THE UNIVERSITY OF CHICAGO

# CONTRIBUTION OF NEUROPEPTIDE F (NPF) NEURONS TO REWARD IN DROSOPHILA MELANOGASTER

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES

AND THE PRITZKER SCHOOL OF MEDICINE

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BIOLOGY

BY

MATHIAS ANDRES SAVER CORTES

CHICAGO, ILLINOIS

JUNE 2017

## Table of Contents

**List of figures** ..... **iv**

**Aknowledgements** ..... **vii**

**ABSTRACT** ..... **ix**

**Chapter 1: INTRODUCTION** ..... **1**

1.1 General definition of Reward Olds & Milner Experiments ..... 1

1.2 Behavioral paradigms to test for reward in mammals ..... 4

1.3 Examples of brain regions from the Reward Circuit in the mammalian (rodent) brain: Role of ventral tegmental area and nucleus accumbens in reward processing ..... 8

1.4 Drosophila as a model organism: Binary expression systems ..... 11

1.5 Behavioral paradigms to test for reward in flies ..... 13

1.6 Example of neurons signaling reward in Drosophila ..... 15

1.6.1. The case of Octopamine vs. Dopamine ..... 15

1.6.2. Kenyon cells and Mushroom Body output neurons ..... 16

1.6.3. NPF neurons as part of Drosophila's reward circuit ..... 20

1.7. Thesis outlook and plan ..... 22

**Chapter 2: Methods** ..... **23**

**Chapter 3: Establishing a simple 2-choice assay for studying reward in Drosophila** ..... **36**

3.1 Description of the 2-choice assay. Preference for optogenetic activation of NPF neurons ..... 36

3.2 NPF neuron activation-induced preference is not dependent on grouping flies ..... 42

3.3 Effect of NPF neuron activation on locomotion ..... 43

3.4 Optogenetic activation of NPF neurons is rewarding ..... 48

**Chapter 4: Characterization of the preference for NPF neuron activation in the 2-choice assay** ..... 53

4.1. Flies withstand an aversive stimulus in order to gain access to activation of NPF neuron ..... 53

4.2. Effect of alcohol exposure on NPF neuron activation-induced preference ..... 55

4.3. Natural vs. Artificial reward competition ..... 56

4.4. NPF is required for the NPF neuron activation preference ..... 59

**Chapter 5: Anatomical and functional subdivision of NPF neurons ..... 62**

5.1 Neuroanatomy of NPF neurons ..... 62

5.2 NPF neuron functional subdivison ..... 66

**Chapter6: NPF neuron post-synaptic elements ..... 74**

6.1 NPF and dopamine neuron behavioral epistasis ..... 75

6.2 NPF and dopamine neurons functional connectivity ..... 78

**Chapter7: Discussion and Future Directions ..... 81**

7.1 Preference for NPF neuron activation ..... 81

7.2 Is Activation of NPF neurons in the 2-choice assay truly rewarding? ..... 83

7.3 The value of activation of NPF neurons as a reward ..... 86

7.4 NPF is required for the NPF neuron activation preference ..... 87

7.5 Functional Subdivision of subsets of NPF neurons ..... 87

7.6 Dopamine neurons as downstream targets of NPF neurons ..... 88

7.7 Future Directions ..... 90

**References ..... 92**

## List of figures

**1.1 Olds & Milner intracranial self-stimulation (ICSS) experiments ..... 3**

**1.2 Behavioral paradigms used in the study of reward ..... 5**

**1.3 The runway model of drug self-administration ..... 7**

**1.4 Schematic representation of the mammalian (rodent) reward circuitry ..... 9**

**1.5 The GAL4/UAS system ..... 12**

**1.6 Olfactory conditioning ..... 14**

**1.7 *Drosophila melanogaster* olfactory pathway ..... 17**

**1.8 *Drosophila* mushroom bodies and pathways for reinforcement ..... 18**

**1.9 The neuropeptide F (NPF) neurons ..... 20**

**3.1 Schematic representation of the 2-choice assay system ..... 37**

**3.2 Flies exhibit a preference for NPF neuron activation ..... 38**

**3.3 Frequency dose-response curve for activation of NPF neurons ..... 39**

**3.4 Preference for NPF neuron activation does not desensitize upon repeated activation ..... 41**

**3.5 Flies avoid activation of Gr66a neurons ..... 42**

**3.6 Preference for NPF neuron activation does not depend on a social (group) context ..... 44**

**3.7 Effect on NPF neuron activation of single flies locomotion ..... 45**

**3.8 Effect of NPF neuron activation on the locomotion of grouped flies ..... 46**

**3.9 Correlation of preference (□Time (%)) and speed during activation for single flies ..... 47**

**3.10 Correlation of preference (activation effect) and speed during activation for grouped flies ..... 47**

**3.11 Optogenetic activation of NPF neurons is rewarding ..... 49**

**3.12 Optogenetic activation of Gr66a neurons is aversive ..... 50**

**3.13 Optogenetic activation of NPF neurons in a circular arena is rewarding ..... 51**

**4.1 Flies withstand an aversive stimulus, when paired with activation of NPF neurons ..... 54**

**4.2 Alcohol exposure sensitizes flies to a subsequent activation of NPF neurons ..... 56**

**4.3 Competition between natural and artificial rewards ..... 58**

**4.4 NPF is required for the NPF activation-induced preference ..... 60**

**4.5 Presence of extra UAS binding sites does not change the preference for NPF neuron activation ..... 61**

**5.1 *Drosophila* neuropeptide F (NPF) neurons ..... 63**

**5.2 NPF neurons cell polarity ..... 64**

**5.3 Neuroanatomy of NPF neurons ..... 66**

**5.4 Expression pattern of ss0020 line ..... 66**

**5.5 Intersection of the NPF and NPFR expression pattern effectively labels the large NPF neurons ..... 67**

**5.6 Preference for the activation of specific subsets of NPF neurons..... 69**

**5.7 Effect on locomotion of the activation of specific subsets of NPF neurons ..... 70**

**5.8 Intersectional strategy for labeling DM and P2 NPF neurons ..... 71**

**5.9 Preference for the activation of the small (DM + P2) NPF neurons ..... 72**

**5.10 Effect on locomotion of the activation of the small (DM + P2) NPF neuron ..... 73**

**6.1 Blocking of dopamine neurons reduces the preference for NPF neuron activation 75**

**6.2 Effect of blocking individual dopamine neuron on the preference for NPF neuron activation ..... 76**

**6.3 Regions of interest (ROIs) employed for quantification of calcium responses in PAM dopamine neurons ..... 78**

**6.4 Calcium responses of PAM dopamine neurons upon activation of NPF neurons .... 78**

**6.5 Quantified calcium responses of PAM dopamine neurons upon activation of NPF neurons..... 79**

**6.6 Quantified calcium responses of PAM-□3 dopamine neurons upon activation of NPF neurons ..... 79**

**Aknowledgements**

First of all, I thank Ulrike for giving me the opportunity to come to Janelia to join her lab. Both professionally and personally the experience has been priceless. I would also like to thank both Ulrike and Dan for recruiting me into the Janelia/UofChicago graduate student program. I would like to also thank Xiaoxi for providing me with mentorship and space in his lab during my time at Chicago. This section would not be complete without acknowledging past and present members of the Heberlein lab at Janelia, Reza, Karla, Galit, Jasper, Pavan, Fran, Yong-Kyu, Clement, Mark, Lisha, Hugh, Phuong, Carmen, Sarah, Cheryl and Emily whom provide/provided and amazing place to come to everyday. Specially, I would like to thank Lisha for contributing with some of the behavioral data presented here, Phuong for making the fly brain stainings shown here, as well as Carmen for her very critical comments about the manuscript of the paper describing part of the results presented here. In particular, I would like to, once again, thank Lisha not only for mentoring me professionally, but also for taking the taking the time to ensure my well-being when I needed it the most. The work presented in this thesis benefited from the help of many people at Janelia including Karen, Todd, Amanda, Vivek, Sung-Soo, Yoshi, Ming, Tanya Wolf, Jasmine, Allan, Tanya Tabachnik, Steve, Igor, Spencer, Peter, Samantha, Lakshmi, Ben and Sean. In particular, I would like to thank Jasmine and Allan for taking the time to help us with the calcium imaging experiments. Not only people, but physical places as well deserve an acknowledgement. I would like to thank the gym at Janelia for being a space where I can go to clear up and exercise my body/mind.

Finally I would like to thank the many friends I made during my time at both Chicago and Janelia, not only for emotional support but also for all the good moments. I thank Eric, Doreen, Brian, Martin, Angela, Maxim, Manuel and Bruno.

## ABSTRACT

In classical experiments performed by Olds & Milner in 1954, it was shown that rats learn to perform an operant lever-pressing behavior in order to receive an electric stimulus in specific brain nuclei, leading to the identification of what we currently know as mammalian "reward centers" and the discovery that neuronal activation in these brain regions is perceived as rewarding. For the fruit fly *Drosophila melanogaster*, it is known that specific subsets of neurons regulate different forms of reward. However, a more extensive circuit level description of the fly's reward circuit is missing. Here we present the development of a novel and high throughput 2-choice assay that employs optogenetic activation of neurons to study reward in *Drosophila*. This new system was characterized using activation of Neuropeptide F (NPF) neurons, a known rewarding stimulation. We showed that NPF neurons could be subdivided into different groups based upon their neuroanatomy and contribution to the NPF neuron activation-induced preference. Finally, we showed that this preference response is dependent on the activity of dopamine neurons.

# Chapter 1: INTRODUCTION

## 1.1. General definition of Reward: Olds & Milner Experiments

Reward is a complex phenomena and providing a comprehensive definition of it falls out the scope of the present thesis. In general, however, rewards could be described as neural representations that bias the behavior of an organism towards a particular stimuli or context (i.e., food, water, or drug consumption) (Kelley & Berridge, 2002). At the same time, rewards are recognized as having three distinct components (Berridge & Robinson, 2003; Berridge et al., 2009a):

- **'Liking'**: it corresponds to objective affective reactions (affective component of reward). For example, positive 'liking' facial expressions (i.e tongue protrusions) elicited by the sweet taste of sucrose.
- **'Wanting'**: it corresponds to incentive salience, an implicit and objective type of motivation. The sensory information from a particular reward, is transformed into desired incentives that promote the approach to and consumption of a particular reward.
- **Learning:** upon presentation of an unconditioned stimulus, the brain assigns to it a particular hedonic value, which is dependent on internal homeostatic signals like thirst or hunger. This hedonic value can then be associated with a conditioned stimulus (CS). During a subsequent presentation, the CS has now gained incentive salience ('wanting'). Conditioned 'liking' could also be associated to the respective CS.

A major breakthrough in the understanding of reward in the mammalian brain came from a series of experiments carried out by James Olds and Peter Milner (Olds & Milner, 1954) (**Figure 1.1A**). They showed that rats implanted with stimulating electrodes in different brain areas learned to press a lever in order to receive intracranial stimulation (ICSS). Rats "became addicted" and preferred to lever-press rather than receiving a natural reward such as food. Furthermore, the rats chose to press the lever even if they had to endure an electric foot-shock in order to receive the ICSS (Olds, 1958) (**Figure 1.1B**). These experiments showed that reinforcement (sustained self-stimulation) could be achieved by electrical stimulation of different brain areas. Although originally the work of Olds & Milner prompted to suggest the discovery of pleasure centers in the brain (Olds, 1956b), it should be noted that the word 'pleasure' denotes the hedonic 'liking' associated with a particular reward (Berridge, 2009b). Furthermore, while both 'wanting' and 'liking' are required for any given reward to occur, these two components can be dissociated both psychologically and neurologically (Berridge et al., 2009a; Berridge, 2009b). For example, specific areas of the brain region known as Nucleus accumbens independently modulate 'wanting' or 'liking (Berridge et al., 2009a; Berridge, 2009b). Thus, it is possible that the lever-pressing behavior observed in the rats from Olds & Milner is a reflection of the activation of brain regions that contribute to 'wanting' or 'liking' specifically or to a combination of both.

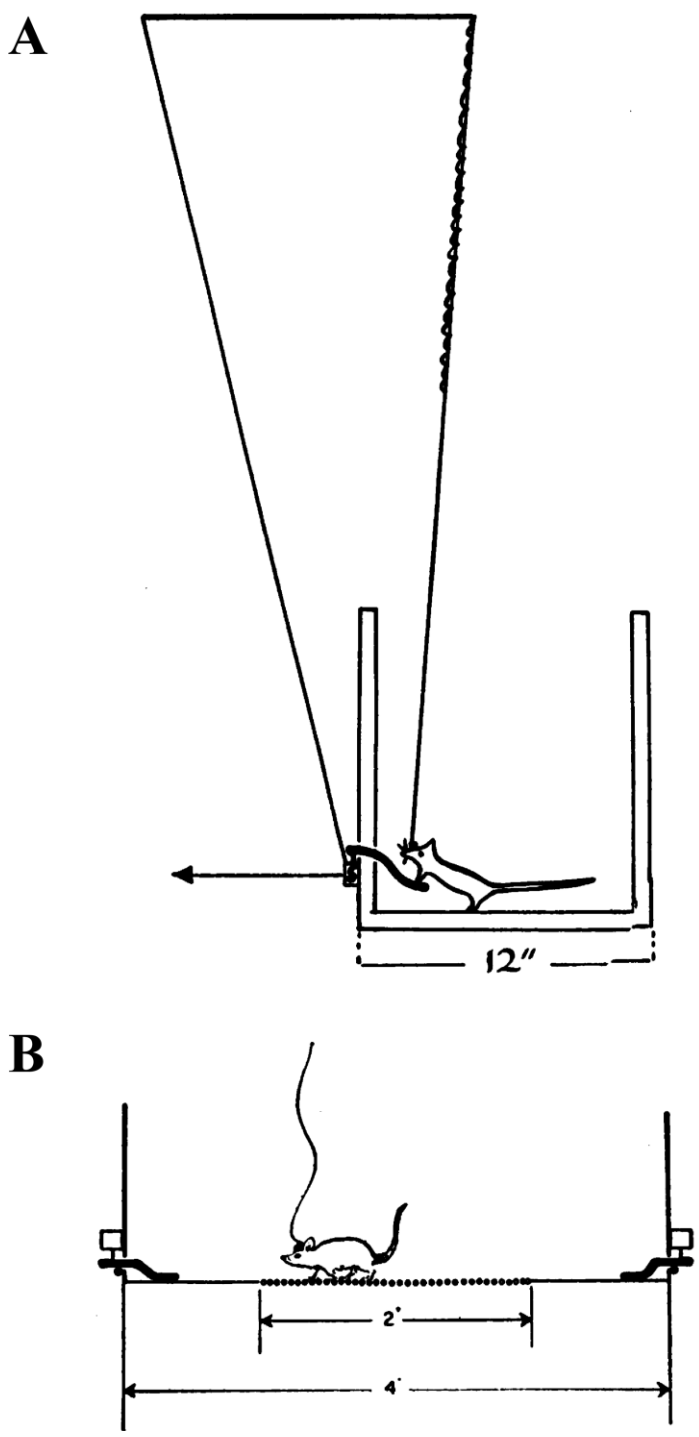


**Figure 1.1. Olds & Milner intracranial self-stimulation (ICSS) experiments**. **(A)** Schematic representation of the behavioral apparatus employed for ICSS experiments. Here, a rat is implanted with an electrode into a specific brain area. The animal is then introduced into an operant conditioning chamber, or Skinner box, containing a lever. Every time the rat presses the lever, it receives an electric shock into the brain area where the electrode is implanted. The effect of stimulation is assessed as the number of lever presses over a period of time. **(B)** Schematic representation of the obstruction box or electric grid experiment. Here, the rat is introduced into a Skinner box containing two lever for self-stimulation, each on opposite sides of the box. In order to continue obtaining the electric stimulation, the rat must alternate between the two sides of the chamber, however, to do so the rat must walk through an electric grid. Interestingly, rats withstood more electric shock to obtain the brain stimulation (artificial reward) than to obtain food (natural reward). Images extracted from: (Olds, 1958).

### 1.2. Behavioral paradigms to test for reward in mammals

The conditioned place preference (CPP) (**Figure 1.2A**) (Tzschentke, 2007), which is a form of classical conditioning (Domjan, 2005), is one of the most commonly used paradigms to study the effects of natural or artificial rewards. In this procedure, the intrinsic motivational and hedonic properties of a particular stimulus serve as an unconditioned stimulus (US) that is repeatedly associated with an, a priori neutral, external cue. Typically, an animal (i.e. a rat) is put in a box with two different sides (contexts), each having a particular cue or conditioned stimulus (CS) (i.e a particular floor texture). The cue that is associated with the US is known as $CS^+$ while the other cue is known as $CS^-$. During the course of the procedure, the $CS^+$ cue acquires the incentive properties of the US. Later, during the test phase, the preference observed for the $CS^+$ is a reflection of this acquired incentive value. It is interesting to mention, as pointed out by Huston et al., 2013, that in addition to 'incentive motivation' two other processes may account for preference that an animal shows for the context in which it experienced the US. For example, while an animal is experiencing the US it could also be spontaneously engaging in a particular behavior that gets reinforced by operant conditioning. Later on when the animal is located in the context in which it experienced the reinforcer, it will engage into that particular behavior, which will result in the animal spending more time in that particular area of the behavioral chamber. In addition, it is also possible that the reinforcer triggers an unconditioned response (UCR), which in the presence of a $CS^+$ becomes a conditioned response (CR). When the animal experiences the $CS^+$ again, it will express the CR that would prevent the animal from leaving a particular compartment giving the impression that the animal is expressing preference.

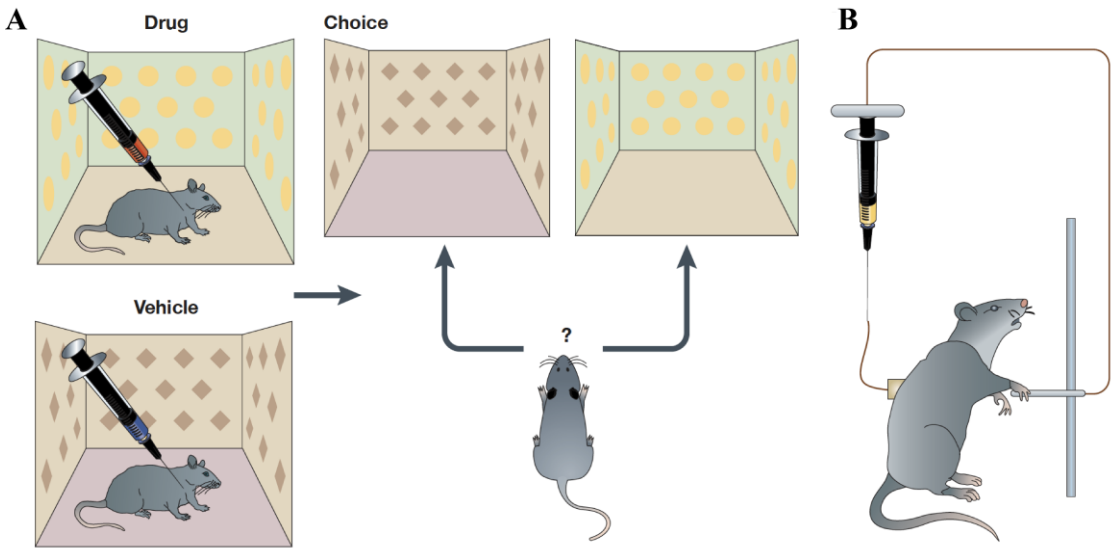


**Figure 1.2. Behavioral paradigms used in the study of reward**. **(A)** The conditioned place preference (CPP) procedure is typically carried out by training the animal to associate one of two different environments with an unconditioned stimulus (US) (natural or artificial reward), in this example, drug delivery. During the testing phase, the animal is presented with the choice of exploring the two environments. The hedonic properties of the US are assessed by quantifying the amount of time the animal spends in the environment previously associated with the US. **(B)** Animals can be trained to perform an operant behavior in order to receive a reinforcer. This type of paradigm is commonly used as a model of drug self-administration where the animal performs an operant behavior such as lever pressing in order to receive an intravenous dose of a drug. Here, the number of times that the animal performs the operant behavior is a reflection of the rewarding properties of the particular reinforcer (i.e food or drug) used in the task. Images extracted from: (Laviolette & van der Kooy, 2004).

Operant conditioning (**Figure 1.2B**) (Staddon & Cerutti, 2003) is a form of learning where, by performing a particular behavior, the organism controls the delivery of a particular reinforcer (natural or artificial reward). Thus, the organism makes an association between the execution of a particular behavior and the delivery of a reinforcer. In this type of assay the assumption is that the association of a specific behavior with a reinforcer, increases the probability of that behavior to happen again. For example, (Skinner, 1948) showed that hungry pigeons can be trained to peck into a disk or to perform turns inside their cages in order to get a food reward. The experiments performed by Olds and Milner (Olds J & Milner P, 1954) are also examples of operant conditioning, where rats had to press a lever in order to get an artificial

reward (i.e ICSS into a particular brain region). In another example, Witten et al., 2011 trained rats to perform a nose poke response in order to obtain photo-stimulation of dopamine neurons. Operant conditioning, however, is more commonly associated with models of drug self-administration (Panlilio & Goldberg, 2007). Here, an animal (i.e., a rat) presses a lever or performs a nose poke in order to receive a dose of a particular drug typically through an intra-venous catheter. The rule under which the reinforcer is delivered, whether a natural or artificial reward, is known as reinforcement schedule (Staddon & Cerutti, 2003). For example, on a Fixed-ratio schedule, the operant behavior has to be executed the same number of times each time the animal obtains the reinforcer. On the other hand, on a Progressive-ratio schedule, the number of times the animal has to perform the operant behavior increases after each time the animal obtains the reinforcer.

Runway assays, perhaps less commonly used than CPP or operant conditioning, have also been employed in the study of the motivational properties of natural (Hull, 1934; Crespi, 1942) or artificial (Olds, 1956a) rewards. More recently, Ettenberg (Ettenberg, 2009) described the use of a modified runway assay that employs intravenous drug self-administration as a reinforcer (**Figure 1.3**). The behavioral chamber consists of an alley or runway that has a start compartment and a goal compartment. During several trials the animal, typically a rat, runs from the start to the goal compartment where it obtains a reward (i.e food pellet, ICSS or intravenous drug dose). The time that it takes for the animal to run the runway is considered a good indicator of the animal's motivation to seek the reward in the goal compartment (Ettenberg, 2009). It should be noted that this type of procedure combines aspects of both CPP and operant conditioning: like the CPP, the animal has to actively look for the environment previously associated with the

reinforcer, and like operant conditioning, the delivery of the reinforcer is contingent upon the execution of a particular behavior (i.e run down the alley).

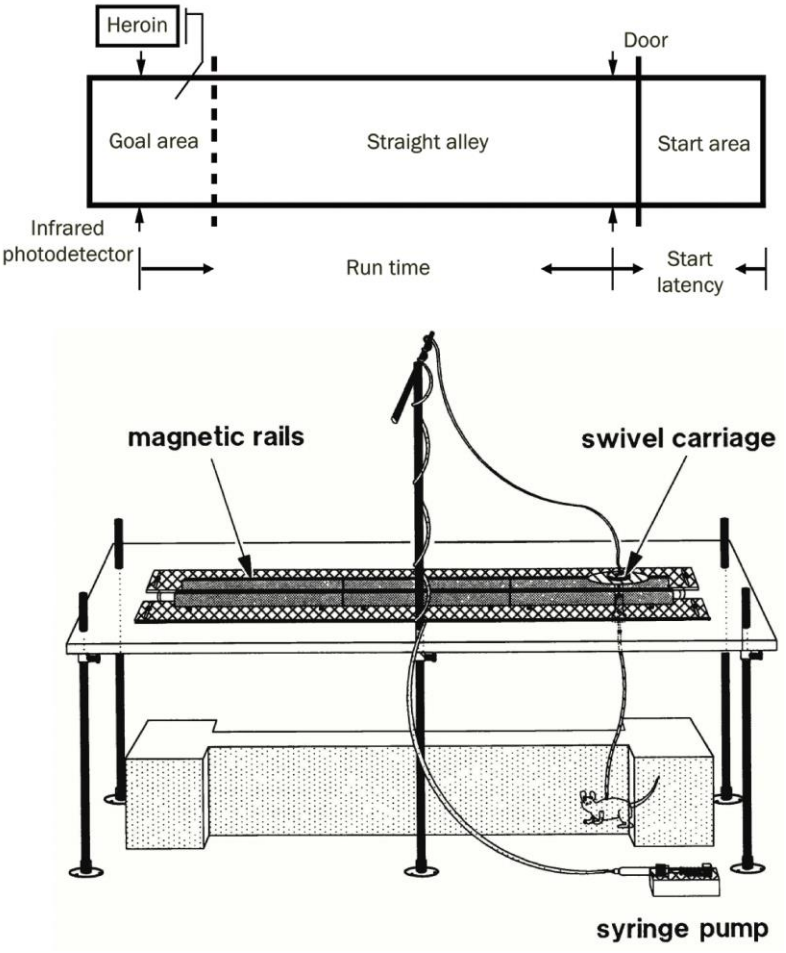


**Figure 1.3. The runway model of drug self-administration.** In this paradigm, the behavioral chambers consist of an alley with a start compartment and goal compartment. During several trials, the animal, typically a rat, has to run the alley from the start compartment towards the goal compartment, where it receives an intravenous dose of a drug, heroin in this example. The runtime, which is the time that it takes for the animal to go from the start to the goal compartment, is considered an indicator of the animal's motivation to obtain the reward (an intravenous dose of heroin in this example). Images extracted from: (Li et al, 2013) and (Cohen, 2014).

## 1.3 Examples of brain regions from the Reward Circuit in the mammalian (rodent) brain: Role of ventral tegmental area and nucleus accumbens in reward processing

The mammalian reward circuitry as been extensively studied, and summarizing every brain area involved in reward falls out of the scope of this introduction. However, examples of the most studied brain areas will be presented here.

Some of the most well characterized neurons involved in reward processing are the dopaminergic neurons located in the ventral tegmental area (VTA), which project to other brain regions such as the nucleus accumbens (NAc), amygdala and prefrontal cortex (PFC) (**Figure 1.4**) (Russo & Nestler, 2013). A distinctive property of the VTA dopaminergic neurons is that they can fire action potentials in two modes: a fast burst spike firing or a tonic single spike firing (Grace & Bunny, 1998a,b). The work from Schultz et al. 1997 showed that dopamine neurons respond with a burst spike firing upon presentation of unexpected rewards, and it has been suggested that this firing mode can act as a reward prediction error signal. The tonic spike firing, on the other hand, has been associated with the differential modulation of D2 dopamine receptors: while the dopamine phasic dopamine release preferentially activates D1 dopamine receptors and facilitates limbic inputs to the NAc, the dopamine tonic release bi-directionally regulates prefrontal cortex inputs into NAc via D2 dopamine receptors (Goto & Grace, 2005).

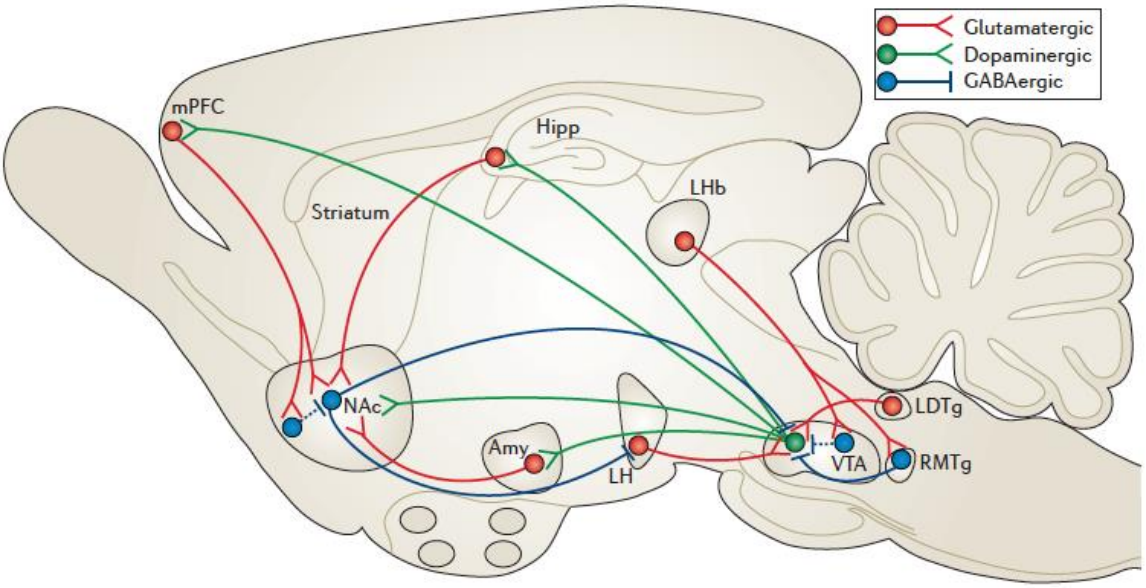


**Figure 1.4. Schematic representation of the mammalian (rodent) reward circuitry.** This picture shows the major glutamatergic (red), dopaminergic (green) and GABAergic (blue) neurons involved in reward. Of all of them, perhaps the dopaminergic neurons located in the ventral tegmental area (VTA) are the ones that are best characterized. From the VTA, these neurons send projections to other regions such as the nucleus accumbens (NAc), the medial prefrontal cortex (mPFC). While the NAc acts as a center of integration of information from cortical and limbic areas, the mPFC acts as a regulator of decision making and executive control. Other areas such as the lateral habenula (LHb), the lateral dorsal tegmentum (LTDg) and rostro medial tegmentum (RMTg) send projections to the VTA, hence, they are able to regulate the activity of the dopamine neurons in this region. Other areas that are involved in reward processing include the amygdala (Amy), which participates in the formation of associative memories related to rewarding events, the lateral hypothalamus (LH), which integrates information from other brain regions and regulates motivated behaviors like feeding and drinking, and the hippocampus (Hipp), which is involved in the formation of declarative memories. Image extracted from: (Russo & Nestler, 2013).

Along with dopaminergic neurons, the VTA also contains a large population of GABAergic interneurons, which regulate the activity of the dopamine neurons (Creed et al., 2014). For example, reducing the GABAergic signaling into dopamine neurons leads to higher rates of reward learning (Parker et al., 2011). On the other hand, optogenetic activation of these local GABAergic interneurons leads to conditioned place aversion (Tan et al., 2012). In addition, the VTA also contain glutamatergic and GABAergic projection neurons that send information into

the NAc. These neurons have been implicated in aversion (Qi et al., 2016) and regulation of associative learning (Brown et al., 2012), respectively.

The NAc, on the other hand, is composed primarily of medium spiny neurons (MSNs), which can be classified as D1-MSN or D2-MSN based on the type of dopamine receptor they express (Carlezon & Thomas, 2009). The MSNs express glutamate receptors, thus enabling the NAc to integrate information of glutamatergic inputs from other brain areas such as the amydala, hippocampus and prefrontal cortex (O'Donnell & Grace, 1995; Kelley, 2004). Most notably natural rewards such as food (Westerink et al., 1997) or water (Yoshida et al., 1992) and artificial rewards such cocaine (Jones et al., 1995) or amphetamine (Badiani et al., 1998), all induce dopamine release in NAc.

