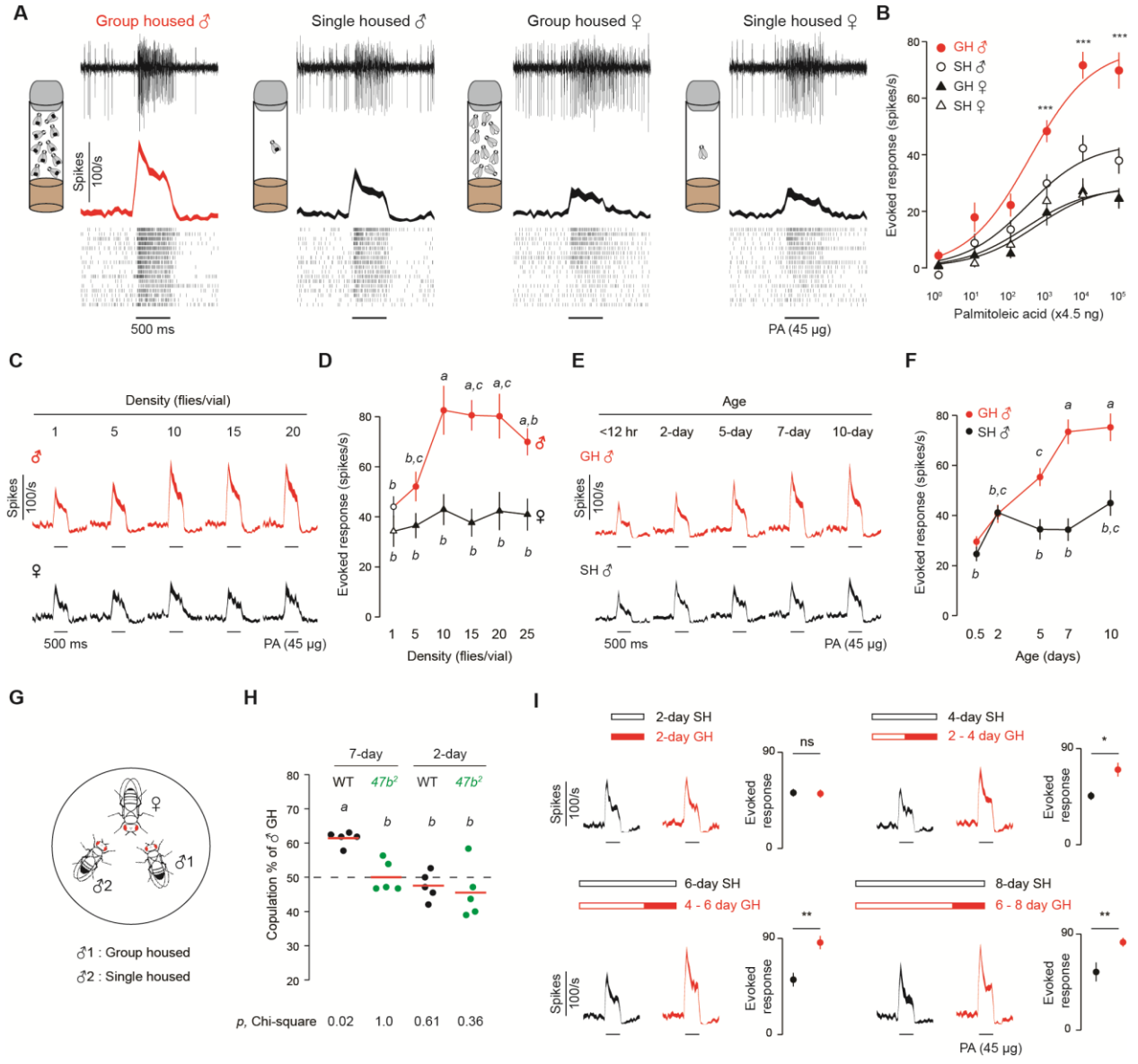
Keleman et al., 2012; Liu et al., 2011). There are several lines of evidence suggesting that plasticity in Or47b ORNs may underlie contextual modulation of courtship behavior in *Drosophila*. Among the ~50 ORN types in the adult olfactory system, Or47b neurons exhibit uncommon anatomical and physiological plasticity (Kayser et al., 2014; Lin et al., 2016; Yamamoto and Kohatsu, 2017). Anatomically, the plasticity lies in the age-dependent size increase in the VA1lm glomerulus, which is innervated by Or47b ORNs (Kayser et al., 2014). Physiologically, Or47b ORN responses increase with age in males, leading to higher pheromone sensitivity in older individuals at their fertility peak (Lin et al., 2016). The age-dependent increase in Or47b response is mediated by juvenile hormone, a pleiotropic hormone that also regulates the reproductive maturity in adult *Drosophila* (Flatt et al., 2005; Lin et al., 2016). Notably, this anatomical and physiological plasticity occurs only in males but not females (Lin et al., 2016; Stockinger et al., 2005). This sex-specific plasticity may arise from the expression of Fru^M, a male-specific isoform of the *fruitless* transcription factor, in Or47b ORNs (Stockinger et al., 2005). Fru^M is required for many aspects of male courtship behavior (Yamamoto and Koganezawa, 2013) and has been implicated in regulating courtship behavior by group housing (Kohatsu and Yamamoto, 2015; Yamamoto and Kohatsu, 2017). Finally, because Or47b ORNs respond to fly pheromones (Dweck et al., 2015; Lin et al., 2016) their neural activity level may encode population density. Given that Or47b ORNs display age-related plasticity and may report population density, it is likely that Or47b ORNs are a common neural substrate regulated by both social context and reproductive maturity such that male courtship behavior is modulated in coordination with these contextual cues.

In this study, we identify a neural substrate which integrates social context and reproductive maturity to regulate pheromone detection. We find that group housing elevates Or47b pheromone responses in mature males, but not in females or young males. Remarkably, the effect of group housing can be mimicked by raising single-housed males in the presence of

palmitoleic acid, a pheromone ligand for Or47b ORNs. Mechanistically, group housing increases intracellular Ca^{2+} levels in Or47b neurons to activate Ca^{2+}/calmodulin-dependent protein kinase I *(CaMKI) and* CREB binding protein (dCBP), creating a permissive intracellular environment which enhances the efficacy of juvenile hormone. Furthermore, we find that Fru^{M} expression levels determine Or47b response and are regulated by juvenile hormone signaling. Our study describes a molecular mechanism by which internal state (age/sexual maturity) and social context (population density) synergistically regulate the expression of the male-specific transcription factor Fru^{M}. This mechanism allows male flies to adjust their sensitivity to aphrodisiac pheromones in accordance with their reproductive maturity and social environment. This flexibility in courtship behavior is likely to enhance the evolutionary fitness of the animal.

Figure 2.1. Group housing enhances pheromone response of Or47b ORNs in mature males.

(A, B) Group housing enhances odor response in Or47b neurons in 7-day old male flies but not in 7-day old female flies. Spike activity of Or47b neurons in at4 sensillum was measured by single-sensillum recording, in which palmitoleic acid (PA) was used as a stimulus. GH: flies were raised in groups of 15 per vial. SH: flies were raised individually with each vial containing one fly. (**A**) Sample traces (top panel), peri-stimulus time histogram (PSTH, middle panel) and spike rasters (bottom panel) of Or47b neurons in WT males and females. (**B**) Dosage-response curves comparing male and female flies under group housing (GH) and single housing (SH) conditions. ***, $p<0.001$, denotes significant difference for a given odor concentration, determined by two-way ANOVA followed by Tukey's post-hoc test. n = 17 flies per condition. Significant differences ($p<0.05$) are denoted by different letters, determined by two-way ANOVA followed by Tukey's post-hoc test (D, F, H). Error bars indicate SEM (B-I). **(C, D)** Effect of housing density on Or47b response. WT flies were raised for 7 days either in isolation or in same-sex groups of varying size from 5 to 25 in increments of 5. Odor stimulation: 45 μg PA. Two-way ANOVA indicated a significant effect of sex ($F=54.13$, $p<0.001$) and housing density ($F=4.01$, $p=0.002$). n = 16 for males, n = 12 for females. **(E, F)** Effect of age and housing condition on Or47b response in WT male flies. Males were raised in groups of 15 or in isolation from eclosion to different ages. Odor stimulation: 45 μg PA. Two-way ANOVA indicated a significant interaction between housing condition and age. $F=3.03$, $p=0.019$ n = 20, for each condition. **(G)** Courtship competition assay with one virgin female and two males. Genotypes and housing conditions of male flies and copulation percentages of ♂1 are shown in (**H**). Chi-square test was used to determine if copulation percentage was different from chance. *p*-values are indicated on the figure. **(H)** Group housing increases the copulation rate of 7-day but not 2-day old WT males. This effect is absent in *Or47b* mutant males. Bars indicate mean copulation percentage. A single dot represents one experiment of 12-26 chambers each containing one GH male, one SH male and one female. n = 5 experiments per condition. Dashed line indicates chance level. Two-way ANOVA indicated a significant effect of age ($F=16.51$, $p=0.0009$) and genotype ($F=8.4$, $p=0.010$). **(I)** Two days of group housing enhances Or47b odor response in 4, 6 and 8 day old but not 2-day old male flies. Spike responses were measured at the end of the two day group housing period. 4, 6 and 8 day old flies were single housed prior to group housing. Odor stimulation: 45 μg PA. Two-tailed unpaired t-test with Bonferroni correction, *, $p<0.012$, **, $p<0.002$; n=15 flies per condition.


A
Group housed ♂
Single housed ♂
Group housed ♀
Single housed ♀
Spikes
100/s
500 ms
PA (45 µg)
B
GH ♂
SH ♂
GH ♀
SH ♀
Evoked response (spikes/s)
Palmitoleic acid (x4.5 ng)
C
Density (flies/vial)
1
5
10
15
20
Spikes
100/s
500 ms
PA (45 µg)
D
Evoked response (spikes/s)
Density (flies/vial)
E
Age
<12 hr
2-day
5-day
7-day
10-day
GH ♂
SH ♂
Spikes
100/s
500 ms
PA (45 µg)
F
GH ♂
SH ♂
Evoked response (spikes/s)
Age (days)
G
♂1 : Group housed
♂2 : Single housed
H
7-day
2-day
WT
47b²
Copulation % of ♂ GH
p, Chi-square
0.02
1.0
0.61
0.36
I
2-day SH
2-day GH
4-day SH
2 - 4 day GH
6-day SH
4 - 6 day GH
8-day SH
6 - 8 day GH
ns
Spikes
100/s
Evoked response
PA (45 µg)

2.3. Results

Group housing enhances pheromone response of Or47b neurons in mature males

We first investigated the effect of housing density on Or47b response. Flies were raised for 7 days either in same-sex groups of 15 (group housing, GH) or in isolation (single housing, SH). We measured odor-evoked spike responses of individual Or47b ORNs to palmitoleic acid, an aphrodisiac pheromone that stimulates male courtship (Lin et al., 2016). We found that group housing increased the pheromone sensitivity of Or47b ORNs in males but not females (**Figures 2.1A and 2.1B; Figure 2S.1**). By varying the group size from 5 to 25 in increments of 5, we found that a minimum group size of 10 was required to induce a higher response in males when compared to single-housed males (**Figures 2.1C and 2.1D; Figure 2S.2A**). In contrast, none of the examined housing conditions altered Or47b responses in females (**Figures 2.1C and 2.1D; Figure 2S.2A**). In addition to Or47b ORNs, we examined Or67d and Or88a ORNs that also respond to fly pheromones (van der Goes van Naters and Carlson, 2007). Responses of Or67d and Or88a neurons did not increase under similar housing conditions, suggesting that Or47b neurons are unique among pheromone-sensing ORNs in their adaptive response to housing density (**Figure 2S.3**).

We next investigated the effect of group housing on males of different ages. Adult males were raised in groups of 15 or in isolation from eclosion up to 10 days of age. In males aged 5–10 days but not younger, we observed that group-housing increased their Or47b responses when compared to single-housed controls (**Figures 2.1E and 2.1F**). This result is consistent with our previous study indicating that Or47b responses are higher in males at the age of 7 days compared to those at 2 days (Lin et al., 2016). In *Drosophila melanogaster* males, the peak of both courtship activity and fertility is reached around 7 days post-eclosion (Kvelland, 1965; Long et al., 1980), an age we therefore refer to as mature.

Does group housing-induced sensitization of Or47b neurons translate into a higher mating drive? Using an established behavioral assay that measures the differential courtship motivation between two males (**Figure 2.1G**; (Lin et al., 2016)), we found that group-housed males had a higher copulation rate than single-housed males at the age of 7 days but not 2 days (**Figure 2.1H**), consistent with the observation that group housing-induced Or47b sensitization occurred in 7-day but not 2-day old males (**Figure 2.1F; Figure 2S.2B**). These results suggest that the effect of group housing on courtship in males is mediated by changes in Or47b pheromone responses. Indeed, we found that group housing did not affect the copulation rate of *Or47b* mutant males at either age (**Figure 2.1H**).

This age-dependent disparity in group-housing effect can in principle arise from differences in the duration of social experience or in the age of flies when group housing takes place. To distinguish between these two possibilities, we first investigated the impact of different group-housing durations. Flies that were group housed for 7 days had higher Or47b responses when compared to flies group housed for only one day, but not for two or more days (**Figure 2S.2C**). This result demonstrates that two days of group housing is sufficient to enhance Or47b response.

Next, to determine the importance of age when social encounter commences, we group housed males of different ages for a fixed duration of 2 days. We found that two days of group housing was sufficient to enhance Or47b responses in 4-day, 6-day and 8-day old males but not in 2-day old males (**Figure 2.1I**). This observation argues for a minimum age at which group housing can sensitize Or47b ORNs. Collectively, these results show that male Or47b ORNs can integrate age and population density information to adapt their pheromone responses. Specifically, group housing enhances Or47b pheromone responses in mature males, allowing for an age-dependent modulation of courtship behavior by prior exposure to other males.

Group-housing effect can be mimicked by chronic exposure to an Or47b ligand

What is the input signal that leads to the adaptive Or47b responses to group housing? Given that Or47b is a pheromone receptor, group-housed flies are likely exposed to higher levels of Or47b ligands such as palmitoleic acid (PA) than single-housed flies. We therefore hypothesized that the group-housing effect is mediated by chronic exposure to Or47b ligands, which trigger activity-dependent plasticity in Or47b ORNs. In support of our hypothesis, single-housed males raised in vials perfumed with palmitoleic acid had higher Or47b responses compared to control males (**Figures 2.2A and 2.2B**). The effect of PA exposure is also age-dependent; it manifested in 7-day old but not 2-day old males (**Figures 2.2C and 2.2D**). This result suggests that in mature males, PA-evoked activity initiates a positive feedback mechanism, which enhances Or47b pheromone sensitivity.

Next, we determined if chronic exposure to PA is sufficient to enhance courtship behavior in single-housed males. In courtship competition assays using wildtype males, we found that flies exposed to PA had a higher copulation rate than control males (**Figure 2.2E**). In contrast, chronic PA exposure did not alter the copulation rate of *Or47b* mutants (**Figure 2.2E**), suggesting that Or47b sensitization is required for the courtship enhancement. Additionally, we found that the effect of PA exposure was sexually dimorphic as with group housing; raising single-housed females in PA-perfumed vials did not result in enhanced Or47b responses (**Figure 2S.4A**). Notably, female and male flies carry similar amounts of PA and other Or47b ligands (Dweck et al., 2015; Lin et al., 2016), suggesting that the dimorphic Or47b plasticity is likely mediated by the genetic differences between the two sexes instead of differing PA levels in the environment. In summary, this series of PA perfuming experiments argues that a heightened level of Or47b ligands in group-housing condition is sufficient to sensitize Or47b ORNs in mature males.

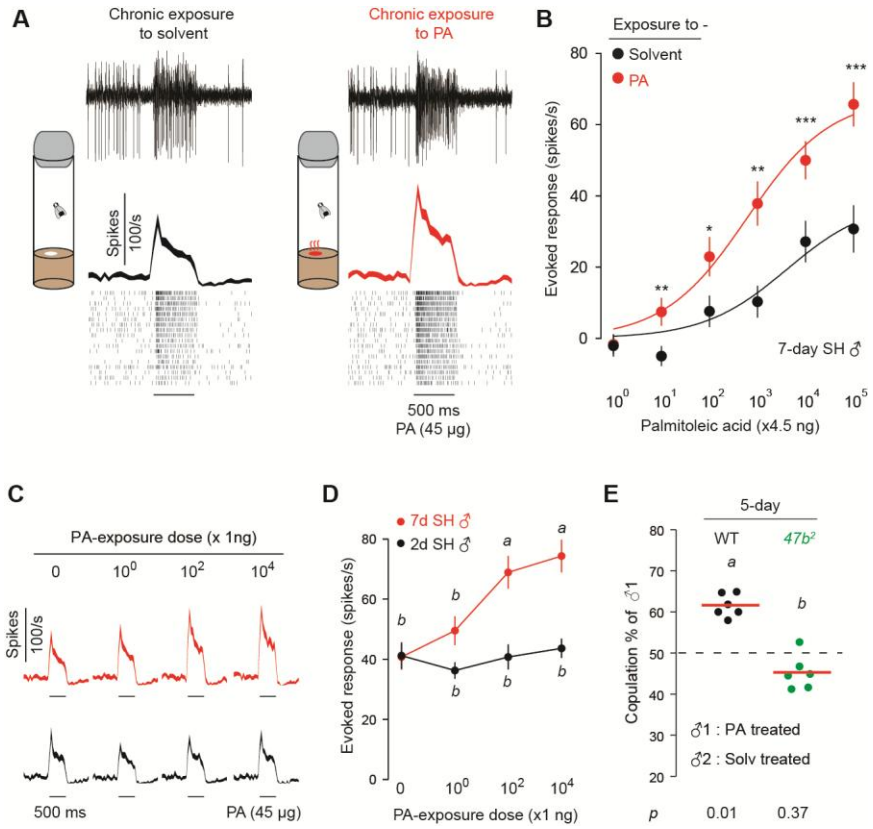


Figure 2.2. Chronic exposure to Or47b ligand is sufficient to enhance pheromone response and mating drive in male flies.

(A, B) Chronic exposure to 1 μg of Or47b ligand (PA, palmitoleic acid) enhances Or47b odor response in male flies. **(A)** Flies were single housed in vials containing filter paper with solvent (left) or 1 μg PA (right) starting from eclosion for 7 days. (**B**) Dose-curve indicating that flies that were previously exposed to 1 μg PA have higher odor responses in Or47b neurons when compared to flies exposed to solvent only. *, p<0.05; **, p<0.01; ***, p<0.001, denotes significant difference for a given odor stimulation concentration, determined by unpaired two-tailed t-test. n=13 flies for each condition. Error bars indicate SEM. **(C, D)** Chronic exposure to palmitoleic acid enhances Or47b response in 7-day old but not 2-day old WT males. Flies were exposed to varying amounts of PA starting from eclosion till the time of the experiment. Odor stimulation for recording experiment: 45 µg PA. Two-way ANOVA indicated a significant interaction between age and PA exposure dose, F=5.42, p=0.001. n=18 flies for each condition. **(E)** Chronic exposure to 1 μg of palmitoleic acid increases copulation rate in WT males but not in *Or47b* mutant males. Flies were exposed to PA for 5 days. A single dot represents one experiment of 12-20 chambers each containing one GH male, one SH male and one female. n = 6 experiments per condition. Comparison between WT and *Or47b* mutant males was made using unpaired t-test (two tailed, n=6, t=7.98, p<0.0001).

CaMKI and dCBP are both required for the group-housing effect

We next sought to determine the molecular mechanisms which underlie the sensitization of Or47b ORNs. Given that calcium signaling is typically required for activity-dependent neuronal plasticity (Zucker, 1999), we first asked whether group-housed males have higher baseline calcium levels in their Or47b neurons. Using CaLexA, a transcriptional reporter of intracellular Ca^{2+} (Masuyama et al., 2012), we observed that group housing enhanced Or47b neuronal calcium levels in 7-day but not 2-day males (**Figure 2.3A**). To determine the subsequent signaling events following rising intracellular Ca^{2+}, we employed a candidate-based approach. We first focused on Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), which are widely recognized for their role in mediating activity-dependent gene expression (Yu et al., 2014; Zucker, 1999). In particular, CaM kinase signaling has been implicated in regulating gene expression downstream of the Or47b receptor (Hueston et al., 2016). We found that a loss-of-function mutation in CaMKI reduced Or47b ORN responses in 7-day group-housed males (**Figure 2.3B**). Furthermore in group-housed males, RNAi-mediated knockdown of CaMKI in Or47b neurons lowered their pheromone responses to a level similar to that of single-housed males (**Figure 2.3C**). Similarly, CaMKI knockdown precluded the effect of chronic PA exposure (**Figure 2S.4B**). These data collectively suggest that group housing elevates Or47b pheromone response by means of activity-dependent CaMKI signaling. In comparison, CaMKII knockdown did not have any effect on Or47b response in 7-day group-housed males (**Figure 2S.4C**).

To determine the signaling molecules downstream of CaMKI in regulating Or47b neuronal properties, we explored the role of *Drosophila* CREB binding protein (dCBP, also called *nejire*). CBP is a lysine acetyltransferase that functions as a transcriptional co-activator in a variety of physiological processes (Goodman and Smolik, 2000). In addition, CBPs can directly acetylate histones and participate in chromatin remodeling, thereby regulating gene expression (Turner and Thangue, 1991). The vertebrate homolog of *Drosophila* CaMKI is known

to phosphorylate and activate CBP, which in turn initiates transcription in response to neuronal activity (Impey et al., 2002). Furthermore, dCBP is also involved in regulating gene expression downstream of the Or47b receptor (Hueston et al., 2016). We found that RNAi-mediated knockdown of dCBP in the Or47b neurons of group-housed males reduced their responses to a level similar to that of single-housed males (**Figure 2.3D**). Moreover in courtship competition assays, dCBP knockdown eliminated the difference in the copulation rate between group- and single-housed males (**Figure 2.3E**). Together, these data support a model in which group housing enhances courtship behavior by activating a CaMKI-dCBP signaling pathway in male Or47b ORNs (**Figure 2.3F**).

Figure 2.3. CaMKI and dCBP are required for the effect of group housing on Or47b pheromone response and courtship behavior.