The medial prefrontal cortex (mPFC) is involved in the regulation of processes such as decision making (Botvinick et al., 2004), error detection (Holroyd et al., 2002), executive control (Posner et al., 2007) and reward-guided learning (Rushworth et al., 2011). In rodents, the mPFC can be subdivided into the anterior cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL) and medial orbital PFC (oPFC) (Ongür & Price, 2000). In particular, the PL projects to the core compartment of the NAc as well as the basolateral and lateral amygdala, while the IL projects to the shell compartment of the NAc as well as the basal, medial and central compartments of the amygdala (Ongür & Price, 2000). The PL and IL cortices have been implicated in the expression and extinction of conditioned fear. For example, activity of the PI cortex is required for conditioned fear expression (Sangha et al., 2014), while inactivation of local inhibitory interneurons enhances the expression of conditioned fear (Courtin et al., 2014). On the other hand, the IL cortex is required for the retention of conditioned fear extinction

(Quirk et al., 2000; Milad et al., 2004). In addition to regulating fear related memories expression and retention, the PL and IL cortices also regulate reward related processes.

For example, activity of the PL cortex is required during the stress and cocaine induced reinstatement of cocaine self-administration (Capriles et al., 2003), which could also be triggered by direct injection of cocaine into the PL cortex (Park et al., 2002). The IL cortex, on the other hand, has been involved in the extinction of drug seeking behavior. For example, when rats that were trained to extinguish a cocaine CPP received optogenetic activation of the IL cortex (Van den Oever et al., 2013), they showed an enhanced extinction of the place preference response. Similarly, rats in which the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) synaptic transmission in the IL cortex was activated (LaLumiere et al., 2010) showed an enhanced extinction of a previously acquired cocaine self-administration behavior. Moreover, activation of the IL cortex AMPAR synaptic transmission reduces the level of reinstatement of a cocaine self-administration behavior (LaLumiere et al., 2012).

### 1.4 Drosophila as a model organism: Binary expression systems

Although much information has been gained by studying reward processing in the mammalian brain, the fruit fly *Drosophila melanogaster* represents an excellent alternative model organism in which to study behaviors, such as reward perception and processing. Some of its advantages include: low maintenance cost, the possibility to work with a large number of genetically identical individuals and a short generational lifespan. However, the greatest advantage of using the fly as a model organism is the availability of an unrivalled number of neurogenetic tools. One of the most commonly used in the fly lab is the binary expression system

GAL4-UAS, developed by Brand & Perrimon, 1993. Briefly, this system employs different genomic enhancers to command the cell-specific

expression of the transcription factor GAL4. In turn, wherever GAL4 is expressed, it will induce the expression of a desired transgene placed downstream of the UAS sequence (**Figure 1.5**). Furthermore, this system can gain temporal specificity by using the temperature-sensitive GAL4 repressor GAL80ts (McGuire et al., 2003). More recently a second binary expression system, the LexA-LexAop system, has been developed (Lai & Lee, 2006). The possibility of having multiple binary expression systems, allows for different neuronal populations to be manipulated independently.

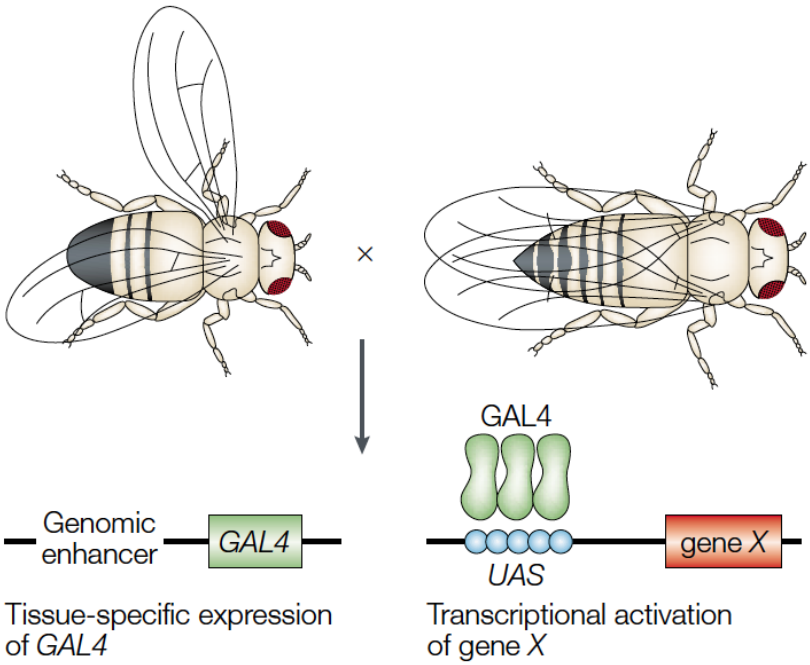


**Figure 1.5. The GAL4/UAS system.** The yeast transcription factor GAL4 can be used to induce the expression of any transgene of interest (gene X), located downstream of the upstream activating sequence (UAS). In turn, the expression of GAL4 of controlled by a tissue specific genomic enhancer, thus restricting the tissue or cells (neurons) where the transgene of interest will be expressed. The gene X construct can range from constructs to manipulate gene expression, such as cDNA or RNAi, constructs to label neurons, such as GFP or *Brainbow*, constructs to monitor cellular activity, such as GCaMP or TANGO, to constructs for activation of neuronal activity, such as CsChrimson or dTrpA1, or inhibition of neuronal activity, such as *shi*$^{ts}$. Image extracted from: (St Johnston, 2013).

By crossing appropriate fly lines, one can use these binary expression systems to target the expression of any transgene of interest, such as temperature sensitive modulators of neuronal activity (*shi*$^{ts}$, dTrpA1) (Kitamoto, 2001; Hamada et al., 2008), light sensitive modulators of neuronal activity (P2X2, ChR2, ReaChR, CsChrimson) (Lima & Miesenböck, 2005; Suh et al, 2007; Inagaki et al., 2014a; Klapoetke et al., 2014), reporters of neuronal activity (TANGO, GCaMPs) (Inagaki et al., 2012; Akerboom et al., 2012) or neuroanatomical markers (mCD8-GFP, *Brainbow*) (Lee and Luo, 1999; Hampel et al., 2012), to a particular neuronal subpopulation. In addition, the possibility of performing different types of intersectional strategies, such as the split-GAL4 system, further restricts the number of cells that can be labeled, thus enhancing the anatomical resolution at which neural circuits can be studied (Simpson, 2009; Venken et al., 2011).

### 1.5 Behavioral paradigms to test for reward in flies

Most of what is known today about reward in *Drosophila*, comes from classical conditioning experiments that use rewarding unconditioned stimuli (US). The most commonly used learning paradigm in the fly remains the Conditioned Odor Preference (COP) assay (**Figure 1.6**), commonly called the olfactory T-maze (Tempel et al., 1983; Tully & Quin, 1985). Briefly, a group of flies is exposed separately to two different odors (conditioned stimuli; CS), one of which is paired with an unconditioned stimulus (US). After the training phase, flies are presented with the choice of the two odors alone, and a "preference" or "learning" index is calculated based on the number of flies that chose each odor.

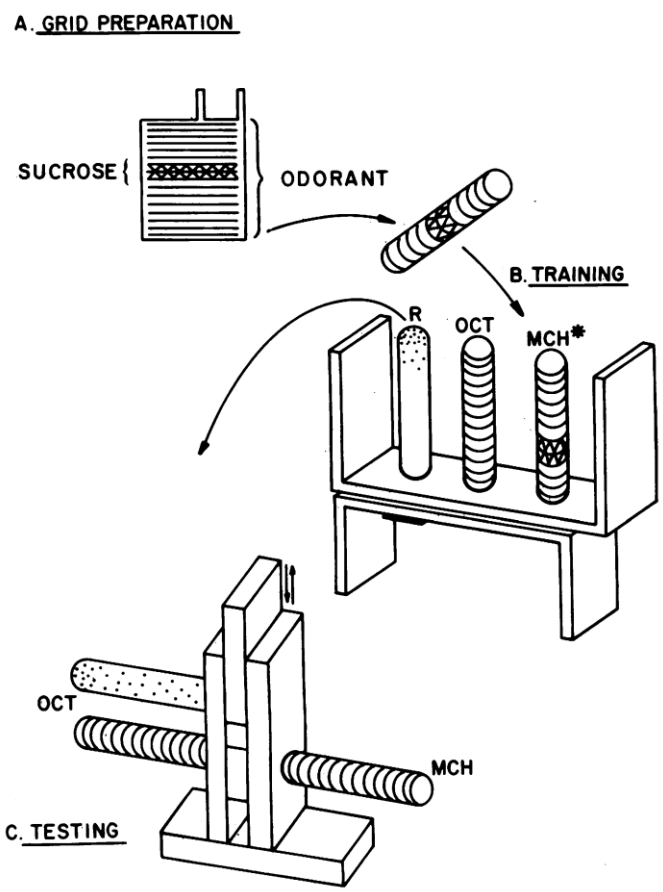


**Figure 1.6. Olfactory conditioning.** In this behavioral paradigm, a naive group of flies is trained (phases A & B) to associate the presence of either of two olfactory cues, 3-octanol (OCT) and 4-methylcyclohexanol (MCH, with the presence of an unconditioned stimulus (US), which in this example corresponds to sucrose. Some time after the training phase, the flies are presented with the choice of the two olfactory cues alone. If more flies approach the odor previously associated with the respective US, then it is said that the US was perceived as rewarding. Conversely, if it happens that more flies approach the odor that had not been previously associated with the US, then it is said that the US was perceived as aversive. Image extracted from: (Tempel et al., 1983).

If more flies prefer the odor previously paired with the US, the US is considered rewarding, while if more flies prefer the unpaired odor, then the US is considered aversive. Using different variations of this procedure, it has been shown that flies perceive sucrose (Tempel et al., 1983), water (Lin et al., 2014), alcohol intoxication (Kaun et al., 2011) and mating (Shohat-Ophir et al., 2012) as rewarding, while noxious stimuli, such as electric shock, are perceived as aversive (Tempel et al., 1983; Tully & Quin, 1985).

Reward in *Drosophila* can also be studied using conditioning of the proboscis extension response (PER) (Chabaud et al., 2006). In this procedure flies are carefully restrained such that they are only able to move their antennae and mouthparts. During the training phase, flies are presented with a particular CS (odor or tastant), which is paired with the presentation of the US

(sucrose). In a control group, the CS is unpaired from the US. Later, during the test phase, flies are presented with the CS alone. If the CS triggers higher proboscis responsiveness in the paired group compared with the unpaired group, the US can be considered as rewarding.

### 1.6 Example of neurons signaling reward in Drosophila

#### 1.6.1. The case of Octopamine vs. Dopamine

For a long time the idea persisted that in the fly, the neurotransmitters octopamine and dopamine were specifically required for appetitive and aversive reinforcement, respectively (Schwaerzel et al., 2003). This was contradicted with the later observations that *dumb* flies, which are mutant for the dopamine receptor DopR, were defective for both appetitive and aversive memory (Kim et al., 2007), and that inhibiting neurotransmission in dopaminergic neurons affected memory for ethanol reward but not aversion (Kaun et al., 2011), thus challenging the notion that dopamine was involved exclusively in aversive learning.

Dopaminergic neurons are organized in 15 distinct clusters across the fly brain, and today it is known that the TH-GAL4 transgene used to block neuronal activity in the experiments of (Schwaerzel et al., 2003) labels the majority of dopaminergic neurons, with the exception of most of the dopaminergic neurons in the protocerebral anterior medial (PAM) cluster (Mao & Davis, 2009). In the last few years, new GAL4 drivers have been identified that label specifically the PAM cluster. Two independent studies showed that the specific neuronal activation of the dopaminergic neurons in the PAM cluster paired with odor conditioning, led to a robust appetitive memory (Liu et al., 2012; Burke et al., 2012). In addition, (Burke et al., 2012) showed that the octopamine-dependent reinforcement requires signaling through dopaminergic neurons. The dopaminergic neurons involved in the formation of aversive memory described by

(Schwaerzel et al., 2003), have been mapped to subsets of neurons from the posterior inferiorlateral protocerebrum (the PPL1 cluster), the type 1 medial lobe and peduncle (MB-MP1), the type 1 vertical lobe (MB-V1) neurons and PAM cluster type 2 medial lobe (MB-M2) and type 3 medial lobe (MB-M3) neurons (Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012). It is interesting to note that the PAM cluster of dopamine neurons is functionally heterogeneous, with some neurons signaling punishment and others signaling reward (Aso et al., 2012; Liu et al., 2012; Burke et al., 2012). Furthermore, two largely non-overlapping subsets of PAM dopamine neurons mediate specifically the formation of short-term or long-term appetitive memories (Huetteroth et al., 2015; Yamagata et al., 2015). This detailed functional characterization shows that, like in the mammalian brain (Lammel et al., 2014), dopamine neurons are heterogenous, in that different subsets are involved in the processing of appetitive or aversive stimuli.

### 1.6.2. Kenyon cells and Mushroom Body output neurons

In *Drosophila* olfactory processing starts in odor sensing neurons located in the antenna and maxillary palps that project to the antennal lobe, where the information is conveyed to the projections neurons (PNs) (**Figure 1.7**). In turn, the PNs send the processed olfactory information to higher order brain structures such as the mushroom body (MB) (Davis, 2011).

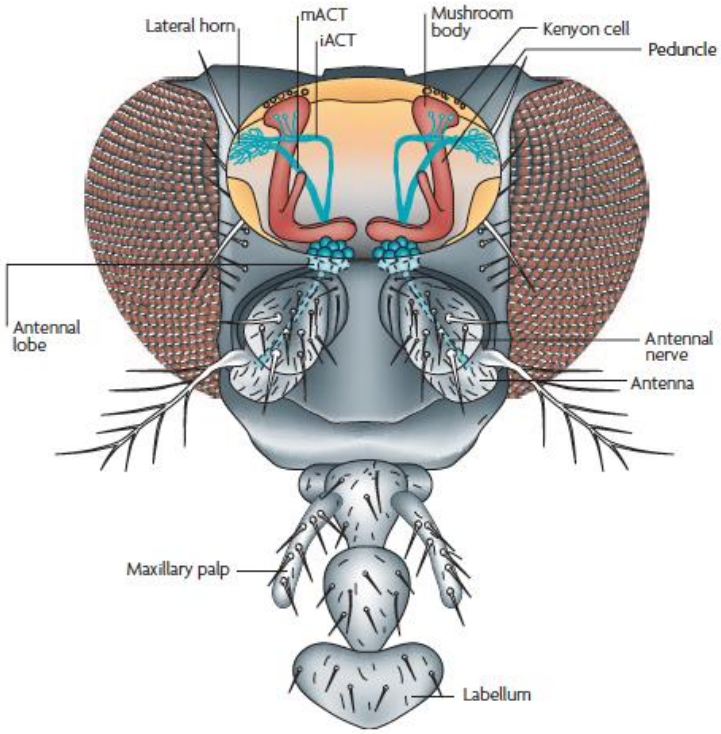


**Figure 1.7 *Drosophila melanogaster* olfactory pathway.** Schematic representation of a fly's olfactory system. Odors are sensed by sensory neurons, located in the antenna, expressing specific odorant receptor. Through the antennal nerve, this sensory neurons will project to the different glomeruli in the antennal lobe. In turn, to projection neurons located in the antennal lobe, will relay the information into higher order brain areas such as the Mushroom Bodies and Lateral horn. mACT: medial antennocerebral tract. iACT: inner antennocerebral tract Image extracted from: (Keene & Waddell, 2007).

The MB is composed of both intrinsic neurons, more commonly known as Kenyon cells (KCs), and extrinsic neurons, composed of mushroom body output neurons (MBONs) and several other neuromodulatory neurons (Tanaka et al., 2008; Aso et al., 2014a). The cell bodies of the KCs form a cluster in the dorsal posterior brain, while axonal projections are sent to the anterior brain forming the peduncle. At its anterior end, the axons bifurcate to form the vertical lobe, which consists of the α and α' lobes, and the horizontal lobe, which consists of the β, β' and γ□□□□□□. In turn, the KCs can be classified as αβ□□α'β'□or□γ□depending on which area of the MBs lobes they project to (Tanaka et al., 2008) (**Figure 1.8A**).

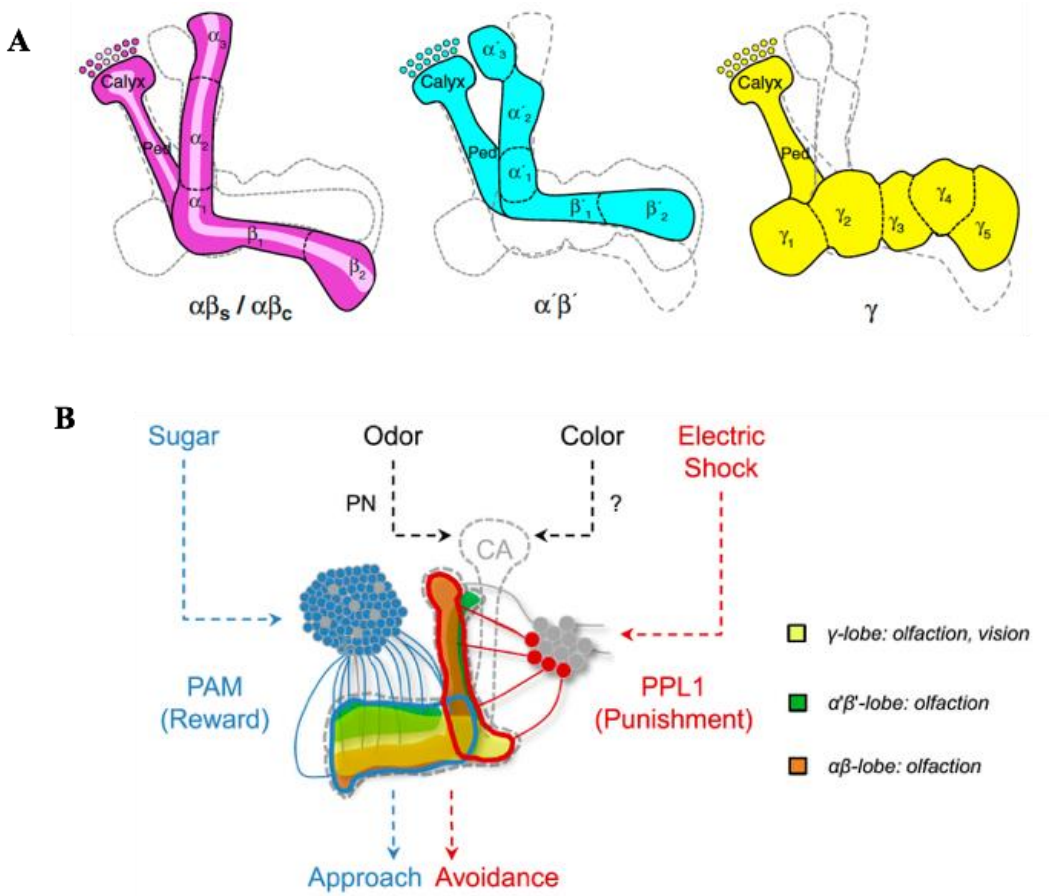


**Figure 1.8.** ***Drosophila*** **mushroom bodies and pathways for reinforcement. (A)** The Kenyon Cells (KC), the mushroom body (MB) intrinsic neurons, have their cell bodies in the posterior brain in what is known as the calyx. From here, they project to the anterior brain, with some cells producing a vertical bifurcation, thus forming the different lobes, α, β and γ, of the MBs. In turn, the KC can be classified as $\alpha\beta_s$, $\alpha\beta_c$, α'β' or γ, depending on which lobes they innervate. Furthermore, the lobes can be subdivided in different regions or compartments based upon the innervation that they receive from extrinsic neurons (Aso et al., 2014a). **(B)** While appetitive reinforcement requires signaling through the dopamine neurons located in the PAM cluster (Liu et al., 2012; Burke et al., 2012), aversive reinforcement requires signaling from dopamine neurons located in the PPL1 cluster, as well as a small subset of dopamine neurons in the PAM cluster (Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012). In addition, olfactory information is conveyed to the calyx by the projection neurons from the antennal lobe, while visual information is conveyed by visual projection neurons from the optic lobes to a specialized subset of KCs which project to the g lobe (Vogt et al., 2016). While all of the KCs play a role in associative memory involving olfaction, only the aforementioned subset of KCs projecting to the γ lobe plays a role in associative learning involving vision. Images extracted from: (Perisse et al., 2013b; Vogt et al., 2014).

While the olfactory (CS) information is conveyed to the MB calyx by the PNs, the US associated with a given odor is conveyed by different dopaminergic inputs to the different lobes of the MB (Perisse et al., 2013b) (**Figure 1.8B**). Aversive reinforcement requires the MB-MP1 and MB-V1 neurons in the PPL1 cluster that project to the heel in the γ lobe, and the small subset of MB-M2 and MB-M3 neurons in the PAM cluster, which project to the γ lobe and tip in the β lobe respectively (Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012). Appetitive reinforcement (**Figure 1.8B**), requires dopaminergic neurons from the PAM cluster that project to the horizontal β, β' and γ lobes (Liu et al., 2012; Burke et al., 2012).

An interesting feature of the KCs is that their activity is sequentially required for memory formation: γ neurons are required for short-term memory formation, α'β' neurons are required during consolidation and αβ□neurons are required for long-term memory retrieval (Kaun et al., 2011; Blum et al., 2009; Krashes et al., 2007; Trannoy et al., 2011). The αβ cells can be further subdivided into posterior ($\alpha\beta_p$), surface ($\alpha\beta_s$) and core cells ($\alpha\beta_c$) (Tanaka et al., 2008). It has been suggested that retrieval of an aversive memory requires output only from $\alpha\beta_s$ cells, while retrieval of an appetitive memory requires a combination of both $\alpha\beta_c$ and $\alpha\beta_s$ neurons (Perisse et al., 2013a). Most notably, an exhaustive neuroanatomical characterization of the MB anatomical structure (Aso et al., 2014a) shows that it can be subdivided into 15 different zones or compartments, each receiving dendritic projection from one particular type of mushroom body output neuron, along with axonal projections from one particular type of dopamine neuron. Consistently with this anatomical description, the dopaminergic modulation exerted over the MB is compartment specific (Hige et al., 2015).

### 1.6.3. NPF neurons as part of Drosophila's reward circuit

Neuropeptide F (NPF) is the *Drosophila* homologue of mammalian neuropeptide Y (**Figure 1.8A & 1.8B**). NPF has pleiotropic functions, as it regulates a wide array of behaviors such as ethanol preference and ethanol reward (Shohat-Ophir et al., 2012), ethanol sensitivity (Wen et al., 2005), courtship (Lee at al, 2006), sucrose sensitivity (Inagaki et al., 2014b), aggression (Dierick & Greenspan, 2007) and sleep (He et al., 2013).

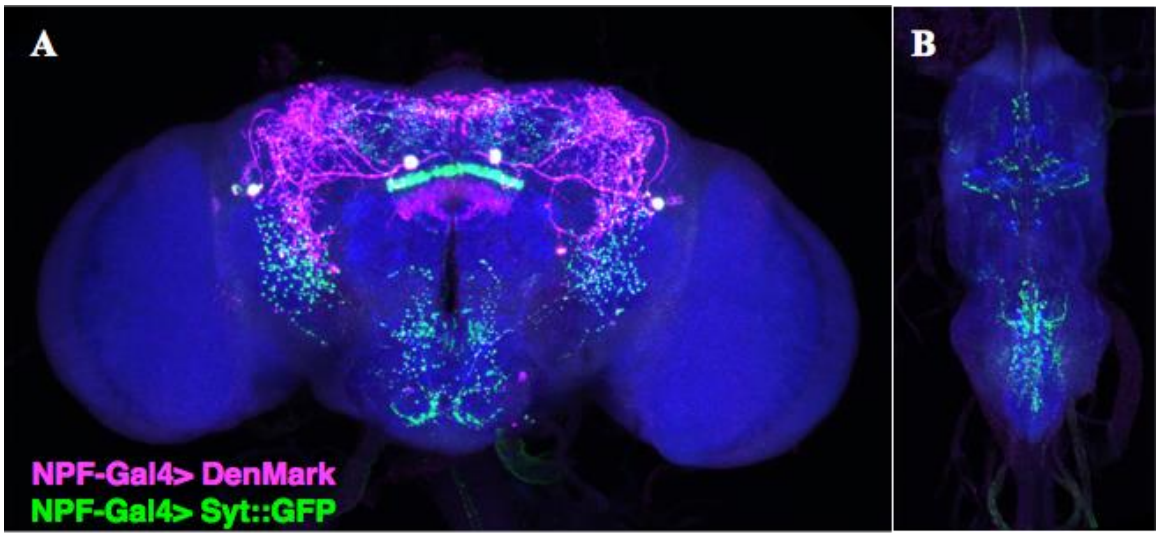


**Figure 1.9. The neuropeptide F (NPF) neurons.** Distribution of NPF neurons in the central brain (A) and ventral nerve cord (B) of the fly, as visualized by the expression of the cell polarity markers DenMark and Syt::GFP (Nicolai et al., 2010; Zhang et al., 2002) driven by a NPF-Gal4 driver .

Interestingly, (Krashes et al., 2009) showed that the activity of the NPF circuit acts as a "gate" for the retrieval of appetitive memories. In flies, expression of an appetitive memory is stimulated by hunger, while it is repressed by satiety: activation of NPF neurons in satiated flies mimics the effect of food deprivation, thus promoting the expression of appetitive memories. In addition, (Krashes et al., 2009) showed that this effect of NPF requires signaling through a subset of MB-MP dopaminergic neurons that project to the heel and peduncle of the MB. While blocking this subset of MB-MP neurons in satiated flies induces expression of appetitive

memories, their stimulation in hungry flies suppresses expression of appetitive memories. Considering the synaptic inhibitory effect described for NPY in mammals (Sah & Geracioti, 2013), the work from Krashes et al., 2009 suggests that in hungry flies NPF suppresses the inhibition that MB-MP have over the expression of appetitive memories stored in the MB. Similarly, two recent studies have also addressed a satiety-state dependent regulation of appetitive memory expression. In one example, Miyamoto et al., 2012 showed that neurons expressing the fructose receptor Gr43a assign opposing valence to feeding experiences in a satiety-state dependent manner: activation of Gr43a neurons in hungry flies is perceived as rewarding and leads to an increased food consumption, whereas activation of Gr43a neurons in satiated flies is perceived as aversive and leads to reduced food consumption. On the other hand, Gruber et al., 2013 showed that activation of adipokinetic hormone (AKH) producing cells, located in the corpora cardiaca, led to a transient suppression conditioned odor approach. It would be interesting to determine if, and how, the Gr43a expressing neurons and AKH producing cells communicate with the NPF system.

The NPF system has also been proposed by Shohat-Ophir et al., 2012, as a mediator of changes induced by sexual experiences: sexual rejection decreases, while mating increases the levels of NPF. Decreased levels of NPF in rejected males are accompanied by an increased preference to consume ethanol-containing food. Shohat-Ophir et al., 2012 also showed that thermogenetic activation of NPF neurons is by itself rewarding. Consistent with the notion that NPF levels are intimately involved in regulating the reward system, activation of NPF cells reduced the rewarding effects of ethanol.

### 1.7. Thesis outlook and plan

Even though we know of some neurons involved in reward processing in *Drosophila*, we are far from a complete picture of its reward circuit. For example, while Kaun et al., 2011 showed that flies perceive ethanol intoxication as rewarding and that retrieval of the memory of the reward required the activity of dopaminergic neurons labeled by the TH-GAL4 driver as well as activity from αβ Kenyon cells, Which are the specific dopaminergic neurons and MB output neurons involved in ethanol reward processing? Another example comes from the work of Shohat-Ophir et al., 2012 and Krashes et al., 2009 that suggests that while NPF neurons form part of the fly's reward circuit, different NPF cells may be responsible for exerting seemingly opposite effects over sucrose and ethanol reward; what may be the mechanisms underlying such disparate results?

In order to have a better understanding of how rewards are processed and remembered in the brain of the fly, and analogously to the Olds & Milner experiments, one could envision unbiased neuronal activation experiments in *Drosophila* in order to uncover new neurons involved in reward processing. To map the entire *Drosophila* reward circuitry, one would need to perform a neuronal activation screen of collections containing a large number of GAL4 drivers, such as the collection described by Pfeiffer et al., 2008 and Jenett et al., 2012. However, application of the standard assays (COP or PER conditioning) would be logistically impractical to test the several thousand independent set of neurons labeled by independent GAL4 expression patterns that we have envisioned for an unbiased neuronal activation screen.

In Chapters 3 and 4 of this thesis, we present the development and characterization of a simple and high-throughput 2-choice assay that employs the red-shifted channel rhodopsin CsChrimson as a means of neuronal activation. Subsequently, Chapter 5 describes the anatomical and functional subdivision of different types of NPF neurons and Chapter 6 will describe dopamine neurons as downstream targets of NPF neurons. Finally, Chapter 7 will present the discussion and future directions for the results presented here.

## Chapter 2: Methods

### Fly lines and culture

Parental lines were raised at room temperature on standard media (cornmeal/yeast/molasses/agar). Experimental flies were raised under constant darkness at 25°C and 70% humidity on standard media containing 0.2 mM all trans-retinal (SIGMA, Cat #: R2500-1G) and collected 0-3 days after eclosion on media containing 0.4 mM all trans-retinal (SIGMA, Cat #: R2500-1G). Flies were 3-6 days old at the moment of experiments. The *$w^{1118}$;NPF-GAL4* (Wu et al., 2003) and *$w^{1118}$;UAS-RNAi$^{NPF}$* (Wen et al., 2005) stocks were a gift of Ping Shen (U. Georgia). The *$w^{1118}$;;NPF-LexA* stock was a gift of Stefanie Hampel (Janelia Research Campus). The *$w^{1118}$;;NPFR-Gal4* was a gift from Michael Texada (Janelia Research Campus). The *$w^{1118}$,20XUAS-stop-CsChrimson; 8XLexAop-FLP* stock was a gift from Marta Zlatic (Janelia Research Campus). The *$w^{1118}$; UAS-Kir2.1/CyO; Sb/TM3,Ser* stock was a gift from Barry Dickson (Janelia Research Campus). The *$w^{1118}$;Gr66a-GAL4* stock was a gift of Kristin Scott (UC Berkeley). The following strains were generated using classical genetic procedures:

- *$w^{1118}$; NPF-GAL4; 20XUAS-CsChrimson-mVenus*
- *$w^{1118}$; NPFR-Gal4/Cyo; NPF-LexA/TM6b*
- *$w^{1118}$,13XLexAop2-myrGFP, 10XUAS-mCD8-RFP; Sp/Cyo; NPF-LexA/MKRS*
- *$w^{1118}$,13XLexAop2-CsChrimson-mVenus; UAS-Kir2.1/Cyo; NPF-LexA/ TM3,Ser*
- *$w^{1118}$,13XLexAop2-CsChrimson-tdT; UAS-opGCaMP6s; NPF-LexA*

**Behavioral chambers**

To allow for high-throughput assays, we built a system containing 20 independent rectangular chambers running in parallel, each of dimensions 10 cm x 1 cm x 0.3 cm (Fig.1A). The top of each chamber is a sliding, transparent acrylic sheet with a small hole through which flies are introduced using a mouth pipet. The floor of each chamber is a 3-mm thick white acrylic diffuser. Positioned 1 cm below the diffuser is a printed circuit board (PCB) that contains infrared (IR) LED lights for back-illumination as well as 617-nm light-emitting diodes (LEDs) (LUXEON Rebel; Luxeon Star LEDs, Bradford Ontario, Canada) for CsChrimson activation. The midline of each chamber is marked with 1mm-wide metallic strip located below the diffuser. To prevent overheating upon repeated use, the PCB board is located on top of an aluminum block connected to a water-cooling system (Fisher Scientific IsoTemp 6200 R20). Experiments are recorded with cameras (Basler AG, Model: A622F) located above the chambers and equipped with an IR long-pass filter (HOYA 49mm Infrared R72 filter).