(A) Group housing elevates intracellular Ca2+ concentration of Or47b neurons in 7-day but not in 2-day old males. Pseudocolor images show GFP fluorescence intensity of Or47b axon terminals in the antennal lobe. Two-way ANOVA indicated a significant interaction between housing condition and age (F=11.71, p=0.003). Significant differences between conditions (p<0.05) were determined using Tukey's post-hoc test and are denoted by different letters, n = 6. Scale bar = 15 μm. GH: flies were raised in groups of 15 per vial. SH: flies were raised in isolation. *, p<0.05; **, p<0.01; ***, p<0.001, denotes significant difference for a given odor concentration, determined by one-way (B) or two-way (C, D) ANOVA followed by Tukey's post-hoc test. Error bars indicate SEM. **(B)** CaMKI mutation suppresses Or47b odor response in 7-day GH males. n=14 per genotype. **(C)** RNAi-mediated knockdown of CaMKI in Or47b neurons eliminates GH-dependent increase in odor response without altering the response in SH males. n=14 per genotype, 7-day males. **(D)** RNAi-mediated knockdown of dCBP in Or47b neurons reduces odor responses in 7-day GH males without altering the response in SH males. n=15 per genotype. **(E)** dCBP knockdown in Or47b neurons abolishes the differences in copulation rate between GH and SH males. A single dot represents one experiment of 13-22 chambers each containing one GH male, one SH male and one female. n = 6 experiments per condition. Different letters denote significant differences between genotypes (p<0.05), one-way ANOVA (F=24.06 p<0.0001) followed by Tukey's post-hoc test. Chi-square test was used to determine if copulation percentages of GH and SH males were different from chance. p-values are indicated on the figure. GAL4: Or47b-GAL4, UAS: UAS-dCBP-RNAi, RNAi: Or47b-GAL4>UAS-dCBP-RNAi. **(F)** Model for molecular pathway involved in effect of group housing on Or47b odor response. Group housed flies are exposed to high PA concentration, which activates CamKI and its target dCBP resulting in elevated Or47b odor response and mating drive in male flies.

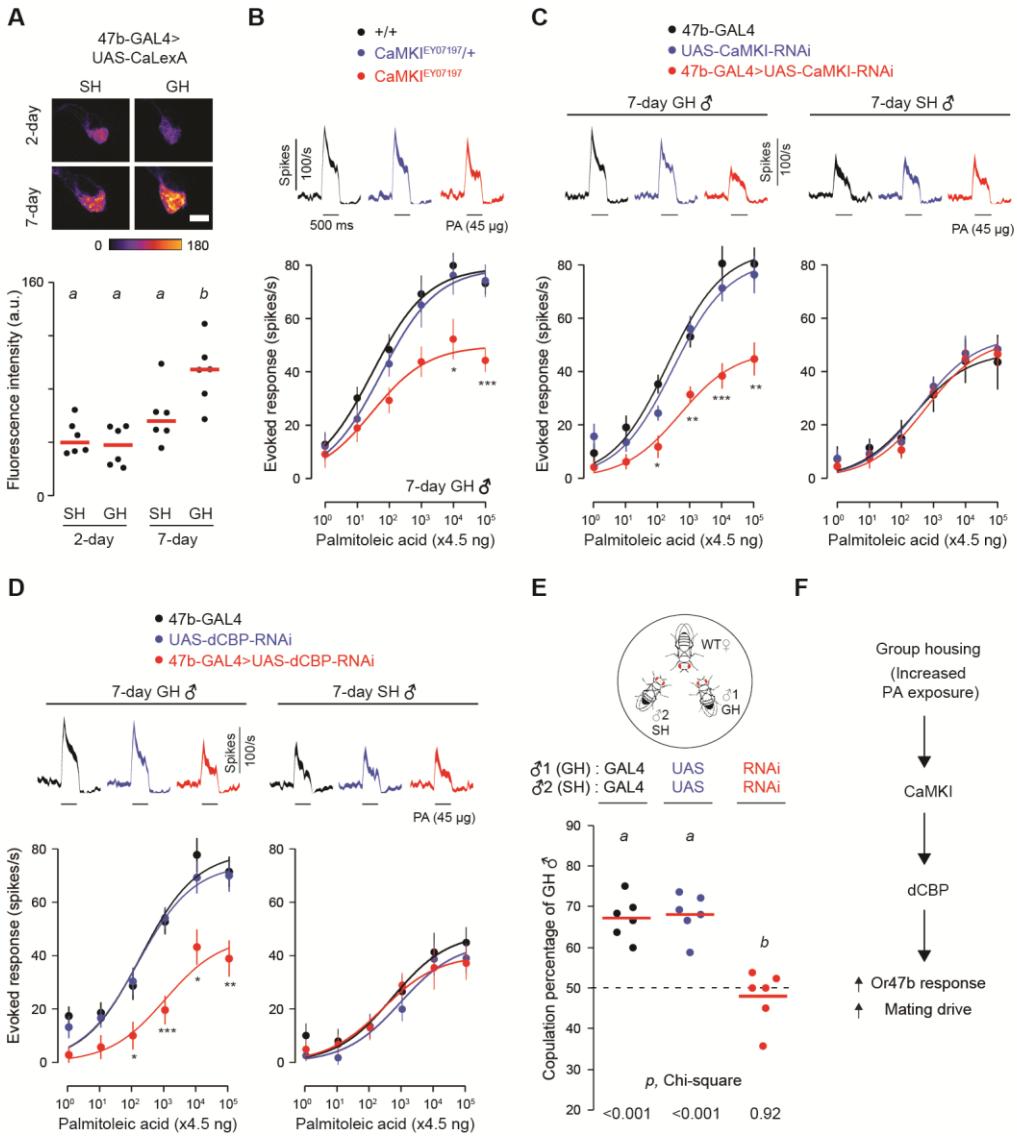
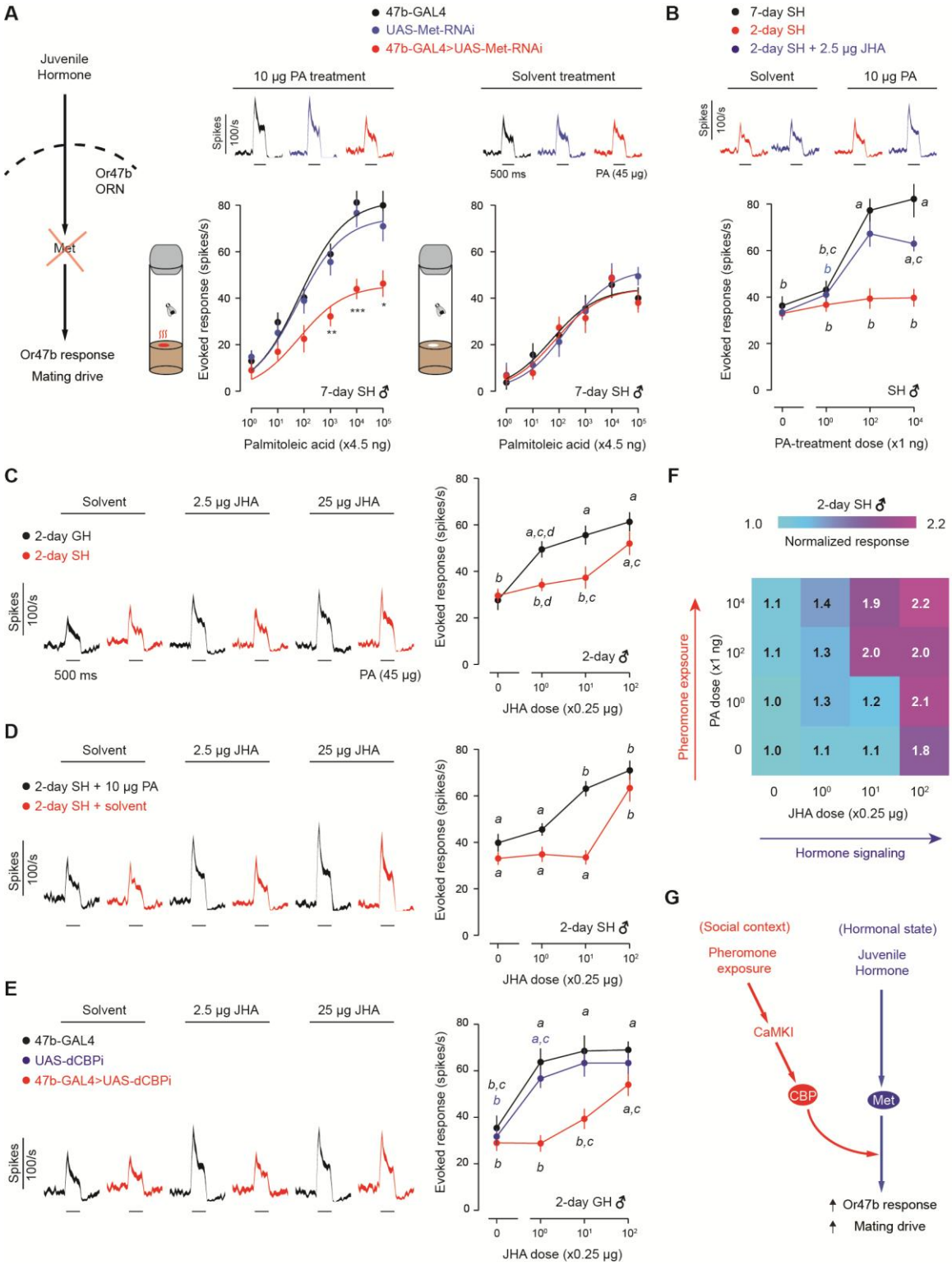

A
47b-GAL4>
UAS-CaLexA
SH
GH
2-day
7-day
0
180
Fluorescence intensity (a.u.)
160
0
a
a
a
b
SH
GH
SH
GH
2-day
7-day
B
+/+
CaMKI EY07197/+
CaMKI EY07197
Spikes
100/s
500 ms
PA (45 μg)
Evoked response (spikes/s)
80
60
40
20
0
7-day GH ♂
10⁰ 10¹ 10² 10³ 10⁴ 10⁵
Palmitoleic acid (x4.5 ng)
C
47b-GAL4
UAS-CaMKI-RNAi
47b-GAL4>UAS-CaMKI-RNAi
7-day GH ♂
7-day SH ♂
Spikes
100/s
PA (45 μg)
Evoked response (spikes/s)
Palmitoleic acid (x4.5 ng)
Palmitoleic acid (x4.5 ng)
D
47b-GAL4
UAS-dCBP-RNAi
47b-GAL4>UAS-dCBP-RNAi
7-day GH ♂
7-day SH ♂
Spikes
100/s
PA (45 μg)
Evoked response (spikes/s)
Palmitoleic acid (x4.5 ng)
Palmitoleic acid (x4.5 ng)
E
WT♀
♂1 GH
♂2 SH
♂1 (GH) : GAL4
♂2 (SH) : GAL4
UAS
UAS
RNAi
RNAi
Copulation percentage of GH ♂
90
80
70
60
50
40
30
20
a
a
b
p, Chi-square
<0.001
<0.001
0.92
F
Group housing
(Increased
PA exposure)
CaMKI
dCBP
Or47b response
Mating drive

Figure 2.4. Group housing enhances the effect of juvenile hormone signaling on Or47b pheromone response.

(A) RNAi-mediated knockdown of juvenile hormone receptor (Methoprene-tolerant, Met) in Or47b neurons eliminates the effect of chronic PA exposure on odor response in 7-day SH male flies without any effect on control males exposed to solvent alone. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, denotes significant difference for a given odor concentration, determined by two-way ANOVA followed by Tukey's post-hoc test. n = 13 flies per condition. Error bars indicate SEM (A-E). GH: flies were raised in groups of 15 per vial. SH: flies were raised in isolation. **(B)** Treating young 2-day old males with 2.5 μg of juvenile hormone analog (JHA, Methoprene) makes them susceptible to the effect of chronic PA exposure on Or47b odor response. Two-way ANOVA indicated a significant interaction between PA exposure dosage and treatment condition (F=6.17, $p<0.0001$), n=18 flies per condition. Odor stimulation: 45 μg PA. Significant differences ($p<0.05$) are denoted by different letters, determined by two-way ANOVA followed by Tukey's post-hoc test (B-E). **(C)** Group housing enhances the effect juvenile hormone signaling on Or47b odor response in 2-day old WT males. Two-way ANOVA indicated a significant effect of housing condition (F=17.16, $p<0.0001$) and JHA dosage (F=13.21, p=0.0004), n=17 flies per condition. Odor stimulation: 45 μg PA. Group or single housed male flies were treated with indicated dosage of JHA (Methoprene) starting from eclosion for two days (C-E). **(D)** Chronic exposure to 10 μg PA enhances the effect of juvenile hormone signaling on Or47b odor response in 2-day old WT males. Two-way ANOVA indicated a significant interaction between PA exposure condition and JHA dosage (F=3.76, p=0.012). n=18 flies per condition. Odor stimulation: 45 μg PA. **(E)** RNAi-mediated knockdown of dCBP in Or47b neurons reduces the effect of juvenile hormone signaling on odor responses in 2-day WT GH males. Two-way ANOVA indicated a significant interaction between genotype and JHA dosage (F=2.33, p=0.033), n=18 flies per condition. Odor stimulation: 45 μg PA. **(F)** JHA signaling and chronic PA exposure have a synergistic effect in elevating Or47b response in 2-day SH male flies. A lower dose of JHA (2.5 μg instead of 25 μg) was required to potentiate Or47b response in males that were chronically exposed to higher levels of PA (10^2–10^4 ng). Mean evoked response for each condition was normalized to the mean response of males that received no hormone or odor treatment. All flies are 2-day SH WT males. **(G)** Model for molecular interaction between group housing and juvenile hormone signaling. Group housing activates dCBP which enhances the effect of juvenile hormone signaling. Lack of juvenile hormone signaling in young flies or in flies lacking juvenile hormone receptor makes the Or47b neuron insensitive to group housing.


A
47b-GAL4
UAS-Met-RNAi
47b-GAL4>UAS-Met-RNAi
Juvenile Hormone
Or47b ORN
Met
Or47b response
Mating drive
10 µg PA treatment
Solvent treatment
Spikes 100/s
500 ms
PA (45 µg)
Evoked response (spikes/s)
7-day SH ♂
Palmitoleic acid (x4.5 ng)
B
7-day SH
2-day SH
2-day SH + 2.5 µg JHA
Solvent
10 µg PA
SH ♂
PA-treatment dose (x1 ng)
C
Solvent
2.5 µg JHA
25 µg JHA
2-day GH
2-day SH
2-day ♂
JHA dose (x0.25 µg)
D
2-day SH + 10 µg PA
2-day SH + solvent
2-day SH ♂
E
47b-GAL4
UAS-dCBPi
47b-GAL4>UAS-dCBPi
2-day GH ♂
F
2-day SH ♂
Normalized response
Pheromone exposure
PA dose (x1 ng)
Hormone signaling
G
(Social context)
(Hormonal state)
Pheromone exposure
Juvenile Hormone
CaMKI
CBP
Met
Or47b response
Mating drive

Group housing enhances the efficacy of juvenile hormone signaling

Why is the group-housing effect observed only in mature males but not in younger, 2-day old males? Given that Or47b ORNs adapt their responses to changes in housing density in an age-dependent manner (**Figure 2.1**), we hypothesize that the signaling events initiated by the two conditions interact to modulate Or47b responses. We note that the age-dependent sensitization in Or47b ORNs is mediated by juvenile hormone (Lin et al., 2016), a pleiotropic hormone that also regulates reproductive maturity in adult flies (Flatt et al., 2005) . To determine if juvenile hormone signaling is required for activity-dependent Or47b plasticity, we knocked down the juvenile hormone receptor, Methoprene-tolerant (Met) (Jindra et al., 2015), in Or47b ORNs. We found that Met knockdown abolished the effect of chronic PA exposure (**Figure 2.4A**), suggesting that juvenile hormone signaling is also required for the group-housing effect. If a low level of juvenile hormone signaling in immature males renders them insensitive to group housing, then treating 2-day old males with methoprene, a juvenile hormone analog (JHA), should make their Or47b ORNs susceptible to modulation by group housing. In support of this hypothesis, treating 2-day old males with 2.5 µg of JHA increased their Or47b responses following chronic PA exposure (**Figure 2.4B**). These results suggest that the two molecular pathways—one signaling population density and the other fly age—are both required for the activity-dependent Or47b neuronal plasticity.

What then is the nature of interaction between these two signaling pathways? Do they operate in synergy? Can strong activation of one pathway compensate for the low signaling level of the other? To address these questions, we first investigated the efficacy of hormonal signaling by systematically manipulating JHA dosage in group- or single-housed young 2-day males. We found that JHA treatment was more effective in enhancing Or47b ORN responses in group-housed males, when compared to single-housed flies (**Figure 2.4C**). This result indicates that these two signaling pathways functionally interact in a synergistic manner. Surprisingly, a

higher dosage (25 μg) of JHA was able to enhance Or47b responses even in single-housed males (**Figure 2.4C**), suggesting that a high level of hormonal signaling can compensate for low levels of neuronal activity. Additionally using chronic PA exposure, we observed a similar synergistic relationship between juvenile hormone signaling and activity-dependent plasticity, the low level of latter can also be compensated for by a high dosage of JHA (**Figure 2.4D**). Given that dCBP is required for the group-housing effect (**Figures 2.3D and 2.3E**), its absence can in principle reduce the efficacy of juvenile hormone signaling.

As expected, RNAi-mediated knockdown of dCBP in Or47b ORNs reduced the effect of JHA treatment in group-housed males (**Figure 2.4E**). These results suggest that in Or47b ORNs, the crosstalk between dCBP and juvenile hormone signaling is central to the integration of housing density and fly age.

To further determine the relative contribution of neuronal activity and juvenile hormone signaling to Or47b response plasticity, we systematically varied the strength of these two inputs in single-housed young flies (**Figure 2.4F**). We raised 2-day old flies in vials containing varying amounts of PA (0-10 μg) and JHA (0-25 μg). In the absence of JHA treatment, increasing PA levels did not alter Or47b response (**Figure 2.4F**). This result suggests that neuronal activity, and by extension dCBP activation, is not sufficient to enhance Or47b response without juvenile hormone signaling. In contrast, a high-dose of JHA treatment was sufficient to sensitize Or47b pheromone response in the absence of exogenous PA exposure (**Figure 2.4F**). Notably, treatment with 2.5 μg JHA was able to enhance odor response when flies were raised in PA-perfumed vials (0.1-10 μg), while each of these conditions alone did not have any effect on Or47b responses (**Figure 2.4F**). This result further argues for a synergistic interaction between juvenile hormone signaling and PA-exposure-evoked activity, while a high level of juvenile hormone can compensate for low activity in Or47b ORNs (**Figure 2.4D**).

Our key observations so far are summarized as follows: (1) A juvenile hormone receptor is required for the effect of chronic PA exposure (**Figure 2.4A**); (2) group housing alone is not sufficient to enhance Or47b response in immature males (**Figure 2.4F**), and (3) dCBP is required for group housing to enhance the efficacy of juvenile hormone (**Figure 2.4E**). Based on these results, we propose a model in Or47b ORNs whereby group housing/dCBP activation plays a permissive role, giving rise to an intracellular environment that facilitates juvenile hormone signaling (**Figure 2.4G**). As such, when juvenile hormone signaling is abolished or at a low level, as in the case of Met knockdown or in 2-day old males, dCBP activation alone is not sufficient to regulate Or47b pheromone responses.

Fru^M expression levels determine Or47b response

A striking feature of the adaptive Or47b pheromone response is its sexual dimorphism; housing density and age only affect the Or47b ORNs of males but not females ((Lin et al., 2016); **Figures 2.1C and 2.1D**). What then could be the molecular basis of this sex difference? Given that Fru^M is expressed in Or47b ORNs ((Stockinger et al., 2005), **Figure 2.5A**), it is likely to play a role in regulating the male-specific adaptive responses. To test this hypothesis, we first feminized Or47b neurons by ectopically expressing the female splicing factor Transformer (Tra^F), and found that this manipulation suppressed odor response in group-housed mature males (**Figure 2.5B**).

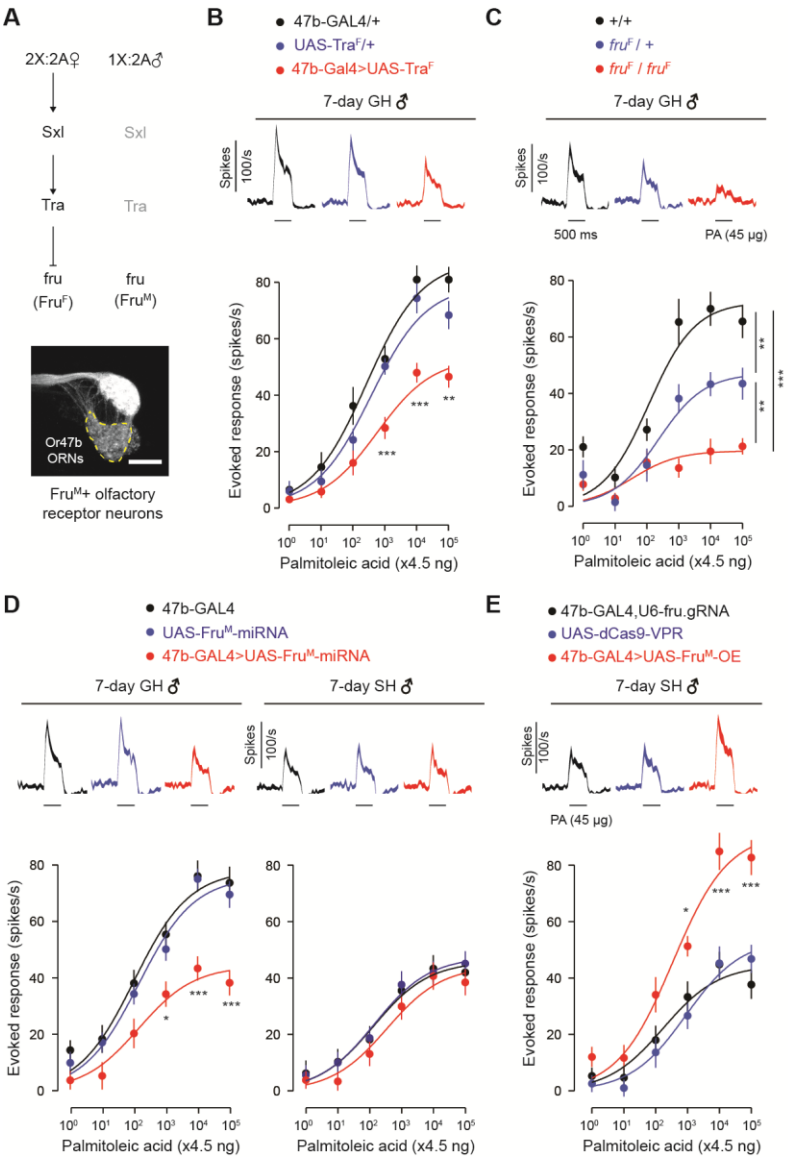


Figure 2.5. Fru^M expression levels determine Or47b response.