**2-choice experiment**

For initial behavioral characterization, experimental flies were obtained by crossing *NPF-GAL4* or *Gr66a-GAL4* males with *20XUAS-CsChrimson-mVenus* (inserted into attP40) females. For subsequent experiments, experimental flies were obtained from crosses between a *GAL4* line males with *20XUAS-CsChrimson-mVenus* (inserted into attP18) females. Crossing the respective UAS-CsChrimson females to $w^{1118}$ males from the appropriate genetic background generated control flies. Flies were 3-6 days old when initially subjected to the 2-choice assay, which was performed at 25°C and 50% humidity. To avoid any potential bias from external visual cues, experiments were performed in a dark room. Male flies were introduced into each chamber,

using a mouth pipet, either as groups of approximately 12 individuals or as single individuals. Flies spent the first 5 min of the assay under IR light alone (617-nm LEDs off) to measure their basal activity, followed by 5 min of optogenetic activation (617-nm LEDs on) in one side of the chambers. Afterwards, flies were given a 5 min-recovery period (617-nm LEDs off). Throughout all experiments, flies were observed under infrared light (IR), to which flies are blind. The activation period consisted of repeated 8 ms pulses of the 617-nm LEDs followed by a variable amount of time, depending on the desired frequency (Fig 1B). For example, for an activation at 40 Hz, the 617-nm LEDs were on for 8 ms followed by 17 ms of being off. The frequency as well as the light intensity employed for each experiments are controlled with MATLAB via a National Instruments Data Acquisition Device (NIDaq), to which the PCB board containing the 617-nm LEDs is connected.

**Quantification of behavior**

Fly behavior in the chambers of our system was recorded with video cameras, and the subsequent video analyzed with custom MATLAB scripts that detect the position of flies within the chamber and thereby allowed us to determine the number of flies on each side of the chamber.

To quantify the distribution of the flies over the course of the experiment, for every frame of the resulting video (time-point i), we calculated a preference-index (PI) defined as:

$$PI_i = \frac{\binom{number\ of\ flies\ in\ 617\ nm\ \boldsymbol{on}}{side\ of\ chamber} - \binom{number\ of\ flies\ in\ 617\ nm\ \boldsymbol{off}}{side\ of\ chamber}}{total\ number\ of\ flies}$$

To describe the relative preference for the activation of a certain group of neurons for a group of flies, based on the PI values obtained, we calculated an activation effect (AE) defined as:

$$AE = \begin{pmatrix} Average\ of\ PI\ values \\ during\ the\ last\ minute\ of\ the\ activation\ phase \end{pmatrix} - \begin{pmatrix} Average\ of\ PI\ values \\ during\ the\ last\ minute\ of\ the\ basal\ activity\ phase \end{pmatrix}$$

In single-fly experiments, for a particular phase, the activation effect (or ΔTime %) was calculated as the difference between the percentage of time spent on the side of activation during the last minute of that particular phase and the last minute of basal activity.

**Conditioned Odor Preference for an odor associated with optogenetic neuronal activation in the 2-choice chambers.**

Olfactory conditioning was performed similarly as previously described (Kaun et al., 2011). For training, groups of 30 flies were introduced into a smaller version of our 2-choice assay, which was at the same time introduced into a bigger plastic box employed for odor delivery. In addition, the top of the chamber was perforated with small holes to allow the odors to reach the inside of the chamber were the flies are exposed to NPF neuronal activation. The olfactory cues used were isoamyl alcohol (IAA) and ethyl acetate (EA). NPF (or Gr66a) neurons were stimulated, on the entire chamber, with 617-nm LED using a frequency of 40 Hz and a light intensity of 5 μW/mm$^2$. A typical T-maze apparatus was used to subsequently test for memory either 10 min or 24 h after training. A one-cycle training protocol was used, in which flies are trained with a 10 min exposure to air, followed by a 10 min exposure to odor A, followed by a 10 min exposure to air, and finally a 10-min exposure to odor B paired with NPF (or Gr66a) neuron

activation. For this group, memory was tested 10 min after end of the end of training. For the olfactory conditioning experiments that used activation of NPF neurons, a massed-training protocol was also tested, in which flies received three cycles of a 30 min exposure to odor A, followed by a 30 min exposure to odor B paired with NPF neuron activation. In this protocol, memory was tested 24 h after the end of training. The conditioned preference index (CPI) for a particular group was calculated as:

$$CPI = \frac{\begin{pmatrix} number\ of\ flies\ in \\ paired\ odor\ t\ maze\ arm \end{pmatrix} - \begin{pmatrix} number\ of\ flies\ in \\ unpaired\ odor\ t\ maze\ arm \end{pmatrix}}{total\ number\ of\ flies}$$

In both cases, one-cycle or massed training, the reciprocal group was performed so as to rule out any inherent bias for either of the olfactory cues, and the resulting Conditioned Preference Index was calculated as the average of the two reciprocal groups.

**Conditioned Odor Preference for an odor associated with optogenetic activation of NPF neurons in a circular arena.**

Olfactory conditioning was performed in a circular arena, described previously (Aso et al., 2014b) that employs 617 nm LEDs for CsChrimson activation, coupled to an odor delivery system that sends odors to each quadrant of the arena (Aso & Rubin, 2016). The olfactory cues used were isoamyl alcohol (IAA) and ethyl acetate (EA). NPF neurons were stimulated, on the entire chamber, using constant 617-nm LED light at an intensity of 20 μW/mm$^2$. Flies were trained as a group of 30 individuals using a single training session consisting of 5 min exposure to air, followed by a 5 min exposure to odor A paired with NPF neuron activation,

followed by a 5 min exposure to air, and finally a 5 min exposure to odor B. In this protocol, the memory was tested 5 min after training, in which the odors A and B are delivered to each pair of opposing quadrants. Experiments are recorded with a camera (Point Grey Research, Model: Flea3 1.3 MP Mono USB3 Vision) located above the chamber and equipped with an IR long-pass filter (Edmund optics, Mounted 800nm Longpass Filter, M30.5 x 0.5mm Mount). The subsequent video was analyzed with custom MATLAB scripts that detect the position of flies within the chamber and thereby allowed us to determine the number of flies on each quadrant of the chamber. For each time point a conditioned preference index ($CPI_i$) was calculated as:

$$CPI_i = \frac{\begin{pmatrix} number\ of\ flies\ in \\ paired\ odor\ quadrants \end{pmatrix} - \begin{pmatrix} number\ of\ flies\ in \\ unpaired\ odor\ quadrants \end{pmatrix}}{total\ number\ of\ flies}$$

The CPI for a particular group corresponds to the average of the $CPI_i$ during the first 2 min of the test phase. The reciprocal group was performed so as to rule out any inherent bias for either of the olfactory cues, and the resulting Conditioned Preference Index was calculated as the average of the two reciprocal groups.

**Electric shock coupled to optogenetic activation of NPF neurons:**

In order to test the effect of coupling an aversive stimulus with the activation of NPF neurons, a small electric grid was introduced in the bottom of the 2-choice assay chambers. To ensure that the flies would stay in contact with the electric grid throughout the experiment, the regular chambers of our system were replaced with chambers of dimensions of dimensions 10 cm x 1 cm x 0.15 cm. In addition, the walls of the arena, as well as the top of the chamber,

were coated with Sigmacote (SIGMA-ALDRICH) to create a slippery surface that prevents the flies from walking on it. The two experimental groups for this experiment were Grid-only and Grid+LEDs. Both started with 5 min of basal activity, followed by 5 min of 617-nm light (40 Hz and 5 $\mu W/mm^2$). During the third phase of the experiment, the Grid-only group received 5 min of electric shock, delivered as 50 ms pulses of 18 V every 1 s using an isolated Pulse Stimulator (AM Systems, model 2100), while the Grid+LEDs group received the electric shock paired with 617-nm light. Finally, flies were given a 3 min recovery period (Grid with 617-nm light off). The side employed for the electric shock, paired or unpaired with 617-nm light, was the same as the side used in the initial activation period. For each phase of the experiment the activation effect is calculated, as described previously, with respect to the basal activity period (initial 5 min).

**Effect of ethanol exposure**

Flies were exposed to ethanol vapors similarly as previously described (Kaun et al., 2011; Shohat-Ophir et al., 2012). Briefly, groups of approximately 12 flies were exposed to three sessions of 53% ethanol vapor for 10 min, spaced by 30 min of air exposure. One hour after the last ethanol exposure, flies were tested in our 2-choice assay. For this experiment, the control for ethanol exposure corresponds to flies that did not undergo the ethanol exposure procedure.

**Natural vs. Artificial reward competition**

For this experiment, we used virgin females (4 days old, grouped housed) expressing CsChrimson in bitter-sensing gustatory neurons expressing Gr66a and virgin males (7 days old, single housed) expressing CsChrimson in NPF neurons or in bitter-sensing gustatory neurons expressing Gr66a. We modified the chambers of our 2-choice assay by introducing a sliding

acrylic divider (middle gate) that separated the two sides of each chambers. A single fly from a particular genotype was introduced, using a mouth pipet, into each side. The experimental group consisted of a male fly with CsChrimson expression in NPF neurons paired with a female fly with CsChrimson expression in Gr66a neurons. On the other hand, the control group consisted of a male fly with CsChrimson expression in NPF neurons paired with a male fly with CsChrimson expression in Gr66a neurons. During the first phase of the experiment, flies were kept isolated into their respective side for a period of 2 min, after which the middle gate was opened. Flies were then given a period of 3 min (pre-Dilemma phase) to explore the entire chamber and interact with each other. During the next 5 min (Dilemma phase), the 617-nm LEDs were turned on, using a frequency of 10 Hz and a light intensity of 5 $\mu W/mm^2$, in only one side of the 2-choice chambers (active side). In this phase, flies with CsChrimson expression in Gr66a neurons will show an aversion to the active side, thus, they will stay on the opposite side. Since normal male flies will show attraction towards a virgin female but not to another male fly, in the experimental group this allowed us to create a dilemma for the male fly with CsChrimson expression in NPF neurons: to approach the active side, for which it would normally show preference, or to approach the opposite side where the virgin female is. Afterwards, the 617-nm LEDs are turned back to off and flies are given an extra 5 min of recovery. The preference ($\Delta$Time %) of each fly for the active side during the Dilemma phase was manually scored and calculated as the difference between the percentage of time spent on the side of activation during the last 2 min of the Dilemma and the percentage of time spent on the side of activation during the last 2 min of the pre-Dilemma phase. For each pair, the mean distance between flies was calculated as the average distance between them during the last 2 min of the Dilemma phase.

**Targeting of NPF expression**

To activate NPF neurons while targeting the expression of NPF using RNAi, we crossed the strain *$w^{1118}$; NPF-Gal4; 20XUAS-CsChrimson* to a *$w^{1118}$; UAS-RNAi$^{NPF}$* strain (a gift from Ping Shen, University of Georgia), or to a *$w^{1118}$* strain to generate the respective positive control group. To control for the effect of extra UAS sequences on the efficacy of the Gal4 protein in driving CsChrimson, we crossed the strain *$w^{1118}$; NPF-Gal4; 20XUAS-CsChrimson* to a *20XUAS-mCD8-GFP* strain.

**NPF neuronal epistasis**

To activate NPF neurons while blocking the synaptic transmission of a candidate postsynaptic group of neurons using expression of Kir2.1 (Baines et al., 2001), we crossed the strain *13XLexAop2-CsChrimson-mVenus; UAS-Kir2.1/Cyo; NPF-LexA/TM3,Ser* to a particular Gal4 driver that labels the neuronal group of interest. The corresponding positive control was generated by crossing the strain *13XLexAop2-CsChrimson-mVenus; UAS-Kir2.1/Cyo; NPF-LexA/ TM3,Ser* to a *$w^{1118}$* strain. The progeny of these crosses was subsequently tested in a 2-choice experiment.

**Immunostaining and imaging**

Fly brains were dissected in cold 1X PBS and fixed in 2% paraformaldehyde made in 1X PBS at 4 °C overnight on a nutator, washed 4 times for 20 min each in PAT (1X PBS, 0.5% Triton, 1% BSA) at room temperature, blocked for 1h at room temperature with blocking buffer (PAT + 3% Normal Goat Serum) and incubated with primary antibodies, diluted in blocking buffer, overnight on a nutator at 4 °C. The primary antibodies used were: Mouse-GFP (SIGMA-

ALDRICH, G6539. 1:500 dilution), Rabbit-DsRed (Clontech, 632496. 1:500 dilution), Rabbit-NPF (RayBiotech, RB-19-0001. 1:200 dilution) and Rat-DN-cadherin (Hybridoma Bank DSHB, DNEX#8) (1:50). This was followed by 4 washes for 20 min each in PAT, and incubation overnight on a nutator at 4°C with secondary antibodies diluted in blocking buffer. The secondary antibodies used were: Alexa Fluor 488 anti-Rabbit (Molecular Probe, A11034. 1:500 dilution), Alexa Fluor 568 anti-Mouse (Molecular Probe, A11031. 1:500 dilution) and Alexa Fluor 633 anti-rat (Molecular Probe, A21094. 1:500 dilution). Brains are then washed 4 times for 20 min each in PAT at room temperature, 1 time for 20 min in 1X PBS and mounted with VECTASHIELD mounting medium (Vector Laboratories, H-1000). Brains were imaged on a Zeiss 880 confocal laser-scanning microscope.

**Functional connectivity experiments**

Flies were raised under constant darkness at 25°C and 70% humidity on standard media containing 0.2 mM all trans-retinal and collected 0-3 days after eclosion on media containing 0.4 mM all trans-retinal (SIGMA, Cat #: R2500-1G). All experiments were performed on male flies, 5-8 days after eclosion. Brains were dissected in a saline bath (103mM NaCl, 3mM KCl, 2mM $CaCl_2$, 4mM $MgCl_2$, 26mM $NaHCO_3$, 1mM $NaH_2PO_4$, 8mM trehalose, 10mM glucose, 5mM TES, bubbled with 95% $O_2$ / 5% $CO_2$). After dissection, the brain was positioned anterior side up on a coverslip in Sylgard dish and perfused with the saline at 20°C.

The sample was imaged with a resonant scanning 2-photon microscope with near-infrared excitation (920nm, Spectra-Physics, INSIGHT DS DUAL) and a 25x objective (Nikon MRD77225 25XW). The microscope was controlled by using ScanImage 2015.v3 (Vidrio Technologies). Volumes were acquired with ~235 μm x ~235 μm x 210 μm field of view at

512x512x42 pixel resolution, approximately 1 Hz per volume. The excitation power for calcium imaging measurement was between 11.2 mW to 15.3 mW.

For the optogenetics manipulation, the light-gated ion channel Chrimson was activated with a 660 nm LED (M660L3 Thorlabs) coupled to a digital micromirror device (Texas Instruments DLPC300 Light Crafter). 525/50 and 625/90 emission filters (Semrock Inc.) were used to filter the light before the photomultiplier. Photoactivation light was delivered in a pulse train that consisted of six 1s pulses (square-wave modulation, 100% duty cycle, 30s inter-pulse interval). The instantaneous light intensity was delivered at 2.47 $mW/mm^2$ (measured using Thorlabs DS2100).

The calcium responses of the genetically targeted neurons were examined as $\Delta F/F$ and the baseline fluorescence (F0); both of them were acquired from the manually defined region of interest (ROI). The $\Delta F/F$ was taken as (F-F0)/F0 where F is the instantaneous mean fluorescence of the ROI. The baseline fluorescence (F0) was taken as the mean fluorescence of the ROI for a 10s period prior to the start of each of the photoactivation period. Integrated responses were further analyzed by determining the peak (minimum or maximum) $\Delta F/F$. For all experiments, the time series $\Delta F/F$ are plotted as the median ± SEM across flies. All data analysis was performed offline using custom MATLAB scripts.

**Statistical analysis**

To determine the statistical significance of our data we employed MATLAB (R2015a) or GraphPad prism (version 6) software package, to perform un-paired t-test or one-way ANOVA followed by Tukey's multiple comparison post-hoc test. The significance of the preference vs. speed correlations, either shown in Figure 3, was tested using the built-in corrcoef function from MATLAB (R2015a). Data is expressed as the mean +/- standard error, along with a scatter plot of the data points. In all figures: ***=$p<0.001$; **=$p<0.01$; *=$p<0.05$.

## Chapter 3: Establishing a simple 2-choice assay for studying reward in Drosophila

### 3.1 Description of the 2-choice assay. Preference for optogenetic activation of NPF neurons

To provide evidence that the activity of particular neurons is perceived as rewarding to the fly, inspired by the work of Olds & Milner, we developed a 2-choice assay (**Figure 3.1A**) in which flies were able to demonstrate whether they had a preference (or aversion) for having a specific subset of neurons experimentally activated. In the fly, neurons can be strongly activated by inducing the activity of recombinant ion channels, such as the temperature-gated TrpA channel or the red light-activated channelrhodopsin CsChrimson (Hamada et al., 2008; Klapoetke et al., 2014). Because of its rapid on-off responsiveness to red light, we chose to use CsChrimson in our assay. We hypothesized that activation of certain neurons would be perceived as rewarding by the flies and consequently induce a preference for red light. As a behavioral readout for potentially perceiving reward, we put a group flies into a chamber in which one side was exposed to LED-generated red light at 617 nm (the activation side) pulsing at a variable frequency (**Figure 3.1B**), while the other side was not (the non-activation side); we then quantitated whether the distribution of flies showed a bias for one side or another after a period of time. A Preference Index (PI) was calculated as the number of flies on the activation side minus the number on the non-activation side, divided by the total number of flies in the chamber (see methods). A positive PI indicates a preference for activation of the neurons, while a negative PI indicates an aversion to activation. We first characterized the efficacy of our two-choice assay by measuring the preference that flies have for CsChrimson-mediated activation of NPF neurons. NPF neurons have been shown to regulate different forms of reward (Shohat-Ophir et al., 2012;

Krashes et al., 2009) and their thermogenetic activation, with dTrpA1 in an olfactory conditioning assay, is rewarding (Shohat-Ophir et al., 2012).

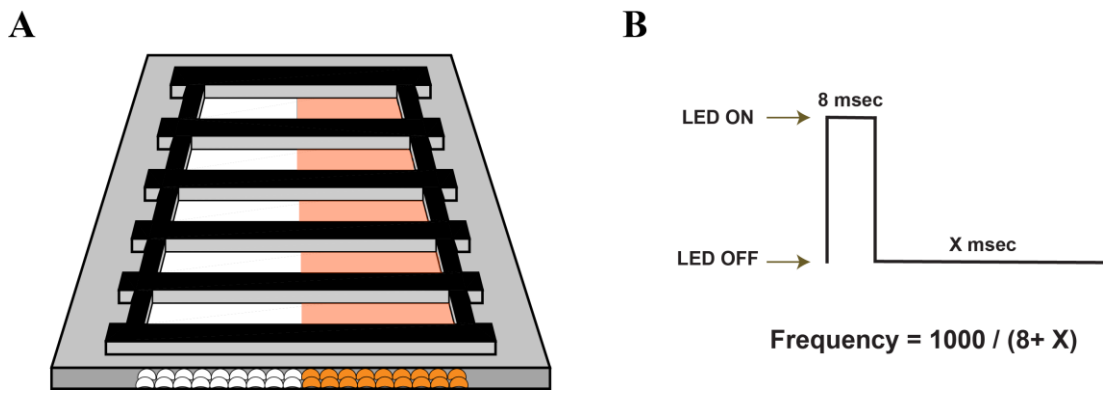


**Figure 3.1. Schematic representation of the 2-choice assay system**. **(A)** During any given experiment, flies are exposed to a 617 nm light in only one side of the chambers. If the activation of a specific subset of neurons is perceived as appetitive, then flies would prefer the side being used for activation. On the contrary, if the activation of a specific subset of neurons is perceived as aversive, flies would avoid the side being used for activation. **(B)** During each pulse, the 617 nm LEDs remain ON for 8 ms followed by a variable amount of time, depending on the desired frequency, in which the 617 nm LEDs remain OFF.

Taking this into account, we asked if flies would find activation of NPF neurons rewarding in our assay. Indeed, as shown in (**Figure 3.2A**), flies expressing CsChrimson in NPF neurons prefer the activation side during the activation period. To quantify how the distribution of flies changes due to the optogenetic activation of a particular subset of neurons when comparing the activation and basal activity period, we defined an Activation Effect (AE, see Methods), which shows a significant preference for the activation of NPF neurons (**Figure 3.2B**).

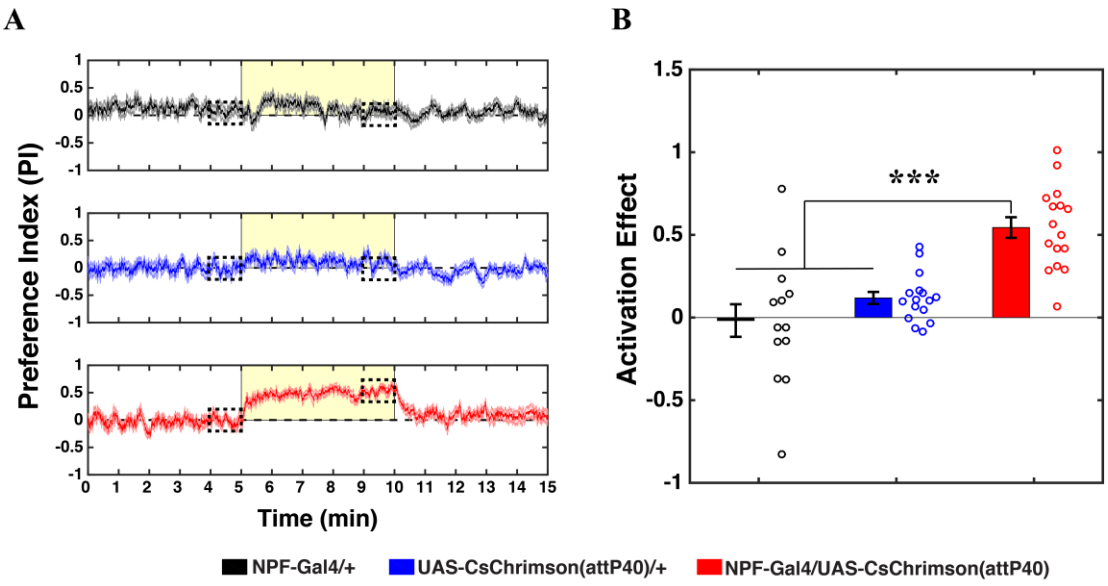


**Figure 3.2. Flies exhibit a preference for NPF neuron activation**. **(A)** Experimental data expressed as the mean +/- standard error of the preference index over time. The yellow box indicates the side and period of activation. The dashed boxes represent the periods of time employed to calculate the activation effect (see methods) (n = 13-16). **(B)** Activation effect for the data shown in (A), showing that flies have a significant preference for the activation of NPF neurons. (n = 13-16; one-way ANOVA with Tukey's post-hoc test; ***: $p<0.001$). (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **5 µW/mm$^2$**).

To see if flies would display different levels of preference when optogenetically activating NPF neurons at different 617 nm LED light frequencies and intensities, we performed a frequency dose-response using a low (**5 µW/mm$^2$**) or high (**20 µW/mm$^2$**) 617 nm LED light intensity (**Figure 3.3**). When the frequency dose-response curve was performed at the low intensity, a minimum frequency of 12.5 Hz is required for the stimulation of NPF neurons to produce a preference response (**Figure 3.3A).**

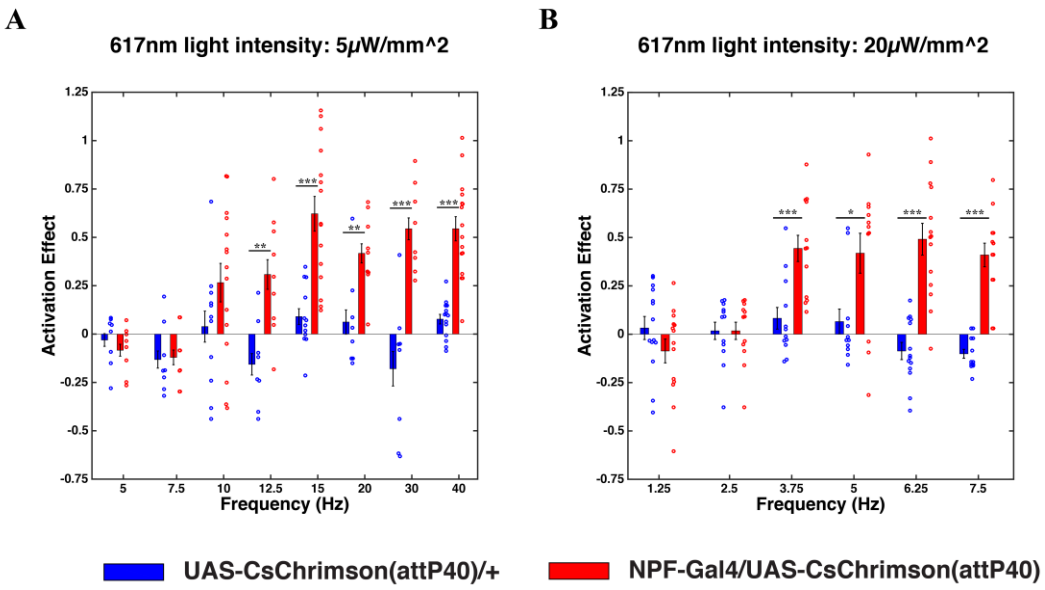


**Figure 3.3. Frequency dose-response curve for activation of NPF neurons**. **(A)** Activation of NPF neurons at different frequencies, using a 617-nm LED light intensity of **5 μW/mm$^2$**, reveals that a minimum of 12.5 Hz is required for the flies to display a significant preference. (Unpaired t-test for each frequency between the inactive control group and the experimental group; n = 7-16; **: p<0.01; ***: p<0.001). No statistical difference was found for preferences at frequencies equal to or higher than 12.5 Hz (n = 7-16; one-way ANOVA with Tukey's post-hoc test). **(B)** Activation of NPF neurons at different frequencies, using a 617-nm LED light intensity of **20 μW/mm$^2$**, reveals that a minimum of 3.75 Hz is required for the flies to display a significant preference. (Unpaired t-test for each frequency between the inactive control group and the experimental group; n = 11-14; *: p<0.05; ***: p<0.001). No statistical difference was found for preferences at frequencies equal to or higher than 3.75 Hz (n = 7-16; one-way ANOVA with Tukey's post-hoc test).

Similarly, when the frequency dose-response curve was performed at the high intensity, a minimum frequency of 3.75 Hz is required for the stimulation of NPF neurons to produce a preference response (**Figure 3.3B**).

It has been shown for other peptidergic neurons, that the effect of a peptide's signaling can desensitize upon repeated activation (Li & van den Pol, 2006). In the context of our 2-choice assay, this raises the question of whether the preference for activation of NPF neurons would decrease after repeated 2-choice trials. To test this, we subjected flies to 5 trials in our 2-choice assay, each consisting of 2 min of activation followed by 3 min of recovery.

As shown in **Figure 3.4A & 3.4C**, flies expressing CsChrimson in NPF neurons showed preference for the activation of NPF neurons for each trial, with no statistical difference in between trials. Thus, for the protocol tested, the preference for the activation of NPF neurons does not desensitize. As expected, no effect was observed for control flies (**Figure 3.4B & 3.4D**).

The design of our 2-choice assay contemplated that activation of a particular group of neurons could lead to aversion to the side of the chambers used for activation. To explore this possibility, we tested flies expressing CsChrimson in the bitter-sensing gustatory neurons expressing Gr66a, the activation of which has been shown to produce aversive responses in other assays (Keene & Masek, 2012). Consistent with previous work, activation of Gr66a neurons in our assay produced an aversion to the activation side (**Figure 3.5A & 3.5B**). In summary, our assay is able to reveal either preference or aversion to the activation of specific neurons in the fly brain.

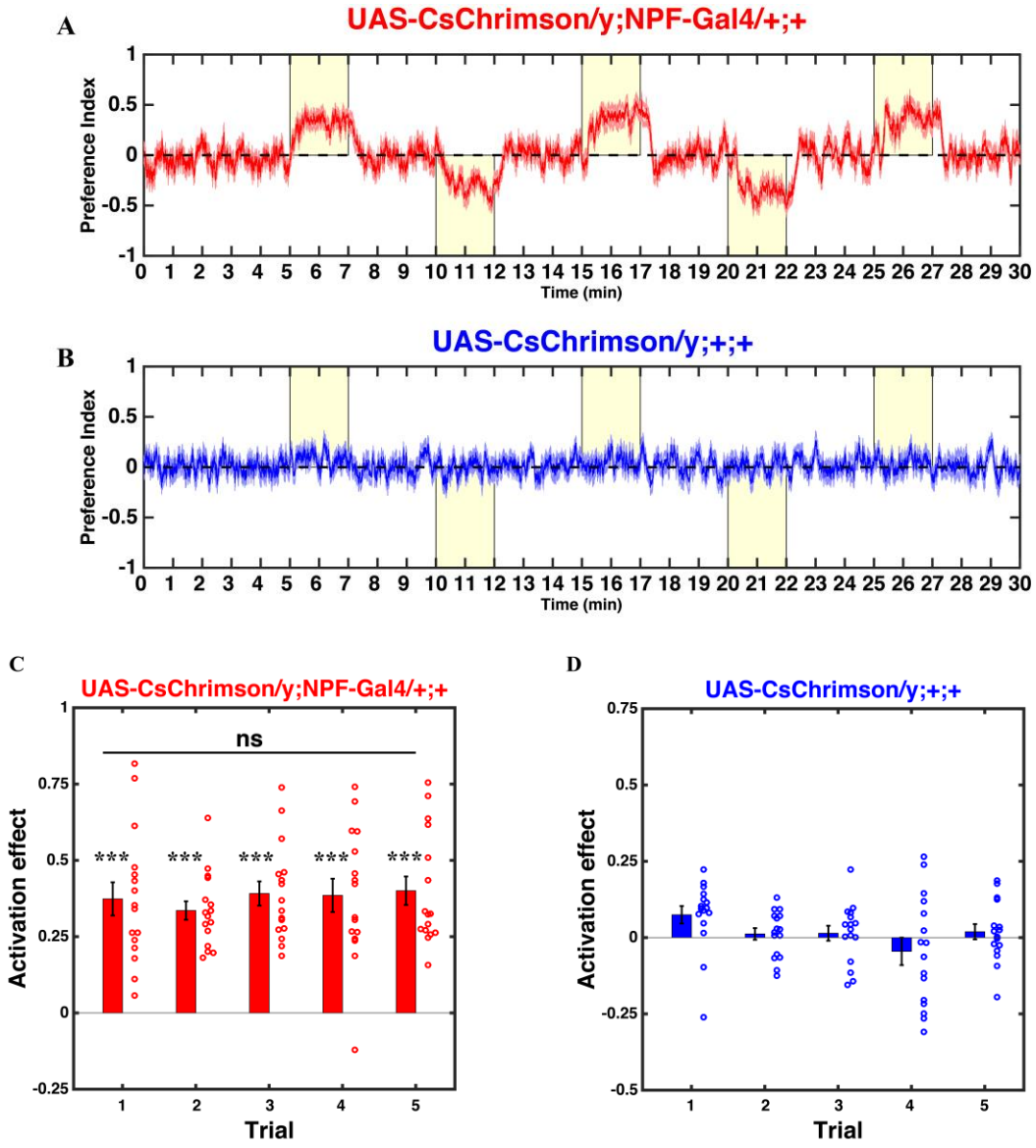


**Figure 3.4. Preference for NPF neuron activation does not desensitize upon repeated activation**. **(A & B)** Experimental data expressed as the mean +/- standard error of the preference index over time for flies expressing CsChrimson in NPF neurons **(A)** or for control flies **(B)**. The yellow boxes indicate the side and period of activation for each trial. **(C & D)** Activation effect for the data shown in (A & B), showing that flies expressing CsChrimson in NPF neurons have a significant preference for the activation of NPF neurons during each of the 5 trials tested (Unpaired t-test for each trial between the inactive control group and the experimental group; n = 16 ***: $p<0.001$). No statistical difference was found for preferences in each trial (n = 16; one-way ANOVA with Tukey's post-hoc test) (617-nm LED light intensity**: 5 µW/mm$^2$.** Frequency of activation: **40 Hz**).