(A) Signaling cascade leading to sexually dimorphic expression of Fruitless in somatic tissues. In females, a functional transformer (Tra) protein splices fruitless into in an inactive form (Fru^F). In males, no functional Tra is present which causes fruitless to be spliced into its active form (Fru^M). Fruitless is expressed in Or47b neurons indicated as the region of interest in male flies of the following genotype - Fru.P1.GAL4, UAS-GFP, ey-FLP, Tub-FRT-GAL80-FRT. Scale bar = 25 µm. **(B)** Feminizing Or47b neurons by ectopic expression of tra^F suppresses odor responses in 7-day GH males. n=14, one each condition. *, p<0.05; **, p<0.01; ***, p<0.001, denotes significant difference for a given odor concentration, determined by one-way (B-E) ANOVA followed by Tukey's post-hoc test. Error bars indicate SEM. **(C)** Fru^M expression level is correlated with odor response in Or47b neurons in GH males. n=13 flies per condition. *fru^F* is a mutant allele that does not produce functional Fru^M. **(D)** RNAi-mediated knockdown of Fru^M in Or47b neurons eliminates GH-dependent increase in odor response without altering the response in SH males. n=14 flies per condition. **(E)** dCas9-mediated overexpression of Fru^M enhances odor response in SH males. n=14 flies per condition. Fru^M-OE = U6-fru.gRNA, UAS-dCas9-VPR.

In addition, we observed a dosage effect of Fru^M expression on Or47b responses; the evoked spike frequency was the lowest in *fru^M* homozygous mutants, followed by heterozygous mutants and then wild type controls (all 7-day old, grouped housed males, **Figure 2.5C**). This haploinsufficiency suggests that Fru^M expression level is a limiting factor in regulating the sensitivity of Or47b ORNs.

This result led us to hypothesize that group housing upregulates Fru^M expression, which in turn gives rise to the elevated pheromone responses in Or47b neurons. This hypothesis is also supported by the observation that Fru^M expression in Or47b ORNs is suppressed in *Or47b* receptor mutant flies, suggesting that neuronal activity is required for sustaining Fru^M expression in Or47b neurons (Hueston et al., 2016). To test if Fru^M expression is necessary for group-housing effect, we knocked down Fru^M specifically in Or47b ORNs and observed reduced responses in group-housed but not single-housed males (**Figure 2.5D**). If Fru^M is a limiting factor that regulates Or47b response plasticity, overexpression of Fru^M in single-housed males should then enhance their pheromone responses. Indeed, dCas9-mediated overexpression of Fru^M ((Ewen-Campen et al., 2017), **Figure 2S.5A**) was sufficient to enhance Or47b response in single-housed males regardless of their age (**Figure 2.5E; Figure 2S.5B**). As an alternative approach, we generated a knock-in UAS transgenic line (**Figure 2S.5C**) to overexpress Fru^M in Or47b ORNs from its endogenous locus, and found that this manipulation yielded a similar outcome (**Figure 2S.5D**). Together, these results indicate that in Or47b ORNs the levels of Fru^M expression determine their pheromone sensitivity.

dCBP and juvenile hormone signaling require Fru^M to elevate pheromone responses

Having determined the relationship between Fru^M expression level and Or47b pheromone sensitivity, we next asked whether Fru^M operates downstream of dCBP (group housing/neuronal activity) and juvenile hormone signaling. This hypothesis is supported by

multiple lines of evidence. Firstly, juvenile hormone signaling can enhance Fru^M expression in adult male neurons (Wu et al., 2018). In addition, the effect of group housing on Or47b response requires CaMKI-dCBP (**Figure 2.3**), a signaling pathway that also modulates Fru^M expression in Or47b neurons (Hueston et al., 2016). Furthermore, dCBP can directly bind to the fru-P1 promoter in adult males ((Nègre et al., 2011), modEncode).

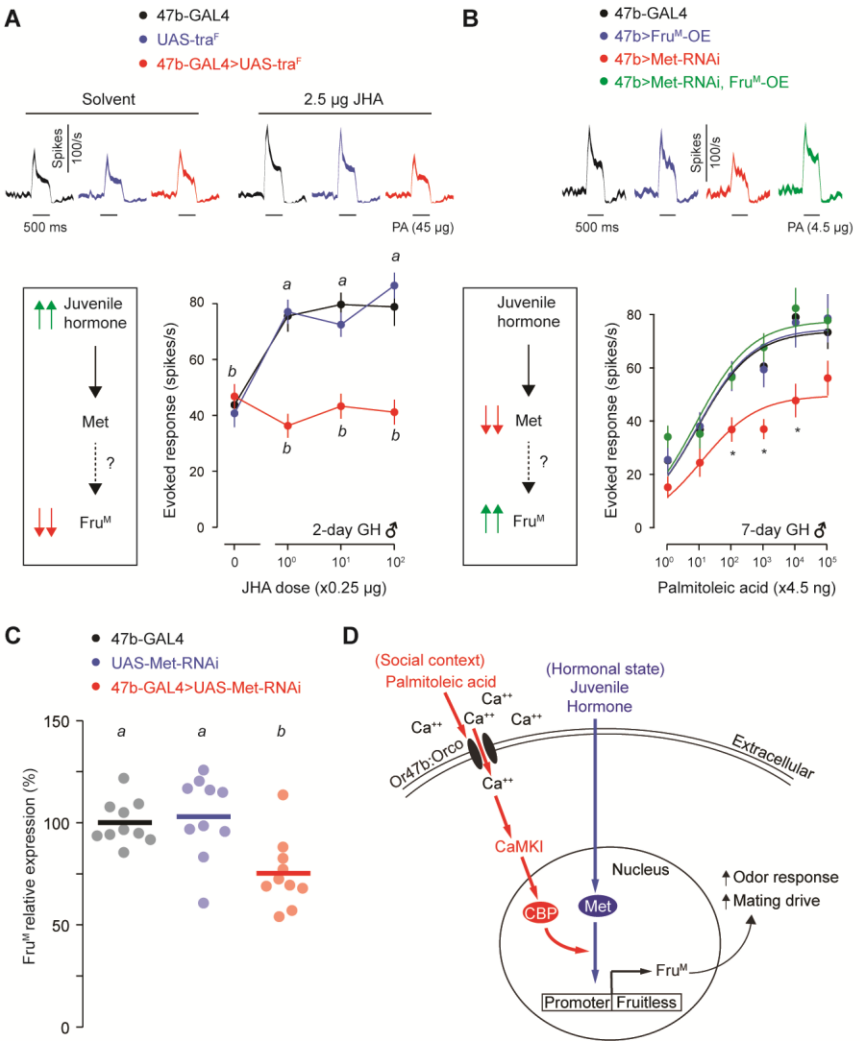


Figure 2.6. Fru^M expression is downstream of juvenile hormone signaling in Or47b neurons.

(A) Feminizing Or47b neurons by ectopic expression of Tra^F eliminates the effect of juvenile hormone signaling on Or47b odor response. Two-way ANOVA indicated a significant interaction between genotype and methoprene dosage (F=7.31, p<0.0001), n=18 flies per condition. Significant differences between conditions (p<0.05) were determined using Tukey's post-hoc test and are denoted by different letters. Odor stimulation: 45 µg PA. **(B)** Fru^M overexpression can enhance odor responses in GH males even in the absence of juvenile hormone signaling. n=14 flies per condition. Fru^M overexpression: blue, juvenile hormone signaling knockdown - Met RNAi: red, both Fru^M overexpression and Met-RNAi: green. *, p<0.05, denotes significant difference for a given odor concentration, determined by one-way ANOVA followed by Tukey's post-hoc test. Error bars indicate SEM. **(C)** RNAi-mediated knockdown of Met in Or47b neurons reduces Fru^M expression (F=8.64, p=0.001, n=10 samples per condition). Data normalized to mean Fru^M expression in Or47b-GAL4 controls. **(D)** Our data support a model where the Fru^M locus acts as a genomic coincidence detector to integrate social context with the hormonal state of a fly. Group housing activates the CaMKI/CBP pathway to establish an intracellular environment that enhances the effect of juvenile hormone signaling in Or47b neurons. Graded levels of Fru^M expression fine-tune neuronal properties and mating behavior.

To test the hypothesis that Fru^M expression is downstream of juvenile hormone signaling, we first feminized Or47b ORNs by ectopically expressing Tra^F and found that this manipulation abolished JHA-induced sensitization (2-day old, group-housed males, **Figure 2.6A**). Conversely, if Fru^M expression is downstream of dCBP or juvenile hormone signaling, then overexpressing Fru^M in Or47b ORNs should rescue the phenotype of dCBP or Met knockdown. As expected, overexpression of Fru^M abolished the reduction in Or47b pheromone response caused by Met knockdown (7-day old, group-housed males, **Figure 2.6B**). Likewise, the response reduction caused by dCBP knockdown was also abolished by Fru^M overexpression (7-day old, group-housed males, **Figure 2S.6A**). Furthermore, knocking down Met expression in Or47b neurons reduced Fru^M expression in the antenna (**Figure 2.6C**). These results support a model in which the two signaling pathways—initiated by group housing and hormonal state respectively—act in concert to regulate the expression of Fru^M in Or47b ORNs. Thus, the Fru^M locus may function as a genomic coincidence detector that integrates social context with reproductive maturity to adapt Or47b pheromone responses in a sexually dimorphic manner (**Figure 2.6D**).

2.4. Discussion

Modulation of physiology and behavior by population density is a conserved feature across animal species (Applebaum and Heifetz, 1999b; Brennan et al., 2017). Here we find that group housing promotes mating in males in a behavioral assay which mimics how multiple males compete for a female in nature. From an evolutionary standpoint, it is beneficial for male flies to upregulate their mating drive in an environment of high population density, likely to gain a competitive edge over an increased number of rivals. Conversely, a low population density may signal an environment of scarce reproductive opportunity. In this scenario, flies may lower their pheromone sensitivity to prevent futile courtship and conserve energy. In addition to its

ethological significance, our study also identifies a critical marker of population density—the levels of a fly pheromone—which alone recapitulates the effect of group housing on Or47b ORN sensitization.

In *Drosophila*, neural circuits underlying male courtship behavior are orchestrated by Fru^M. Most efforts in understanding sex-typical behavior have focused on the developmental role of Fru^M (Ito et al., 2012; Yamamoto and Koganezawa, 2013). Fru^M promotes male fate by inhibiting cell death, altering dendritic arborization and instructing synaptic connectivity (Ito et al., 2012; Kimura et al., 2005; Kohl et al., 2013). Although Fru^M is expressed in the adult brain (Stockinger et al., 2005), its function in mature neurons is largely unknown. In this study, we find that Fru^M directly regulates the responses to aphrodisiac pheromones in adult ORNs. Our results suggest that Fru^M expression level is regulated by social context, thereby allowing neuronal properties to be fine-tuned in mature neurons. These findings are in agreement with the observation that Fru^M regulates the expression of several genes that are known to control neuronal physiology (Dalton et al., 2013). Taken together, our results highlight a novel function for Fru^M in adulthood, which has a direct impact on social behavior beyond its role during development.

Our study uncovers an interaction between dCBP and juvenile hormone signaling that underlies the integrative effect of age and social context on courtship behavior. The critical role of dCBP in the adaptive Or47b response suggests an epigenetic mechanism that allows for modulation of pheromone detection by social context. Such neuronal plasticity induced by high population density may enable the animals to adapt to environmental changes at a rate faster than that afforded by genetic changes under selection pressure (Cardoso et al., 2015). How may dCBP regulate juvenile hormone signaling? One possibility is that dCBP activation leads to chromatin modification by means of histone acetylation, thereby enhancing the accessibility of the *fruitless* P1 promoter to juvenile hormone receptors. dCBP may also function as a

transcriptional co-activator that forms a complex with juvenile hormone receptors to enhance the expression of Fru^M in Or47b ORNs. A similar interaction between CBP and juvenile hormone signaling is likely at play in the red flour beetle, where knockdown of CBP reduces expression of several juvenile hormone-responsive genes (Xu et al., 2018). In mammals, CBP can enhance the transcriptional activity of sex hormone receptors (Frønsdal et al., 1998; Smith et al., 1996). Thus, activity-dependent modulation by CBP may represent a conserved mechanism for generating hormone-mediated polyphenism, a phenomenon where distinct phenotypes are produced by the same genotype (Hartfelder and Emlen, 2012). As such in the regulation of hormonal signaling, our study suggests a novel means by which social experience impacts reproductive behaviors through modulating the interaction between a hormone receptor and its target genes (Ball and Balthazart, 2008).

Sex-typical behaviors in mammals are controlled by sex hormones that organize the developing brain and control the activation of the underlying neural circuits in adults. In comparison, genetic sex plays a minor role in modulating the display of sexual behaviors (Yang and Shah, 2014). In insects, the display of sexual behaviors is thought to be determined entirely by genetic sex (Yamamoto and Koganezawa, 2013). However, a growing body of evidence points to the sex-specific activational effects of juvenile hormone on insect reproductive behaviors (Elekonich and Robinson, 2000). Here we resolve this apparent conflict by identifying a molecular pathway, in which juvenile hormone activates the Or47b olfactory circuit exclusively in males through promoting expression of Fru^M. Based on this study, we propose that genetic sex plays a dominant role and reproductive hormones modulate the vigor of sex-typical behaviors in *Drosophila.*

2.5. Methods

Fly husbandry

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). Glass vials were filled with 8 to 10 mL of fly food and plugged with cotton balls. Flies were collected within 12 hours of eclosion, separated by sex and raised either in isolation (1 per vial) or in groups of 15 (unless otherwise indicated). All flies were flipped every 24 hours to avoid the potential effect of odor build up on single housed flies. All wild-type males were in the Berlin background. The following transgenes were used in this study - or47b2 (BDSC_51306) (Wang et al., 2011), Or47b-GAL4 (RRID:BDSC_9984, BDSC_9983) (Fishilevich and Vosshall, 2005), 13XLexAop2-6XGFP (RRID:BDSC_52265) (Shearin et al., 2014), 10XUAS-mLexA-VP16-NFATdC (this study), UAS-CaMKI-RNAi (TRiP.JF02268 - BDSC_26726) (Perkins et al., 2015), UAS-dCBP-RNAi (RRID:BDSC_32576) (Smolik and Jones, 2007), UAS-Met-RNAi (VDRC_100638), ey-FLP (RRID:BDSC_5580), Tb-(FRT-GAL80-FRT)-STOP (RRID:BDSC_38880) (Gordon and Scott, 2009), UAS-traF (RRID:BDSC_4590) (Ferveur et al., 1995), fruF (RRID:BDSC_66873) (Demir and Dickson, 2005), UAS-fru-miRNA (Meissner et al., 2016), U6-fru-sgRNA (RRID:BDSC_80225) (Ewen-Campen et al., 2017), UAS-dCas9-VPR (RRID:BDSC_66561) (Ewen-Campen et al., 2017), CaMKI[EY07197] (RRID:BDSC_16799) (Bellen et al., 2004), UAS-CaMKII-RNAi (RRID:BDSC_29401) (Perkins et al., 2015), fru[GAL4.P1] (RRID:BDSC_66696)(Stockinger et al., 2005), fru[UAS.P1] (this study). Detailed genotypes and raising conditions of flies for every experiment is listed in Table S1.

Generation of transgenic reagents

To generate 10XUAS-mLexA-VP16-NFATΔC fly, mLexA-VP16-NFATΔC was cloned from the original CaLexA plasmid (Masuyama et al., 2012) and inserted into the pJRC81 plasmid. The original *XbaI* site in the pJFRC81 plasmid (Pfeiffer et al., 2012) was converted to a

SpeI site using site-directed mutagenesis. mLexA-VP16- NFATΔC was cloned with 5' Syn21-*NotI* and 3' *SpeI* overhangs. The NFATΔC domain is composed of 1-588 amino acids of the human NFATc1 protein. The construct was transformed using phiC31 integrase-mediated recombination into the attP2 landing site by Bestgene Inc. (Chino Hills, CA).

To generate the knock-in fru[UAS.p1] line, CRISPR-mediated mutagenesis was performed by WellGenetics Inc. (Taipei City, Taiwan) based on a previously published strategy with some modifications (Kondo and Ueda, 2013). In brief, gRNA sequence CACATAAACGCAGTACATGG[TGG] was cloned into U6 promoter plasmid(s). Cassette RFP-20xUAS containing two loxP sites, 3XP3-RFP, 20xUAS, hsp70 promoter, intervening sequences (IVS, introns) and two homology arms were cloned into pUC57-Kan as donor template for repair. *fru*/CG14307-targeting gRNAs and hs-Cas9 were supplied in DNA plasmids, together with donor plasmid for microinjection into embryos of control strain w1118. F1 flies carrying selection marker of 3xP3-RFP were further validated by genomic PCR and sequencing. CRISPR generates a 73 bp deletion in *fru*/CG14307 and is replaced by cassette RFP-20xUAS.

Single-sensillum recording

Sharp aluminosilicate glass electrodes (AF100-64-10, Sutter Instrument Co., CA) containing artificial hemolymph solution (Wang et al., 2003) were inserted in the sensillum to record odor-evoked changes in local field potential and spike responses. Odor cartridges were prepared by placing filter paper disks in truncated 200 µL pipette tips (53508-810, VWR, Radnor, PA). 4.5 µL of individual odorants of different concentrations was added to the filter paper disk and the cartridge was placed in a vacuum desiccator (VX-06514-30, Cole-Parmer, Vernon Hills, IL USA) for 60 minutes to evaporate the solvent. Ethanol (E7023, Sigma-Aldrich) was used as a solvent for all odorants. Odor cartridges were positioned with filter disks placed at a distance of 6-7 mm and pointed directly at the antenna. Odor stimulus was delivered via a 500-ms pulse of air (500 mL/min) directed through the odor cartridge towards the antenna in the

presence of humidified air flow at 2 L/min from a different direction. A step-by-step protocol for the recording setup has been described previously (Ng et al., 2017). The following odorants were used in this study - trans-palmitoleic acid (Cayman Chemical*,* 9001798, CAS 10030-73-6*),* methyl palmitate (Sigma-Aldrich, P5177, CAS 112-39-0), and 11-cis Vaccenyl Acetate (Cayman Chemical, 10010101, CAS 6186-98-7).

LFP traces were obtained by applying a low-pass filter (15 Hz, 8-pole Butterworth) in Clampfit 10.7. Individual spikes were sorted based on spike amplitude using Clampfit 10.7 (Molecular Devices). Sorted spikes were manually inspected to ensure accuracy. Evoked response was calculated as (2 x number of spikes during the 500 ms of odor stimulation) - (number of spikes during the period of 1000 ms before odor stimulation). Peri-stimulus time histograms (PSTHs) were calculated by averaging spike numbers in 50 ms time bins.

Courtship competition assay

Courtship experiments were conducted in a cylindrical mating chamber (2 cm in diameter and 1 cm in height) with a wire mesh bottom placed over a petri-dish containing standard fly food. The courtship chamber has been previously described in detail (Lin et al., 2016). All experiments were conducted under 660-nm dim red light at 25°C and 50% relative humidity. Two virgin males were placed in a courtship chamber with one 2-day old virgin wild-type Canton-S female. To facilitate identification of the males, one of the two males was dusted with a fluorescent dye (UVXPBR, LDP LLC, Carlstadt, NJ) 48 hours prior to experiment. Dye application was alternated between group- and single-housed males on a trial-by-trial basis to minimize possible dye-induced behavioral bias. The identity of the male which copulated first with the female and the latency to copulation were manually recorded during a 2-hour observation period. Courtship chambers in which neither of the males copulated with the female during the 2-hour period were excluded from further analysis. Each trial consisted of 20-29 courtship chambers set up in parallel. Mating advantage was indicated by the percentage of

chambers in which males of a given condition copulated first with the female. 5-6 independent trials were conducted for each experiment to allow statistical analysis of mating advantage among the two males.

Chronic odor exposure

A piece of filter paper (approximately 8x8 mm) was placed directly on the surface of fly food in standard fly vials. 1 µL of a given odorant at the specified concentration was applied to the filter paper. Ethanol (E7023, Sigma-Aldrich) was used as a solvent to dilute the odorants. Flies were flipped into fresh vials containing the odorants every 24 hours.

Pharmacological manipulations of juvenile hormone

20 µL of methoprene (33375, Sigma-Aldrich) at given concentrations (between 0.00125% to 0.125% v/v) was applied to the surface of fly food. Ethanol was used as a solvent. The fly vials were placed in the fume hood for 2 hrs to evaporate the solvent. Flies were flipped into fresh vials containing methoprene or the solvent every 24 hours.

Histology

Fly brain samples were dissected in cold PBS and fixed using 4% (w/v) paraformaldehyde for 3 min, followed by 4% (w/v) paraformaldehyde containing 0.25% Triton-X-100 for 3 min on ice in a microwave. Next, samples were placed in blocking solution (2% Triton X-100, 0.02% sodium azide and 10% normal goat serum in PBS) and degassed in a vacuum chamber 6 times for 15 minutes each to expel tracheal air. Samples were not immunostained for quantification of native GFP in CaLexA experiments. Samples were mounted in Focusclear (Cedarlane Labs, Canada). All samples for one experiment were prepared and imaged on the same day. Samples were imaged with a 20X/0.75 objective using a Zeiss LSM 510 confocal microscope to collect Z-stacks at 2-µm intervals. Laser power and gain were held constant to

allow for comparison among samples from different conditions during one experiment. Maximum intensity Z-projections were prepared using ImageJ (NIH). Background-subtracted images were used to quantify GFP expression.

RT-qPCR

Male flies were collected within 24 h of eclosion and aged 7 days in group housing condition. Anesthetized flies were dipped in liquid nitrogen and antennae were brushed off using a needle into a 1.5 ml centrifuge tube filled with liquid nitrogen. Antennae were collected from 10 sample sets of 50 flies each for each genotype and stored at -80°C. mRNA was extracted from each antennal sample using the QIAGEN RNeasy Mini and QIAGEN QIAshredder kits. A hand-held pestle drill was used during extraction. RNA yield was determined using a ThermoFisher Scientific NanoDrop spectrophotometer and cDNA was prepared using Invitrogen SuperScript VILO™ MasterMix. RT-qPCR was performed on the Bio-Rad CFX machine using iQ™ SYBR® Green Supermix from Bio-Rad and the following primer sets: Fru^M (forward primer - GCCACGCCCACTCGCATTAC, reverse primer - TGGTCAGTGTTGTACCTAG); and rp49 (forward primer - AGGGTATCGACAACAGAGTG, reverse primer - CACCAGGAACTTCTTGAATC). rp49 served as a reference gene to which Fru^M was normalized. RT-qPCR was performed with each primer pair in three technical replicates for each cDNA sample. CT values were recorded with Bio-Rad CFX Manager software to determine relative transcript levels.

Statistical analysis

Statistical results (p-value, t-statistic or F-statistic, n) are indicated in figure legends corresponding to each experiment. In cases where a dosage curve for odor concentration was performed, statistical tests comparing the experimental groups were performed for each concentration and the p-value is indicated on the figure for conciseness. All statistical analyses were performed in Igor Pro (V.6, Wavemetrics). To determine the minimum sample size for SSR

recording experiments, we used power analysis based on pilot data (GPower 3.1, (Faul et al., 2009)). For experiments with one factor (f=0.68, α=0.05, power=0.95): 13 samples each are required (3 conditions); 14 samples each are required (4 conditions). For experiments with two factors, n=13 per condition is required to determine interaction between the two factors for 12 (4x3) conditions (f=0.3, α=0.05, power=0.80, df=6); n=16 per condition is required to determine interaction between the two factors for 8 (4x2) conditions (f=0.26, α=0.05, power=0.80, df=3).

To fit dosage response curves to the experimental data, we used Hill equation fitting (Igor Pro V.6, Wavemetrics). Using free fitting parameters on the pooled data showed in Figure 2S.1, we determined that group housed and single housed Or47b neuron dosage-response curves have the same rate but differ in their V_{max}. Based on this initial characterization, we fit all dosage-response curves using mean responses at each odor concentration with the same parameters (base=0, rate=0.5). The dosage-response curves were made for illustration purpose only and were not used for any statistical analysis.

2.6. Acknowledgments

We thank members of the Wang, Su and Volkan labs for advice on experiments and comments on the manuscript; Dr. Jonathan Zirin and Dr. Norbert Perrimon for sharing the fruitless gRNA fly line; Dr. Yishi Jin for sharing the confocal microscope facility.

Chapter 2, in full, is material that is unpublished and coauthored by Sethi S., Lin, H.H., Shepherd, A.K., Volkan, P.C., Su, C.Y. and Wang, J.W. The dissertation author was the primary researcher of this material.

Appendix 2.1: Supplemental Figures

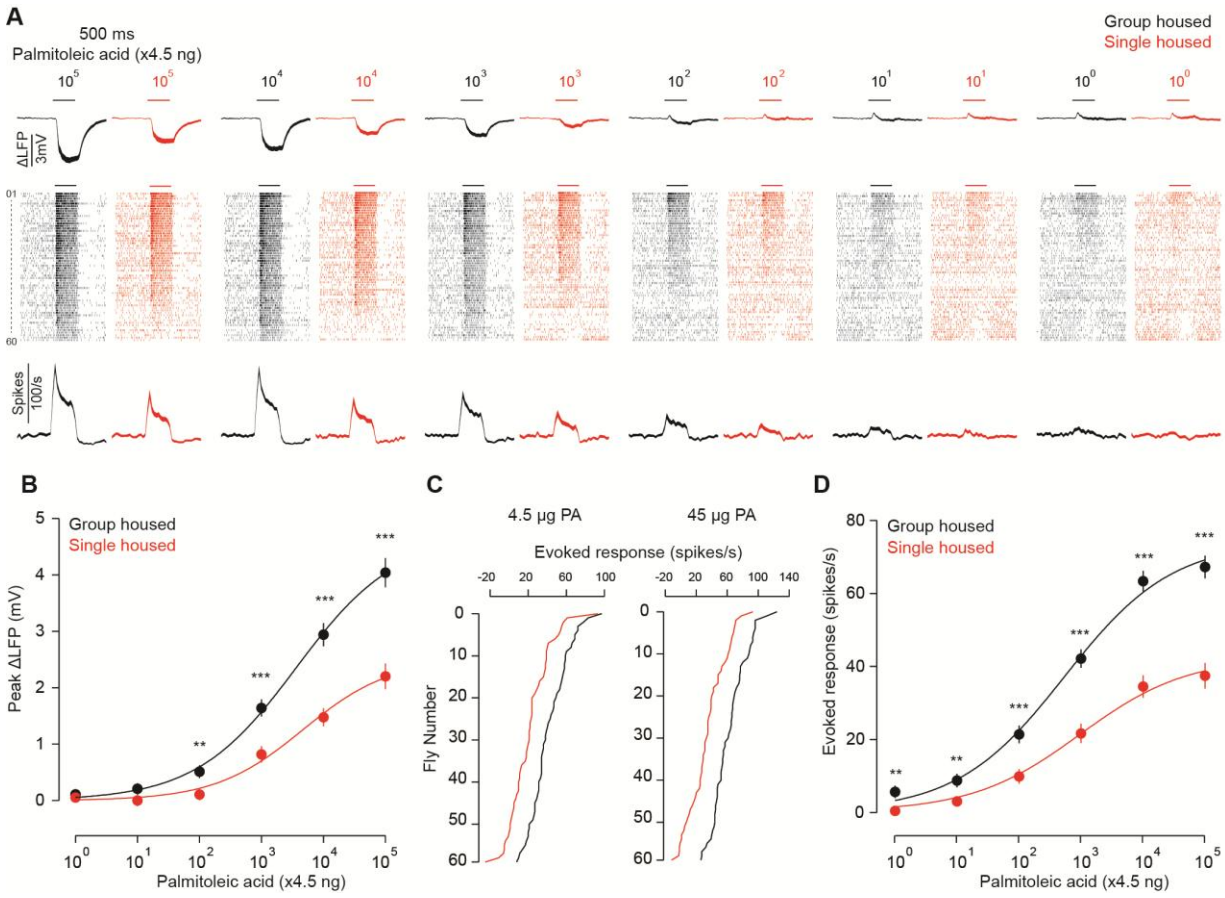


Figure 2S.1. Group housing enhances pheromone response of Or47b ORNs in mature males.

(A) Group housing enhances odor response in Or47b neurons in 7-day old GH but not SH male flies. Average LFP traces (top panel), spike rasters (middle panel) and peri-stimulus time histogram (PSTH, bottom panel) from Or47b neurons in group and single housed flies in response to odor presentation. n=60 for spike raster and PSTH traces, n=47 for LFP traces. GH: flies were raised in groups of 15 per vial. SH: flies were raised individually with each vial containing one fly. *, p<0.05; **, p<0.01; ***, p<0.001, denotes significant difference for a given odor concentration, determined by unpaired two-tailed t-test (B, D). Error bars indicate SEM. **(B)** Group housing enhances the LFP change in response to PA stimulation in 7-day WT males. n=47 for each condition. **(C)** Distribution of spike response in 7-day GH and SH males in response to 4.5 µg PA and 45 µg PA stimulation. n=60 for each condition. **(D)** Group housing enhances the evoked spike responses in 7-day WT males. n=60 for each condition.

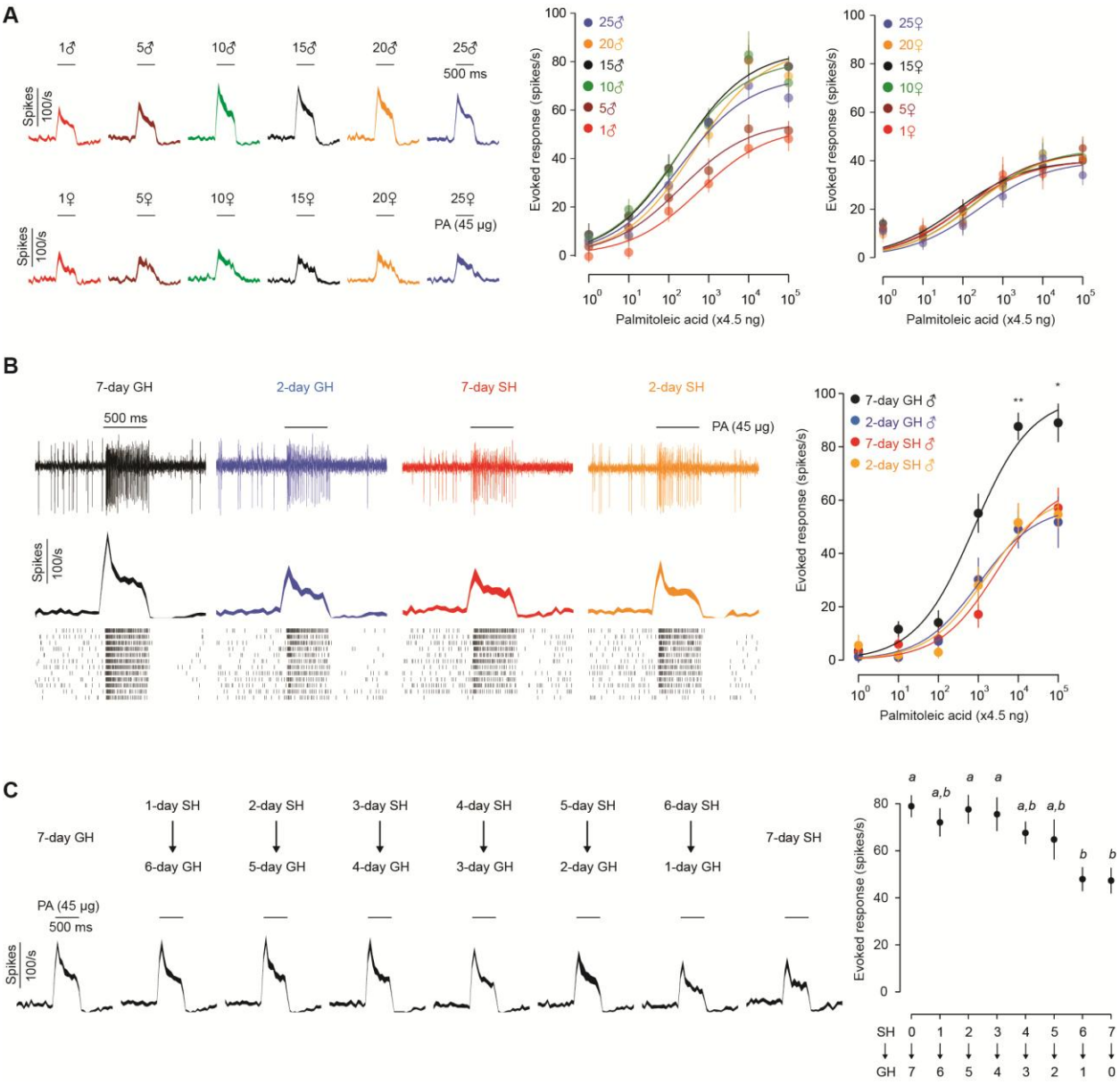


Figure 2S.2. Group housing enhances pheromone response of Or47b ORNs in mature males.

(A) Effect of housing density and sex on Or47b response. WT flies were raised for 7 days either in isolation or in same-sex groups of varying size from 5 to 25 in increments of 5. n = 16 for males, n=12 for females (related to Figure 1C). Peri-stimulus time histogram (PSTH, left panels) and dosage-response curves (right panels) of Or47b neurons in WT males and females. **(B)** Group housing enhances Or47b odor response in 7-day old but not in 2-day old WT males. n=12 flies for each condition. *, p<0.05; **, p<0.01; denotes significant difference for a given odor concentration, determined by two-way ANOVA followed by Tukey's post-hoc test. Error bars indicate SEM. **(C)** Fewer than two days of group housing is not sufficient to enhance odor response in Or47b neurons. Significant differences (p<0.05) are denoted by different letters, determined by one-way ANOVA followed by Tukey's post-hoc test (F=4.26, p<0.001), n=18 flies per condition. Flies were single housed for varying durations prior to being group housed. All flies were 7 days old at the time of the experiment.

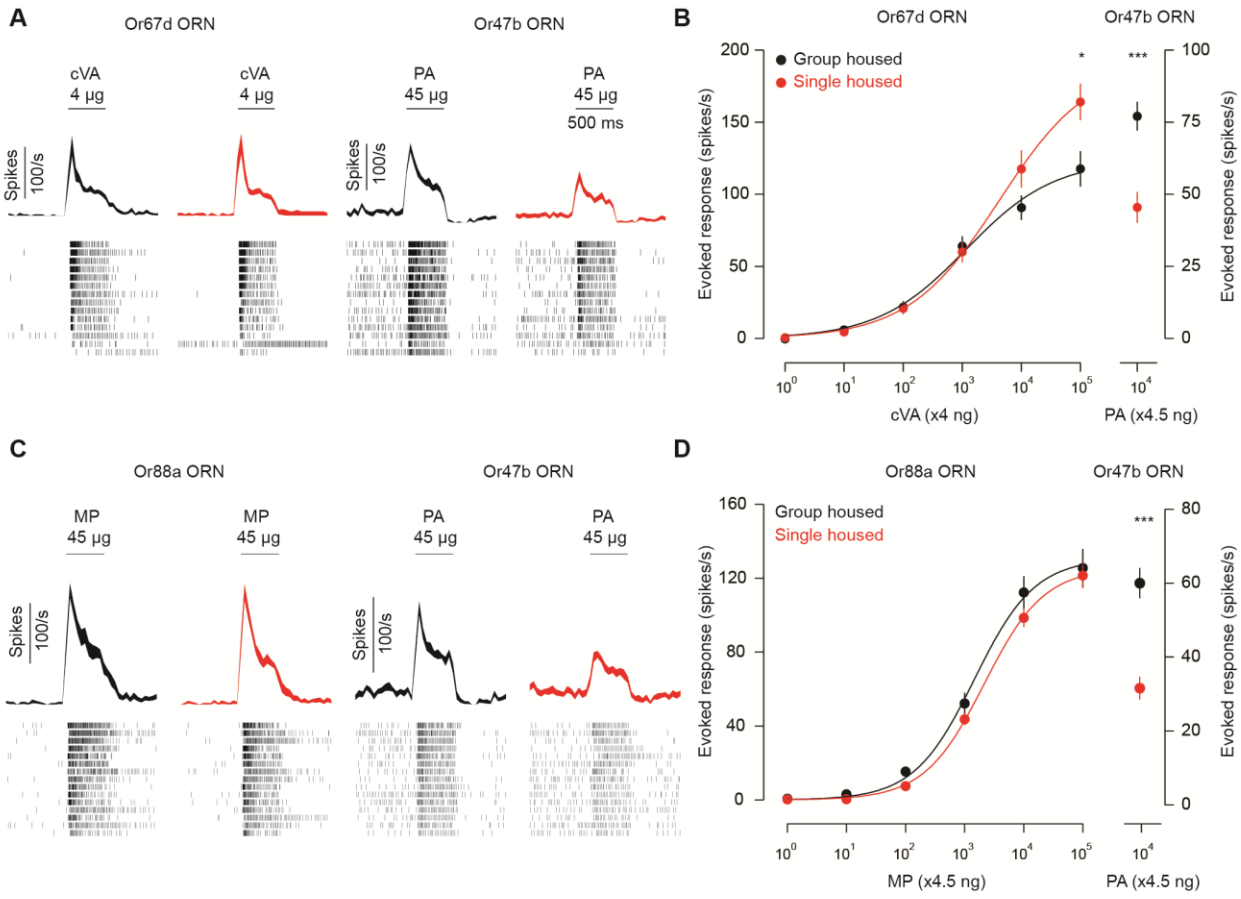


Figure 2S.3. Group housing does not enhance pheromone response of Or67d or Or88a ORNs.

(A, B) Group housing enhances odor evoked response in Or47b neurons but not in Or67d neurons (n=14, Berlin WT male flies, grouped 15 flies per vial, two-tailed unpaired t-test, Or47b neurons: t=4.33, p=0.0002, Or67d neurons: t=1.7291, p=0.1, one recording each from both neurons per fly). **(C, D)** Group housing enhances odor evoked response in Or47b neurons but not in Or88a neurons (n=15, Berlin WT male flies, grouped 15 flies per vial, two-tailed unpaired t-test, Or47b neurons: t=5.543, p<0.0001, Or67d neurons: t=1.3492, p=0.19, one recording each from both neurons per fly). Error bars indicate SEM. cVA - cis-vaccenyl acetate, MP – methyl palmitate, PA – palmitoleic acid.

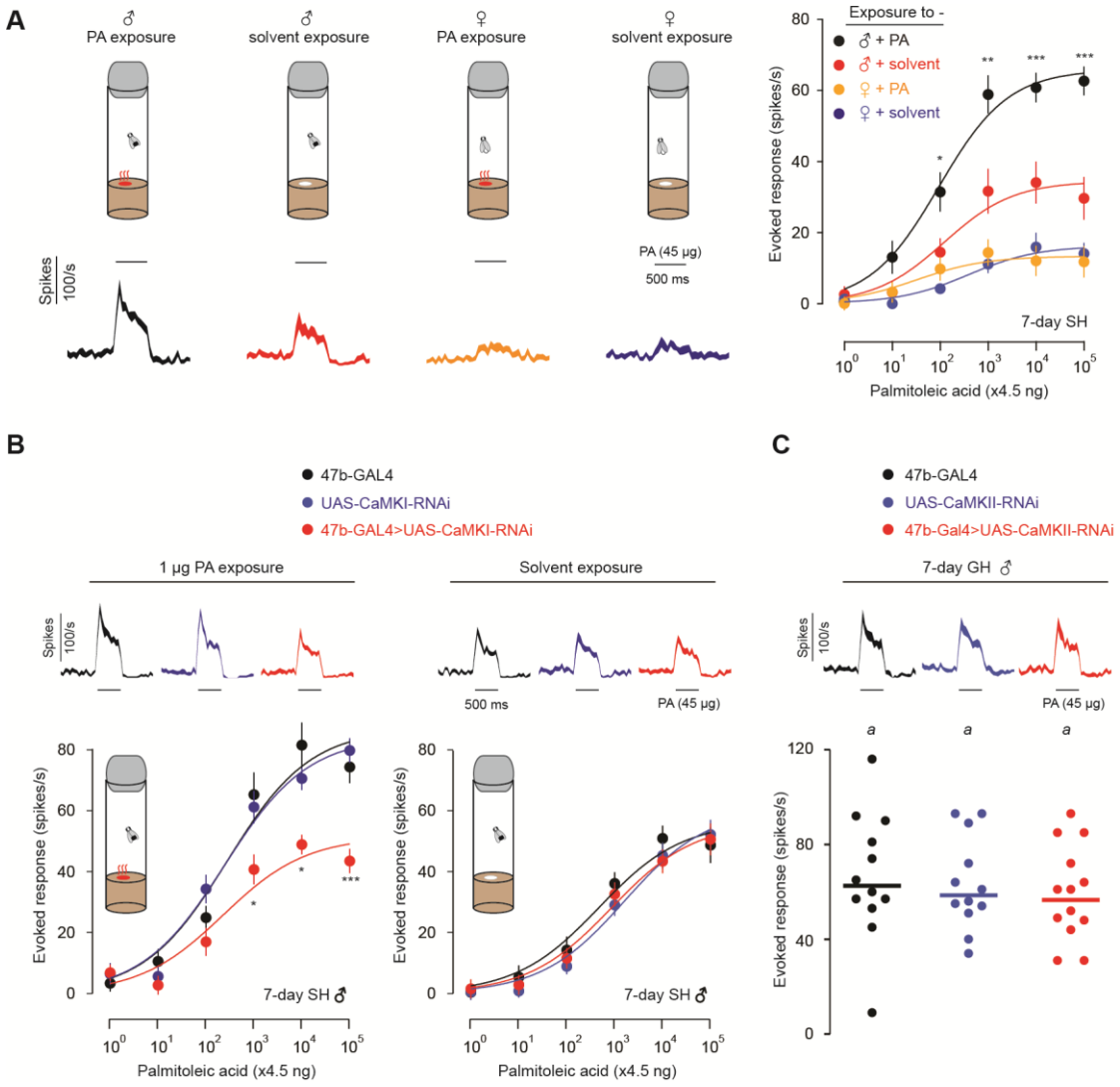


Figure 2S.4. CaMKI, but not CaMKII, is required for the effect of group housing on Or47b response in mature males.

(A) Chronic exposure to 1 µg PA enhances Or47b response in 7-day old WT SH males but not females. n=14 flies for each condition. *, p<0.05; **, p<0.01; ***, p<0.001, denotes significant difference for a given odor concentration, determined by two-way (A, B) or one-way (C) ANOVA followed by Tukey's post-hoc test. Error bars indicate SEM. **(B)** CaMKI mutation suppresses Or47b odor response in 7-day GH males. n=14 flies for each condition. **(C)** RNAi-mediated knockdown of CaMKII does not alter Or47b odor response in 7-day GH males, one-way ANOVA with Tukey's post-hoc test (F=0.284, p=0.75). n=12, for each genotype. Odor stimulation: 45 µg PA. GH: flies were raised in groups of 15 per vial. SH: flies were raised individually with each vial containing one fly.