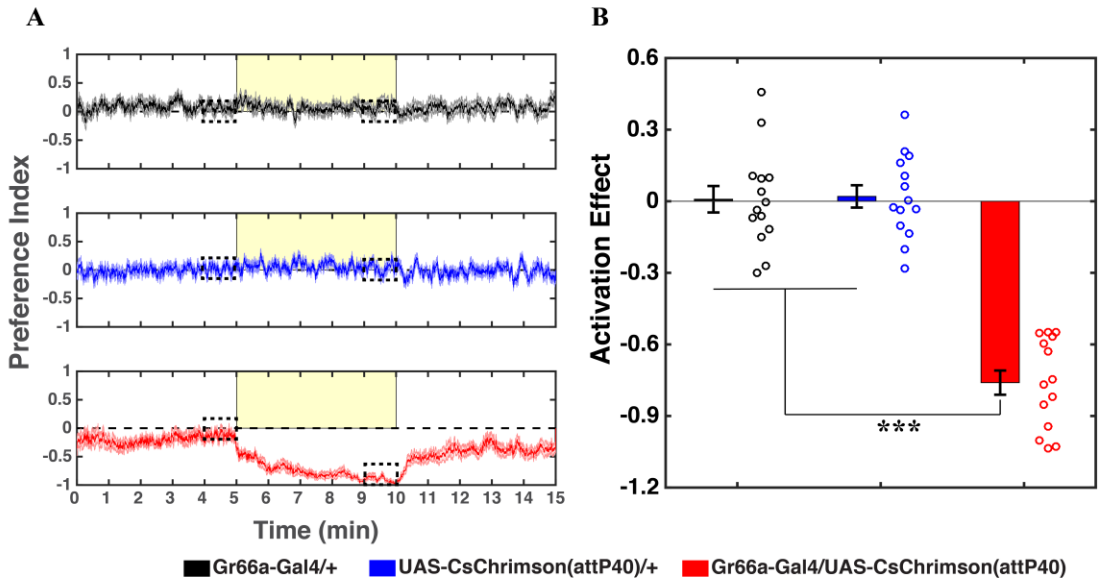


**Figure 3.5**. **Flies avoid activation of Gr66a neurons**. **(A)** Experimental data expressed as the mean +/- standard error of the preference index over time. The dashed boxes represent the periods of time employed to calculate the activation effect (see methods) (n = 14) **(B)** The activation effect for the data in (A) shows that flies display a significant avoidance for the activation of Gr66a neurons (n = 14; one-way ANOVA with Tukey's post-hoc test. ***: $p<0.001$) (617-nm LED light intensity**: 5 µW/mm$^2$.** Frequency of activation: **40 Hz**).

### 3.2 NPF neuron activation-induced preference is not dependent on grouping flies

Although *Drosophila* is a "simple" model organism, it is capable of various forms of social behaviors (Sokolowski, 2010). For example, it has been shown that the size of a group of fruit flies has an effect on the extent to which flies avoid $CO_2$ in an olfactory 2-choice assay (Ramdya et al., 2015) or the rate at which flies aggregate on a food resource (Lihoreau et al., 2016). These observations raise the question of whether the preference displayed by the flies upon activation of NPF neurons may be influenced by the fact that flies are tested as groups of individuals. To investigate this idea, we tested flies expressing CsChrimson in the NPF neurons as single

individuals in our 2-choice assay. During the activation phase, flies displayed a preference for the activation of NPF neurons (**Figure 3.6A & 3.6B**). As expected, the same CsChrimson-activating stimulation had no effect on individual control flies (**Figure 3.6A & 3.6B**). During the recovery phase, neither single control nor single experimental flies showed a preference response. We also speculated that if a single fly is experiencing a reward through activation of NPF neurons on the active side and then wanders into the inactive side, it should travel a shorter distance than a control fly before returning to the active side. Indeed, during the activation phase, single flies expressing CsChrimson in NPF neurons travel a shorter distance into the inactive side, when compared with control flies (**Figure 3.6C**).

### 3.3 Effect of NPF neuron activation on locomotion

We noted that, while activation of NPF neurons triggers a preference response in single flies, it also reduces the speed of the flies during the activation phase (**Figure 3.7A**). This effect was also observed when flies were tested as groups of individuals (**Figure 3.8A**). In fact the preference and speed during the activation phase, either for flies tested as individuals (**Figure 3.9A**) or as groups (**Figure 3.10A**), are negatively correlated. As expected, the same stimulation had no effect on the speed of single control flies or on the speed of groups of control flies, as revealed by the lack of correlation between preference and speed during the period of activation phase (**Figure 3.9B & 3.10B**). Both experimental and control flies (**Figure 3.7 & 3.8**) respond (rapid and transient changes in speed) to the onset of illumination, while only control flies respond to the off set of illumination. In synthesis, flies show a preference for the activation of NPF neurons even if they are tested as single individuals. In addition, flies also display a reduction in locomotion upon NPF neuron activation.

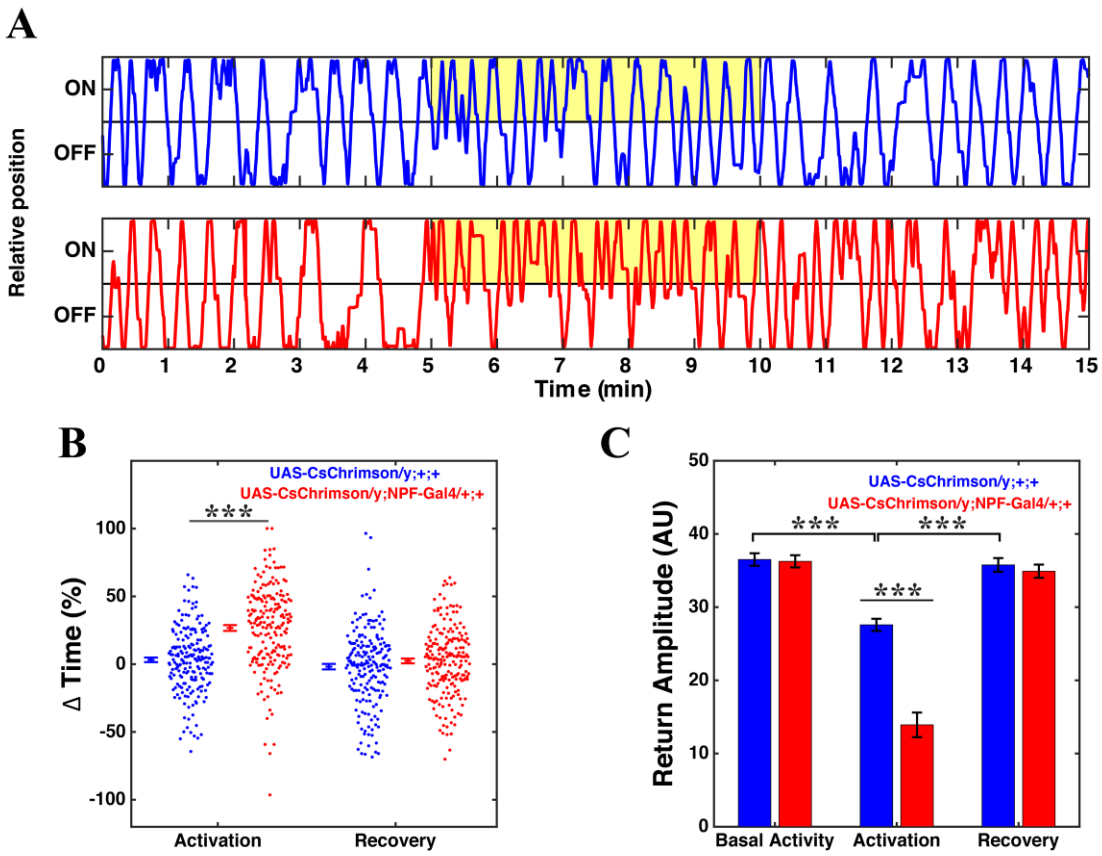


**Figure 3.6**. **Preference for NPF neuron activation does not depend on a social (group) context**. **(A)** Example traces for the relative position over time for a single control fly (upper panel; blue) or a single fly expressing CsChrimson in NPF neurons (test flies) (lower panel; red). OFF indicates the inactive side of the chamber, while ON indicates the side of the chamber that was used for CsChrimson activation. The yellow box indicates the period of activation. **(B)** ΔTime (%) (Preference; see methods) data, showing that single flies have a significant preference for the activation of NPF neurons. (control flies: n=198; test flies: n=203) (Unpaired t-test; ***: p<0.001). **(C)** Return amplitude data showing that, during the period of activation, flies expressing CsChrimson in NPF neurons (test flies) wander shorter distances into the inactive side, when compared with control flies. This analysis excluded those test flies (n = 21) that never left the active side during the activation phase, leaving a total of n = 182 test flies (Unpaired t-test; ***: p<0.001). (617-nm LED light intensity**: 5µ W/mm$^2$.** Frequency of activation: **40 Hz**).

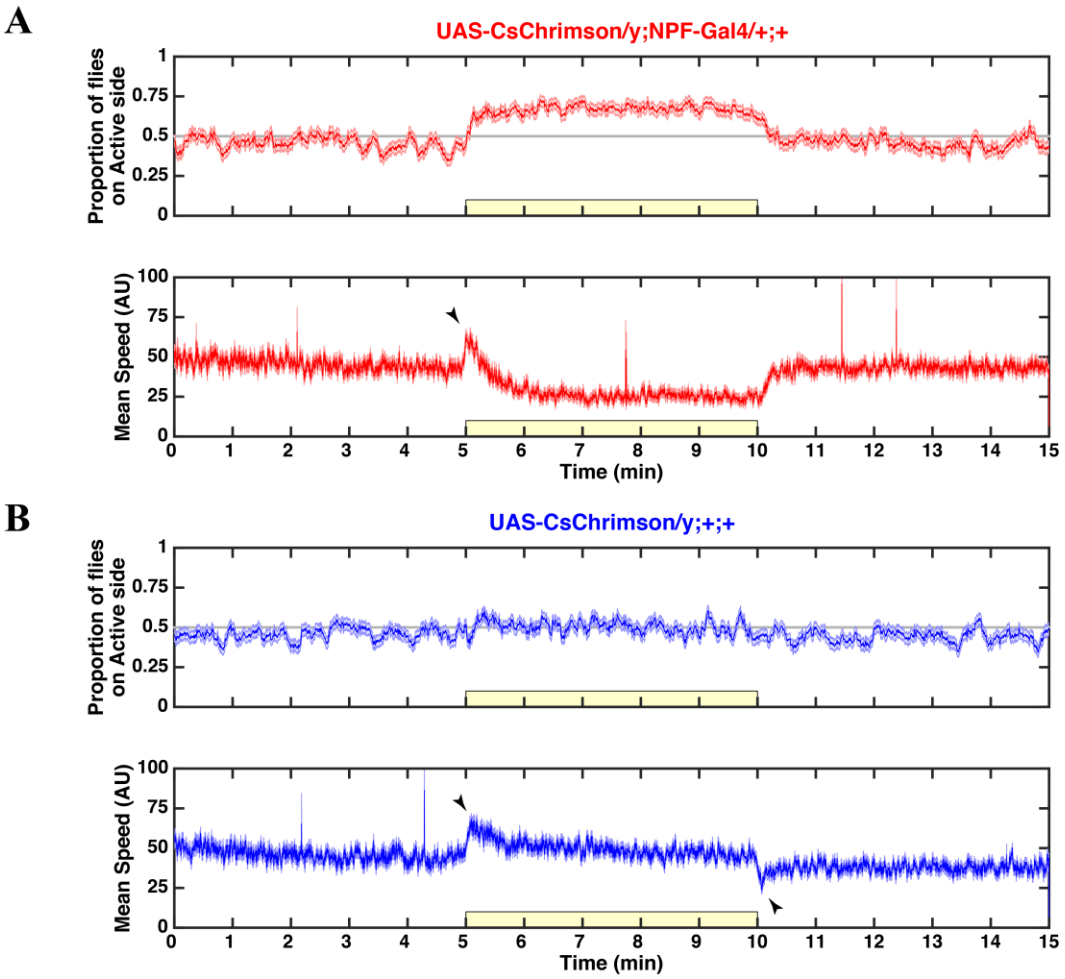


**Figure 3.7**. **Effect of NPF neuron activation on single flies locomotion**. **(A)** Experimental data for flies expressing CsChrimson in NPF neurons (n=203) expressed as the mean +/- standard error of the proportion of flies on active side over time (upper pannel) or speed over time (lower pannel). **(B)** Experimental data for control flies (n=198) expressed as the mean +/- standard error of the proportion of flies on active side over time (upper pannel) or speed over time (lower pannel). (617-nm LED light intensity**: 5µ W/mm$^2$.** Frequency of activation: **40 Hz**). In both (A) & (B), the black arrows indicate the lights-on and lights-off response of the flies to the onset and offset of the light stimulation.

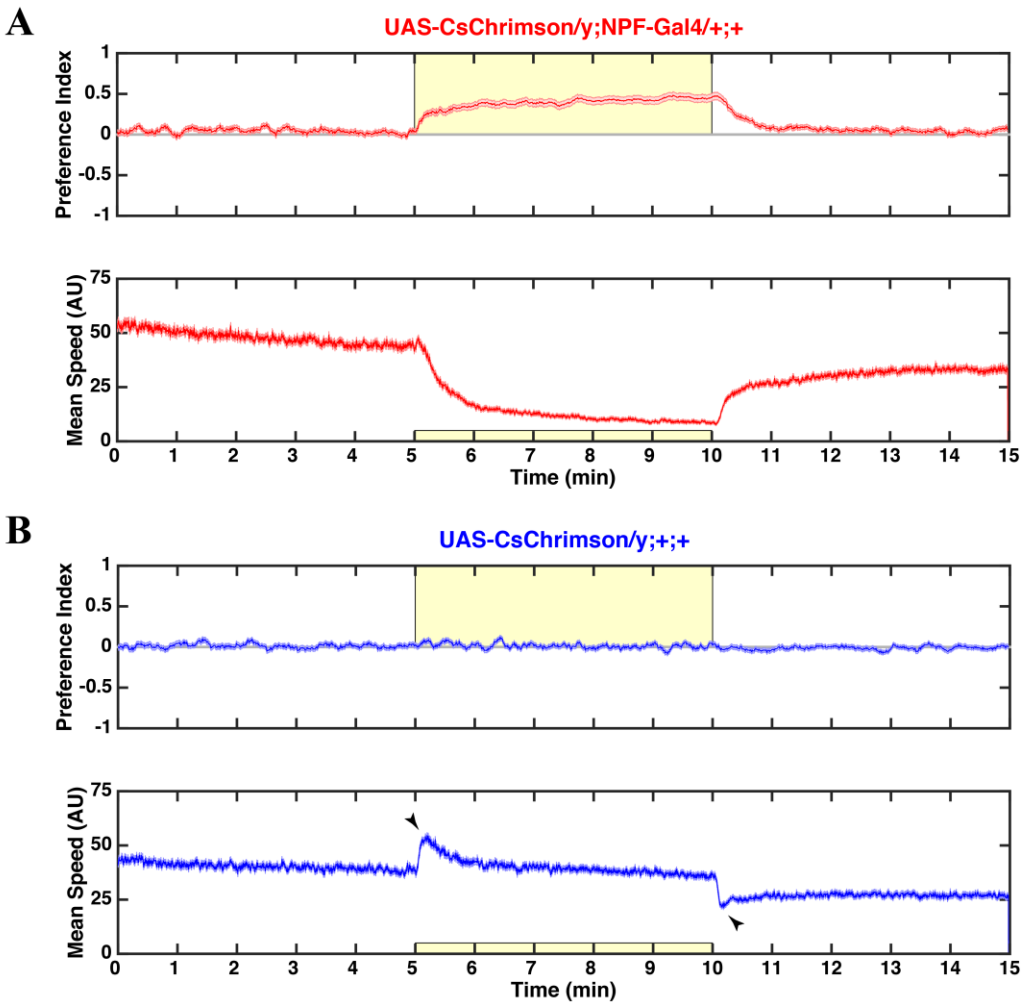


**Figure 3.8**. **Effect of NPF neuron activation on the locomotion of grouped flies**. **(A)** Experimental data for flies expressing CsChrimson in NPF neurons (n=186) expressed as the mean +/- standard error of the preference index over time (upper panel) or speed over time (lower panel). **(B)** Experimental data for control flies (n=179) expressed as the mean +/- standard error of the preference index over time (upper panel) or speed over time (lower panel). (617-nm LED light intensity**: 5µ W/mm$^2$.** Frequency of activation: **40 Hz**). In both (B), the black arrows indicate the lights-on and lights-off response of the flies to the onset and offset of the light stimulation.

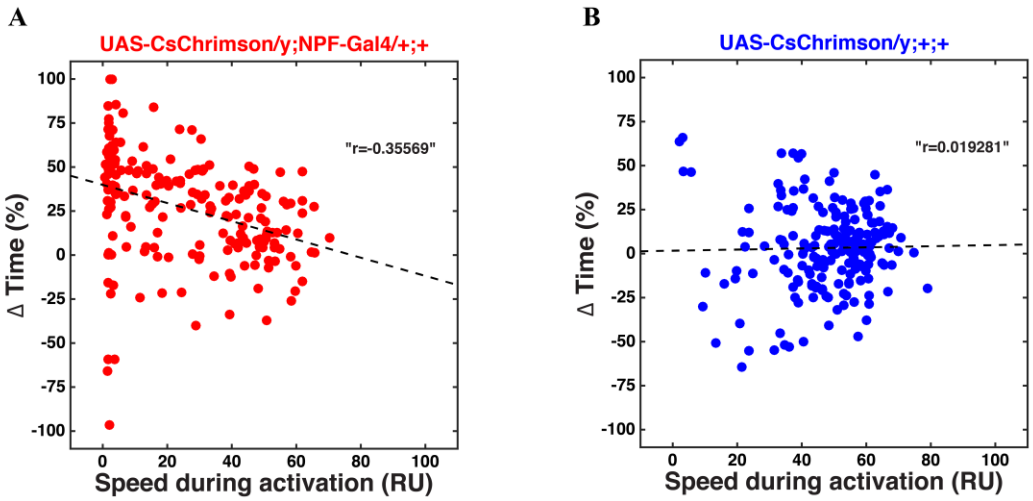


**Figure 3.9**. **Correlation of preference (ΔTime (%)) and speed during activation for single flies**. Scatterplot of the preference (ΔTime (%)) vs. speed during activation for single flies expressing CsChrimson in NPF neurons (total of n=203) **(A)** or single control flies (total of n=198) **(B)**. Each figure also shows the respective, correlation coefficient (r) and the least-squares line (dashed line) (617-nm LED light intensity**: 5µW/mm$^2$.** Frequency of activation: **40 Hz**).

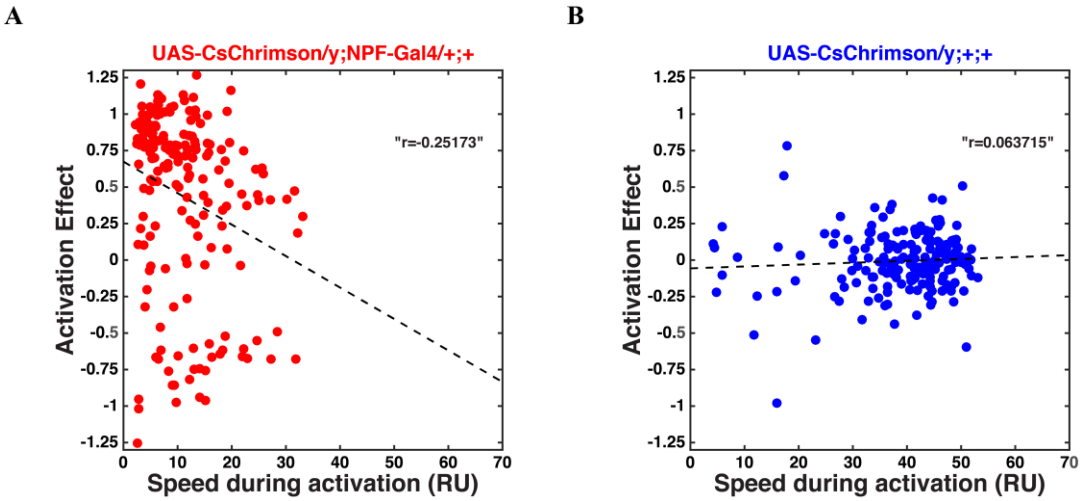


**Figure 3.10**. **Correlation of preference (activation effect) and speed during activation for grouped flies**. Scatterplot of the preference (activation effect) vs. speed during activation for grouped flies expressing CsChrimson in NPF neurons (total of n=186) **(A)** or grouped control flies (total of n=179) **(B)**. Each figure also shows the respective, correlation coefficient (r) and the least-squares line (dashed line) (617-nm LED light intensity**: 5µW/mm$^2$.** Frequency of activation: **40 Hz**).

### 3.4 Optogenetic activation of NPF neurons is rewarding

As it was shown previously, activation of NPF neurons not only produces a preference effect in the 2-choice assay, but is also accompanied by a decrease in speed. This raises the question of whether the NPF neuron activation-induced preference is a reflection of its "rewarding" effects or is merely reducing the locomotion of the flies, thereby "trapping" them on the active side. We reasoned that if the activation of NPF neurons in our assay is truly rewarding, flies should be able associate this experience with an olfactory cue in a COP assay. In order to test this, we performed an olfactory conditioning assay using a smaller version of our system. In a first series of experiments (**Figure 3.11A**), where a one-cycle training protocol was used (10 min of exposure to odor 1 followed by 10 min of exposure to odor 2 coupled with optogenetic activation of NPF neurons), activation of NPF neurons was not remembered as an appetitive experience when the memory was tested 10 min after training (**Figure 3.11B**). However, flies expressing CsChrimson in Gr66a neurons that underwent this same training protocol, remembered the experience as aversive (**Figure 3.12**) when the memory was tested 10 min after training, consistent with previous work (Keene & Masek, 2012). To explore the possibility that activation of NPF neurons may induce a memory only when a more extended conditioning protocol is used, we designed a massed training protocol (**Figure 3.11C**) (three consecutive sessions of 30 min of exposure to odor 1 followed by 30 min of exposure to odor 2 coupled with optogenetic activation of NPF) similar to the one used by (Shohat-Ophir et al., 2012). When the memory was tested 24 h after training, activation of NPF neurons with this protocol was indeed remembered as an appetitive experience (**Figure 3.11D**).

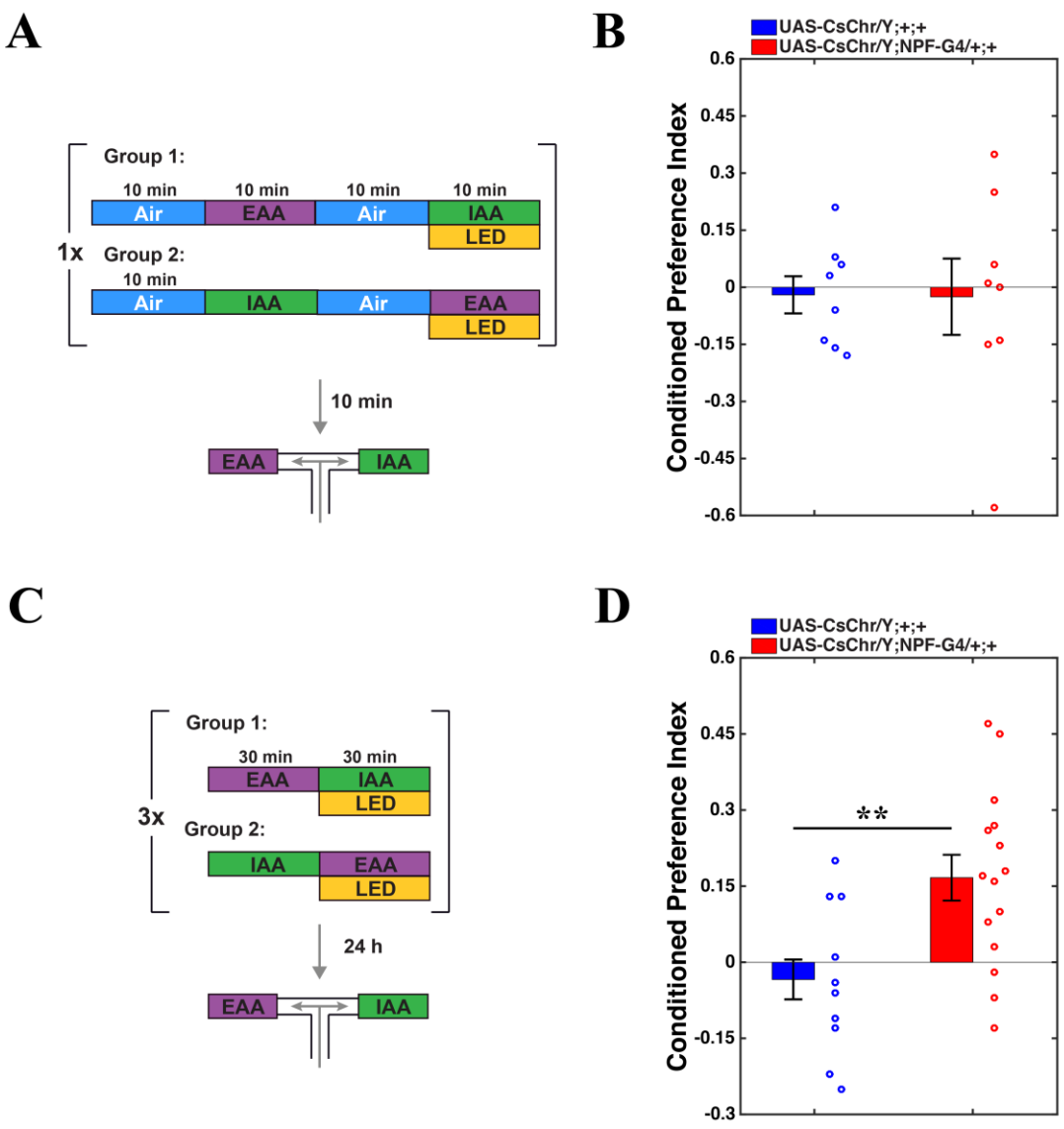


**Figure 3.11**. **Optogenetic activation of NPF neurons is rewarding. (A)** Flies that were trained to associate optogenetic activation of NPF neurons with the presence of an odor, using a single training session consisting of 10-min exposure to air, followed by a 10-min exposure to odor A, followed by a 10-min exposure to air, and finally a 10-min exposure to odor B paired with NPF neuron activation, showed no conditioned odor preference **(B)**, when the memory was tested 10 min after training. **(C)** Flies that were trained to associate an odor with the optogenetic activation of NPF neurons in three consecutive sessions, each consisting of 30 min of exposure to odor 1, followed by 30 min of exposure to odor 2 coupled with optogenetic activation of NPF neurons (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **5 µW/mm$^2$**) showed a significant conditioned odor preference **(D)** (n = 13-15; unpaired t-test; **: p<0.01) when the memory was tested 24 h after training.

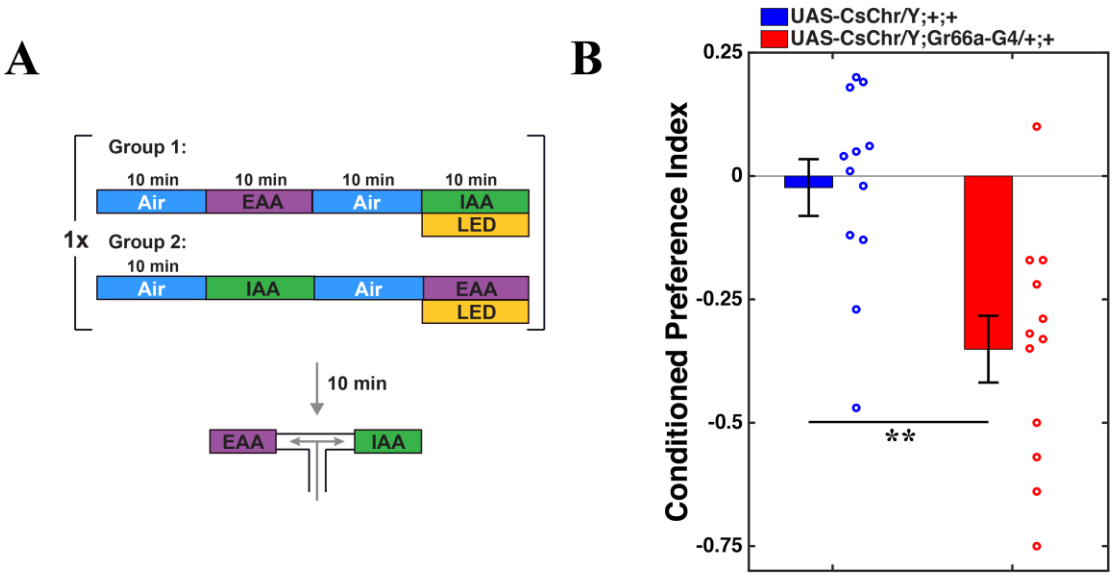


**Figure 3.12**. **Optogenetic activation of Gr66a neurons is aversive. (A)** Flies that were trained to associate an odor with the optogenetic activation of Gr66a neurons using a single training session consisting of 10-min exposure to air, followed by a 10-min exposure to odor A, followed by a 10-min exposure to air, and finally a 10-min exposure to odor B paired with Gr66a neuron activation (Frequency of activation: 40 **Hz**; 617-nm LED light intensity: **5 µW/mm$^2$**) showed a significant conditioned odor aversion **(B)** (n = 12; unpaired t-test; **: p<0.01) when the memory was tested 10 min after training.

During a typical olfactory conditioning protocol, flies have to be transferred from the training apparatus to the testing apparatus (typically a t-maze). This not only makes the procedure very laborious, but it also introduces the possibility of loosing some individuals, thus lowering the resolution at which the memory can be quantified. To overcome these drawbacks, we implemented in our laboratory a new circular arena that uses optogenetic activation of neurons, coupled with an odor delivery system (Aso & Rubin, 2016) to perform automated olfactory conditioning experiments. We tested this system with flies expressing CsChrimson in NPF neurons, using a training protocol consisting of 5 min exposure to air, followed by a 5 min exposure to odor A paired with NPF neuron activation, followed by a 5 min exposure to air, and finally a 5 min exposure to odor B. The memory was tested 5 min after training.