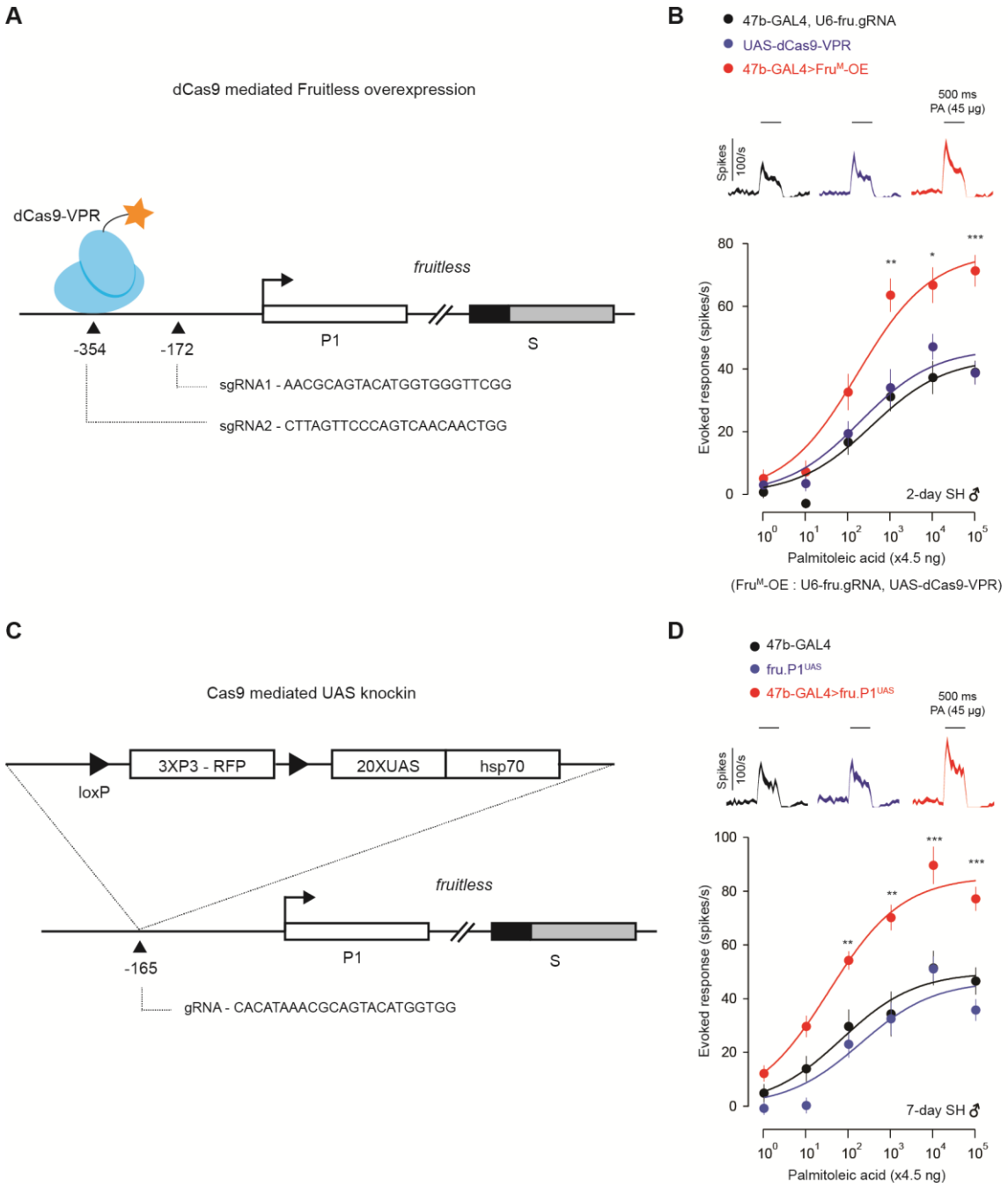


Figure 2S.5. Fru^M overexpression enhances odor response in SH males.

(A) dCas9-mediated overexpression of Fru^M. Two single-guide RNAs (sgRNAs) target a dead Cas9 molecule fused to the transcriptional activator VPR to the fruitless P1 promoter. **(B)** dCas9-mediated overexpression of Fru^M in Or47b neurons enhances odor response in 2-day SH males. n=14 flies per genotype. *, p<0.05; **, p<0.01; denotes significant difference for a given odor concentration, determined by one-way ANOVA followed by Tukey's post-hoc test **(B, D)**. Error bars indicate SEM. **(C)** Cas9-mediated knock-in strategy to insert 20XUAS-hsp70 cassette upstream of the fruitless P1 promoter which allows GAL4 mediated overexpression from the endogenous locus. 3XP3-RFP was used as a positive selection marker. **(D)** UAS-knockin-mediated overexpression of Fru^M in Or47b neurons enhances odor response in 7-day SH males. n=13 flies per genotype.

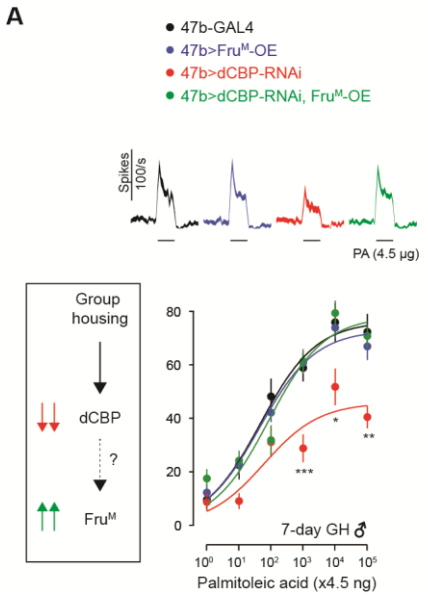


Figure 2S.6. Fru^M expression is downstream of dCBP signaling in Or47b neurons.

(A) Fru^M overexpression can enhance odor responses in GH males even in the absence of dCBP signaling. n=14 flies per condition. Fru^M overexpression: blue, RNAi mediated dCBP knockdown: red, both Fru^M overexpression and dCBP-RNAi: green. *, $p<0.05$, **, $p<0.01$ denotes significant difference for a given odor concentration, determined by one-way ANOVA followed by Tukey's post-hoc test. Error bars indicate SEM.

Appendix 2.2: Supplemental Tables

Table 2.1. List of fly genotypes and raising conditions by experiment

Figure	Genotype	Genetic background	Experimental conditions
2.1 A, B	Wild-type	Berlin	All flies are 7-days old. Group-housed males and females raised in same-sex groups of 15 flies per vial. Single housed flies were raised in isolation- one fly per vial.
2.1 C, D	Wild-type	Berlin	All flies are 7-days old. Group-housed males and females raised in same-sex groups with indicated number of flies per vial.
2.1 E, F	Wild-type	Berlin	Age of flies indicated in figure. Group-housed males - 15 flies per vial. Single housed males - one per vial.
2.1 H	Male - Wild-type or w*; *Or47b2[mw+]/Or47b2* [mw+]; + Female - Wild-type	Male - Berlin ; Female - Canton-S	Age of male flies indicated in figure. Group-housed males - 15 flies per vial. Single housed males - one per vial. Female flies were 2-days old and raised in group housed condition.
2.1 I	Wild-type	Berlin	Age of male flies indicated in figure. Flies were either single housed or group housed for 2 days. Group-housed males - 15 flies per vial. Single housed males - one per vial.
2.2 A, B	Wild-type	Berlin	All male flies are 7-days old. Male flies were single housed with 1 µg PA or solvent from eclosion to experiment.
2.2 C, D	Wild-type	Berlin	Age of flies indicated in figure. All flies were single housed with odor of indicated dosage from eclosion to experiment.
2.2 E	Male - Wild-type or w*; *Or47b2[mw+]/Or47b2* [mw+]; + Female - Wild-type	Male - Berlin ; Female - Canton-S	All male flies are 5-days old. Male flies were single housed with 1 µg PA or solvent from eclosion to experiment. Female flies were 2-days old and raised in group housed condition.
2.3 A	+; LexAop-6xGFP/+; Or47b-GAL4/10XUAS-mLexA-VP16-NFATdC	Berlin	Age of flies indicated in figure. Group-housed males - 15 flies per vial. Single housed males - one per vial.
2.3 B	Wild-type or + ; + ; + ; CaMKI[EY07197]/+ or + ; + ; + ; CaMKI[EY07197]	Unknown	All flies were group-housed 7-day old males. Group-housed males - 15 flies per vial.
2.3 C	+; + ; Or47b-GAL4/UAS-CaMKI-RNAi or	Berlin	All flies are 7-days old. Group-housed males - 15 flies per vial. Single housed males - one per vial.

	+ ; + ; 47b-GAL4/+ or + ; + ; UAS-CaMKI-RNAi/+		
2.3 D	w*, UAS-dCBP-RNAi ; + ; 47b-GAL4/+ or w*, UAS-dCBP-RNAi ; + ; + or + ; + ; 47b-GAL4/+	Berlin	All flies are 7-days old. Group-housed males - 15 flies per vial.
2.3 E	GAL4/UAS: w*, UAS-dCBP-RNAi ; + ; 47b-GAL4/+ or UAS: w*, UAS-dCBP-RNAi ; + ; + or GAL4: + ; + ; 47b-GAL4/+	Male - Berlin ; Female - Canton-S	All flies are 7-days old. Group housed males - 15 flies per vial. Single housed males - one per vial. Female flies were 2-days old and raised in group housed condition.
2.4 A	+ ; + ; 47b-GAL4/UAS-Met-RNAi or + ; + ; UAS-Met-RNAi/+ or + ; + ; 47b-GAL4/+	Berlin	All flies are 7-days old. Male flies were single housed with 1 µg PA or solvent from eclosion to experiment.
2.4 B	Wild-type	Berlin	Flies of indicated age were single housed with PA of indicated dosage or solvent from eclosion to experiment.
2.4 C	Wild-type	Berlin	All flies are 2-days old. Group housed males - 15 flies per vial. Single housed males - one per vial. Flies were housing in vials containing methoprene of indicating dosage on the surface of fly food from eclosion to experiment.
2.4 D	Wild-type	Berlin	All flies are 2-days old single housed males. Flies were housed with 10 µg PA or solvent from eclosion to experiment. Flies were housing in vials containing methoprene of indicating dosage on the surface of fly food from eclosion to experiment.
2.4 E	GAL4/UAS: w*, UAS-dCBP-RNAi ; + ; 47b-GAL4/+ or UAS: w*, UAS-dCBP-RNAi ; + ; + or GAL4: + ; + ; 47b-GAL4/+	Berlin	All flies are 2-days old, Group housed males - 15 flies per vial. Flies were housing in vials containing methoprene of indicating dosage on the surface of fly food from eclosion to experiment.
2.4 F	Wild-type	Berlin	All flies are 2-days old single housed males. Flies were housed with varying amounts of PA and methoprene from eclosion to experiment.
2.5 A	ey-FLP; UAS-GFP/Tb-(FRT-GAL80-FRT)-STOP; fru.p1.GAL4/+	Unknown	All flies were group-housed 7-day old males.
2.5 B	+ ; UAS-tra.F/+ ; 47b-GAL4/+ or	Berlin	All flies were group-housed 7-day old males.

	+ ; + ; 47b-GAL4/+ or + ; UAS-tra.F/+ ; +		
2.5 C	Wild-type or + ; + ; fru[F]/+ or + ; + ; fru[F]	Berlin	All flies were group-housed 7-day old males.
2.5 D	+ ; + ; 47b-GAL4/UAS-fru-miRNA1 or + ; + ; UAS-fru-miRNA1/+ or + ; + ; 47b-GAL4/+	Berlin	All flies are 7-days old. Group housed males - 15 flies per vial. Single housed males - one per vial.
2.5 E	+; U6-fru-sgRNA/UAS-dCas9-VPR; 47b-GAL4/+ or +; U6-fru-sgRNA/+; 47b-GAL4/+ or +; UAS-dCas9-VPR/+ ; +	Berlin	All flies were single-housed 7-day old males.
2.6 A	+ ; UAS-tra.F/+ ; 47b-GAL4/+ or + ; + ; 47b-GAL4/+ or + ; UAS-tra.F/+ ; +	Berlin	All flies are 2-days old, Group housed males - 15 flies per vial. Flies were housing in vials containing methoprene of indicating dosage on the surface of fly food from eclosion to experiment.
2.6 B	+ ; 47b-GAL4/+; + or +; U6-fru-sgRNA/47b-GAL4; UAS-dCas9-VPR/+ or +; 47b-GAL4/+ ; Met-RNAi/+ or +, U6-fru-sgRNA/47b-GAL4; UAS-dCas9-VPR/UAS-Met-RNAi	Berlin	All flies were group-housed 7-day old males.
2.6 C	+ ; + ; 47b-GAL4/UAS-Met-RNAi or + ; + ; UAS-Met-RNAi/+ or + ; + ; 47b-GAL4/+	Berlin	All flies are 7-days old. Group housed males - 15 flies per vial.
2S.1	Wild-type	Berlin	All flies are 7-days old. Group housed males - 15 flies per vial. Single housed males - one per vial.
2S.2 A	Wild-type	Berlin	All flies are 7-days old. Group housed males and females raised in same-sex groups with indicated number of flies per vial.
2S.2 B	Wild-type	Berlin	Age of flies indicated in figure. Group housed males - 15 flies per vial. Single housed males - one per vial.
2S.2 C	Wild-type	Berlin	All flies are 7-days old. Flies were single housed for varying durations, then switched to group housing vials.

2S.3	Wild-type	Berlin	All flies are 7-days old. Group housed males - 15 flies per vial. Single housed males - one per vial.
2S.4 A	Wild-type	Berlin	All flies are 7-days old single housed with 1 µg PA or solvent from eclosion to experiment.
2S.4 B	+; + ; Or47b-GAL4/UAS-CaMKI-RNAi or + ; + ; 47b-GAL4/+ or + ; + ; UAS-CaMKI-RNAi/+	Berlin	All male flies are 7-days old. Male flies were single housed with 1 µg PA or solvent from eclosion to experiment.
2S.4 C	+; + ; Or47b-GAL4/UAS-CaMKII-RNAi or + ; + ; 47b-GAL4/+ or + ; + ; UAS-CaMKII-RNAi/+	Berlin	All flies were group-housed 7-day old males.
2S.5 B	+; U6-fru-sgRNA/UAS-dCas9-VPR; 47b-GAL4/+ or +; U6-fru-sgRNA/+; 47b-GAL4/+ or +; UAS-dCas9-VPR/+ ; +	Berlin	All flies were single-housed 2-day old males.
2S.5 D	+ ; + ; 47b-GAL4/fru.P1.UAS or + ; + ; fru.P1.UAS/+ or + ; + ; 47b-GAL4/+	Berlin	All flies were single-housed 7-day old males.
2S.6 A	+ ; + ; 47b-GAL4/+ or +; U6-fru-sgRNA/+; UAS-dCas9-VPR/47b-GAL4 or w*, UAS-dCBP-RNAi ; + ; 47b-GAL4/+ or w*, UAS-dCBP-RNAi; U6-fru-sgRNA/+; UAS-dCas9-VPR/47b-GAL4	Berlin	All flies were group-housed 7-day old males.

Chapter III.
A versatile genetic tool for post-translational control of gene expression in *Drosophila melanogaster*

"Every once in a while, a new technology, an old problem, and a big idea turn into an innovation."

— Dean Kamen

3.1. Abstract

Several techniques have been developed to manipulate gene expression temporally in intact neural circuits. However, the applicability of current tools developed for *in vivo* studies in *Drosophila* is limited by their incompatibility with existing GAL4 lines and side effects on physiology and behavior. To circumvent these limitations, we adopted a strategy to reversibly regulate protein degradation with a small molecule by using a destabilizing domain (DD). We show that this system is effective across different tissues and developmental stages. We further show that this system can be used to control *in vivo* gene expression levels with low background, large dynamic range, and in a reversible manner without detectable side effects on the lifespan or behavior of the animal. Additionally, we engineered tools for chemically controlling gene expression (GAL80-DD) and recombination (FLP-DD). We demonstrate the applicability of this technology in manipulating neuronal activity and for high-efficiency sparse labeling of neuronal populations.

3.2. Introduction

Tools for precise spatial and temporal control of gene expression are essential for understanding how neuronal circuits develop and function. For example, limiting genetic manipulation of a target gene to a specific time and a defined neuronal population permits the

separation of the developmental role of the gene from its contribution to circuit function in the adult stage. In the vinegar fly, *Drosophila melanogaster*, bipartite expression systems (GAL4/UAS, LexA/LexAop, QF/QUAS) provide a powerful means to control gene expression in a spatially selective manner (Brand and Perrimon, 1993; Lai and Lee, 2006; Potter et al., 2010). Several modifications of these expression systems have been made to permit temporal control over the exogenous transcription factors (GAL4, LexA or QF) (Chan et al., 2015; McGuire et al., 2003; Osterwalder et al., 2001; Potter et al., 2010). Much of the effort has been focused on using temperature or chemicals as means to control the gene expression systems. Temperature-dependent expression systems have been previously engineered by the direct fusion of a heat-inducible promoter to a gene of interest (Lis et al., 1983), or by using a temperature sensitive allele of GAL80, $GAL80^{ts}$. In the GAL4/UAS system, $GAL80^{ts}$ suppresses GAL4-induced gene expression at a low temperature (18 °C) but not at a high temperature (29 °C) (McGuire et al., 2003). Chemical-dependent tools include tetracycline-inducible systems (Bello et al., 1998; Bieschke et al., 1998), steroid hormone-inducible GAL4/LexA hormone receptor chimeras (Han et al., 2000; Osterwalder et al., 2001; Roman et al., 2001) and the quinic acid-inducible QS/QF/QUAS system (Potter et al., 2010). For example, an RU486-inducible GAL4 was made by fusing the GAL4 DNA-binding domain to the human progesterone receptor and the p65 transcriptional activation domain (Roman et al., 2001). However, there are limitations associated with the existing tools for temporal control of gene expression. First, temperature can have a significant impact on the physiology and behavior of a fly (Parisky et al., 2016; Sigrist et al., 2003), which may prevent detection of the phenotype of interest. For example, temperature-dependent tools are unlikely to be suitable for the study of thermosensory circuits and related behaviors. Second, temporal control of gene expression using $GAL80^{ts}$ can only be achieved with transcription factors containing the GAD-activation domain, making it incompatible with a majority of the driver lines (LexA, QF and split-GAL4) that do not have the GAD domain. Third, application of the current chemical-dependent tools requires the generation

of new transgenic stocks, such as new promoter-GAL4 lines. Additionally, RU486, a chemical used to induce gene expression in the Geneswitch system, has been reported to cause developmental lethality in flies with pan-neuronal expression of the RU486-sensitive GAL4 (Li and Stavropoulos, 2016).

We propose an alternative chemically inducible system, in which gene expression is controlled at the post-translational stage, making it compatible with the existing library of GAL4 stocks. We adopted the destabilizing domain (DD) derived from dihydrofolate reductase (ecDHFR) of *E. coli* to control protein stability in a ligand-inducible manner (Cho et al., 2013; Iwamoto et al., 2010), a strategy that has been used to control gene expression in mice and worms (Cho et al., 2013; Iwamoto et al., 2010; Sando et al., 2013). On fusing the destabilizing domain to a protein of interest, the chimeric protein is degraded by the proteasome, but its degradation is blocked by trimethoprim (TMP), a cell-permeable ligand for DD (**Figure 3.1A**) (Iwamoto et al., 2010). Thus, the protein of interest can be temporally controlled by TMP. A recent study demonstrated that TMP can regulate activity of the yeast I-SceI endonuclease in *Drosophila* larvae expressing the fusion protein I-SceI-DD (Janssen et al., 2016). Here, we characterized the efficiency and dynamics of this technology *in vivo* in the fly brain. We further use the DD technology to develop tools for mapping and manipulating neural circuits in *Drosophila*. As a proof of its utility, we fused DD to GAL80 and controlled GAL4-dependent gene expression in a TMP-dependent manner. We show that TMP can activate GAL80-DD to manipulate neuronal activity in behaviorally relevant sensory neurons. Additionally, by fusing DD to the FLP recombinase, we devised a strategy to control the recombination frequency within a neuronal population by controlling the concentration of TMP in fly food. We further used the destabilized FLP recombinase to refine the expression pattern arising from the intersection of two transgenic lines by temporally limiting the availability of TMP. In summary, we present a chemically inducible system optimized for neurogenetics in *Drosophila* with broader utility than comparable conventional tools.

3.3. Results

Destabilized GFP. We first tested whether the ecDHFR-derived destabilizing domain (DD) can be used to control GFP expression levels. DD was genetically fused to the C-terminus of GFP and cloned into a 10XUAS construct (**Figure 3.1A, Figure 3S.1**) to make *UAS-GFP-DD* transgenic flies. We reasoned that GFP expression should be conditioned on both the presence of the transcriptional activator GAL4 and the availability of the stabilizing ligand TMP. The expression of GAL4 in select neuronal populations affords spatial specificity. Feeding these flies with TMP at a specific time could provide a temporal control of GFP expression. Using the pan-neuronal *nsyb-GAL4* to drive GFP-DD expression, we observed robust GFP expression throughout the brain of adult flies fed with TMP (**Figure 3.1B**). In the absence of TMP, GFP expression was low throughout the brain; this is consistent with the previous studies in mice and nematodes showing that unbound DD is an effective tag to mark the fusion protein for degradation (Cho et al., 2013; Sando et al., 2013) (**Figure 3.1B**). However, TMP levels may start to decline at the beginning of pupation, during which flies do not feed. To determine the efficiency of TMP-dependent GFP-DD stabilization during development, we measured GFP-DD expression throughout the brain from the larval to the adult stage (**Figure 3S.2A**). We found substantial differences in GFP-DD expression between flies fed with solvent versus TMP during the larval (44 fold), 48 hr APF (19 fold) and three day old adult (27 fold) stages. Notably, even though flies do not feed for several days leading up to the late pupal stage (96 hr APF) and eclosion (<12 hr adult), we observed a five-fold difference in GFP-DD expression between flies raised on solvent versus TMP (**Figure 3S.2A**). In summary, the TMP-inducible DD system is maximally effective during the larval, early pupal and adult stages. During the late pupal stage, however, the utility of the system may be limited by unavailability of TMP.

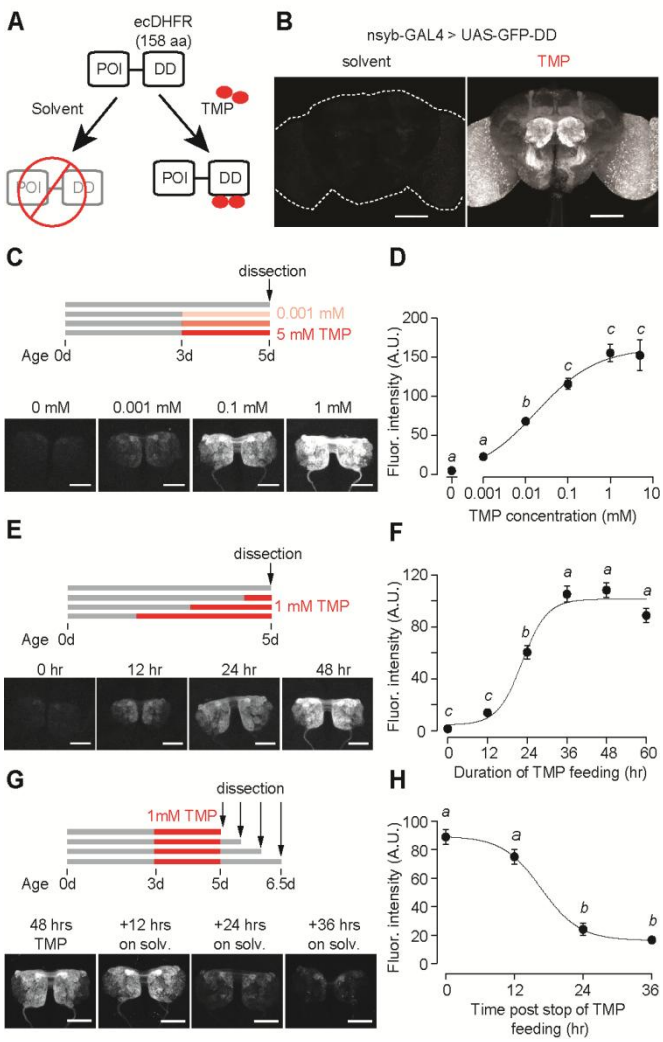


Figure 3.1. GFP-DD expression and degradation kinetics.

(A) Schematic showing the destabilizing domain (DD) system. ecDHFR = *E. coli.* Dihydrofolate reductase, POI = protein of interest, TMP = Trimethoprim. (B) TMP-dependent GFP expression in the adult brain. Flies were fed 1 mM TMP-containing food from embryo stage up to dissection. (C, D) Dose-dependent change in GFP-DD expression in the axonal terminals of olfactory sensory neurons. *Orco-Gal4, UAS-GFP-DD* flies were fed with TMP (0 – 5 mM) for 48 hours before dissection (n=5-6, p<0.001, F=41.37, one-way ANOVA with Tukey's post-hoc test). (E, F) GFP-DD expression is dependent on duration of TMP feeding. All flies were fed with fly food containing 1 mM TMP (n=5-6, p<0.001, F= 87.34, one-way ANOVA with Tukey's post-hoc test). (G, H) GFP degradation kinetics. Flies were fed with 1 mM TMP for 48 hours and then switched to standard fly food. GFP-DD expression in the antennal lobe was observed at 12-hour intervals following the switch. (n=8-10, p<0.001, F=71.43, one-way ANOVA with Tukey's post-hoc test). Error bars indicate SEM. Significant differences between conditions (p<0.05) are denoted by different letters. Scale bar = 100 μm (B), 50 μm (C, E, G).

Because TMP-dependent protein stabilization acts through post-translational modification of protein levels, it should be compatible with any GAL4, LexA, QF or split-GAL4 line. To illustrate this principle, we visualized TMP-dependent GFP-DD expression across several different cell types in the adult brain using previously characterized driver lines (**Figure 3S.3A**). We observed a significant difference in GFP-DD expression (ranging from 4 to 64 fold) between solvent and TMP feeding for every cell type that we tested. Maximum differences were observed in the axon terminals of sensory neurons (at least 45 fold). Interestingly, different cell types innervating the same anatomical region showed differential susceptibility to the DD system. For example, olfactory sensory neurons showed a much larger difference between TMP and solvent feeding (45 fold) compared to olfactory projection neurons (6 fold), even though both cell types innervate the antennal lobe. Similarly, PAM dopamine neurons showed a larger difference in GFP-DD expression (12 fold) compared to Kenyon cells (4 fold), even though both cell types innervate the mushroom body. Based on these results, we conclude that at least some of the differences between cell types arise from the variability in their proteasome activity levels. We also observed that the DD system was effective in non-neuronal tissues, such as ovaries (**Figure 3S.3B**). Finally, to demonstrate that the DD system can be used in combination with other binary systems apart from GAL4/UAS, we generated a *13XLexAop-GFP-DD* transgenic fly line and observed similar TMP-dependent GFP expression in olfactory sensory neurons using the *Orco-LexA* driver line (**Figure 3S.3D**).

We next carried out experiments to determine the kinetics and dynamic range of this chemical induction system using *Orco-GAL4* to drive GFP-DD expression in olfactory sensory neurons. Feeding adult flies with food containing varying concentrations (0-5 mM) of TMP for 48 hours resulted in a dose-dependent change in GFP expression in the antennal lobe (**Figure 3.1C, D**). The maximum GFP expression, induced by 1 mM TMP, was between 34 and 45 times higher than that of control flies fed with the solvent-containing food (**Figure 3.1D, Figure 3S.3A**). Results from an experiment in which flies were fed for varying durations (0-60 hrs) with

food containing 1 mM TMP show that GFP levels increase initially but plateau within 36 hours (**Figure 3.1E, F**). To test if TMP-dependent GFP expression is reversible, we fed flies with food containing 1 mM TMP for 48 hours and then switched them to regular food (**Figure 3.1G**). We found that the GFP intensity was reduced by 73% within 24 hours (**Figure 3.1H**). In sum, using GFP as a test molecule, we show that genetic fusion of the ecDHFR-derived destabilizing domain confers instability to a protein of interest in *Drosophila*. Feeding flies with TMP can control protein levels in a reversible and dose-dependent manner with a large dynamic range.

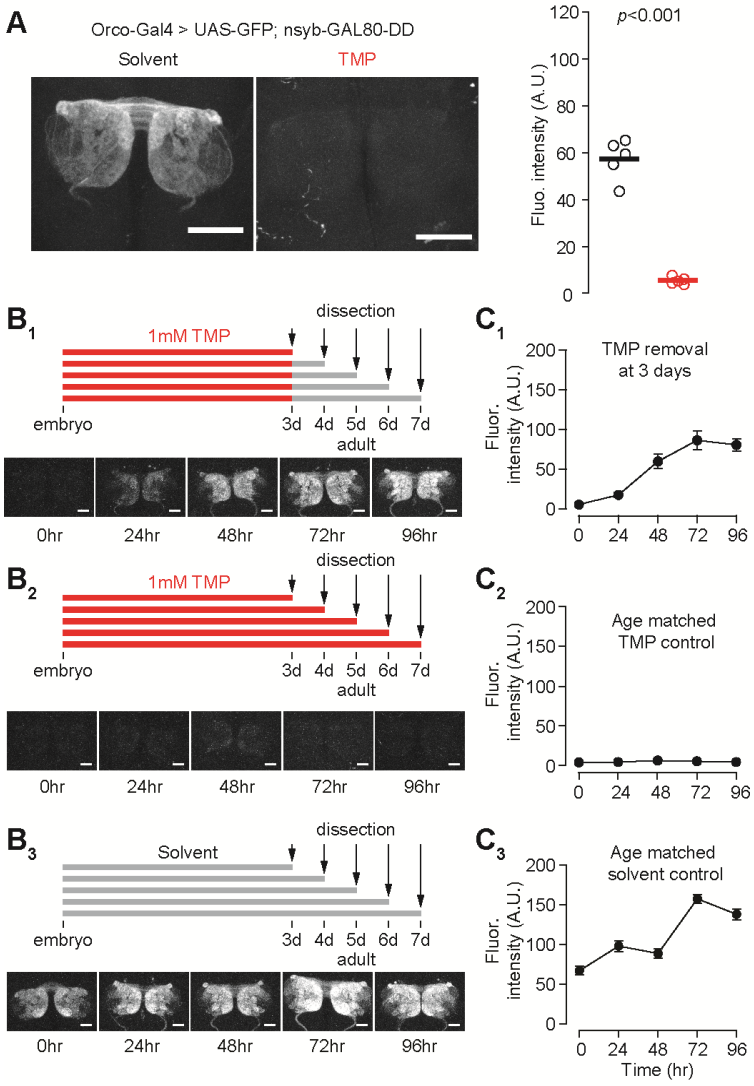
We then investigated whether TMP has adverse effects on survival and behavior. Feeding adult flies with a defined medium containing TMP ranging from 0 to 10 mM did not have any detectable effect on their survival (**Figure 3S.4A**). Moreover, feeding adult flies with 1 mM TMP for 48 hours did not alter their locomotion speed (**Figure 3S.4C**) or their ability to locate an odor source (**Figure 3S.4D**) during foraging. We then tested if TMP affects the development of the fly. Flies raised on TMP ranging from 0 to 10 mM throughout development were equally likely to survive to adulthood (**Figure 3S.4B**). However, we observed a delay in the developmental time for flies raised on 1 mM TMP (11.2 +/- 0.1 days to eclosion) and 10 mM TMP (15.3 +/- 0.3 days) as compared to solvent (10.2 +/- 0.1 days) (**Figure 3S.4B**). In summary, feeding adult flies TMP at 1 mM can induce GFP-DD expression at saturation level (**Figure 3.1D**) without any observable side effects on their survival or behavior. Notably, however, the same dosage of TMP delays development by about one day. Thus, we settled on the concentration of 1 mM TMP for further experiments except for cases when a lower level of induction was desirable.

Destabilized GAL80. To evaluate the utility of the DD system for manipulating circuit function, we next investigated whether expression of GAL80 could be controlled by TMP. Binding of GAL80 to GAL4 prevents GAL4-mediated transcriptional activation in the GAL4/UAS expression system (Lee and Luo, 1999). We engineered a chemically inducible GAL80 by fusing DD to the C-terminus of GAL80. GAL80-DD was cloned downstream of a pan-neuronal

promoter, *n-synaptobrevin* (*nsyb*). Addition of GAL80-DD to the GAL4/UAS expression system could allow TMP to control gene expression. Indeed, we found that *nsyb-GAL80-DD* was able to suppress GAL4-dependent GFP expression in olfactory sensory neurons (**Figure 3.2A**). This suppression of GFP expression in flies carrying the *Orco-GAL4*, *UAS-GFP* and *nsyb-GAL80-DD* transgenes was TMP-dependent (**Figure 3.2A**). This feature can be used to control gene expression to perturb neuronal function in a stage-dependent manner. For example, RNAi expression could be targeted to specific neurons during the adult stage by removing TMP from the food, which causes the degradation of GAL80. As a proof-of-concept experiment, we fed flies with TMP throughout development and up to 3 days post-eclosion (**Figure 3.2B, C**). When flies were moved from TMP-containing food to regular food, GFP expression started to increase after 24 hours, and peaked at 72 hours post-TMP removal (**Figure 3.2B$_1$, C$_1$**). In contrast, flies fed with TMP continuously, from embryo to adult, showed low GFP expression throughout the course of the experiment (**Figure 3.2B$_2$, C$_2$**). Furthermore, flies that were raised on regular fly food throughout showed high GFP expression (**Figure 3.2B$_3$, C$_3$**). To determine how soon after eclosion it is possible to activate gene expression, we raised flies on TMP during development up to eclosion and measured GFP expression in the brains of young adult flies (**Figure 3S.5A**).

Figure 3. 2. Chemically inducible control of GAL4-dependent expression using destabilized GAL80.

(A) GAL4-driven GFP expression in olfactory sensory neurons can be suppressed by destabilized GAL80 (*nsyb-GAL80-DD*) in a TMP-dependent manner (n=5, unpaired t-test, two-tailed, t=13.25). (B, C) GAL80-DD can be used to temporally control GFP expression. (B_1, C_1) *Orco-Gal4, UAS-GFP, nsyb-GAL80-DD* flies were fed with food containing 1 mM TMP up to 3 days post-eclosion, following which flies were switched to standard fly food and dissected for quantification. GFP expression was compared to flies fed with 1 mM TMP throughout (B_2, C_2) or solvent throughout (B_3, C_3) (n=4-5). 0 hour time point in C_1 and C_2 represent the same sample. (D) Tetanus toxin expression in the V antennal lobe glomerulus of flies fed with 1 mM TMP or solvent. (E) CO_2 avoidance index of flies fed with 1 mM TMP or solvent. One arm of the T-maze contained 0.28% (v/v) CO_2 and the other arm had air. GAL80-DD can restore CO_2 aversion by suppressing TNT expression in the presence of TMP. n=11 per condition, two-way ANOVA indicated a significant interaction between feeding condition and genotype, F=23.66, p<0.001. Significant differences between conditions (p<0.05) are denoted by different letters (Tukey's post-hoc test). All flies were between 4-7 days old. Error bars indicate SEM. Scale bar = 50 μm (A), 25 μm (B,D).


A
Orco-Gal4 > UAS-GFP; nsyb-GAL80-DD
Solvent
TMP
p<0.001
Fluo. intensity (A.U.)
B1
1mM TMP
dissection
embryo
3d 4d 5d 6d 7d
adult
0hr 24hr 48hr 72hr 96hr
C1
TMP removal at 3 days
Fluor. intensity (A.U.)
B2
1mM TMP
Age matched TMP control
B3
Solvent
Age matched solvent control
Time (hr)
D
Anti-TNT
Gr21a-Gal4
UAS-TNT, nSyb-GAL80-DD
Gr21a-Gal4 UAS-TNT, nSyb-GAL80-DD
Solvent
TMP
E
Avoidance index
a
a
b
a
c
a,b

We observed a difference between flies raised on TMP and those switched to solvent-containing food starting at one day post-eclosion (**Figure 3S.5B, C**). However, we did not detect any GFP expression in flies younger than eight hours for either condition (**Figure 3S.5B, C**). This lack of GFP expression even in the absence of TMP is most likely due to the time that is required to inactivate GAL80-DD upon TMP withdrawal (**Figure 3.1H**), although it is formally possible that the expression level of *Orco-Gal4* is low at eclosion. Either way, these results suggest that fusion of the ecDHFR-derived destabilizing domain to GAL80 permits TMP to control GAL80 activity, providing a chemically inducible system to control gene transcription in a temporal manner.

We further tested if GAL80-DD can be used to manipulate neuronal activity underlying behavior. We focused on the innate olfactory aversion to CO_2 in a T-maze assay. Olfactory aversion to CO_2 can be abolished by silencing Gr21a-expressing sensory neurons (Suh et al., 2004). We controlled the expression of tetanus toxin, a potent inhibitor of synaptic transmission (Sweeney et al., 1995), in Gr21a neurons using *nsyb-GAL80-DD* and TMP feeding (**Figure 3.2D**). *Gr21a-GAL4*-derived tetanus toxin expression was blocked in the presence of GAL80-DD when flies were fed TMP, but not when they were fed the solvent (**Figure 3.2D**). Accordingly, aversion to CO_2 was observed only when flies expressing *Gr21a-GAL4, UAS-TNT, nsyb-GAL80-DD* were fed with TMP, and not when they were fed with the solvent (**Figure 3.2E**). In comparison, control flies with GAL4 alone had high avoidance for both solvent and TMP feeding conditions. We observed a difference between solvent- and TMP-fed flies expressing *UAS-TNT, nsyb-GAL80-DD* (**Figure 3.2E**). However, the avoidance indices of both solvent- and TMP-fed *UAS-TNT, nsyb-GAL80-DD* flies were significantly higher than the avoidance index of *Gr21a-GAL4, UAS-TNT, nsyb-GAL80-DD* flies fed with the solvent (**Figure 3.2E**). In summary, we show that GAL80-DD can be used to manipulate GAL4-dependent expression of neuronal effectors and thereby alter the function of neuronal circuits underlying behavior.

Destabilized flippase. Flippase-mediated removal of a stop cassette has been widely used for lineage analysis and sparse neuronal labeling (Lee and Luo, 1999; Marin et al., 2002; Wong et al., 2002). Lineage analysis requires transient high-level expression of flippase (FLP) at specific developmental stages. On the other hand, sparse neuronal labeling requires low-level FLP expression in post-mitotic neurons for the stochastic removal of a stop cassette. Owing to the large dynamic range of the DD system, we reasoned it could be used to control FLP expression at different levels by varying TMP concentrations in fly food, thereby

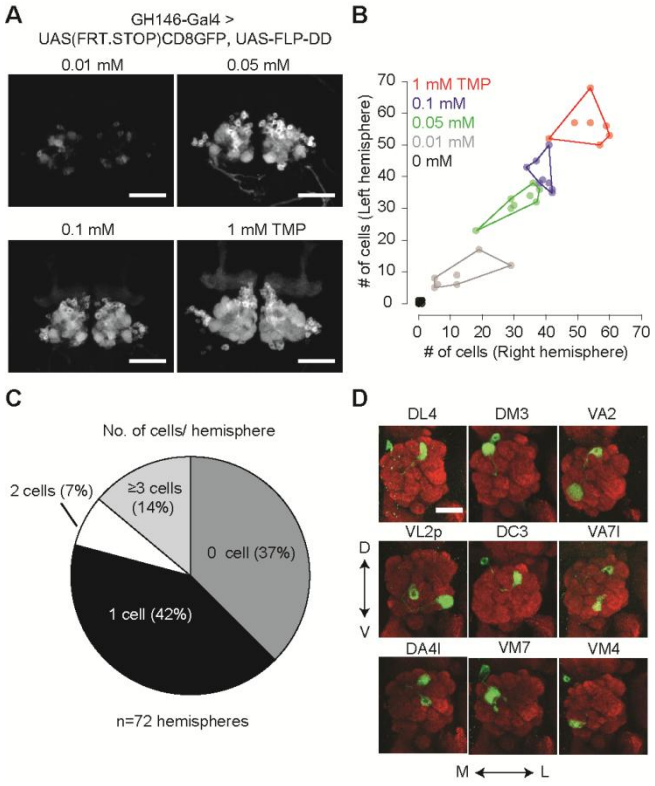


Figure 3.3. Chemical control of recombination frequency using destabilized flippase.

(A) GFP expression in a sub-population of olfactory projection neurons following excision of the STOP cassette by FLP-DD. Scale bar = 50 μm. (B) Number of GFP-positive projection neurons can be controlled by varying TMP dosage. The number of GFP-labeled cells within a sample is similar across both brain hemispheres. Each point represents number of cells in one brain. (C) Pie chart indicating the number of labeled projection neurons for flies fed with solvent. 42% of all hemispheres had a single GFP-labeled cell. (D) Examples of labeled single projection neurons (D - dorsal, V - ventral, M - medial, L - lateral). Red = anti-Bruchpilot nc82, Green = GFP. Scale bar = 25 μm.

accommodating both sparse labeling and lineage mapping. The heat-shock promoter has been used previously to drive different levels of FLP expression by varying the duration of the heat-shock pulses. However, heat-shock driven FLP activity cannot be limited to a subset of cells due to the ubiquitous expression of the heat shock promoter. This limitation restricts the utility of *hs-FLP* for lineage analysis in an intersectional manner.

We fused DD to the C-terminus of FLP and incorporated the coding sequence into a 10XUAS construct (*10XUAS-FLP-DD*). We tested the destabilized flippase in olfactory projection neurons using *GH146-GAL4* to drive *UAS-FLP-DD* and a GFP stop-cassette reporter, *UAS(FRT.STOP)CD8GFP*. In these flies, stabilization of FLP-DD by TMP should permit FLP-mediated excision of the stop cassette, resulting in GFP expression in certain projection neurons. We observed that the number of GFP-positive olfactory projection neurons was correlated to the TMP dosage (**Figure 3.3A, B**). By varying the concentration of TMP (0.01 – 1 mM) in fly food, we could control the number of labeled projection neurons (**Figure 3.3A**). Furthermore, there were similar numbers of labeled neurons in both brain hemispheres for a given sample (**Figure 3.3B**). For flies fed with standard fly food without TMP, 42% of the brain hemispheres had only one GFP-positive cell (**Figure 3.3C**). This feature of FLP-DD can be used to generate single-cell clones at a reasonable probability for connectomics applications. As a proof-of-concept, we analyzed GFP-labeled neurons in the brains of 36 flies fed with solvent only. Out of 72 brain hemispheres, 30 had only a single GFP-positive projection neuron (see **Figure 3.3D** for examples). In summary, dose-dependent expression of FLP-DD can be used to control the number of genetically manipulated cells within a population.

Restricting the activity of FLP-DD in a spatial and temporal manner should further refine expression patterns which arise from the intersection of two expression systems (eg. GAL4/UAS and QF/QUAS). To illustrate this principle, we focused on the intersection of *GH146-QF* and *NP21-GAL4*. It has been reported that the expression patterns for *NP21-GAL4* and *GH146-GAL4* overlap only in the DA1 lateral projection neurons (lPNs) in the adult brain (Potter et al.,

2010), which we validated (**Figure 3.4A$_1$, A$_2$**). However, when UAS-FLP expression is driven by *NP21-GAL4*, the adult intersection pattern includes additional olfactory projection neurons, ellipsoid body neurons and neurons with cell bodies close to the lateral horn (**Figure 3.4B$_1$, B$_2$**). Similarly, when *QUAS-FLP* is driven by *GH146-QF*, the adult intersection pattern includes additional neurons (visual projection neurons in this case) (**Figure 3.4C$_1$, C$_2$**). This discrepancy between the overlap and the intersection patterns arises because of the broader expression patterns for *GH146-QF* and *NP21-GAL4* before the adult stage. Thus, the stop cassette is prematurely excised during development in neurons outside of the overlapping adult pattern.

We reasoned that the adult expression pattern can be recapitulated by limiting TMP

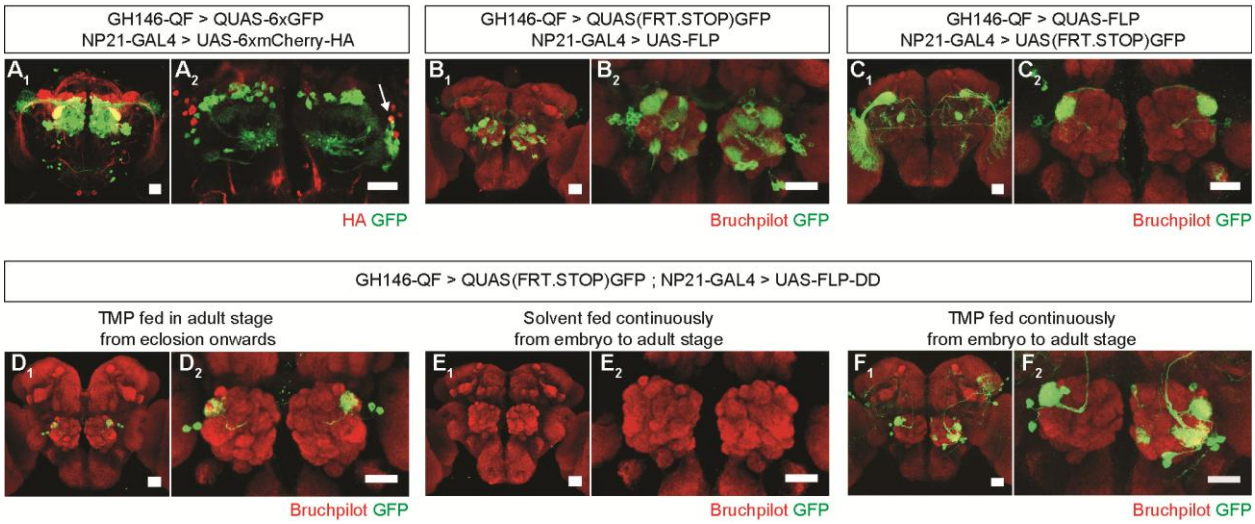


Figure 3.4. Refining intersection patterns by temporally limiting FLP-DD expression.