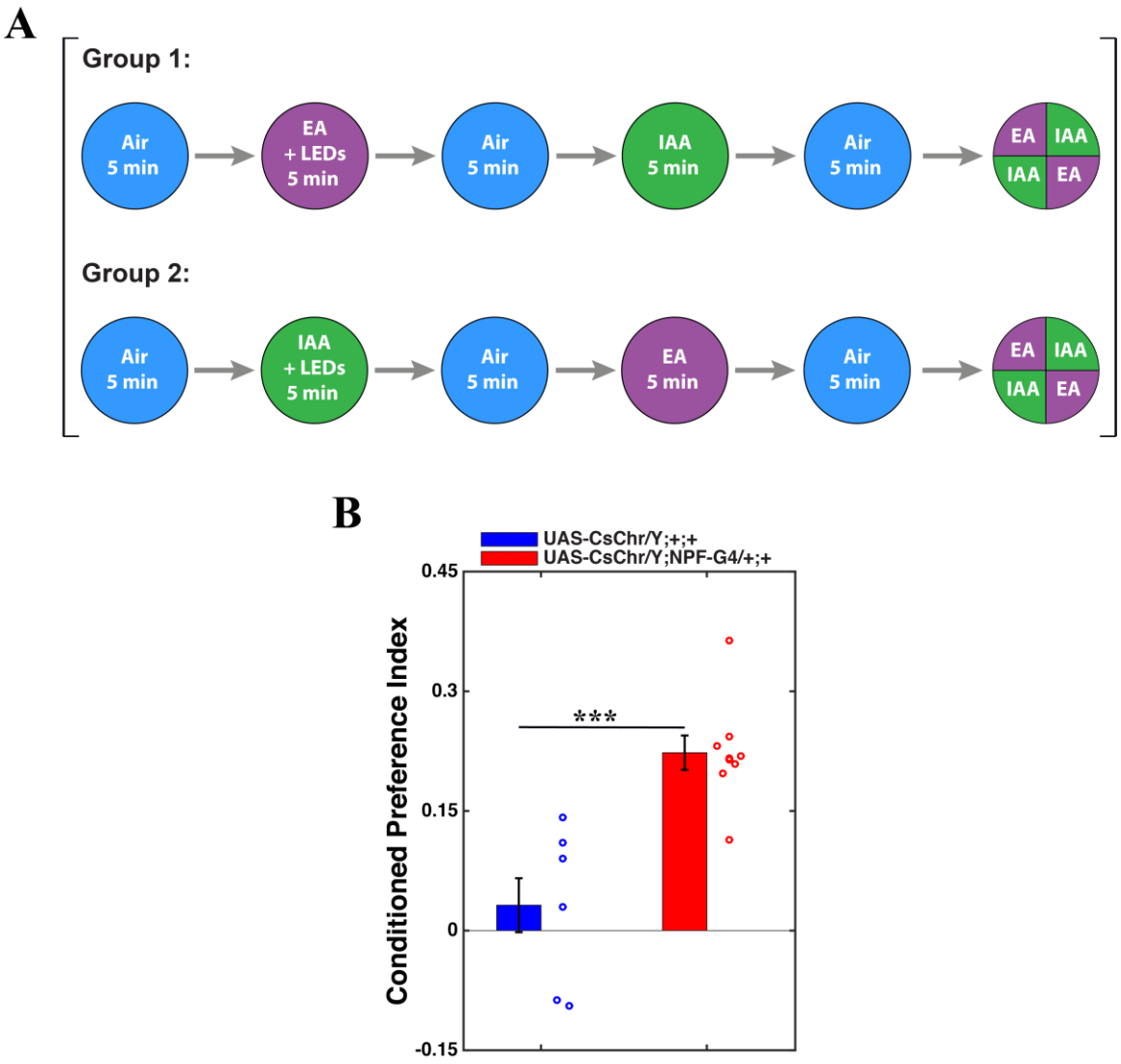


**Figure 3.13**. **Optogenetic activation of NPF neurons in a circular arena is rewarding. (A)** Flies that were trained to associate optogenetic activation of NPF neurons with the presence of an odor, using a single training session consisting of 5-min exposure to air, followed by a 5-min exposure to odor A paired with NPF neuron activation, followed by a 5-min exposure to air, and finally a 5-min exposure to odor B, showed no conditioned odor preference **(B)**, showed a significant conditioned odor preference (n = 13-15; unpaired t-test; ***: $p<0.01$) when the memory was tested 5 min after training (activation performed at constant light; 617-nm LED light intensity: **20 µW/mm$^2$**).

Of note, in this arena we employed a higher 617-nm LED light intensity (**20 µW/mm$^2$**), than the one used in the previous olfactory conditioning experiments (**5 µW/mm$^2$**). As shown in (**Figure 3.13**), activation of NPF neurons using the aforementioned protocol is able to induce the formation of an appetitive memory. Overall, these olfactory conditioning experiments show that flies perceive the optogenetic activation of NPF neurons as rewarding.

## Chapter 4: Characterization of the preference for NPF neuron activation in the 2-choice assay

### 4.1. Flies withstand an aversive stimulus in order to gain access to activation of NPF neurons

Flies are willing to overcome an electric grid in order to approach an odor previously associated with an alcohol reward (Kaun et al., 2011). We therefore tested whether flies may be willing to withstand an electric shock if it was paired with optogenetic activation of NPF neurons.

We subjected two groups of test flies expressing CsChrimson in NPF neurons to our standard two-choice assay, with 5 min of basal activity followed by 5 min of LED exposure on one side of the chamber (Phase I). As expected, the flies showed a preference for the activation side (**Figure 4.1**). During the next 5 min (Phase II), one group (Grid only; blue) of flies was exposed to an electric shock via an electric grid on the floor of one side of the chamber, and the flies showed aversion to the electrified region as represented by the negative AE. The second group (Grid + LEDs; red) was exposed to the same electric shock, but it was paired with NPF neuronal activation. During the last 5 min of the experiment (Phase III), both the LEDs and the electric grid are switched back to off. A one-way ANOVA analysis showed that there is a significant interaction among the groups of phase II and phase III ($P = 0.0079$). Interestingly, a post-hoc comparison revealed a significant difference between the "Grid only" and "Grid + LEDs" groups during phase II ($p < 0.01$; Tukey's post-hoc test). That is, flies did not show aversion towards the electrified grid when it was paired with activation of NPF neurons.

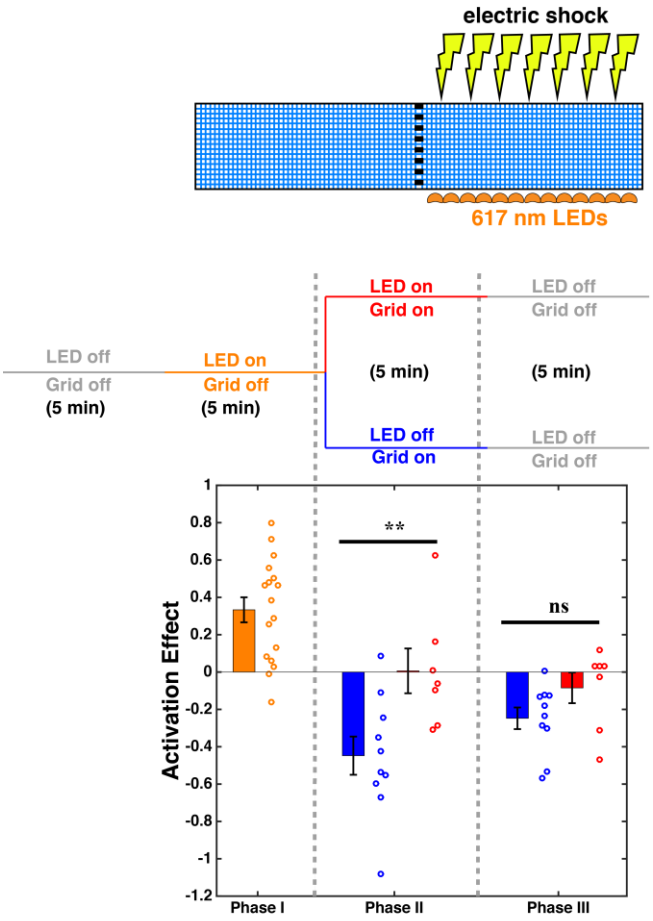


**Figure 4.1**. **Flies withstand an aversive stimulus, when paired with activation of NPF neurons**. The activation effect for each particular phase was calculated as the average of the preference indexes of the last minute of that particular phase, minus the average of the preference indexes during the last minute of the activation phase. Activation of NPF neurons was performed as in previous experiments (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **5 µW/mm$^2$**). Electric shocks were delivered as 50 ms pulse every 1 s, at an intensity of 18 V. The "Grid only" and "Grid + LEDs" showed a significant interaction ($P = 0.0079$, one-way ANOVA) with a significant difference during phase II ($p < 0.01$, Tukey's post-hoc test). No difference was observed during phase III ($p > 0.05$, Tukey's post-hoc test). The preference shown during Phase I, corresponds to the combined preferences of the Grid-only ($n = 10$) and Grid + LEDs groups ($n = 7$) during this particular phase of the experiment.

Of note, the ICSS from Olds and Milner had a similar property, that is, rats were willing to cross an electrified grid in order to access stimulation of certain brain regions (Olds, 1958). Thus, the results observed in our electric grid experiments are consistent with the optogenetic activation of NPF neurons in our assay being a rewarding experience.

### 4.2. Effect of alcohol exposure on NPF neuron activation-induced preference

The ICSS paradigm of Olds and Milner can be used to assess the addictive potential of multiple chemical compounds. Exposure to drugs of abuse, such as cocaine or amphetamine, is able to reduce the frequency or intensity of ICSS required to sustain the self-stimulatory behavior (Wise, 1996; Carlezon & Chartoff, 2007). Thus, drugs of abuse can facilitate the rewarding effects of the electrical stimulation of specific brain regions. In flies, not only is alcohol exposure rewarding, but it also increases the levels of NPF expression (Kaun et al., 2011; Shohat-Ophir et al., 2012). Taking this into account, we asked whether alcohol exposure would affect the sensitivity of flies to subsequent activation of NPF neurons. Flies expressing CsChrimson in NPF neurons were exposed to a moderately intoxicating dose of alcohol as previously described (Kaun et al., 2011; Shohat-Ophir et al., 2012), and tested one hour later in the 2-choice assay (Figure 4.2). Flies were tested at 1.25, 2.5 or 3.75 Hz and at a 617nm-light intensity of 20 µW/mm^2. Remarkably, flies that were exposed to alcohol (red) started showing a preference for NPF neuron activation at lower LED frequencies than unexposed flies (blue), indicating that alcohol exposure sensitizes flies to the effects of a subsequent NPF neuron activation (Figure 4.2). As expected, alcohol exposure has no effect on control flies that do not express CsChrimson (**Figure 4.2**).

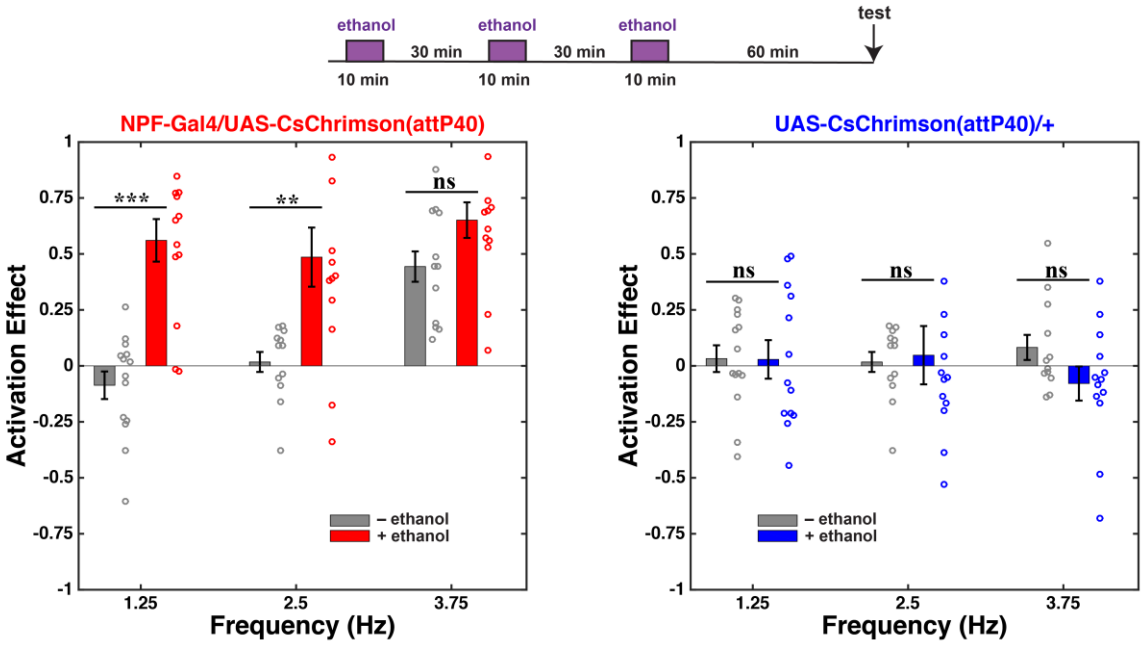


**Figure 4.2. Alcohol exposure sensitizes flies to a subsequent activation of NPF neurons**. Flies expressing CsChrimson on the NPF neurons were exposed to alcohol as previously described (Kaun et al., 2011; Shohat-Ophir et al., 2012). Briefly, flies were exposed to three consecutive session each consisting of a 10 min exposure to alcohol vapors, followed by a 30 min exposure to air. One hour after the end of the last session, flies were tested in a 2-choice experiment. Flies that were exposed to alcohol show preference for NPF neuron activation at lower frequencies than un-exposed flies (n = 12-14; unpaired t-test; **: $p<0.01$; ***: $p<0.001$). As expected, alcohol exposure has no effect on control flies (unpaired t-test) (617-nm light intensity: **20 µW/mm$^2$**).

### 4.3. Natural vs. Artificial reward competition

When flies are presented with multiple rewards, they base their preference for a particular option based on its relative value. For example, flies initially prefer to ingest food based on its taste alone, however, over time they shift their preferences towards foods with higher nutritional value (Stafford et al., 2012). In addition, female flies show an increased preference to lay eggs in a substrate with higher alcohol concentrations, however, when given the choice to lay eggs in two substrates with two different alcohol concentrations, females show a preference to lay eggs

for the substrate with ecologically relevant alcohol content (Azanchi et al., 2013). In the context of our 2-choice assay, and given that both mating and artificial activation of NPF neurons are perceived as rewarding (Shohat-Ophir et al., 2012), we asked whether the preference for the optogenetic activation of NPF neurons would be affected by the presence of a mating reward (virgin female). To test this idea we modified the chambers of our 2-choice assay by introducing a sliding acrylic divider (middle gate), which allowed us to introduce a single fly into each side of the chamber and keep them isolated at the beginning of the experiment. Our control group consisted of a single male expressing CsChrimson in NPF neurons paired with a single male expressing CsChrimson in bitter sensory neurons expressing Gr66a. On the other hand, our experimental group consisted of a single male expressing CsChrimson in NPF neurons paired with a single virgin female expressing CsChrimson in bitter sensory neurons expressing Gr66a. Flies expressing CsChrimson in NPF neurons are from here on referred to as NPF flies, while flies expressing CsChrimson in the bitter sensory neurons expressing Gr66a as Gr66a flies (male or female).

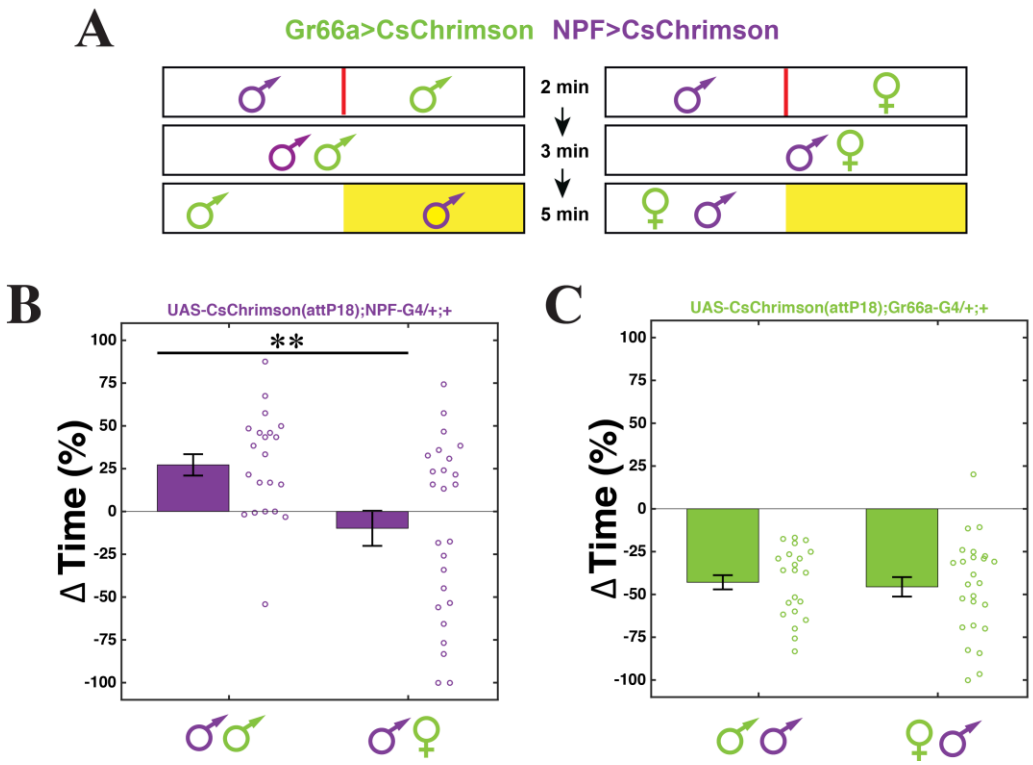


**Figure 4.3**. **Competition between natural and artificial rewards**. **(A)** The chambers from our 2-choice assay were modified by introducing an acrylic divider, thereby isolating the two sides of the chamber. A single fly (male or virgin female), expressing CsChrimson in NPF neurons or Gr66a neurons, was introduced at each side of the chamber and kept isolated from its respective pair for 2 min, after which the divider was removed and flies had the possibility to interact with each other for 3 min. This was followed by CsChrimson stimulation (yellow area) in only one side of the chambers for a period of 5 min (Frequency of activation: **10 Hz**; 617-nm LED light intensity: **5 µW/mm$^2$**). **(B)** Activation effect (ΔTime (%)) data for single test flies expressing CsChrimson in NPF neurons when paired with a single male (control group) or single female (experimental group) expressing CsChrimson in Gr66a neurons, showing that when paired with a virgin female, male flies have a reduced preference for the activation of NPF neurons (n = 21-25; Unpaired t-test, **: $p<0.01$). **(C)** Activation effect (ΔTime (%)) data for single test flies expressing CsChrimson in Gr66a neurons, for both control and experimental groups. As expected, in both cases flies avoid the activation of Gr66a neurons (n = 21-25; Unpaired t-test, **: $p<0.01$) with no difference between them (n = 21-25; Unpaired t-test, $p>0.05$)).

Since the Gr66a flies are expected to avoid the active side of the arena, this would create a dilemma for the NPF male between two rewards: access to optogenetic activation of NPF neurons on the active side or access to a natural reward (virgin female) on the inactive side

(**Figure 4.3A**). During the first 2 min of the experiment flies were kept isolated (middle gate closed). This was followed by 3 min (pre-dilemma phase) during which the middle gate was open, allowing the flies to explore the entire chamber and to interact with each other. During the next 5 min (dilemma phase), the 617nm LED light was turned on thereby allowing for activation of CsChrimson. During this phase, when paired with a Gr66a male, NPF males show a preference for the active side (ΔTime (%) = 27.2 +/- 6.8) (**Figure 4.3B**). Notably, when paired with a Gr66a female, NPF males showed a reduced preference for the active side (ΔTime (%) = -8.8 +/- 10.5). During this phase Gr66a males and Gr66a females showed the same level of aversion towards the active side (**Figure 4.3C**). Overall, we conclude that the drive of NPF males to approach a natural reward (virgin female) is comparable to their drive to approach the active side of the chamber under the standard conditions of illumination used in the experiment.

### 4.4 . NPF is required for the NPF neuron activation preference

Activation of CsChrimson will trigger a cation influx into the presynaptic terminals of the particular neurons into which it is being expressed, thereby inducing the release of the synaptic vesicles' content (neuropeptides and/or neurotransmitter) into the synaptic space (Owald et al., 2015b). Because certain neurons can express more than one neuropeptide and/or fast-acting neurotransmitter (van den Pol, 2012), the functional outcome of the activation of a particular group of neurons may be due to a combination of multiple neurotransmitters and/or neuropeptides. In the context of the preference for NPF neuron activation in our 2-choice experiments, this points to the question: To which extent does NPF play a role in the preference for NPF neuron activation? To answer this question, we expressed CsChrimson in NPF neurons along with an RNA interference (RNAi) construct targeted against NPF. The positive control

group consisted of flies expressing only CsChrimson in NPF neurons. Flies with targeted expression of NPF have a lower degree of preference for the activation of NPF neurons (**Figure 4.4**). Because expression of both the RNAi and CsChrimson transgenes relies on the ability of their UAS sequences to bind GAL4, it was formally possible that, if GAL4 were a limiting factor, the presence of the UAS-RNAi construct could reduce expression of CsChrimson and thus account for the reduced preference of the RNAi-expressing flies. However, this is not the case, as introducing a 20XUAS-mCD8::GFP construct has no effect on the preference for NPF neuron activation (**Figure 4.5**). Overall, we conclude that the preference for the activation of NPF neurons requires NPF.

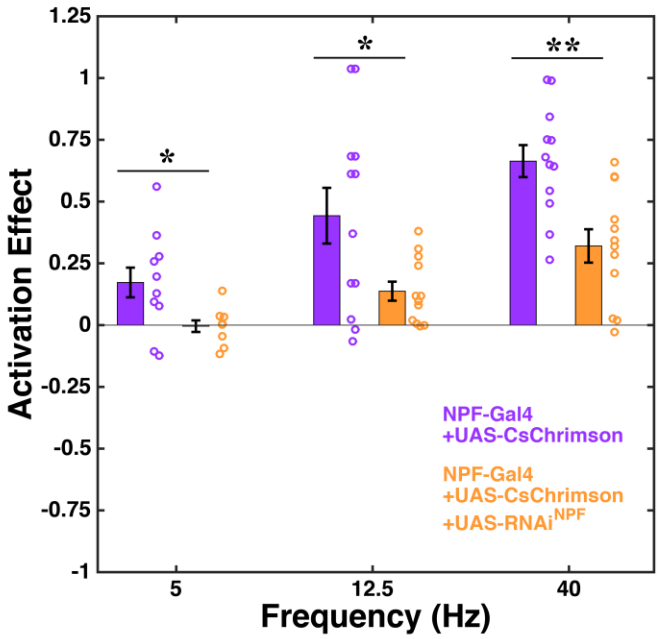


**Figure 4.4**. **NPF is required for the NPF activation-induced preference.** Flies, in which the expression of NPF was targeted using an RNAi construct, show a reduced preference for the activation of NPF neurons (617-nm LED light intensity: 5 **μW/mm²**) (n= 8-12; Unpaired t-test *: $p<0.05$; **: $p<0.01$).

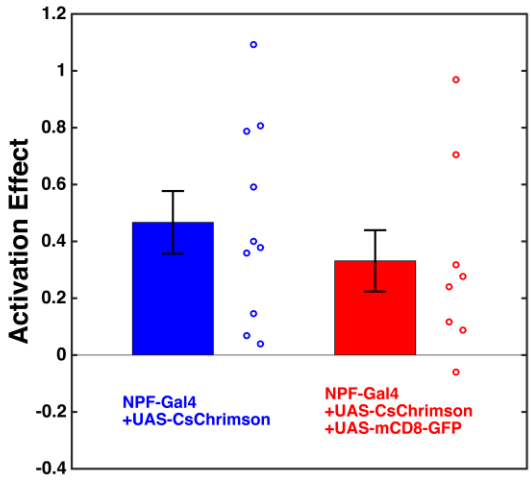


**Figure 4.5**. **Presence of extra UAS binding sites does not change the preference for NPF neuron activation.** (617-nm LED light intensity: 5 **µW/mm$^2$**) (n = 10-12; Unpaired t-test, p>0.05).

## Chapter 5: Anatomical and functional subdivision of NPF neurons

### 5.1 Neuroanatomy of NPF neurons

An inspection of the NPF-GAL4 expression pattern in the central brain (i.e. Full NPF-Gal4) (**Figure 5.1**) revealed labeling of distinct subsets of NPF neurons in each hemisphere: two large neurons that project to the dorsolateral brain (**Figure 5.1A**) and a group of several small NPF neurons located in the posterior brain, some of which have projections in dorsal brain (**Figure 5.1 B-D**), while others project to the fan-shaped body of the central complex (**Figure 5.1 E-G**). In order to get a better understanding of the polarity of this neurons, we used the NPF-Gal4 to drive expression of the cell polarity markers DenMark (labeling postsynaptic compartments) (Nicolai et al., 2010) and Syt::GFP (labeling pre-synaptic compartments) (Zhang et al., 2002). As shown in (**Figure 5.2**), NPF neurons receive information (dendritic compartment) through their projection in the dorsal lateral brain (**Figure 5.2B & 5.2D**), while their output is located primarily in the dorso-medial and ventral-lateral brain (**Figure 5.2C & 5.2D**). The subset of small neurons with cell bodies in the posterior brain (cell bodies not shown in figure 4.8), have both their dendritic and presynaptic compartment in different layers of the fan-shaped body of the central complex.

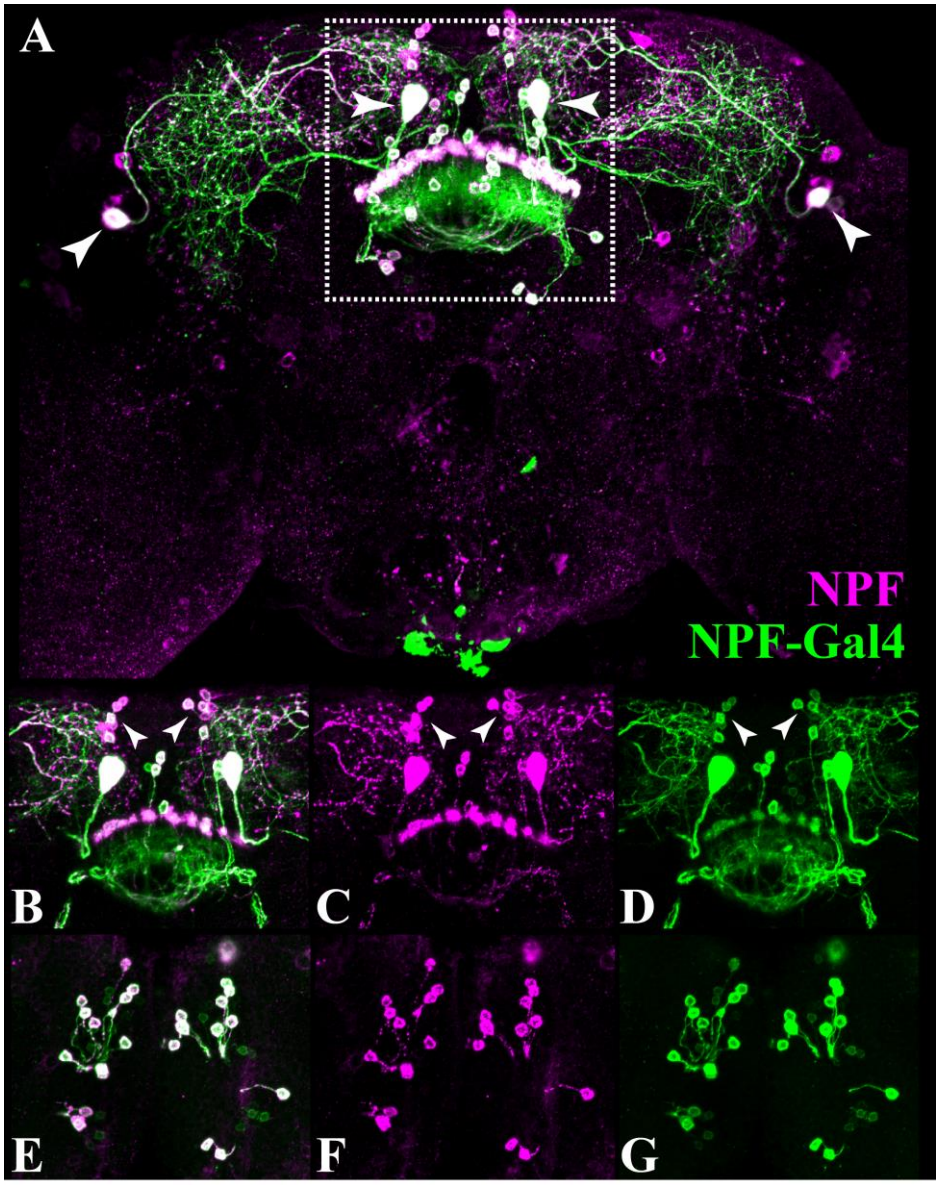


**Figure 5.1**. ***Drosophila*** **neuropeptide F (NPF) neurons. (A)** The NPF-Gal4 expression pattern shows that NPF is expressed in 2 large neurons (arrows) per hemisphere that project to the dorsolateral brain. **(B-D)** Zoom in to the area indicated by the dashed box in (A), showing that NPF is also expressed in several small neurons, located in the dorsal medial brain (arrows). Images correspond to the second third of the confocal stack. **(E-G)** Same are as shown in (B-D), however, images correspond to the first third of the confocal stack, which allows for the visualization of the cell bodies of the small neurons projecting to the central complex. Images correspond to the maximum intensity projection of different portions of a confocal stack collected from the posterior to the anterior end of the brain. Magenta: endogenous NPF expression. Green: NPF-Gal4 expression pattern.

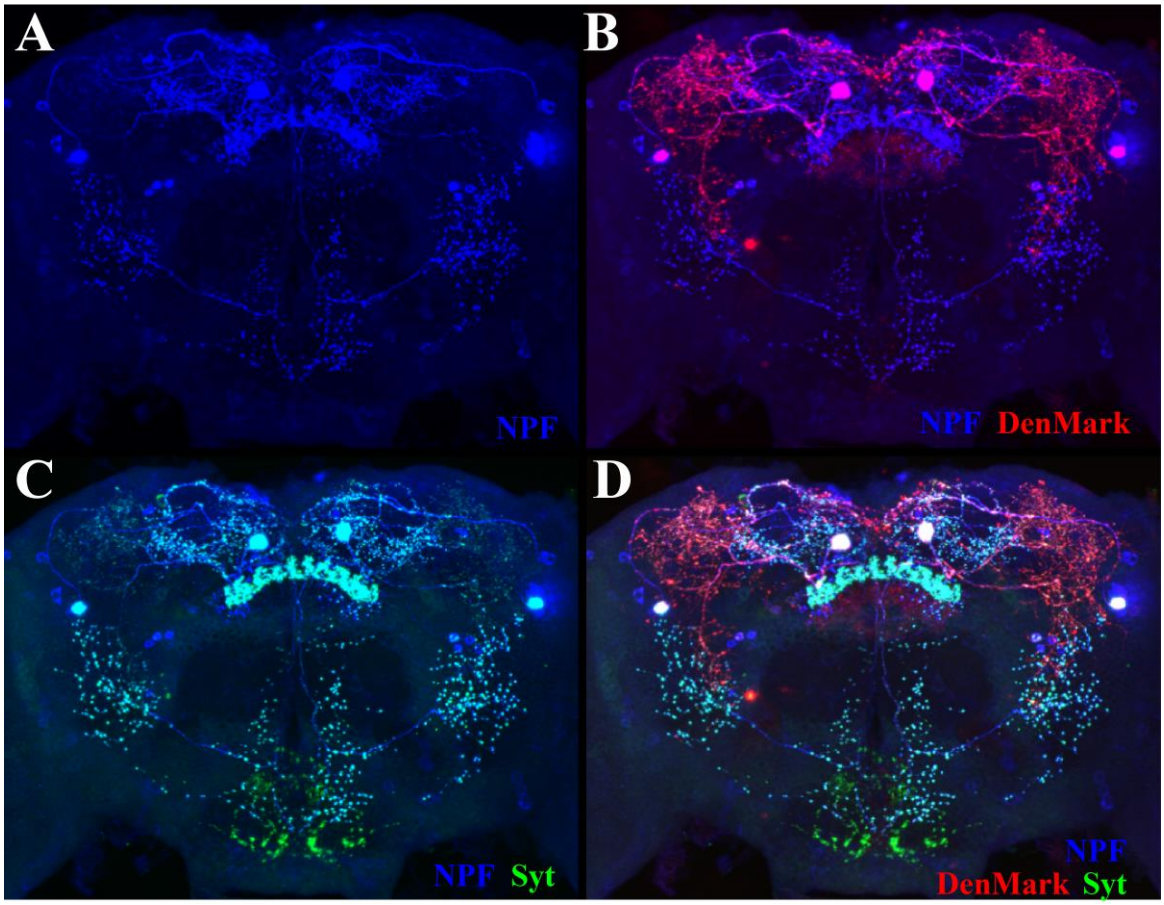


**Figure 5.2**. **NPF neurons cell polarity. (A)** Endongenous NPF expression. **(B)** Overlay of DenMark and endogenous NPF expression. **(C)** Overlay of Syt::GFP and endogenous NPF expression. (D) Overlay of endogenous NPF, DenMark and Syt::GFP expression. Blue: endogenous NPF. Red: DenMark. Green: Syt::GFP.