(A) Z-stack projections showing expression patterns of *GH146-QF* (green) and *NP21-GAL4* (red). Both transgenic lines overlap in a single population of DA1 lPNs (arrow in A$_2$). Between one to three overlapping neurons can be observed across all samples. Antenna was ablated from the brain sample shown in A$_2$ to visualize projection neurons in the absence of sensory neuron axon terminals in the antennal lobe. (B-C) Intersection using constitutively expressed flippase generates expanded patterns with additional expression in other olfactory (B) or visual (C) projection neurons. (D) Temporally limiting FLP-DD expression by feeding 1 mM TMP exclusively in adult stage results in GFP expression only in DA1-lPNs. (E) No GFP expression is observed in the absence of TMP. (F) GFP expression in additional olfactory projection neurons can be observed using FLP-DD if TMP is fed continuously. Scale bar = 25 μm.

feeding to the adult stage thereby inactivating FLP during development. Indeed, when *UAS-FLP-DD* was expressed by the *NP21-GAL4* line and 1 mM TMP was fed to flies only during the adult stage, GFP expression was limited only to DA1 lPNs in the whole brain (**Figure 3.4D$_1$, D$_2$**). In comparison, flies fed with solvent alone did not have GFP expression in any neurons in the brain (**Figure 3.4E$_1$, E$_2$**). Furthermore, flies fed with 1 mM TMP throughout development have GFP expression in additional olfactory projection neurons (**Figure 3.4F$_1$, F$_2$**). We noted that the expression pattern in *UAS-FLP-DD* flies fed with 1 mM TMP throughout development was a subset of that observed with *UAS-FLP* flies (**Figure 3.4B, F**). It is possible that TMP levels decline in the fly brain during metamorphosis after the larvae stop feeding. In fact, similar results have been observed in the context of RU486-induced FLP activity (Harris et al., 2015). To mitigate this potential decline of TMP, we fed flies with 10 mM TMP throughout the larval stage and obtained a larger portion of the *UAS-FLP* expression pattern (**Figure 3.4B, Figure 3S.6**). In sum, we show that TMP can be used to limit FLP-DD activity temporally in a way such that the intersection pattern is identical to the overlap in the adult expression patterns.

3.4. Discussion

Here we report a chemically inducible system to control gene expression in a dose-dependent and reversible manner. The DD system broadens the functionality of the *Drosophila* genetic toolkit as it provides an independent axis of control which can be used in combination with existing reagents. The DD system provides several advantages over existing chemical-dependent tools. First, TMP-induced DD stabilization is dose-dependent over several orders of magnitude of TMP concentration. This dose-dependency can be exploited for titration of *in vivo* gene expression levels. Here, we use this dose-dependent relationship to predictably alter the number of labeled projection neurons in the fly brain. Second, in contrast to existing chemical reagents, tools such as GAL80-DD can be combined with existing GAL4 lines to knockdown targeted genes by RNAi or to perform neuronal silencing screens in a temporally refined

manner. Third, when TMP is withdrawn, the degradation kinetics of the DD fusion protein are most likely faster than those of the native protein. Thus, it is possible that the DD system offers fast temporal control in experiments which require reversible gene expression. Finally, it is worth noting that the cost of TMP is almost 150 times less than RU486 or quinic acid at their respective working concentrations, making the DD system conducive to large scale behavioral screens.

The applicability of the DD system for a given cell type is limited by two factors: 1) the cell type should have an active proteasome; 2) orally-fed TMP should be able to reach the cell. In the absence of TMP, the level of proteasome activity in a given cell type may influence the residual level of a DD chimeric protein. For instance, low proteasome activity may result in high residual levels of the chimeric protein. It may be possible to reduce the residual expression by co-expressing components of the protein degradation machinery, similar to how co-expression of Dicer enhances RNAi efficiency (Dietzl et al., 2007). As it is, the background accumulation of the DD-fusion protein and the sensitivity of the downstream targets should be taken into consideration while designing experiments using the DD-system. As TMP is a cell-permeable ligand which can cross the blood-brain barrier, it should be accessible to all tissues during the adult and larval stages. However, the effectiveness of TMP in stabilizing the protein of interest during late pupal stages is reduced due to lack of feeding for several days leading up to this stage (**Figure 3S.2**). This may lead to undesirable leaky expression when GAL80-DD is used with a GAL4 line that is highly expressed during the late pupal stage. Therefore, the level of gene expression controlled by GAL80-DD should always be determined using reporters before manipulating neuronal activity with effector transgenes. Due to the nature of chemical delivery, the utility of the DD-system is also limited to applications which can tolerate gene expression at a relatively slower rate. We anticipate that it will take roughly 24 hours to activate or inactivate the DD-system to an appreciable degree. However, the optimal delay from the start or stop of drug feeding is dependent on the level of gene expression required for a specific application. It

may also be possible to achieve faster induction by using photocaged forms of trimethoprim (Ballister et al., 2015). During the course of our experiments, we observed a detrimental effect of the solvent DMSO (greater than 0.1%) on the survival of larvae (see Methods). This toxicity can be circumvented by using a water-soluble form of TMP- trimethoprim lactate or by mixing pure TMP directly into the food. Finally, as trimethoprim is an antibiotic, experiments using the DD system should incorporate appropriate controls to rule out the effect of gut microbes on the phenotype of interest.

DD-based tools are conducive for mapping and manipulating neural circuits underlying behavior. We illustrate this concept by using destabilized GAL80-DD to chemically manipulate neural activity in olfactory sensory neurons. To our knowledge, GAL80-DD is the first construct that allows control of GAL80 activity *in vivo* by a small molecule. Conventional experimental designs utilize the heat-inducible $GAL80^{ts}$ to suppress GAL4-depdendent transcription at low temperatures. While $GAL80^{ts}$ may still be preferable in experiments that require low background expression, GAL80-DD is a useful alternative for experiments that are disrupted by temperature manipulations. To further illustrate the utility of the DD-system for circuit mapping, we engineered FLP-DD for sparse neuronal labeling at high-efficiency and temporally controlled genetic intersection. In a previous study, an RU486-inducible FLP recombinase was constructed by fusing it with the human progesterone receptor (Flp-Switch) (Harris et al., 2015). Although this construct can be chemically induced similar to FLP-DD, further experiments will be required to compare the efficacy and dose-dependency of the two recombinases. All DD-fusion proteins presented in this study are soluble molecules which function in the cytoplasm or the nucleus. However, previous studies have used ecDHFR-derived destabilized domains to conditionally alter membrane protein expression (Iwamoto et al., 2010). Therefore, we predict that this tool can be used to temporally control expression of membrane proteins such as ion channels and G-protein-coupled receptors.

In addition to chemically inducible forms of GAL80 and FLP, the DD technology can be used in flies for several other applications. DD can be knocked-in and fused to endogenous proteins to control their expression by limiting TMP feeding. This can be done using custom-designed genome editing strategies or by integration into the large number of available MiMIC sites within coding introns (Venken et al., 2011). DD can also be fused to a variety of effector genes for the purpose of inducible neuronal silencing or genome editing (Maji et al., 2016). Due to its inducible nature, GFP-DD can be coupled with knock-in GAL4 lines to compare gene expression in individual cells across time points spanning only a few hours, such as circadian fluctuation of gene expression. GFP-DD may also be useful as a sensor for proteasome activity. In conclusion, we have developed a new set of tools for chemical control of gene expression in *Drosophila* which has broad-ranging applications and several advantages over existing tools of a similar nature. We characterized its efficiency and temporal limitations, and demonstrated its utility by engineering tools for chemical control of gene expression, recombination and neuronal activity.

3.5. Methods

Fly husbandry

Flies were raised on standard fly food (unless otherwise noted) at 25°C in a 12:12 light-dark cycle. The following transgenes were used in this study - nsyb-GAL4 (Riabinina et al., 2015) (BDSC_51941), Orco-GAL4 (Kreher et al., 2005) (BDSC_23292), UAS-GFP, GH146-GAL4 (Stocker et al., 1997)(BDSC_30026), Gr5a-GAL4 (Thorne et al., 2004), R58E02-GAL4 (Liu et al., 2012), $P1^{a}$-split GAL4 (Hoopfer et al., 2015), MB434B-split GAL4 (Aso et al., 2014), Tdc2-GAL4 (Cole et al., 2005), MB247-GAL4 (Zars et al., 2000), UAS-(FRT.STOP)mCD8-GFP (Potter et al., 2010) (BDSC_30032) and UAS-(FRT.STOP)GFP.myr (BDSC_55810), UAS-6XmCherry-HA(Shearin et al., 2014) (BDSC_52267), QUAS-6xGFP(BDSC_52264)(Shearin et al., 2014), 20XUAS-FLPD5 (Nern et al., 2011)(BDSC_55805), GH146-QF(Potter et al., 2010)

(BDSC_30014), QUAS(FRT.STOP)GFP (Potter et al., 2010) (BDSC_30134), NP21-GAL4 (Hayashi et al., 2002) (BDSC_30027), Actin5C-GAL4 (BDSC_4414), Orco-LexAVP16 (Lai et al., 2008), Gr21-GAL4 (Scott et al., 2001), UAS-TNT (Sweeney et al., 1995), 10XUAS-GFP-DD (this study), 10XUAS-FLP-DD (this study), nsyb-GAL80-DD (this study), 13XLexAop-GFP-DD (this study). See supplementary information for list of fly genotypes for every experiment.

Transgenic fly generation

Drosophila codon optimized destabilized domain (DD) was synthesized with 5' *XhoI* and 3' *XbaI* overhangs by Genewiz, Inc. (La Jolla, CA). Plasmids were generated using standard protocols for PCR, restriction digestion and ligation.

Destabilized GFP: To generate the 10XUAS-GFP-DD fly, DD was ligated to the c-terminus of GFP in the pJFRC81 vector (Pfeiffer et al., 2012). GFP was subcloned from the pJFRC81 plasmid using primer P1 and P2. DD was ligated to the c-terminus of GFP using the *XhoI* cut site. GFP-DD was ligated into the pJFC81 vector between the *PshAI* and *XbaI* cut sites. To generate the 13XLexAop2-GFP-DD fly, GFP-DD was cut from the 10XUAS-GFP-DD and ligated to the pJFRC95 plasmid(Pfeiffer et al., 2012) between the *NotI* and *XbaI* sites. Both GFP-DD constructs were transformed using phiC31 integrase mediated recombination into the attP2 landing site(Groth et al., 2004) by Genetic Services Inc. (Cambridge, MA).

Destabilized GAL80: To generate the nsyb-GAL80-DD fly, DD was ligated to the c-terminus of GAL80. GAL80 was subcloned with 5' *EcoRI* and 3' *XhoI* overhangs from pAC-GAL80 plasmid (Addgene #24346) using primers P3 and P4. DD was subcloned from the 10XUAS-GFP-DD plasmid with 5' *XhoI* and 3' *AatII* overhangs using primers P5 and P6. GAL80-DD was triple ligated between *EcoRI* and *AatII* sites in the cut nsyb-GAL4-hsp70 plasmid (Addgene #46107)(Riabinina et al., 2015). The resulting construct was transformed using phiC31 integrase mediated recombination into the VK00005 landing site(Groth et al., 2004) by Genetic Services Inc. (Cambridge, MA).

Destabilized FLP: The 10XUAS-FLP-DD plasmid was generated by ligating DD to the c-terminus of FLPD5. FLPD5 was subcloned with 5' *NotI* and 3' *XhoI* overhangs from pCaSpeR-DEST5 (DGRC #1031) using primers P7 and P8. FLP was ligated between the *NotI* and *XhoI* sites in the cut 10XUAS-GFP-DD plasmid. The construct was transformed using phiC31 integrase-mediated recombination into the attP2 landing site by Bestgene Inc. (Chino Hills, CA).

Primers:

P1- GGAGTAGTCCCGATATTGGTTG

P2- TTCATCTCGAGCTTGTAGAGCTCATCCATGCCGT

P3- ATCATCGACAGCCGAATTCCAACATGGACTACAACAAGAGATCTTCG

P4- GCGGCAATCAGGGAGATCTCGAGTAAACTATAATGCGAGATATT

P5- CTGGTTTCCAAACTGATCGGTC

P6- CGACGGTATCGATAGACGTCTATTAACGGCGCTCCAGAATCTCGAA

P7-

TACTTCAGGCGGCCGCGGCTGGAGGGTACCAACTTAAAAAAAAAAATCAAAATGCCACAATTTGATATATTATGT

P8- ATCAGGGAGATCTCGAGTATGCGTCTATTTATGTAGGATG

Recommended steps for generation of new POI-DD constructs: To generate new protein of interest (POI)-DD fusion constructs driven by UAS, the POI can be cloned in a non-directional manner (between *XhoI* restriction sites) or in a directional manner (between *NotI* and *XhoI*) (See **Figure 3S.1**) using the UAS-GFP-DD plasmid as template. Note that for directional cloning between *NotI* and *XhoI*, the *XhoI* site upstream of Syn21 must be mutated during primer design (see P7 for example).The Syn21 sequence (21 bp) can be included within the primer if desired (see P7 for example).

TMP feeding

Trimethoprim (Teknova Inc., CA) was maintained as a 100 mM stock solution in DMSO. To prepare food containing TMP for adult flies, standard fly food was heated to a liquid state. After cooling, TMP (or pure DMSO) was added to the food and vortexed to achieve a homogenous mixture of the required concentration. Food was poured into standard fly vials and allowed to solidify. Adult flies were transferred to new vials with TMP-containing food every 3 days. 1% DMSO was found to severely affect survival of larvae. Therefore, to feed flies with TMP from the embryo stage, pure TMP in powder form was mixed in fly food to attain the required concentrations. Detailed information on the feeding regimen for every experiment can be found in the supplementary information.

Histology

Tissue samples were prepared for imaging using protocols that have been previously described (Lin et al., 2013). Tissues were dissected in cold PBS and fixed in 4% (w/v) paraformaldehyde for 3 minutes on ice in a microwave. Next, tissues were fixed in 4% (w/v) paraformaldehyde containing 0.25% Triton-X-100 for 3 minutes on ice in a microwave. Fixed tissues were placed in blocking solution (2% Triton X-100, 0.02% sodium azide and 10% normal goat serum in PBS) and degassed in a vacuum chamber for 6 x 15 mins to expel tracheal air. For the purpose of quantification in Figures 1 and 2, samples were not immunostained. All samples for a given experiment were prepared and imaged in parallel to allow for comparison among them. Rabbit anti-GFP (Invitrogen A-11122, 1:200), mouse anti-bruchpilot nc82 (DSHB AB_2314866, 1:50), mouse anti-HA (Biolegend 901501, 1:500) and rabbit anti-TeTx antibody (POL 016, Statens Serum Institut, 1:1000) were used as primary antibodies in this study. Alexa Fluor 488 anti-rabbit immunoglobulin G (Invitrogen A-31628; 1:100) and Alexa Fluor 647 anti-mouse immunoglobulin G (Invitrogen A-21235, 1:100) were used as secondary antibodies. Brains were incubated in primary antibodies in dilution buffer (1% normal goat serum, 0.02%

sodium azide and 0.25% Triton X-100 in PBS) for 48 hours at 4°C, rinsed for 3 x 15 mins in washing buffer (1% Triton X-100, 3% NaCl in PBS), incubated in secondary antibodies in dilution buffer for 24 hours at 4°C, and rinsed again for 3 x 15 mins in washing buffer. Samples were mounted in Focusclear (Cedarlane Labs) between glass coverslips separated by spacer rings.

Samples were imaged with a 10X/0.3 or 20X/0.75 objective using a Zeiss LSM 510 confocal microscope to collect Z-stacks at 2-µm intervals. During the course of an experiment, the laser power and gain were held constant to allow for comparison among images from different experimental conditions. To quantify GFP expression, maximum intensity Z-projections were prepared using ImageJ (NIH). Average fluorescent intensity in the background was subtracted from the sample fluorescent intensity and the result was used as a proxy for GFP expression.

T-maze assay

Flies were raised in standard fly food or food containing 1 mM TMP from embryo to adult stages up to the time of the experiment. Behavioral tests were performed as described previously (Su et al., 2012). About 30 flies were transferred from food vials into a 15 mL centrifuge tube (Fisher scientific, 14959B) using a funnel. The tube containing the flies was connected to the T-maze apparatus and the flies were transferred into a horizontal elevator in the dark. Flies were held in the elevator for one minute before being pushed forward to choose between the test and the control arm. A fluorescent lamp was switched on at this point to phototactically draw flies out of the elevator. Flies were given one minute to choose between either arm, following which the elevator was retracted to separate the flies in the test arm from those in the control arm. The tubes serving as the test and the control arms were detached and flies in them were counted.

Flies were forced to choose between the control arm containing air and the test arm containing 0.28% CO_2. 400 μL of 10% CO_2 was injected into the test arm using a syringe (Becton Dickinson, 10 mL). The positions of the test arm and the control arm were alternated for each trial. The avoidance index was calculated as (no. of flies in the control arm - no. of flies in the test arm) /(no. of flies in the test arm + no. of flies in the control arm).

Survival assay

Adult flies were raised on a defined medium (1 M sucrose, 1% agar) with 0-10 mM TMP from eclosion to death. Each experimental vial contained 5 male and 10 female flies. Flies were transferred to new vials every two days. Number of living flies was recorded every day. To quantify survival during development, 20 eggs were manually placed using forceps in a vial of fly food containing 0-10 mM TMP. Vials were observed daily to quantify the developmental timing for puparium formation and time to eclosion.

Odor localization and locomotion assay

Odor localization ability and walking speed were measured using a setup described previously (Root et al., 2011; Zaninovich et al., 2013). Single flies were introduced in custom built chambers (60 mm diameter, 6 mm height) and tracked at 2 Hz under 660 nm LED illumination using custom software written in Labview (V.8.5, National Instruments, code available from Zaninovich et al., 2013). Wild type flies were fed with regular fly food containing 1 mM TMP or 1% DMSO for 48 hours before the experiment. The average walking speed of each fly during the first 50 seconds of each trial was determined using a custom macro with Igor Pro (V.6, Wavemetrics, Inc.). To perform the odor localization experiment, flies were transferred to starvation vials containing water with 1 mM TMP or 1% DMSO in Kimwipes (Kimberly-Clark) 24 hours prior to the experiment. 1% apple cider vinegar in low melting agarose was used as the odor source. Latency to localization is defined as the elapsed time before a fly spends more

than 5 seconds within 5 mm of the odor source.

Statistical Analysis

Statistical results (*p* value, effect size, n) are indicated in figure legends corresponding to each experiment. All statistical analyses were performed in Igor Pro (V.6, Wavemetrics, Inc.). Sample size for each experiment was pre-determined based on variation in experimental groups in pilot experiments. Most experiments were performed at least twice to confirm results. Data from one representative experiment is shown.

3.6. Acknowledgements

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Chapter 3, in full, is material as it appears in *eLife*, 2017, Sethi, Sachin, Wang, Jing W.. The dissertation author was a primary researcher and author of this paper.

Appendix 3.1: Supplemental Figures

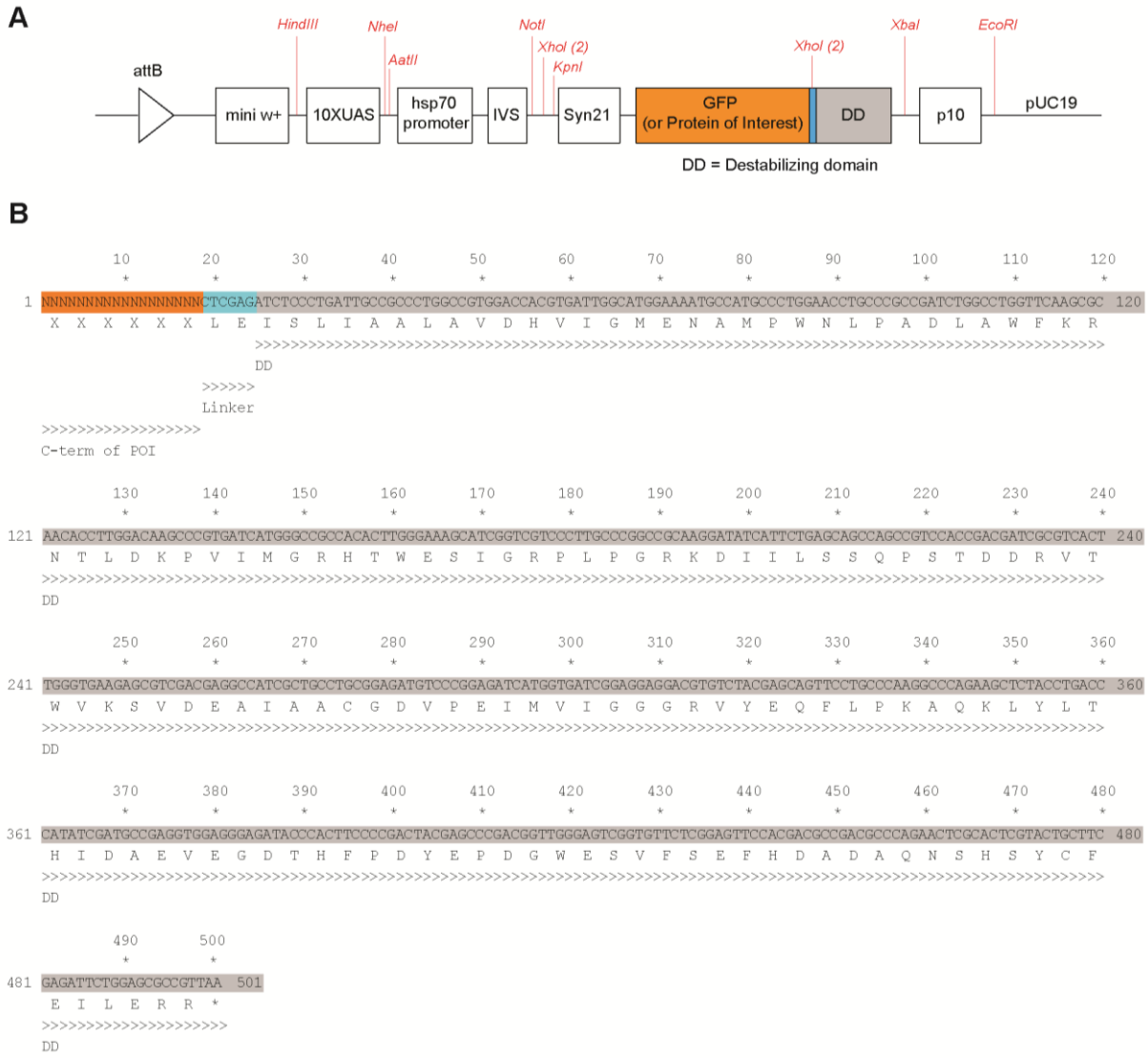

Figure 3S.1. Schematic and sequence of DD construct

(A) Schematic of the *10XUAS-GFP-DD* construct. Restriction sites are shown in red to facilitate cloning. All shown sites are unique apart from *XhoI* which has two cut sites (both shown). (B) DNA and protein sequence of the destabilizing domain.