NPF neurons, as shown in both (**Figure 5.1**) and (**Figure 5.2**), have projections in the vast majority of the brain, with a particular high density in the dorsal brain. To further disentangle these projections from one another, that is, to identify which projections correspond to each neuron, we used the NPF-Gal4 to drive expression of a MultiColor FlpOut transgene (MCFO) (Nern et al., 2015), which allowed for the stochastic multicolor labeling of the NPF neurons. (**Figure 5.3**) shows a set of representative MCFO results for the NPF neurons. For example, (**Figure 5.3A**) shows that the projections from the dorsal-lateral (L1-l) and dorsal-medial (P1) NPF neurons are spatially segregated from each other. In addition, the projections that appear in

the ventral brain originate from the contralateral dorsal-medial (P1) NPF neuron. On the other hand, (**Figure 5.3B**) shows a single dorsal-lateral (L1-l) neuron indicating that all of its projections, aside from its axon, reside in the dorsal-medial brain. In addition, (**Figure 5.3B**) shows several small NPF neurons (P2), located in the dorsal brain, that project to the fan-shaped body. (**Figure 5.3C**) shows, in addition to some P2 neurons, a subset of small NPF neurons, which we named DM, with their cell bodies in the dorsal-medial brain and projections towards the dorsal-lateral brain. A schematic representation of the neuroanatomy of these different neurons is provided in (**Figure 5.3D**). Of note, to our knowledge, the DM neurons have not been previously described. Except for the DM neurons, the respective nomenclature used here is in accordance with previous neuroanatomical reports for the NPF neurons (Lee et al., 2006; He et al., 2013b).

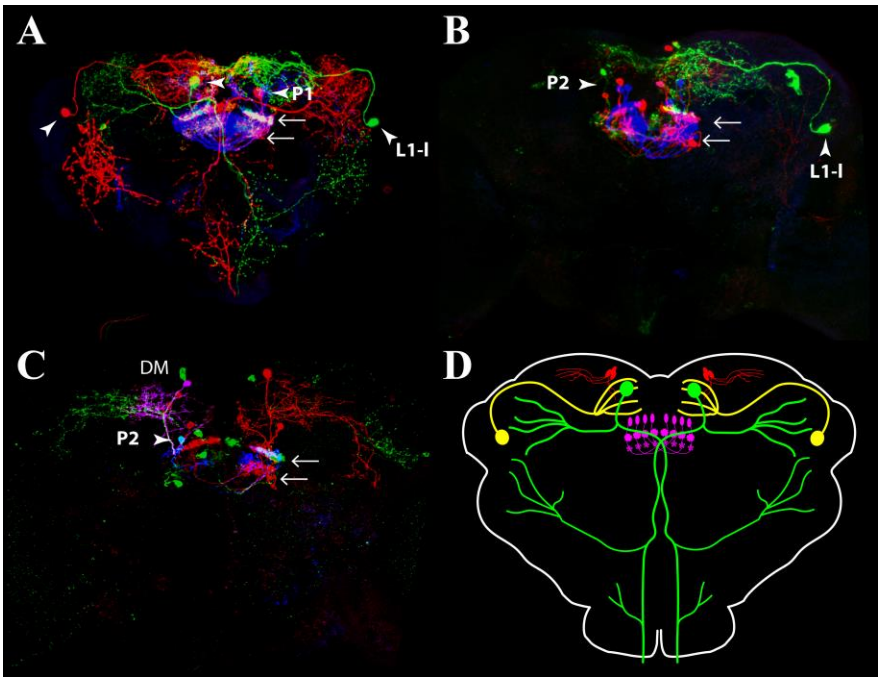


**Figure 5.3**. **Neuroanatomy of NPF neurons.** Representative MCFO results, showing the different types of NPF neurons: large dorsal medial (P1) neuron (**A**); large dorsal lateral (L1-l) neuron (**A & B**); small fan-shaped body (P2) projecting neuron (**B & C**); small dorsal medial (DM) neuron. (D) Schematic representation of the different subtypes of NPF neurons. In all images, small arrows indicate the projections from P2 neurons in the fan-shaped body, while big arrows indicate the neuron of interest in each panel.

### 5.2 NPF neuron functional subdivison

Considering that the anatomy of the different NPF neurons is very distinctive from each other, we wondered whether particular subsets might be differentially involved in the preference for NPF neuron activation. To address this, we first sought genetic tools with which to independently label the large and small NPF neurons.

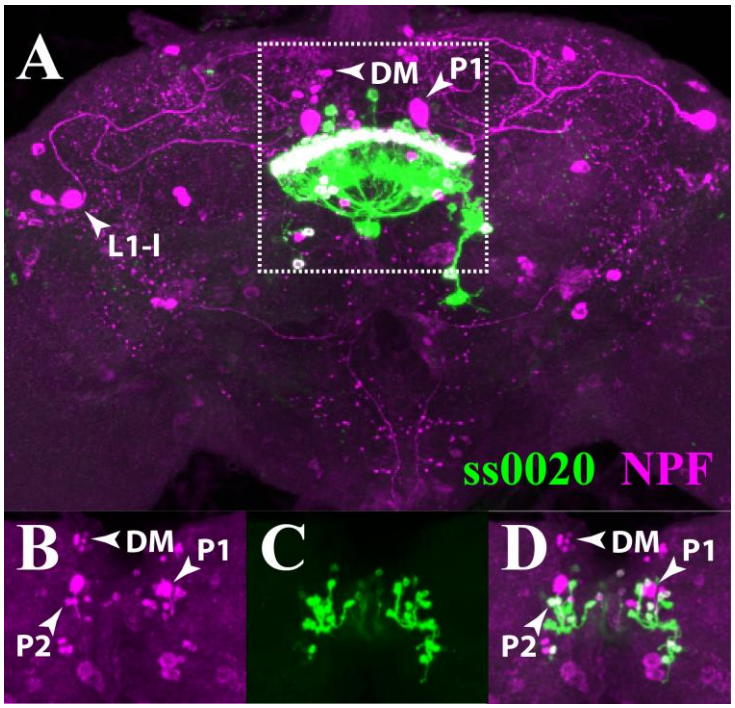


**Figure 5.4**. **Expression pattern of ss0020 line.** (**A**) Colocalization of the expression patterns of ss0020 (green) and endogenous NPF (red). **(B, C & D)** Zoom in to the area indicated by the dashed box in (A). Images in (B, C & D) correspond to only the first third of the confocal. Brain was imaged from posterior to anterior. Magenta: endogenous NPF. Green: mVenus.

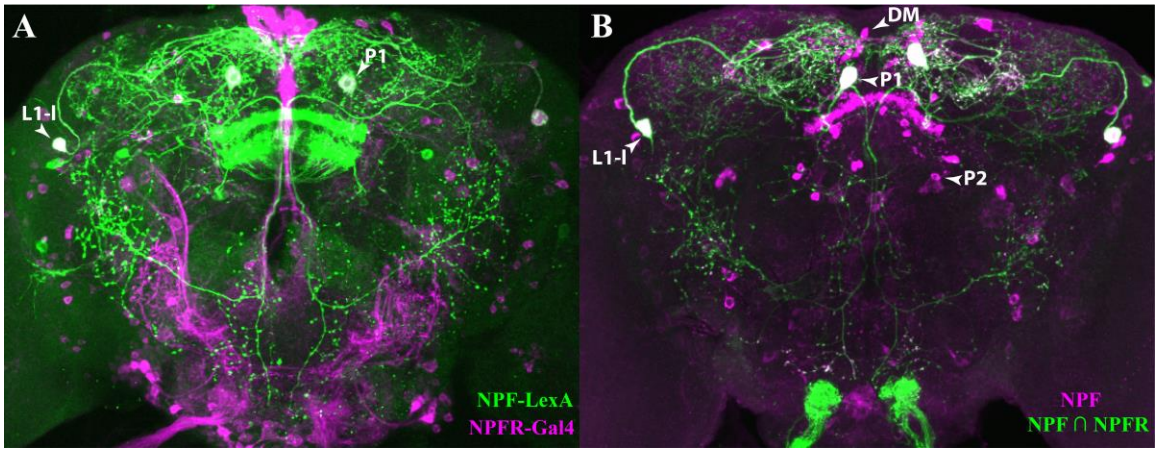


**Figure 5.5**. **Intersection of the NPF and NPFR expression pattern effectively labels the large NPF neurons.** (**A**) The NPF-LexA and NPFR-Gal4 drivers are co-expressed in the large NPF neurons (L1-l & P1). Green: NPF-LexA. Magenta: NPFR-Gal4. Green: NPF-LexA. Magenta: NPFR-Gal4. **(B)** Intersection of the drivers shown in (A) labels the large NPF neurons only. Brain was imaged from posterior to anterior. Magenta: endogenous NPF. Green: NPF-LexA ∩ NPFR-Gal4.

By inspecting the expression patterns of a collection of "Split Gal4" lines that produce functional GAL4 in neurons that project to the fan-shaped body (Tanya Wolf, personal communication), we identified line ss0020 labeling a subset of the P2 NPF neurons without labeling any of the DM, P1 or L1-l NPF neurons (**Figure 5.4**). To label the large NPF neurons, P1 and L1-l, we exploited the observation that these neurons also express the NPF receptor (NPFR) (**Figure 5.5A**). Here, we employed a FRT-FLP mediated recombination to intersect the NPF-LexA and NPFR-Gal4 expression patterns, which effectively labels only the big NPF neurons (**Figure 5.5B**). With the ability to now manipulate the large NPF neurons (L1-l & P1) and a subset of P2 neurons, we tested their contributions to the NPF neuron activation-induced preference. We found that activating neither (L1-l + P1) (big neuron NPF-Gal4) nor a subset of P2 (small neuron NPF-Gal4) NPF neurons was sufficient to induce a preference response (**Figure 5.6A & 5.6B**). Surprisingly, activation of only the big NPF neurons was able to recapitulate the effect on locomotion of the NPF neuron activation (**Figure 5.7A & 5.7B**).

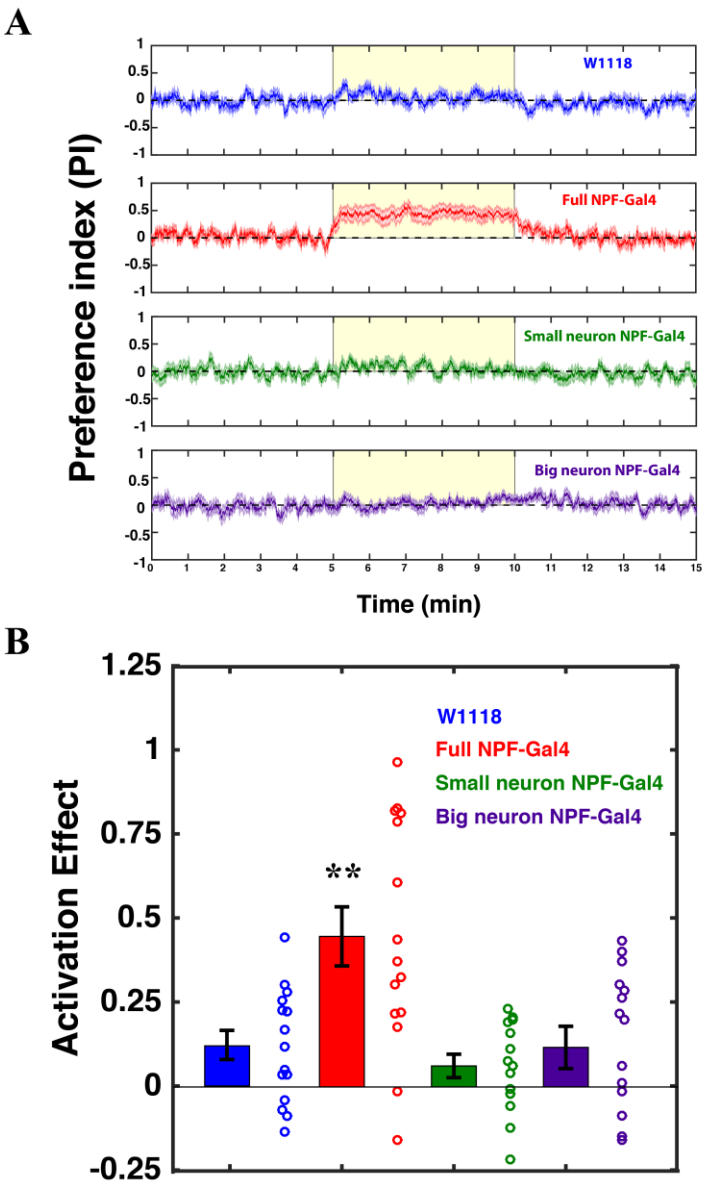


**Figure 5.6. Preference for the activation of specific subsets of NPF neurons**. **(A)** Experimental data expressed as the mean +/- standard error of the preference index over time. The yellow box indicates the side and period of activation (n = 14-15). **(B)** Activation effect for the data shown in (A), indicating that neither activation of the Big (L1-l + P1) or small (subset of P2) NPF neurons is sufficient to recapitulate the effect of activating all of the NPF neurons. (n = 14-15; Unpaired t-test against control (W1118); **: $p<0.01$). (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **20 µW/mm$^2$**).

To label all of the small NPF neurons (P2 + DM), in a second set of experiments we used the NPF-LexA driver to express CsChrimson, while using the NPFR-Gal4 driver to expression of an RNAi against LexA. Here, the expected result would be the lack of expression of CsChrimson anywhere the NPF-LexA and the NPFR-Gal4 expression patterns intersect (i.e the big NPF neurons). As shown in (**Figure 5.8**), this intersectional strategy successfully prevents expression of the reporter, CsChrimson::mVenus, in the L1-l NPF neurons, while leaving residual levels of reporter expression in the P1 neurons. Under these conditions, when flies were tested in our 2-choice assay, they still showed a preference response (**Figure 5.9**) and reduced locomotion (**Figure 5.10**) similar to that shown after activating the entire NPF expression pattern.

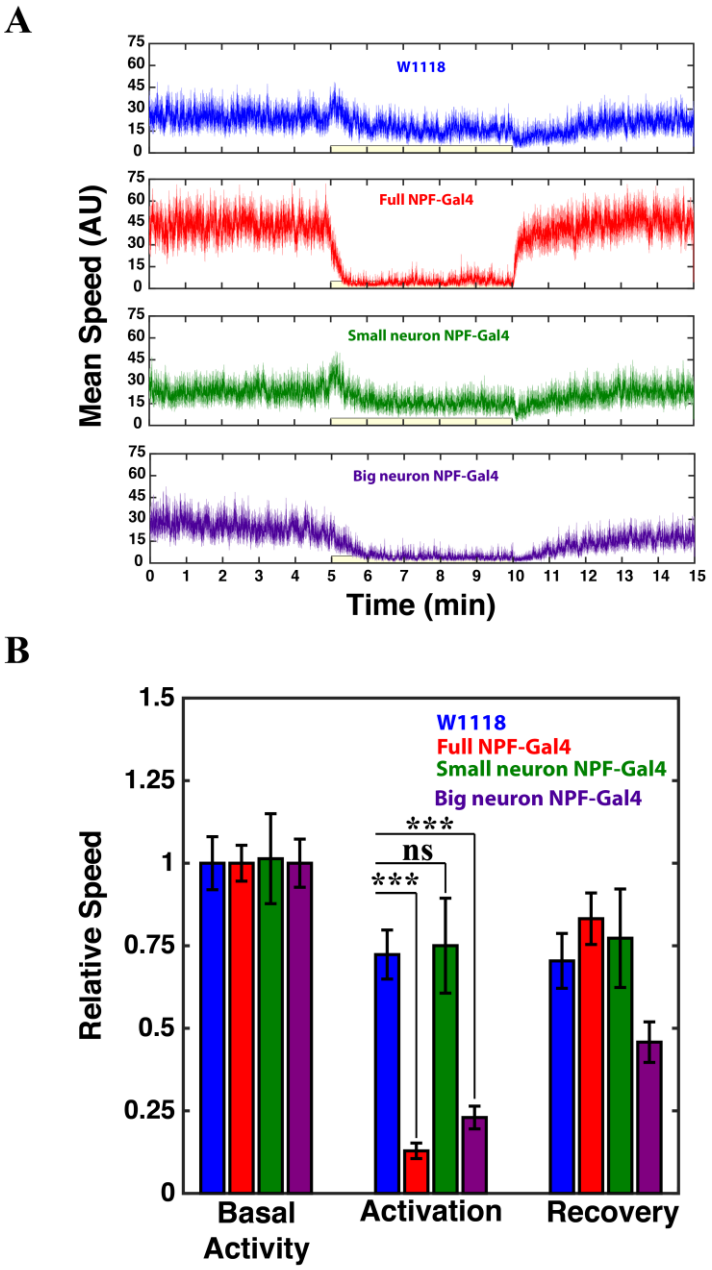


**Figure 5.7. Effect on locomotion of the activation of specific subsets of NPF neurons**. **(A)** Experimental data expressed as the mean +/- standard error of the speed over time. The yellow box indicates period of activation (n = 14-15). **(B)** Speed data from (A) expressed as the average speed during each period of the 2-choice experiment. For each group, the data was normalized by the average of the basal activity period. (n = 14-15; Unpaired t-test against control (W1118); **: $p<0.01$; ***: $p<0.01$). (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **20 µW/mm$^2$**).

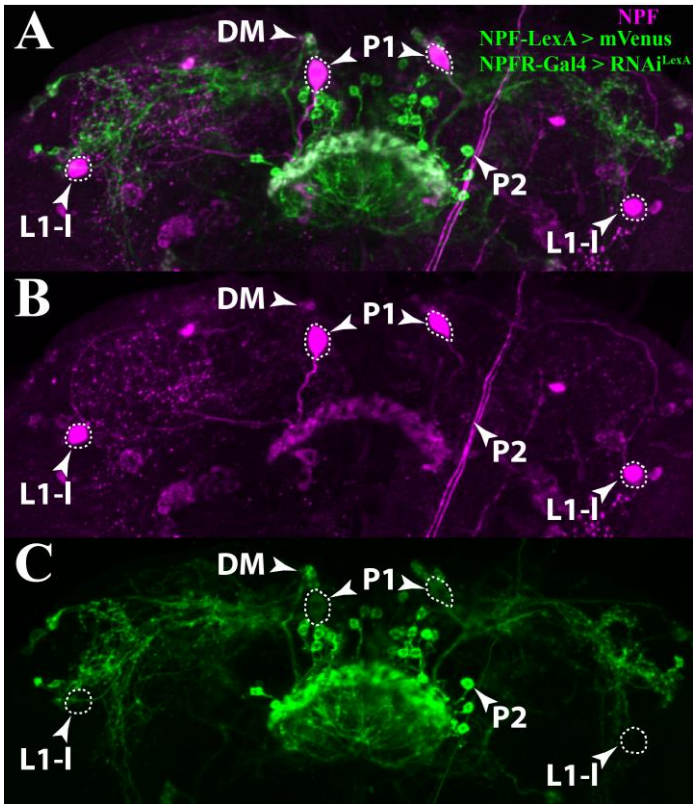


**Figure 5.8**. **Intersectional strategy for labeling DM and P2 NPF neurons.** (**A**) Colocalization of the expression patterns of ss0020 (green) and endogenous NPF (red). **(B, C & D)** Zoom in to the area indicated by the dashed box in (A). Images in (B, C & D) correspond to only the first third of the confocal. Brain was imaged from posterior to anterior. Magenta: endogenous NPF. Green: mVenus.

Because of the residual levels of expression of CsChrimson::mVenus on the P1 neurons, it is impossible to rule out a possible interaction of P1 with either P2 or DM neurons. However, since activation P1 neurons or a subset of P2 neurons (ss0020) triggers no preference response, this suggests that he DM neurons are responsible for the NPF neuron (Full NPF-Gal4) activation-induced preference.

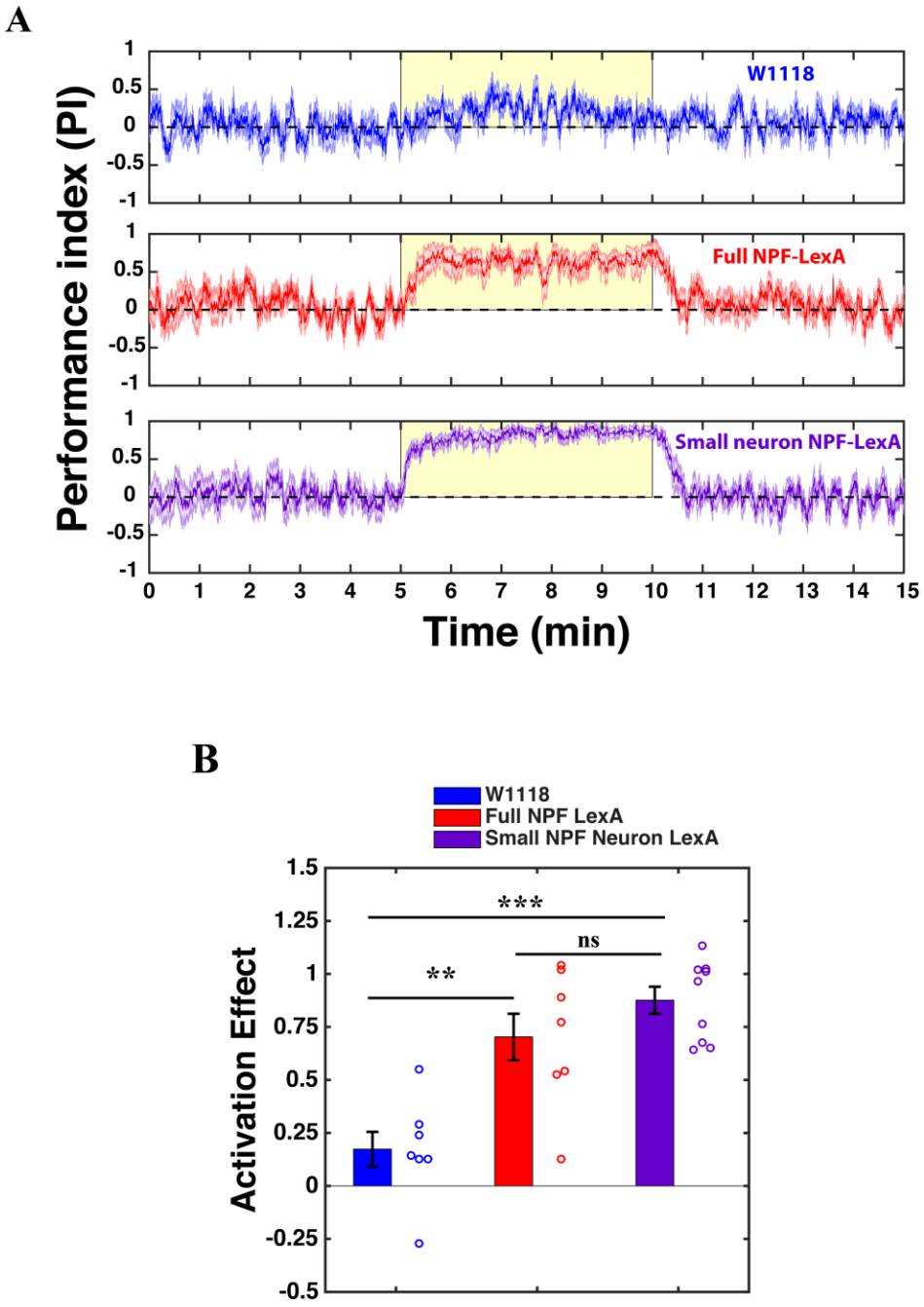


**Figure 5.9. Preference for the activation of the small (DM + P2) NPF neurons**. **(A)** Experimental data expressed as the mean +/- standard error of the preference index over time. The yellow box indicates the side and period of activation (n = 7-8). **(B)** Activation effect for the data shown in (A), indicating that the activation of small (P2 + DM) NPF neurons is sufficient to recapitulate the effect of activating all of the NPF neurons. (n = 7-8; Unpaired t-test against control (W1118); **: $p<0.01$; ***: $p<0.001$). (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **20 µW/mm$^2$**).

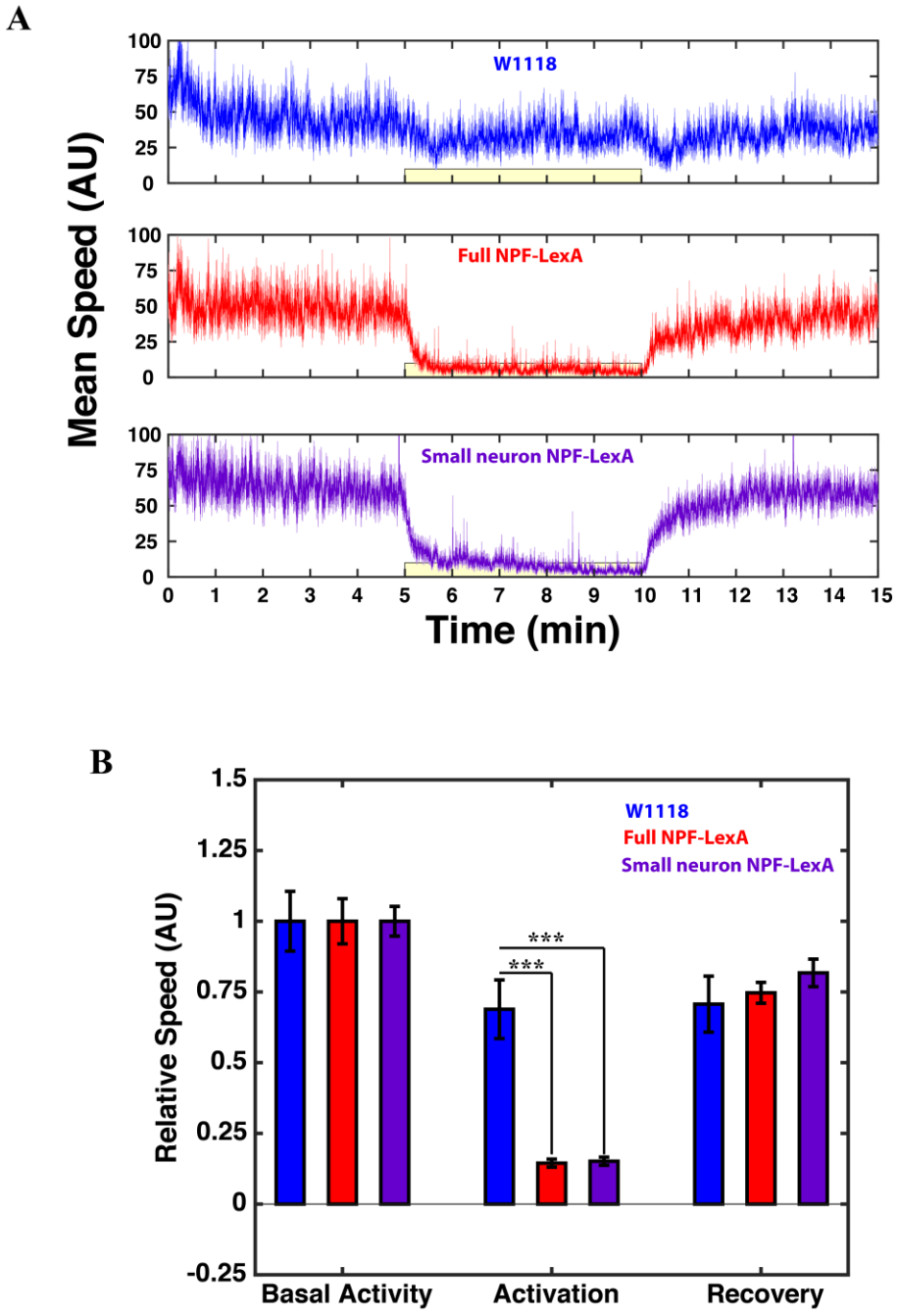


**Figure 5.10. Effect on locomotion of the activation of the small (DM + P2) NPF neurons**. **(A)** Experimental data expressed as the mean +/- standard error of the speed over time. The yellow box indicates period of activation (n = 7-8). **(B)** Speed data from (A) expressed as the average speed during each period of the 2-choice experiment. For each group, the data was normalized by the average of the basal activity period. (n = 7-8; Unpaired t-test against control (W1118); **: $p<0.01$; ***: $p<0.01$). (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **20 µW/mm$^2$**).

## Chapter6: NPF neuron post-synaptic elements

### 6.1 NPF and dopamine neuron behavioral epistasis

The evidence presented so far shows that the optogenetic activation of NPF neurons in our 2-choice assay is a rewarding stimulus for the fly. In addition, dopamine neurons in the fly have been shown to be involved in the processing, for example, of alcohol (Kaun et al., 2011), food (Liu et al., 2012; Burke et al., 2012) or water (Lin et al., 2014) reward. Taking this into account, we asked whether dopamine neurons act downstream of NPF neurons to mediate the NPF-neuron activation-induced preference observed in our 2-choice assay. To do this, we employed classical genetic procedures to generate the following strain:

**"CombKir2.1"**

*13XLexAop2-CsChrimson-mVenus; UAS-Kir2.1/Cyo; NPF-LexA/ TM3,Ser*

For convenience, the strain mentioned above will be referred as "Comb-Kir2.1". Crossing this strain with an appropriate GAL4 driver allows for the activation of NPF neurons with CsChrimson, while blocking a candidate postsynaptic neuronal population by means of Kir2.1 expression (Baines et al., 2001). To block dopamine neurons we crossed Comb-Kir2.1 virgins with either TH-Gal4 or R58E02-Gal4 males. The TH-Gal4 driver labels most of the dopamine neurons except for the ones located in the PAM cluster (Mao & Davis, 2009), which are strongly labeled by the R58E02 driver (Liu et al., 2012). The positive control flies were generated by crossing Comb-Kir2.1 virgins to a $w^{1118}$ stock. As shown in **Figure 6.1**, blocking dopamine neurons in either the TH or R58E02 patterns reduces the preference for NPF neuron activation.

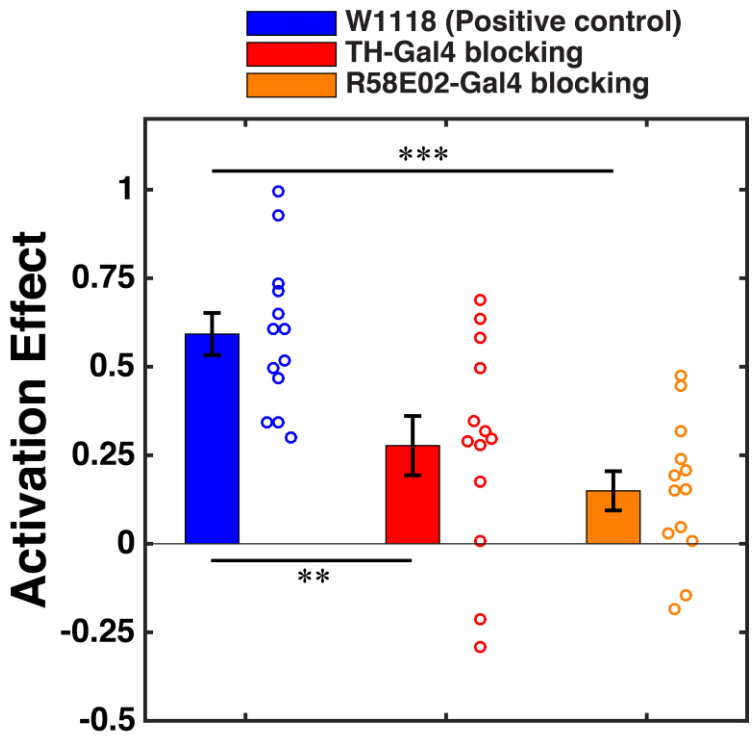


**Figure 6.1. Blocking of dopamine neurons reduces the preference for NPF neuron activation**. Flies expressing CsChrimson on the NPF neurons and Kir2.1 in either of two dopamine neuronal populations show a reduced preference for activation of NPF neurons when compared with positive control flies. (n = 14-15; unpaired t-test; **: p<0.01; ***: p<0.001). (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **5 µW/mm$^2$**)

An anatomical characterization shows that dopamine neurons can be classified based on the area of the mushroom body that they innervate (Aso et al., 2014a). To test if a particular type of dopamine neurons contributes to the behavioral phenotype shown in **Figure 6.1**, we used our CombKir2.1 strain along with specific split-Gal4 drivers (Aso et al., 2014a) to blocked individual types of dopamine neurons while activating NPF neurons in our 2-choice assay. As shown in **Figure 6.2**, blocking individual types of PAM dopamine neurons does not have an effect on the activation of NPF neurons.

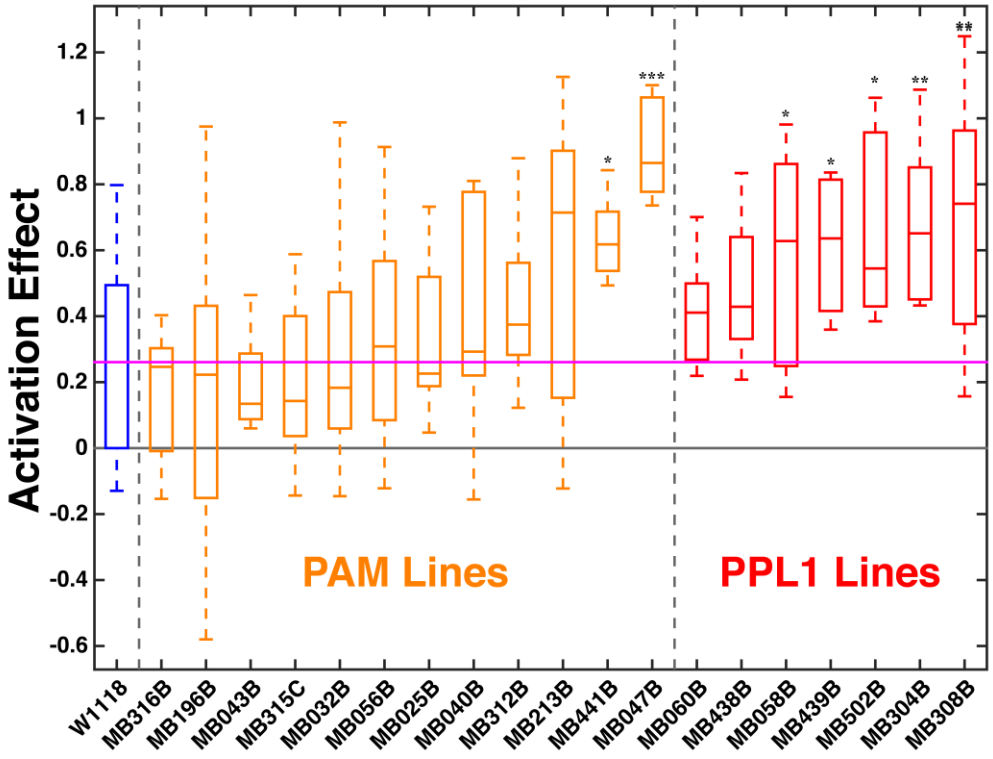


**Figure 6.2. Effect of blocking individual dopamine neuron on the preference for NPF neuron activation**. Flies expressing CsChrimson on the NPF neurons and Kir2.1 in either of two dopamine neuronal populations show a reduced preference for activation of NPF neurons when compared with positive control flies. (n = 7-10 unpaired t-test against control (W1118); *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). (Frequency of activation: **40 Hz**; 617-nm LED light intensity: **5 µW/mm$^2$**)

However, blocking PAM-γ3 neurons (MB441B) or a combination of PAM-α1, PAM-β1, PAM-γ4, PAM-β'2p and PAM-β'2m (MB047B), led to an enhanced preference for NPF neuron activation (**Figure 6.2**). Surprisingly, blocking different groups of PPL1 dopamine neurons also produced an enhanced preference for NPF neuron activation (**Figure 6.2**). Overall, this data suggests that while multiple PAM dopamine neurons may be required downstream of NPF neurons in order to promote a preference response in our 2-choice assay, other PAM dopamine neurons (lines MB441B and MB047B) along with PPL1 dopamine neurons normally repress this preference response.

## 6.2 NPF and dopamine neurons functional connectivity

To further demonstrate the functional connection between NPF and dopamine neurons, we performed functional connectivity experiments. Using classical genetic procedures, we generated the following strain:

*13XLexAop2-CsChrimson-mVenus; UAS-opGCaMP6s; NPF-LexA*

Crossing females of this strain to R58E02-Gal4 males, allowed us to activate NPF neurons with CsChrimson, while expressing GCaMP6s in PAM dopamine neurons to monitor for calcium activity. For this experiments, we decided to focus on PAM dopamine neurons since they are responsible for appetitive reinforcement in the fly (Liu et al., 2012; Burke et al., 2012). Considering that specific dopamine neurons innervate particular compartments of the mushroom body, and that the R58E02-Gal4 is expressed in multiple dopamine neurons belonging to the PAM cluster, by selecting the appropriate regions of interest for the quantification of calcium responses, as shown in **Figure 6.3**, one can infer the effect of NPF neuron activation over each kind of dopamine neuron belonging to this cluster. **Figure 6.4** shows the different dopamine neurons react differently to NPF neuron activation, with some showing inhibition (PAM-γ3 and PAM-β'1) and others showing activation (PAM-α1, PAM-β1, PAM-β2, PAM-β'2, PAM-γ4 and PAM-γ5). Most notably, both PAM-β'2 and PAM-γ4 respond with a strong activation to the stimulation of NPF neurons.

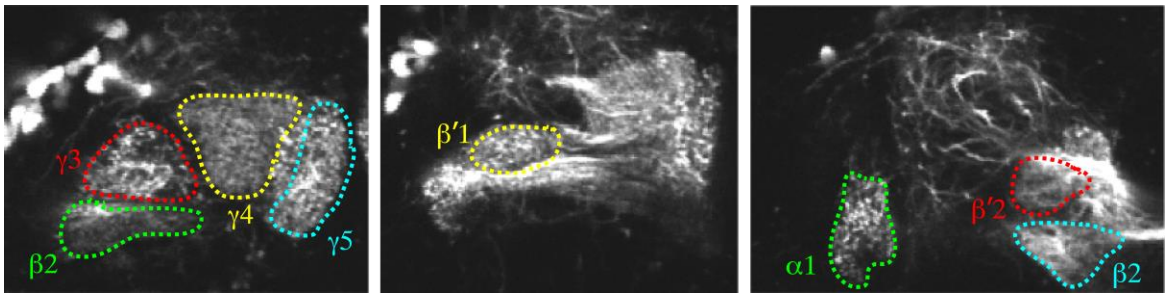


**Figure 6.3. Regions of interest (ROIs) employed for quantification of calcium responses in PAM dopamine neurons**. Images correspond to the raw fluorescence of GCaMP6s expressed in in the PAM cluster of dopamine neurons using the R58E02-Gal4 drivers. For each panel, the dashed lines represent particular compartments of the mushroom bodies.

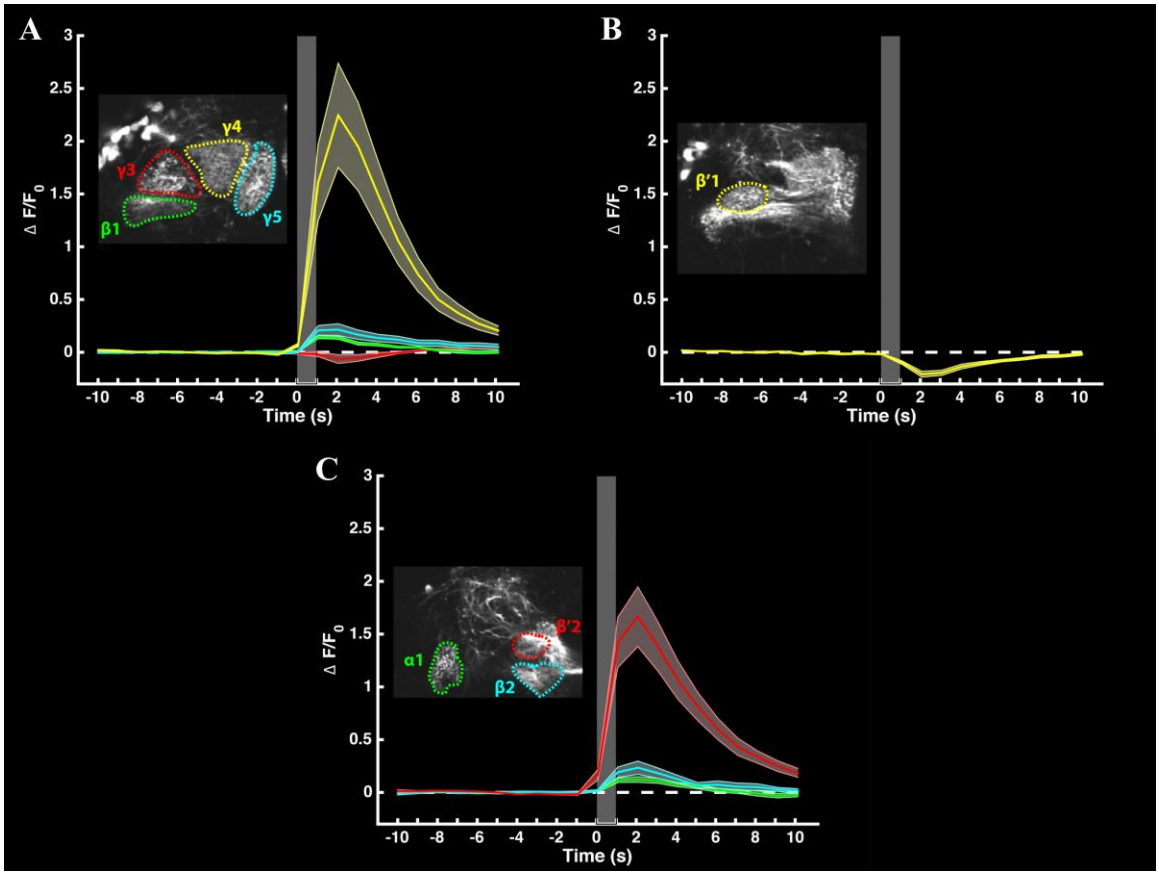


**Figure 6.4. Calcium responses of PAM dopamine neurons upon activation of NPF neurons**. Calcium imaging data expressed as the mean +/- standard error of the $\Delta F/F_0$ over time for the ROIs shown in (Figure 6.3), indicating that different PAM dopamine neurons respond differently to the activation of NPF neurons. The traces shown here correspond to average response for each brain, averaged across brains (n = 6). In each panel, the grey box indicates the time of stimulation.

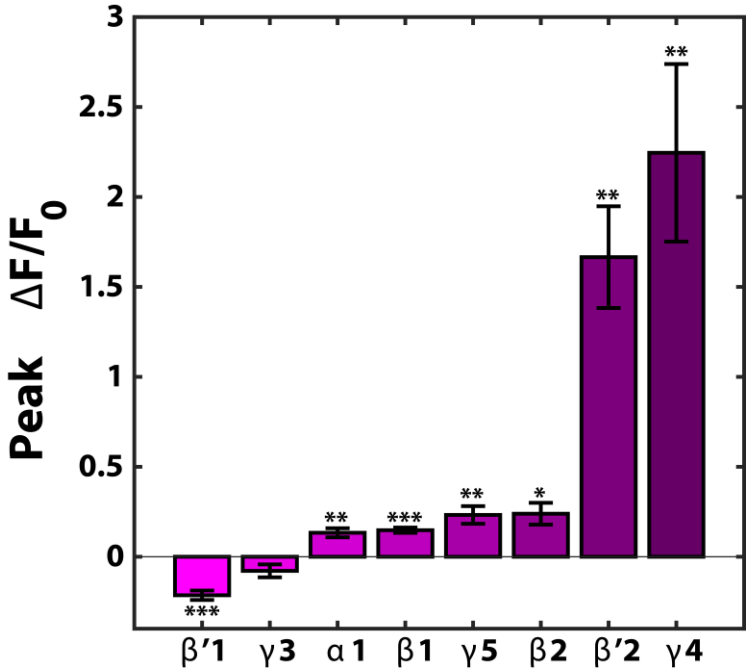


**Figure 6.5. Quantified calcium responses of PAM dopamine neurons upon activation of NPF neurons**. Peak responses (minimum or maximum) for the traces shown in (**Figure 5.4**) expressed as the mean +/- standard error of the $\Delta F/F_0$ over time traces for the ROIs shown in (**Figure 5.3**) (n = 6; Unpaired t-test against 0; *: p<0.05; **: p<0.01; ***: p<0.001).

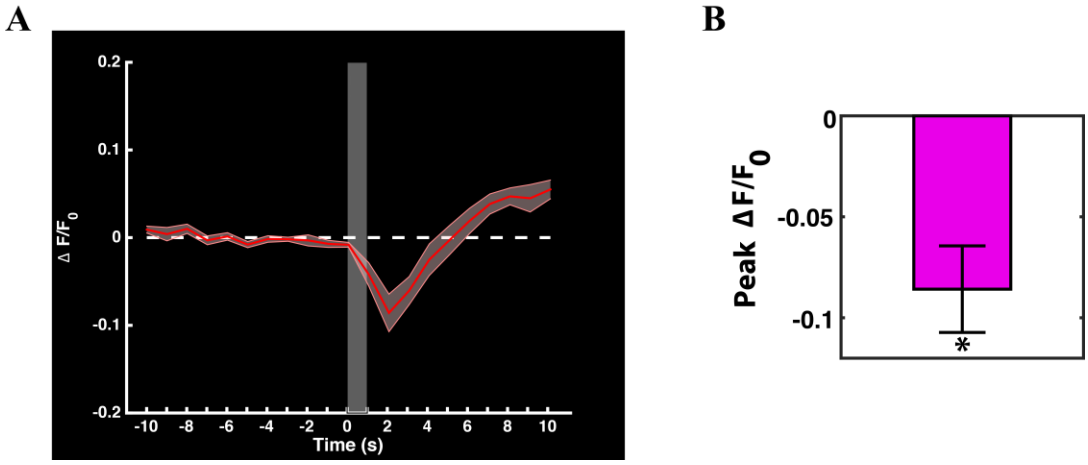


**Figure 6.6. Quantified calcium responses of PAM-γ3 dopamine neurons upon activation of NPF neurons**. **(A)** The traces shown in the left panel correspond to average response of each brain, averaged across brains (n = 5). In panel (A), the grey box indicates the time of stimulation. **(B)** Peak responses for the traces shown in (A). All data is expressed as the mean +/- standard (n = 5; Unpaired t-test against 0; *: p<0.05).

Calculating the peak (minimum or maximum) response for each $\Delta F/F_0$ trace, as shown in (**Figure 5.5**), indicated that all of the PAM dopamine neurons labeled by the R58E02-Gal4 respond (inhibition or activation) to the stimulation of NPF neurons, with the exception of PAM-γ3, which only showed a trend towards inhibition. However, on a different set of experiments where we used the MB441B-Gal4 driver (Aso et al., 2014a) to express GCaMP6s specifically in PAM-γ3 we were able to observe a significant inhibition of this dopamine neuron. We conclude that all of the dopamine neurons in the PAM cluster respond, either with inhibition or activation, to the activation of NPF neurons.

## Chapter7: Discussion and Future Directions

Here we presented the characterization of a novel 2-choice assay that employs optogenetic activation to identify neurons involved in reward processing in *Drosophila*. We designed this assay under the assumption that, if given the choice to avoid or occupy a side of the chambers where a specific group of neurons could be activated, flies would prefer the area of activation if such an activation (of NPF neurons for example) is perceived as rewarding, or avoid the area of activation if such an activation (of Gr66a neurons for example) is perceived as aversive. This assay was designed with the intent of ultimately performing a large neuronal activation screen to probe for unidentified neurons that might be involved in reward. In fact, our system consists of four groups of chambers shown in **Figure 3.1A**, allowing the testing of up to 20 groups of flies in a single round of the 2-choice experiment. It should also be noted that, since our assay does not involve an olfactory conditioning step, it is able to reveal what the flies are experiencing in real time (attraction or aversion) upon activation of a particular group of neurons. This makes our assay particularly useful, for example, if one wanted to study the behavior of single flies, which would be extremely laborious by means of olfactory conditioning.

### 7.1 Preference for NPF neuron activation

Neuropeptide Y (NPY), the mammalian homologue of NPF, is a widely expressed neuropeptide across the brain (Reichmann & Holzer, 2016) and is involved in regulating behaviors such as feeding (Aponte et al., 2011) and alcohol consumption (Pleil et al., 2015). In addition, NPY neurons in the central nucleus of the amygdala and the arcuate nucleus of the hypothalamus send projections to the nucleus accumbens (Borkar et al., 2015), and intra-accumbens injections of NPY are able to produce a place preference response in rats (Josselyn &

Beninger, 1993). Similarly in flies, the neuropeptide F (NPF) neurons regulate several reward related behaviors, such as retrieval of appetitive memories (Krashes et al., 2009), alcohol preference and alcohol reward (Shohat-Ophir et al., 2012). In addition, thermogenetic activation of these neurons is *per se* rewarding (Shohat-Ophir et al., 2012). Given the functional similarities between the NPY system in mammals and the NPF system in flies, and considering that NPY neurons project to the nucleus accumbens, which in mammals has a central role in reward processing, we speculated that the characterization of the NPF/NPFR system in *Drosophila* will serve as an entry point to the study of the flies' reward system. With this in mind we sought to characterize our assay using optogenetic activation of this group of neurons, under the expectation that flies would show a preference for the activation of NPF neurons. Indeed, in our assay, flies expressing CsChrimson on the NPF neurons, display a preference for the side of our chambers in which the 617 nm LEDs are turned on. Interestingly, the preference for NPF neuron activation showed by the flies resembles an "all or nothing" response, in that, above a certain frequency (threshold), flies start to show the same degree of preference (**Figure 3.3**). In the intracranial self-stimulation experiments from Olds & Milner, increasing levels of current applied into the stimulating electrode would progressively recruit more "reward" neurons from a given brain region, thus the increasing preference at higher currents of stimulation (Olds, 1958). In our 2-choice experiments, however, the "all or nothing" preference that we observed can be explained by considering that during the period of activation, the entire fly brain is exposed to the 617-nm light, thus, every neuron expressing CsChrimson would be activated at the same time, provided that the appropriate frequency and 617-nm light intensity are used.

In this type of experiments it is also possible to argue that testing the flies as a group of individuals provides context (social) specific cues that contribute to the display of a preference response. However, as we showed here, this is not the case for the activation of NPF neurons as individual flies also showed a preference for the activation of these neurons.

### 7.2 Is Activation of NPF neurons in the 2-choice assay truly rewarding?

As shown for the grouped and single fly 2-choice experiments (**Figure 3.7 & 3.8**), activation of NPF neurons is also accompanied with a decrease in the fly's locomotion (speed). This raises the following question: Is the activation of NPF neurons truly rewarding? Or, by activating the NPF neurons, one is merely reducing the speed of the flies, therefore trapping them on the active side of our chambers? We reasoned that, if activation of NPF neurons in our assay is in fact rewarding, flies should be able to retain the memory of that reward in an olfactory conditioning assay. Indeed, this is the case for NPF neurons (**Figure 3.11C & 3.11D**). When instead of using a massed-training protocol, we tried a shorter version consisting of only 1 session of a 10-min exposure to odor 1 followed by a 10-min exposure to odor 2 coupled with optogenetic activation of NPF neurons (**Figure 3.11A & 3.11B**), we did not observe a conditioned odor preference when the memory was tested 10 min after the end of training. How is it possible that the activation of NPF neurons leads to a strong preference in our 2-choice assay, but is not able to induce a memory of such experience? . Considering neuropeptide release requires a more intense neuronal activity than fast-acting neurotransmitters (Tallenk, 2012), it seems reasonable to suggest that while acute activation of NPF neurons leads to preference response in our 2-choice assay, the induction of an appetitive memory in an olfactory

conditioning assay may require a higher amount of released NPF, which could be achieved by a more prolongued (**Figure 3.11C & 3.11D**) or intense (**Figure 3.13**) activation of NPF neurons.

In addition, a well-known characteristic of drug addiction is that drug-seeking behavior can persist despite the presence of negative consequences. Rats, for example, are willing to cross an electric grid in order to self-administer a dose of cocaine (Barnea-Ygael et al., 2012). In a similar way, flies overcome an electric grid to get access to an odor that was previously associated with alcohol (Kaun et al., 2011). Analogously, as we showed here (**Figure 4.1**), flies withstood the presence of electric shock stimulation when it was paired with optogenetic activation of NPF neurons, which supports the idea that activation of NPF neurons is a rewarding experience.

On the other hand, NPF neurons have been involved in behaviors such as sucrose reward (Krashes et al., 2009), sucrose perception (Inagaki et al., 2014b), courtship (Lee et al., 2006) and mating (Shohat-Ophir et al., 2012; Gao et al., 2015), as well as alcohol sensitivity (Wen et al., 2005) and alcohol preference and alcohol reward (Shohat-Ophir et al, 2012). In addition, it has been suggested that the state of the fly's reward system may be represented in the NPF neurons (Shohat-Ophir et al., 2012). With this in mind, it makes sense to suppose that, in *Drosophila*, the neural substrates regulating the reward for food and the reward for alcohol are intimately interconnected with the NPF-circuit. We speculated that pre-exposing the flies to a rewarding experience, such as alcohol intoxication (Kaun et al., 2011), would change the sensitivity of the flies for the preference for NPF neuron activation in our assay. Even more, because it is known that exposing flies to alcohol increases the levels of expression of NPF (Shohat-Ophir et al.,

2012), we expected the alcohol exposure to facilitate the preference for NPF neuron activation in our assay, which was indeed the case (**Figure 4.2**).

What is the nature of the preference that the flies show for the activation of NPF neurons? As mentioned earlier, both 'wanting' and 'liking' are required for a reward to happen. In mammals, 'liking' has been studied with facial reactions that occur upon reward presentation (Berridge et al., 2009a; Berridge, 2009b). In the fly, however, there is no current methodology or behavioral paradigm that can differentiate 'wanting' from 'liking'. Thus, it could be argued that the rewarding effects of activating NPF neurons (preference in our 2-choice assay; learning in olfactory conditioning) (present work; Shohat-Ophir et al., 2012) are a combination of both 'wanting' and 'liking'. Similarly, the lever-pressing behavior observed in the rats during the ICSS experiments from Olds & Milner, likely reflects the modulation of either 'wanting' or 'liking' or a combination of both. It should also be considered that unlike the ICSS from Olds & Milner, which is a form of operant conditioning or instrumental learning, our 2-choice assay does not directly address the possibility that the activation of a group of neurons induces learning. Nevertheless, our 2-choice assay shares elements with both classical and operant conditioning: like in a COP (or CPP) the flies show a preference for a region of the behavioral arena, and like in operant conditioning (ICSS), it could be argued that as the flies freely navigate through arena, after several times of entering the active side they would 'learn' that on this part they experience a reward.

More generally, it is interesting to note that the optogenetic activation of NPF neurons has the same characteristics as the Olds & Milner ICSS, that is, presence of a frequency dose-response, sensitization by other rewarding activation and the overcoming of adverse side effects. Overall, we conclude that the preference response observed in our 2-choice assay is indeed a result of the rewarding effects caused by the activation of NPF neurons.

### 7.3 The value of activation of NPF neurons as a reward

When flies are faced with multiple rewards, they base their preference for a given option based on its relative value. For example, in the context of 2-choice feeding experiments, flies show a preference for different food sources based on the relative nutritional value of each option (Stafford et al., 2012; Ribeiro et al., 2010). In addition, when choosing two sites for egg-laying, flies have the capacity to rank the perceived value of each option (Azanchi et al., 2013; Yang et al., 2008; Yang et al., 2015). Considering that both mating and activation of NPF neurons are rewarding to the flies (present work & Shohat-Ophir et al., 2012) and that NPF neurons are activated during mating (Gao et al., 2015), it makes sense to speculate that the mating experience is processed by the fly's reward system, at least in part, through the NPF neurons. Thus, it is possible that the optogenetic activation of NPF neurons in our 2-choice assay mimics what the fly would experience during a mating reward. We observed that single male flies expressing CsChrimson in the NPF neurons, when paired with a single female expressing CsChrimson in Gr66a neurons, showed no preference (nor avoidance) for the active side (**Figure 4.3**). This indicates that, on average, they spend equal amounts of times exploring the active side (activation of NPF neurons) or the inactive side (presence of a virgin female). That is, the value

of the artificial reward achieved by optogenetic activation of NPF neurons is similar to the value of the presence of a virgin female as a natural reward.

### 7.4 NPF is required for the NPF neuron activation preference

In the NPF neurons, Neuropeptide F is the only known present neuromodulator. As it was mentioned earlier, certain neurons express more than one neuropeptide and/or fast-acting neurotransmitter (Van den Pol, 2012). In the context of the preference for NPF neuron activation, this points to the question: Does NPF play a role in the preference for NPF neuron activation? This is in fact the case, as flies where the expression of NPF was targeted with an RNAi construct have a lower degree of preference (**Figure 4.4**). The extent to which the preference for NPF neuron is affected could be explained by the fact that the RNAi transgene employed here may not completely reduce the expression of NPF. Alternatively, the residual preference observe in flies with targeted NPF expression may be due to the presence of a yet unidentified fast-acting neurotransmitter or neuropeptide that might also be present in NPF neurons, thus contributing to preference for the activation of these neurons.

### 7.5 Functional Subdivision of subsets of NPF neurons

As mentioned earlier, the NPF-Gal4 driver that we employed here labels two very distinctive subpopulations of NPF neurons (**Figure 5.1, 5.2 & 5.3**): four prominent neurons (L1-l & P1) that project to the dorsolateral and dorsoventral brain, along with several smaller NPF neurons located in the posterior brain (P2 + DM). By making use of different intersectional methods, we were able to activate specific subsets of NPF neurons in our 2-choice assay. By doing so, we showed that activating the large NPF neurons alone or a subset of P2 neurons was

sufficient to trigger a preference response. However, activation of all small NPF neurons (P2 + DM) produced a preference response comparable to that of activating all NPF neurons. Of note, the RNAi transgene used in this last intersection does not completely abolish the expression of CsChrimson-mVenus in the P1 NPF neurons (**Figure 5.8C**). Nevertheless, and considering the lack of effect of activating a subset of P2 neurons, these data suggests that the DM small NPF neurons are responsible for the preference response upon activation. Although NPF neurons have been implicated in a wide array of different behavior such as ethanol sensitivity (Wen et al., 2005), courtship (Lee at al, 2006), aggression (Dierick & Greenspan, 2007), ethanol preference and ethanol reward (Shohat-Ophir et al., 2012), sleep (He et al., 2013) and sucrose sensitivity (Inagaki et al., 2014b), no study has ever analyzed the potential specific effects a particular subset of NPF neurons might have on a particular behavior. Furthermore, while only the P2 small NPF neurons have been briefly described before (Lee at al, 2006), to our knowledge the work presented here describes for the first the DM small NPF neurons, both from an anatomical and behavioral perspective.

### 7.6 Dopamine neurons as downstream targets of NPF neurons

Dopamine neurons are involved in reward processing in multiple organisms, including flies, rodents and humans (Scaplen & Kaun, 2016). In flies, for example, the PAM cluster of dopamine neurons is involved in sugar (Hueteroth et al., 2015; Yamagata et al., 2015) and water (Lin et al., 2014) reward processing. Furthermore, artificial activation of these neurons is perceived as rewarding (Liu et al., 2012; Burke et al., 2012; Hueteroth et al., 2015; Yamagata et al., 2015). Likewise, artificial activation of NPF neurons is also perceived as rewarding (present work & Shohat-Ophir et al., 2012). This point to the question: Are PAM dopamine neurons

acting downstream of NPF neurons to promote the preference response that we see in our 2-choice assay? This is indeed the case (**Figure 6.1**), as blocking PAM dopamine neurons reduces the preference for the activation of NPF neurons, although we also observed the same effect by blocking dopamine neurons with a different driver (TH-Gal4) (Mao & Davis, 2009) that labels all PPL1 dopamine neurons and but only a few PAM dopamine neurons. Although PPL1 dopamine neurons are required for aversive reinforcement (Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012), the dopamine neurons labelled by the TH-Gal4 driver are required for the processing of alcohol reward, thus it is not surprising, as we saw, that they contribute to the 2-choice preference for the activation of NPF neurons.

On the other hand, specific dopamine neurons are known to innervate particular compartments of the mushroom bodies (Aso et al., 2014a). Could a particular type of dopamine neuron be the one responsible for the preference for the activation of NPF neurons? Our results indicate that the role of dopamine in this context is not straightforward (**Figure 6.2**). For instance, blocking individual PAM dopamine neurons showed no effect, suggesting that multiple PAM dopamine neurons may be required in parallel to promote preference in the 2-choice assay. In a similar way, the work of Masek et al., 2015 suggests that multiple PPL1 dopamine neurons are required for the formation of an aversive taste memory. On the other hand, blocking PAM-γ3 neurons (MB441B) or a combination of PAM-α1, PAM-β1, PAM-γ4, PAM-β'2p and PAM-β'2m (MB047B), led to an enhanced preference for NPF neuron activation (**Figure 6.2**), which indicates that function of PAM dopamine neurons is to repress appetitive (reward) responses.