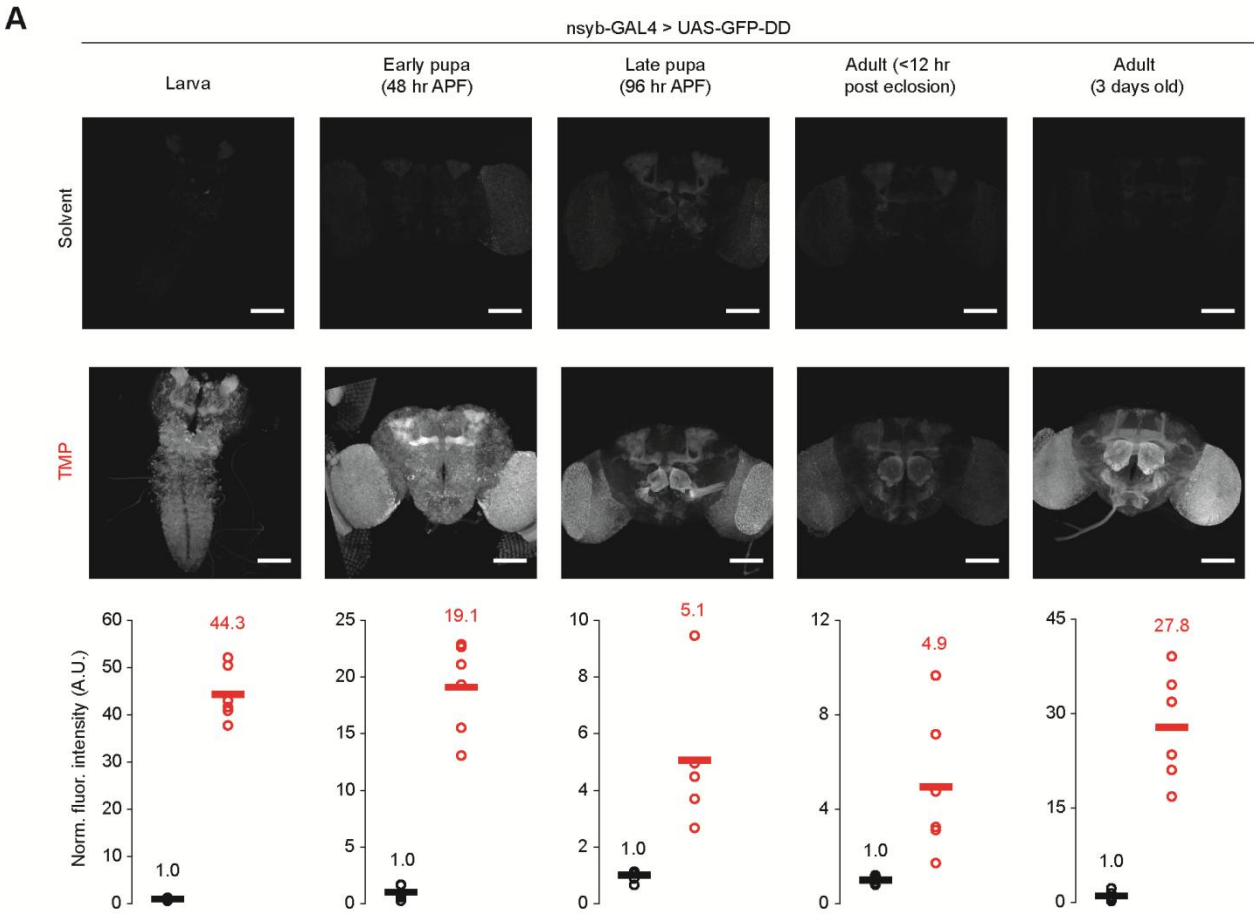

Figure 3S.2. Function of DD system across developmental stages.

TMP-dependent GFP expression in the whole brain across developmental stages – (from left to right) – Larva, Early pupa (48 hr APF), Late pupa (96 hr APF), Adult (<12 hr post eclosion), Adult (3 day old). Fluorescence intensity has been normalized to the mean fluorescence intensity from the brains of flies fed with the solvent. Scale bar = 100 μm. Differences in GFP expression between solvent and TMP fed flies are significant (unpaired t-test, two-tailed) across all developmental stages - Larva (p<0.001, t=18.6, n=6), Early pupa (p<0.001, t=10.89, n=5-6), Late pupa (p=0.025, t=3.46, n=5-6), Adult at eclosion (p=0.022, t=3.25, n=6), 3 day old adult (p<0.001, t=7.55, n=6).

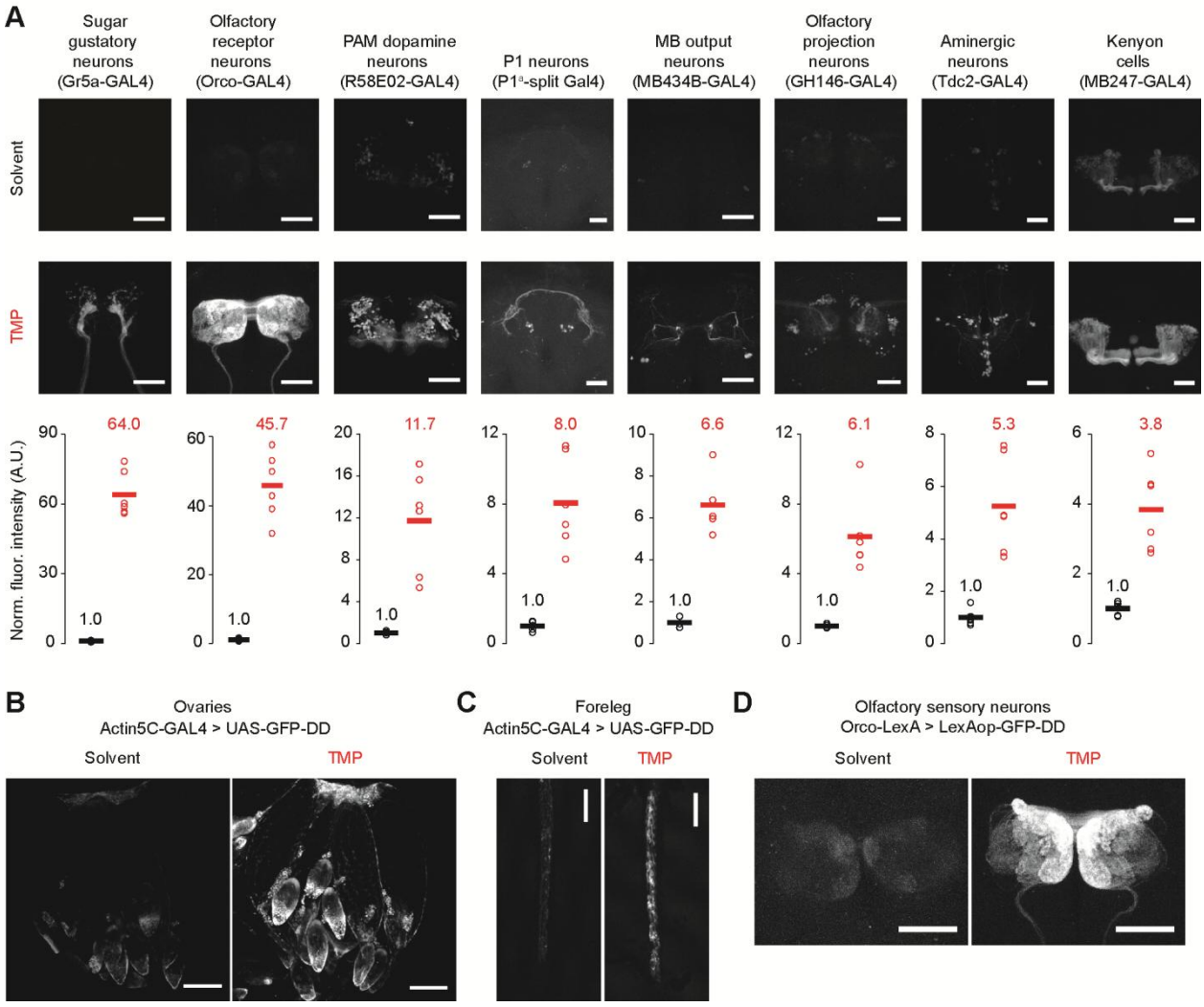

Figure 3S.3. Function of DD system across different cell-types

(A) TMP-dependent GFP expression in different cell types in the adult brain. Fluorescence intensity has been normalized to the mean fluorescence intensity from the brains of flies fed with the solvent. Flies were fed with solvent or TMP-containing food from embryo stage to five days post-eclosion. Differences in GFP expression between solvent and TMP-fed flies are significant for all cell-types shown (n=4-6, p<0.003, t>5.3, unpaired t-test, two-tailed, for all cell types). (B, C) TMP-dependent GFP expression in ovaries (B) and the foreleg (C). (D) TMP-dependent GFP expression in olfactory sensory neurons using the LexA/LexAop system. Scale bar = 50 μm (A, D), 100 μm (B), 150 μm (C).

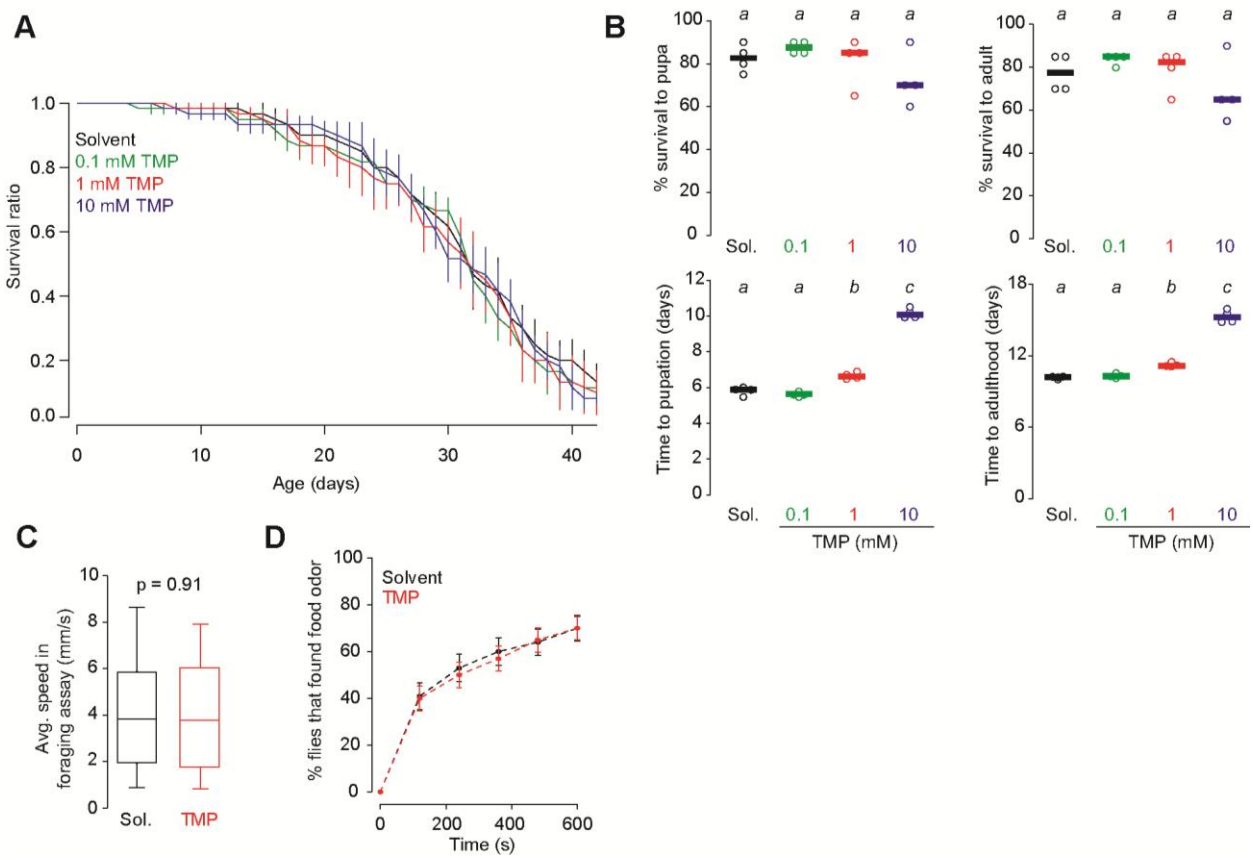

Figure 3S.4. Effect of TMP on survival and behavior

(A) Survival of wild-type flies fed with 0-10 mM TMP from eclosion to death. 15 flies were placed in each vial, four vials per condition. Error bars indicate SEM. (B) Survival (top) and developmental timing (bottom) of flies raised on 0-10 mM TMP. 20 eggs were placed in each vial, four vials per condition, one-way ANOVA with Tukey's post-hoc test. Significant differences between conditions (p<0.05) are denoted by different letters. Survival to pupa (n=4, p=0.186, F=1.88), survival to adult (n=4, p=0.245, F=1.58), time to pupation (n=4, p<0.0001, F=389.04), and time to adulthood (n=4, p<0.0001, F=251.73) (C) Walking speed of wild-type (CS) flies fed with 1 mM TMP or solvent for 48 hours prior to assay (n=148-161, unpaired t-test, two-tailed). Bar indicates median. Whiskers indicate 90% percentile. (D) Percentage of flies reaching the food odor is plotted against time. Food odor: 1% apple cider vinegar. Error bars indicate SEM.

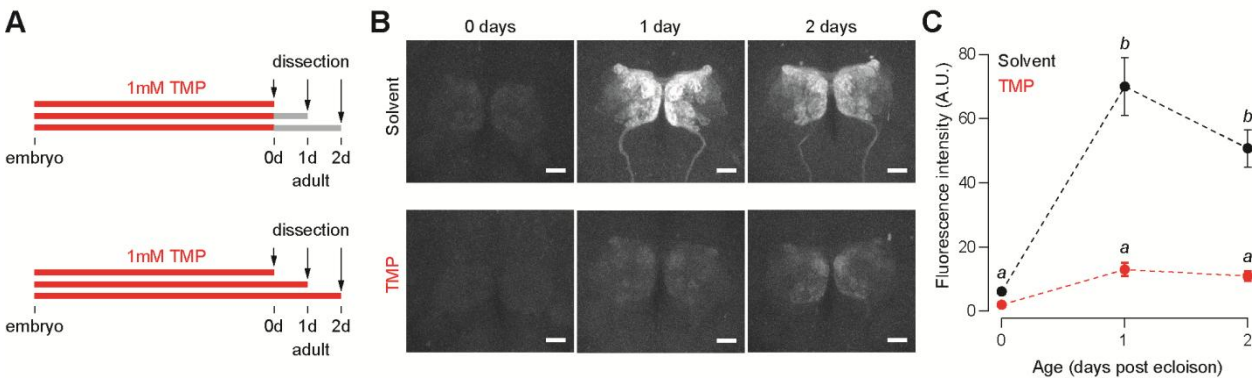

Figure 3S.5. Function of GAL80-DD in early adulthood.

(A) *Orco-Gal4, UAS-GFP, nsyb-GAL80-DD* flies were fed with food containing 1 mM TMP up to eclosion, following which flies were switched to standard fly food or maintained on 1 mM TMP. (B,C) After switching to standard fly food, GFP expression is visible in the antennal lobe starting at 1 day post-eclosion. No GFP is observed in either TMP or solvent-fed flies right after eclosion (<8 hr adults). n=5-6 per condition, two-way ANOVA indicated a significant interaction between feeding condition and age, F=16.22, p<0.001. Significant differences between conditions (p<0.05) are denoted by different letters (Tukey's post-hoc test). Error bars indicate SEM. Scale bar = 25 μm.

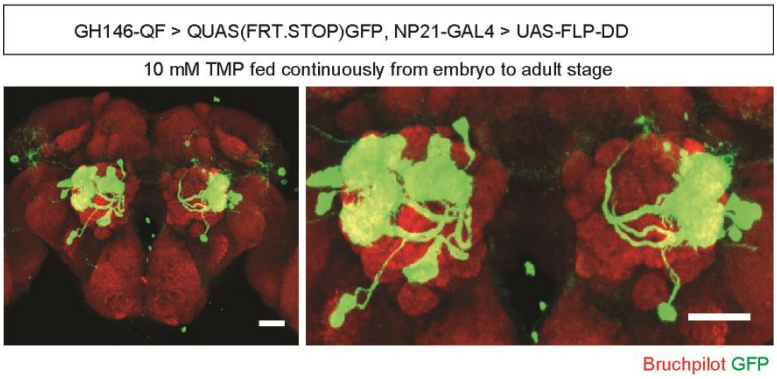

Figure 3S.6. FLP-DD efficiency with high TMP dosage.

Intersection pattern between *GH146-QF* and *NP21-GAL4* in flies fed with 10 mM TMP continuously. Scale bar = 25 μm.

Appendix 3.2: Supplemental Tables

Table 3.1. List of genotypes and feeding condition by experiment

Figure	Genotype	Feeding condition
3.1 B	w^- ; + ; nsyb-GAL4/10XUAS-GFP-DD	Flies fed with standard fly food or food containing 1 mM TMP from embryo stage to adult up to dissection
3.1 C, D	w^- ; + ; Orco-GAL4/10XUAS-GFP-DD	Flies fed with standard fly food containing 0 – 5 mM TMP for 48 hours
3.1 E, F	w^- ; + ; Orco-GAL4/10XUAS-GFP-DD	Flies fed with standard fly food containing 1 mM TMP for 0 - 60 hours
3.1 G, H	w^- ; + ; Orco-GAL4/10XUAS-GFP-DD	Flies fed with standard fly food containing 1 mM TMP for 48 hours, and then moved to standard fly food without TMP for 0-36 hours
3.2 A	w^- ; UAS-GFP/+ ; Orco-GAL4/nsyb-GAL80-DD	Flies fed with standard fly food or food containing 1 mM TMP from embryo stage to adult up to dissection
3.2 B, C	w^- ; UAS-GFP/+ ; Orco-GAL4/nsyb-GAL80-DD	B1, C1: Flies fed with food containing 1mM TMP from embryo stage to 3 days post eclosion. Flies were then transferred to standard food without TMP up to dissection. B2, C2: Flies fed with standard food from embryo stage to adult up to dissection. B3, C3: Flies fed with food containing 1mM TMP from embryo to adult up to dissection.
3.2 D, E	w^-/+ ; Gr21a-GAL4/+ ; + w^-/+ ; UAS-TNT/+ ; nsyb-GAL80-DD/+ w^-/+; Gr21a-GAL4, UAS-TNT/+; nsyb-GAL80-DD/+	Flies fed with standard fly food or food containing 1 mM TMP from embryo stage to adult up to behavioral assay
3.3 A, B	w^- ; GH146-GAL4/+ ; UAS(FRT.STOP)mCD8GFP/ 10XUAS-FLP-DD	Flies fed with standard fly food containing 0 – 1 mM TMP from embryo stage up to dissection.
3.3 C, D	w^- ; GH146-GAL4/+ ; UAS(FRT.STOP)mCD8GFP/ 10XUAS-FLP-DD	Flies fed with standard fly food without TMP from embryo stage up to dissection.
3.4 A	w^-; GH146-QF/UAS-6xmcherry-HA; NP21-GAL4/QUAS-6xGFP	Flies fed with standard fly food from embryo stage up to dissection

3.4 B	w^-; GH146-QF/ QUAS(FRT.STOP)mCD8GFP ; NP21-GAL4/ UAS-FLP	Flies fed with standard fly food from embryo stage up to dissection
3.4 C	w^-; GH146-QF/ UAS(FRT.STOP)GFP.myr; NP21-GAL4/ QUAS-FLP	Flies fed with standard fly food from embryo stage up to dissection
3.4 D-F	w^-; GH146-QF/ QUAS(FRT.STOP)mCD8GFP ; NP21-GAL4/ UAS-FLP-DD	D: Flies fed with standard fly food without TMP from embryo stage up to eclosion. After eclosion, flies were switched to food containing 1 mM TMP for 4-5 days before dissection. E: Flies fed with standard fly food from embryo stage up to dissection F: Flies fed with fly food containing 1mM TMP from embryo stage up to dissection.
3S.2 A	w^- ; + ; nsyb-GAL4/10XUAS-GFP-DD	Flies fed with standard fly food or food containing 1 mM TMP from embryo up to dissection.
3S.3 A	Sugar gustatory neurons: w^- ; + ; Gr5a-GAL4/10XUAS-GFP-DD Olfactory receptor neurons: w^- ; + ; Orco-GAL4/10XUAS-GFP-DD PAM dopamine neurons: w^- ; + ; R58E02-GAL4/10XUAS-GFP-DD $P1^a$ neurons: w^- ; R15A01-AD/+ ; R71G01-DBD /10XUAS-GFP-DD MB output neurons: w^- ; R30E08-p65AD/+; R53C10-DBD /10XUAS-GFP-DD Olfactory projection neurons: w^- ; GH146-GAL4/+; 10XUAS-GFP-DD/+ Aminergic neurons:	Flies fed with standard fly food or food containing 1 mM TMP from embryo up to dissection.

	w^- ; Tdc2-GAL4/+; 10XUAS-GFP-DD/+ <u>Kenyon cells:</u> w^- ; + ; MB247-GAL4/10XUAS-GFP-DD	
3S.3 B,C	w^-; Actin5C-GAL4/+ ; 10XUAS-GFP-DD/+	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 48 hours before dissection.
3S.3 D	w^-; + ; Orco-LexA/13xLexAop-GFP-DD/+	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 48 hours before dissection.
3S.4 A	Wild-type (w1118)	Flies fed with 0 -10 mM TMP in 1 M sucrose, 1% agar from eclosion to death
3S.4 B	Wild-type (w1118)	Flies raised in 0-10 mM TMP in standard fly food from embryo onwards.
3S.4 C	Wild-type (Canton-S)	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 48 hours before behavioral assay.
3S.4 D	Wild-type (Canton-S)	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 24 hours, following which flies were transferred to starvation vials containing water with 1 mM TMP or 1% DMSO for 24 hours before behavioral assay.
3S.5 B,C	w^- ; UAS-GFP/+ ; Orco-GAL4/nsyb-GAL80-DD	Flies raised on food containing 1 mM TMP from embryo stage up to eclosion. Flies were then transferred to standard food without TMP (B, top) or maintained on food containing 1 mM TMP (B, bottom).
3S.6	w^-; GH146-QF/ QUAS(FRT.STOP)mCD8GFP ;	Flies fed with fly food containing 10 mM TMP from embryo stage up to dissection.

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