Our functional connectivity experiments also exemplify the fact that NPF neurons do not necessarily have a simple role in regulating PAM dopamine neurons. For example PAM-β'2 and PAM-γ4 are strongly activated by NPF neuron activation, while PAM-α1, PAM-β1, PAM-β2,

and PAM-γ5 respond with only a moderate activation. Interestingly, high activity of PAM-γ4 neurons has been associated with behavioral quiescence (Cohn et al., 2015), an effect that we also see (reduced locomotion) in our 2-choice assay upon activation of NPF neurons. On the other hand, NPF neuron activation also produced the inhibition of PAM-β'1 and PAM-γ3. This, as well as the behavioral effect (2-choice preference enhancement) of blocking PAM-γ3, indicates that NPF inhibits this dopamine neuron in order to promote preference. A similar relationship has been observed for PAM-γ3 and Allatostatin-A neurons: sugar ingestion would normally activate Allatostatin-A, in turn, inhibiting PAM-γ3 neurons (Yamagata et al., 2016). Furthermore, transient inhibition of this dopamine neuron is perceived as rewarding (Yamagata et al., 2016).

Although we did not perform functional connectivity experiments to test how PPL1 dopamine neurons respond to the activation of NPF neurons, the fact that blocking different groups of them leads to an enhanced preference (like with PAM-γ3 neurons) for NPF neuron activation suggests that NPF might have an inhibitory effect on them. In fact, an example of this is the PPL1-γ1pedc (MB-MP1): inhibition of this neuron by NPF gates the retrieval of appetitive memories (Krashes et al., 2009).

### 7.7 Future Directions

How is the acute activation of NPF neurons translated into the preference response that we see in our assay? Our behavioral epistasis and functional connectivity experiments show that dopamine neurons are required downstream of NPF neuron. Interestingly, the effect of NPF neuron activation over the PAM dopamine neurons goes in the opposite direction as the behavioral valence induced by the respective mushroom body output neurons (MBONs): NPF

strongly activates PAM-β'2 and PAM-γ4 while activation of the respective MBONs leads to aversion; NPF neurons also inhibit PAM-β'1 and PAM-γ3 while activation of the respective MBONs leads to approach. The plasticity generated by pairing an olfactory cue with dopamine neuron activation (aversive or appetitive stimuli) leads to a reduced odor induced activity in the respective MBONs. For example, pairing the activation of PPL1-γ1pedc, known to be involved in aversive learning (Aso et al 2012; Vogt et al., 2014), leads to a reduction in the odor evoked activity of the γ1pedc MBON, which when activated induces aversion (Hige et al., 2015). In a similar way, MBON-β'2mp has a reduced odor-induced activity after an appetitive olfactory conditioning, while blocking this MBON can shift the flies response from avoidance to attraction for a particular odor (Owald et al., 2015a). Thus, associative learning (appetitive or aversive) would involve reducing the odor-induced activation of MBONs that whose activation leads to the opposite valence. Furthermore, activation of more than one MBON can have additive effects for either preference or aversion (Aso et al., 2014b). Therefore it seems conceivable that the acute activation of NPF neurons is being translated into a particular pattern of MBON activation that translates into the preference response we see in our 2-choice assay. Functional connectivity experiments will ultimately show how the activation of NPF neurons is affecting the activity of particular MBONs. Likewise, behavioral epistasis experiments will show if a single or a combination of MBONs are required for NPF neuron activation-induced preference.

## References


Akerboom J, Chen TW, Wardill TJ, Tian L, Marvin JS, Mutlu S, Calderón NC, Esposti F, Borghuis BG, Sun XR, Gordus A, Orger MB, Portugues R, Engert F, Macklin JJ, Filosa A, Aggarwal A, Kerr RA, Takagi R, Kracun S, Shigetomi E, Khakh BS, Baier H, Lagnado L, Wang SS, Bargmann CI, Kimmel BE, Jayaraman V, Svoboda K, Kim DS, Schreiter ER, Looger LL (2012). **Optimization of a GCaMP calcium indicator for neural activity imaging**. *J Neurosci* **32**: 13819-40.

Aponte Y, Atasoy D, Sternson SM (2011). **AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training**. *Nat Neurosci* **14**: 351-5.

Aso Y, Siwanowicz I, Bräcker L, Ito K, Kitamoto T, Tanimoto H (2010). **Specific dopaminergic neurons for the formation of labile aversive memory.** *Curr Biol* **20**: 1445-51.

Aso Y, Herb A, Ogueta M, Siwanowicz I, Templier T, Friedrich AB, Ito K, Scholz H, Tanimoto H (2012). **Three dopamine pathways induce aversive odor memories with different stability.** *PLoS Genet* **8:** e1002768.

Aso Y, Hattori D, Yu Y, Johnston RM, Iyer NA, Ngo TT, Dionne H, Abbott LF, Axel R, Tanimoto H, Rubin GM (2014a). **The neuronal architecture of the mushroom body provides a logic for associative learning.** *Elife* **3**: e04577.

Aso Y, Sitaraman D, Ichinose T, Kaun KR, Vogt K, Belliart-Guérin G, Plaçais PY, Robie AA, Yamagata N, Schnaitmann C, Rowell WJ, Johnston RM, Ngo TT, Chen N, Korff W, Nitabach MN, Heberlein U, Preat T, Branson KM, Tanimoto H, Rubin GM (2014b). **Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila**. *Elife* **3**: e04580.

Aso Y, Rubin GM (2016). **Dopaminergic neurons write and update memories with cell-type-specific rules**. *Elife* **5**: e16135.

Azanchi R, Kaun KR, Heberlein U (2013). **Competing dopamine neurons drive oviposition choice for ethanol in Drosophila**. *Proc Natl Acad Sci U S A* **110**: 21153-8.

Badiani A, Oates MM, Day HE, Watson SJ, Akil H, Robinson TE (1998). **Amphetamine-induced behavior, dopamine release, and c-fos mRNA expression: modulation by environmental novelty**. *J Neurosci* **18**: 10579-93.

Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M (2001). **Altered electrical properties in Drosophila neurons developing without synaptic transmission**. J Neurosci **21**: 1523-31.

Barnea-Ygael N, Yadid G, Yaka R, Ben-Shahar O, Zangen A (2012). **Cue-induced reinstatement of cocaine seeking in the rat "conflict model": effect of prolonged home-cage confinement.** *Psychopharmacology (Berl)* **219**: 875-83.

Brand AH, Perrimon N (1993). **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* **118**: 401-15.

Berridge KC, Robinson TE (2003). **Parsing reward.** *Trends Neurosci* **26**: 507-13.

Berridge KC, Robinson TE, Aldridge JW (2009a). **Dissecting components of reward: 'liking,' 'wanting,'and learning**. *Curr Opin Pharmacol* **9**: 65–73.

Berridge KC (2009b). **'Liking' and 'wanting' food rewards: brain substrates and roles in eating disorders**. *Physiol Behav* **97**: 537-50.

Bindra D (1978). **How adaptive behavior is produced: a perceptual–motivational alternative to response reinforcements**. *Behav Brain Sci* **1**: 41–52.

Botvinick MM, Cohen JD, Carter CS (2004). **Conflict monitoring and anterior cingulate cortex: an update**. *Trends Cogn Sci* **8**: 539-46.

Brown MT, Tan KR, O'Connor EC, Nikonenko I, Muller D, Lüscher C (2012). **Ventral tegmental area GABA projections pause accumbal cholinergic interneurons to enhance associative learning**. *Nature* **492**: 452-6.

Borkar CD, Upadhya MA, Shelkar GP, Subhedar NK, Kokare DM (2016). **Neuropeptide Y system in accumbens shell mediates ethanol self-administration in posterior ventral tegmental area**. *Addict Biol* **21**: 766-75.

Burke CJ, Huetteroth W, Owald D, Perisse E, Krashes MJ, Das G, Gohl D, Silies M, Certel S, Waddell S (2012). **Layered reward signalling through octopamine and dopamine in Drosophila**. *Nature* **492**: 433-7.

Capriles N, Rodaros D, Sorge RE, Stewart J (2003). **A role for the prefrontal cortex in stress- and cocaine-induced reinstatement of cocaine seeking in rats**. *Psychopharmacology (Berl)* **168**: 66-74.

Carlezon WA Jr, Chartoff EH (2007). **Intracranial self-stimulation (ICSS) in rodents to study the neurobiology of motivation.** *Nat Protoc* **2**: 2987-95.

Carlezon WA Jr, Thomas MJ (2009). **Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis**. *Neuropharmacology* **56**: 122-32.

Chabaud MA, Devaud JM, Pham-Delègue MH, Preat T, Kaiser L (2006). **Olfactory conditioning of proboscis activity in Drosophila melanogaster.** J Comp Physiol A Neuroethol Sens Neural Behav Physiol **192**: 1335-48**.**

Claridge-Chang A, Roorda RD, Vrontou E, Sjulson L, Li H, Hirsh J, Miesenböck G (2009). **Writing memories with light-addressable reinforcement circuitry**. *Cell* **139**: 405-15.

Clark JT, Kalra PS, Crowley WR, Kalra SP (1984). **Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats.** *Endocrinology* **115:** 427-9.

Cohen J (2014). **Cocaine and the Pleasure Principle. <http://www.news.ucsb.edu/2014/013995/cocaine-and-pleasure-principle>. Last accessed: August 1st, 2016.**

Cohn R, Morantte I, Ruta V (2015). **Coordinated and Compartmentalized Neuromodulation Shapes Sensory Processing in Drosophila**. *Cell* **163**: 1742-55.

Courtin J, Chaudun F, Rozeske RR, Karalis N, Gonzalez-Campo C, Wurtz H, Abdi A, Baufreton J, Bienvenu TC, Herry C (2014). **Prefrontal parvalbumin interneurons shape neuronal activity to drive fear expression**. *Nature* **505**: 92-6.

Creed MC, Ntamati NR, Tan KR (2014). **VTA GABA neurons modulate specific learning behaviors through the control of dopamine and cholinergic systems**. *Front Behav Neurosci* **8**: 8.

Crespi, LP (1942). **Quantitative variation of incentive and performance in the white rat**. *Am J Psychol* **55**: 46–517.

Davis RL (2011). **Traces of Drosophila memory.** *Neuron* **70**: 8-19.

Domjan M (2005). **Pavlovian conditioning: a functional perspective**. Annu Rev Psychol **56**: 179-206.

Dierick HA, Greenspan RJ (2007). **Serotonin and neuropeptide F have opposite modulatory effects on fly aggression**. *Nat Genet* **39**: 678-82.

Ettenberg A (2009). **The runway model of drug self-administration**. *Pharmacol Biochem Behav* **91**: 271-7.

Gao XJ, Riabinina O, Li J, Potter CJ, Clandinin TR, Luo L (2015). **A transcriptional reporter of intracellular Ca(2+) in Drosophila.** *Nat Neurosci* **18**: 917-25.

Goto Y, Grace AA (2005). **Dopaminergic modulation of limbic and cortical drive of nucleus accumbens in goal-directed behavior**. *Nat Neurosci* **8**: 805-12.

Grace AA, Bunney BS (1984a). **The control of firing pattern in nigral dopamine neurons: burst firing**. *J Neurosci* **4**: 2877-90.

Grace AA, Bunney BS (1984b). **The control of firing pattern in nigral dopamine neurons: single spike firing**. *J Neurosci* **4**: 2866-76.

Gruber F, Knapek S, Fujita M, Matsuo K, Bräcker L, Shinzato N, Siwanowicz I, Tanimura T, Tanimoto H (2013). **Suppression of conditioned odor approach by feeding is independent of taste and nutritional value in Drosophila**. *Curr Biol* **23**: 507-14.

Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla TJ, Garrity PA (2008). **An internal thermal sensor controlling temperature preference in Drosophila**. *Nature* **454**: 217-20.

Hampel S, Chung P, McKellar CE, Hall D, Looger LL, Simpson JH (2011). **Drosophila Brainbow: a recombinase-based fluorescence labeling technique to subdivide neural expression patterns**. *Nat Methods* **8**: 253-9.

He C, Yang Y, Zhang M, Price JL, Zhao Z (2013a). **Regulation of Sleep by Neuropeptide Y-Like System in Drosophila melanogaster**. *PLoS One* **8**: e74237

He C, Cong X, Zhang R, Wu D, An C, Zhao Z (2013b). **Regulation of circadian locomotor rhythm by neuropeptide Y-like system in Drosophila melanogaster**. *Insect Mol Biol* **22**: 376-88.

Hige T, Aso Y, Modi MN, Rubin GM, Turner GC (2015). **Heterosynaptic Plasticity Underlies Aversive Olfactory Learning in Drosophila**. *Neuron* **88**: 985-98.

Holroyd, CB, Coles, MG, and Nieuwenhuis, S (2002). **Medial prefrontal cortex and error potentials**. *Science* **296**: 1610-1.

Huetteroth W, Perisse E, Lin S, Klappenbach M, Burke C, Waddell S (2015). **Sweet taste and nutrient value subdivide rewarding dopaminergic neurons in Drosophila**. *Curr Biol* **25**: 751-8.

Hull, CL (1934). **The rats speed of locomotion gradient in the approach to food**. *J Comp Psychol* 17: 393–422.

Huston JP, Silva MA, Topic B, Müller CP (2013). **What's conditioned in conditioned place preference?** *Trends Pharmacol Sci* **34**: 162-6.

Inagaki HK, Ben-Tabou de-Leon S, Wong AM, Jagadish S, Ishimoto H, Barnea G, Kitamoto T, Axel R, Anderson DJ (2012). **Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing**. *Cell* **148**: 583-95.
Inagaki HK, Jung Y, Hoopfer ED, Wong AM, Mishra N, Lin JY, Tsien RY, Anderson DJ (2014a). **Optogenetic control of Drosophila using a red-shifted channelrhodopsin reveals experience-dependent influences on courtship**. *Nat Methods* **11**: 325-32.

Inagaki HK, Panse KM, Anderson DJ (2014b). **Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in Drosophila**. *Neuron* **84**: 806-20.

Jenett A, Rubin GM, Ngo TT, Shepherd D, Murphy C, Dionne H, Pfeiffer BD, Cavallaro A, Hall D, Jeter J, Iyer N, Fetter D, Hausenfluck JH, Peng H, Trautman ET, Svirskas RR, Myers EW, Iwinski ZR, Aso Y, DePasquale GM, Enos A, Hulamm P, Lam SC, Li HH, Laverty TR, Long F, Qu L, Murphy SD, Rokicki K, Safford T, Shaw K, Simpson JH, Sowell A, Tae S, Yu Y, Zugates CT (2012). **A GAL4-driver line resource for Drosophila neurobiology**. *Cell Rep* **2**: 991-1001.

Ji H, Shepard PD (2007). **Lateral habenula stimulation inhibits rat midbrain dopamine neurons through a GABA(A) receptor-mediated mechanism**. *J Neurosci* **27**: 6923-30.

Josselyn SA, Beninger RJ (1993). **Neuropeptide Y: intraaccumbens injections produce a place preference that is blocked by cis-flupenthixol**. *Pharmacol Biochem Behav* **46**: 543-52.

Kaun KR, Azanchi R, Maung Z, Hirsh J, Heberlein U (2011). **A Drosophila model for alcohol reward.** *Nat Neurosci* **14**: 612–619.

Keene AC, Masek P (2012). **Optogenetic induction of aversive taste memory.** *Neuroscience* **222**: 173-80.

Keene AC, Waddell S (2007). **Drosophila olfactory memory: single genes to complex neural circuits**. *Nat Rev Neurosci* **8**: 341-54.

Kelley AE, Berridge KC (2002). **The neuroscience of natural rewards: relevance to addictive drugs.** *J Neurosci* **22**: 3306-11.

Kelley AE (2004). **Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning**. *Neurosci Biobehav Rev* **27**: 765-76.

Kitamoto, T (2002). **Targeted expression of temperature-sensitive dynamin to study neural mechanisms of complex behavior in *Drosophila***. *J Neurogenet* **16**, 205–228.

Klapoetke NC, Murata Y, Kim SS, Pulver SR, Birdsey-Benson A, Cho YK, Morimoto TK, Chuong AS, Carpenter EJ, Tian Z, Wang J, Xie Y, Yan Z, Zhang Y, Chow BY, Surek B, Melkonian M, Jayaraman V, Constantine-Paton M, Wong GK, Boyden ES (2014). **Independent optical excitation of distinct neural populations**. *Nat Methods* **11**: 338-46.

Krashes MJ, Keene AC, Leung B, Armstrong JD, Waddell S (2007). **Sequential use of mushroom body neuron subsets during drosophila odor memory processing**. *Neuron* **53**: 103-15.

Krashes MJ, DasGupta S, Vreede A, White B, Armstrong JD, Waddell S (2009). **A neural circuit mechanism integrating motivational state with memory expression in Drosophila.** *Cell* **139**: 416-27.

Lai SL, Lee T (2006). **Genetic mosaic with dual binary transcriptional systems in Drosophila**. *Nat Neurosci* **9**: 703–709

LaLumiere RT, Niehoff KE, Kalivas PW (2010). **The infralimbic cortex regulates the consolidation of extinction after cocaine self-administration**. *Learn Mem* **17**: 168-75.

LaLumiere RT, Smith KC, Kalivas PW (2012). **Neural circuit competition in cocaine-seeking: roles of the infralimbic cortex and nucleus accumbens shell**. *Eur J Neurosci* **35**: 614-22.

Lammel S, Lim BK, Malenka RC (2014). **Reward and aversion in a heterogeneous midbrain dopamine system.** *Neuropharmacology* **76 Pt B:** 351-9.

Laviolette SR, van der Kooy D (2004). **The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour.** *Nat Rev Neurosci* **5**: 55-65.

Lee T, Luo L (1999). **Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis**. *Neuron* **22**: 451-61.

Lee G, Bahn JH, Park JH (2006). **Sex- and clock-controlled expression of the neuropeptide F gene in Drosophila**. *Proc Natl Acad Sci U S A* **103**: 12580-5.

Li M, Liu WJ, Lu B, Wang YH, Liu JG (2013). **Differential expression of Arc in the mesocorticolimbic system is involved in drug and natural rewarding behavior in rats.** *Acta Pharmacol Sin* **34**: 1013-24.

Li Y, van den Pol AN (2006). **Differential target-dependent actions of coexpressed inhibitory dynorphin and excitatory hypocretin/orexin neuropeptides**. *J Neurosci* **26**: 13037-47.

Lihoreau M, Clarke IM, Buhl J, Sumpter DJ, Simpson SJ (2016). **Collective selection of food patches in Drosophila**. *J Exp Biol* **219**: 668-75.

Lin S, Owald D, Chandra V, Talbot C, Huetteroth W, Waddell S (2014). **Neural correlates of r reward in thirsty Drosophila.** *Nat Neurosci* **17**: 1536-42.

Liu C, Plaçais PY, Yamagata N, Pfeiffer BD, Aso Y, Friedrich AB, Siwanowicz I, Rubin GM, Preat T, Tanimoto H (2012). **A subset of dopamine neurons signals reward for odour memory in Drosophila**. *Nature* **488:** 512-6.

Madsen HB[1], Brown RM, Short JL, Lawrence AJ (2012). Investigation of the neuroanatomical substrates of reward seeking following protracted abstinence in mice. *J Physiol* **590**: 2427-42.

Mao Z, Davis RL (2009). **Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity**. *Front Neural Circuits* **3**: 5.

Masek P, Worden K, Aso Y, Rubin GM, Keene AC (2015). **A dopamine-modulated neural circuit regulating aversive taste memory in Drosophila**. *Curr Biol* **25**: 1535-41.

McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL (2003). **Spatiotemporal rescue of memory dysfunction in Drosophila**. Science **302**: 1765-8.

Milad MR, Vidal-Gonzalez I, Quirk GJ (2004). **Electrical stimulation of medial prefrontal cortex reduces conditioned fear in a temporally specific manner**. *Behav Neurosci* **118**: 389-94.

Miyamoto T, Slone J, Song X, Amrein H (2012). **A fructose receptor functions as a nutrient sensor in the Drosophila brain**. *Cell* **151**: 1113-25.

Nern A, Pfeiffer BD, Rubin GM (2015). **Optimized tools for multicolor stochastic labeling reveal diverse stereotyped cell arrangements in the fly visual system**. *Proc Natl Acad Sci U S A* **112**: E2967-76.

Nicolaï LJ, Ramaekers A, Raemaekers T, Drozdzecki A, Mauss AS, Yan J, Landgraf M, Annaert W, Hassan BA (2010). **Genetically encoded dendritic marker sheds light on neuronal connectivity in Drosophila**. *Proc Natl Acad Sci U S A* **107**: 20553-8.

O'Donnell P, Grace AA (1995). **Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input**. *J Neurosci* **15**: 3622-39.

Olds J, Milner P (1954). **Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain**. *J Comp Physiol Psychol* **47**: 419-27.

Olds J (1956a). **Runway and maze behavior controlled by basomedial forebrain stimulation in the rat**. *J Comp Physiol Psychol* **49**: 507-12.

Olds J (1956b). **Pleasure centers in the brain**. *Sci Am* **195**: 105-116.

Olds J (1958). **Self-stimulation of the brain; its use to study local effects of hunger, sex, and drugs**. *Science* **127**: 315-24.

Ongür D, Price JL (2000). **The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans**. *Cereb Cortex* **10**: 206-19.

Owald D, Felsenberg J, Talbot CB, Das G, Perisse E, Huetteroth W, Waddell S (2015a). **Activity of defined mushroom body output neurons underlies learned olfactory behavior in Drosophila**. *Neuron* **86**: 417-27.

Owald D, Lin S, Waddell S (2015b). **Light, heat, action: neural control of fruit fly behaviour**. *Philos Trans R Soc Lond B Biol Sci* **370**: 20140211.

Panlilio LV, Goldberg SR (2007). **Self-administration of drugs in animals and humans as a model and an investigative tool**. *Addiction* **102**: 1863-70.

Park WK, Bari AA, Jey AR, Anderson SM, Spealman RD, Rowlett JK, Pierce RC (2004). **Cocaine administered into the medial prefrontal cortex reinstates cocaine-seeking behavior by increasing AMPA receptor-mediated glutamate transmission in the nucleus accumbens**. *J Neurosci* **22**: 2916-25.

Parker JG, Wanat MJ, Soden ME, Ahmad K, Zweifel LS, Bamford NS, Palmiter RD (2011). **Attenuating GABA(A) receptor signaling in dopamine neurons selectively enhances reward learning and alters risk preference in mice**. *J Neurosci* **31**: 17103-12.

Perisse E, Yin Y, Lin AC, Lin S, Huetteroth W, Waddell S (2013a). **Different kenyon cell populations drive learned approach and avoidance in Drosophila**. *Neuron* **79**: 945-56.

Perisse E, Burke C, Huetteroth W, Waddell S (2013b). **Shocking revelations and saccharin sweetness in the study of Drosophila olfactory memory**. *Curr Biol* 23: R752-63.

Pfeiffer BD, Jenett A, Hammonds AS, Ngo TT, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Laverty TR, Mungall C, Svirskas R, Kadonaga JT, Doe CQ, Eisen MB, Celniker SE, Rubin GM (2008). **Tools for neuroanatomy and neurogenetics in Drosophila**. *Proc Natl Acad Sci U S A* **105**: 9715-20.

Pleil KE, Rinker JA, Lowery-Gionta EG, Mazzone CM, McCall NM, Kendra AM, Olson DP, Lowell BB, Grant KA, Thiele TE, Kash TL (2015). **NPY signaling inhibits extended amygdala CRF neurons to suppress binge alcohol drinking**. *Nat Neurosci* **18**: 545-52.

Posner MI, Rothbart MK, Sheese BE, Tang Y (2007). **The anterior cingulate gyrus and the mechanism of self-regulation**. *Cogn Affect Behav Neurosci* **7**: 391-5.

Qi J, Zhang S, Wang HL, Barker DJ, Miranda-Barrientos J, Morales M (2016). **VTA glutamatergic inputs to nucleus accumbens drive aversion by acting on GABAergic interneurons**. *Nat Neurosci* **19**: 725-33.

Quirk GJ, Russo GK, Barron JL, Lebron K (2000). **The role of ventromedial prefrontal cortex in the recovery of extinguished fear**. *J Neurosci* **20**: 6225-31.

Ramdya P, Lichocki P, Cruchet S, Frisch L, Tse W, Floreano D, Benton R (2015). **Mechanosensory interactions drive collective behaviour in Drosophila**. *Nature*, **519**: 233-6.

Reichmann F, Holzer P (2016). **Neuropeptide Y: A stressful review**. *Neuropeptides* **55**: 99-109.

Ren Q, Awasaki T, Huang YF, Liu Z, Lee T (2016). **Cell Class-Lineage Analysis Reveals Sexually Dimorphic Lineage Compositions in the Drosophila Brain**. *Curr Biol* **26**: 2583-2593.

Ribeiro C, Dickson BJ (2010). **Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila**. *Curr Biol* **20**: 1000-5.

Rushworth MF, Noonan MP, Boorman ED, Walton ME, Behrens TE (2011). **Frontal cortex and reward-guided learning and decision-making**. *Neuron* **70**: 1054-69.

Russo SJ, Nestler EJ (2013). **The brain reward circuitry in mood disorders**. *Nat Rev Neurosci* **14**: 609-25.

Sangha S, Robinson PD, Greba Q, Davies DA, Howland JG (2014). **Alterations in reward, fear and safety cue discrimination after inactivation of the rat prelimbic and infralimbic cortices**. *Neuropsychopharmacology* **39**: 2405-13.

Scaplen KM, Kaun KR (2016). **Reward from bugs to bipeds: a comparative approach to understanding how reward circuits function**. *J Neurogenet* **30**: 133-48.

Schultz W, Dayan P, Montague PR (1997). **A neural substrate of prediction and reward**. *Science* **275**: 1593-9.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003). **Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila***. *J. Neurosci* **23**:10495–502.

Shohat-Ophir G, Kaun KR, Azanchi R, Mohammed H, Heberlein U (2012). **Sexual deprivation increases ethanol intake in Drosophila.** *Science* **335**:1351-5.

Simpson JH (2009). **Mapping and manipulating neural circuits in the fly brain.** *Adv Genet* **65**: 79-143.

Skinner BF (1948). **Superstition in the pidgeon.**

St Johnston D (2002). **The art and design of genetic screens: Drosophila melanogaster.** *Nat Rev Genet* **3**: 176-88.

Staddon JE, Cerutti DT (2003). **Operant conditioning**. *Annu Rev Psychol* **54**: 115-44.

Stafford JW, Lynd KM, Jung AY, Gordon MD (2012). **Integration of taste and calorie sensing in Drosophila**. *J Neurosci* **32**: 14767-74.

Suh GS, Ben-Tabou de Leon S, Tanimoto H, Fiala A, Benzer S, Anderson DJ (2007)**. Light activation of an innate olfactory avoidance response in Drosophila.** *Curr Biol* **17**: 905-8.

Tallent MK (2008). **Presynaptic inhibition of glutamate release by neuropeptides: use-dependent synaptic modification.** *Results Probl Cell Differ*, **44**: 177-200.

Tan KR, Yvon C, Turiault M, Mirzabekov JJ, Doehner J, Labouèbe G, Deisseroth K, Tye KM, Lüscher C (2012). **GABA neurons of the VTA drive conditioned place aversion**. *Neuron* **73**: 1173-83.

Tanaka NK, Tanimoto H, Ito K (2008). **Neuronal assemblies of the Drosophila mushroom body**. *J Comp Neurol* **508**: 711-55.

Tempel BL, Bonini N, Dawson DR, Quinn WG (1983). **Reward learning in normal and mutant drosophila.** *Proc Natl Acad Sci U S A* **80**:1482-6**.**

Trannoy S, Redt-Clouet C, Dura JM, Preat T (2011). **Parallel processing of appetitive short- and long-term memories in Drosophila**. *Curr Biol* **21**: 1647–1653

Tully T, Quin WG (1985). **Classical conditioning and retention in normal and mutant Drosophila melanogaster.** *J Comp Physiol A* **157**: 263-77.

Tzschentke TM (2007). **Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade**. *Addict Biol* **12**: 227-462.

Van den Oever MC, Rotaru DC, Heinsbroek JA, Gouwenberg Y, Deisseroth K, Stuber GD, Mansvelder HD, Smit AB (2013). **Ventromedial Prefrontal Cortex Pyramidal Cells Have a Temporal Dynamic Role in Recall and Extinction of Cocaine-Associated Memory**. *J Neurosci* **33**: 18225-33.

Van den Pol AN (2012). **Neuropeptide transmission in brain circuits.** *Neuron* **76**: 98-115.

Venken KJ, Simpson JH, Bellen HJ (2011). **Genetic manipulation of genes and cells in the nervous system of the fruit fly.** *Neuron* 2011, 72: 202-30**.**

Vogt K, Schnaitmann C, Dylla KV, Knapek S, Aso Y, Rubin GM, Tanimoto H (2014). **Shared mushroom body circuits underlie visual and olfactory memories in Drosophila.** *Elife* **3**:e02395.

Vogt K, Aso Y, Hige T, Knapek S, Ichinose T, Friedrich AB, Turner GC, Rubin GM, Tanimoto H (2016). **Direct neural pathways convey distinct visual information to Drosophila mushroom bodies**. *Elife* **5**: e14009.

Wen T, Parrish CA, Xu D, Wu Q, Shen P (2005). **Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity**. *Proc Natl Acad Sci U S A* **102**: 2141-6.

Westerink BH, Kwint HF, de Vries JB (1997). **Eating-induced dopamine release from mesolimbic neurons is mediated by NMDA receptors in the ventral tegmental area: a dual-probe microdialysis study**. *J Neurochem* 69: 662-8.

Wise RA (1996). **Addictive drugs and brain stimulation reward.** *Annu Rev Neurosci* **19**: 319-40.

Witten IB, Steinberg EE, Lee SY, Davidson TJ, Zalocusky KA, Brodsky M, Yizhar O, Cho SL, Gong S, Ramakrishnan C, Stuber GD, Tye KM, Janak PH, Deisseroth K (2011). **Recombinase-driver rat lines: tools, techniques, and optogenetic application to dopamine-mediated reinforcement.** *Neuron* **72**: 721-33.

Q, Wen T, Lee G, Park JH, Cai HN, Shen P (2003). **Developmental control of foraging and social behavior by the Drosophila neuropeptide Y-like system**. *Neuron* **39**: 147-61.

Yamagata N, Ichinose T, Aso Y, Plaçais PY, Friedrich AB, Sima RJ, Preat T, Rubin GM, Tanimoto H (2015). **Distinct dopamine neurons mediate reward signals for short- and long-term memories**. *Proc Natl Acad Sci U S A* **112**:578-83.

Yamagata N, Hiroi M, Kondo S, Abe A, Tanimoto H (2016). **Suppression of Dopamine Neurons Mediates Reward**. *PLoS Biol* **14**: e1002586.

Yang CH, Belawat P, Hafen E, Jan LY, Jan YN (2008). **Drosophila egg-laying site selection as a system to study simple decision-making processes**. *Science* **319**: 1679-83.

Yang CH, He R, Stern U (2015). **Behavioral and circuit basis of sucrose rejection by Drosophila females in a simple decision-making task**. *J Neurosci* **35**: 1396-410.

Yoshida M, Yokoo H, Mizoguchi K, Kawahara H, Tsuda A, Nishikawa T, Tanaka M (1992). **Eating and drinking cause increased dopamine release in the nucleus accumbens and ventral tegmental area in the rat: measurement by in vivo microdialysis**. *Neurosci Lett* **139**: 73-6.

Zhang YQ, Rodesch CK, Broadie K (2002). **Living synaptic vesicle marker: synaptotagmin-GFP**. *Genesi*s **34**: 142-5.