SEROTONERGIC MODULATION OF BEHAVIOURAL CHOICE IN *DROSOPHILA MELANOGASTER*

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SEROTONERGIC MODULATION OF BEHAVIOURAL CHOICE IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

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The monoamine modulator, serotonin (5-HT) has previously been shown to be involved in sleep, feeding, learning and memory, and anxiety in fruit flies. In this study, we used a behavioural paradigm where the flies choose to respond to either to a looming visual stimulus or an appetitive tastant. Using the GAL4-UAS binary system, selectively we activated or inactivated serotonergic neurons in the fly brain to understand the mechanisms of competitive inhibition between appetitive/feeding and aversive/escape behavioural systems. Unlike the results of previous experiments that involved acute manipulations of serotonergic neurons, our results failed to yield a reduction of feeding when we inactivated the same serotonergic neurons chronically using Gal4-R50H05>UASKir2.1. Likewise, we failed to observe overfeeding when we activated the same serotonergic neurons chronically using Gal4-R50H05>UAS-NaChBac. This discrepancy can be accounted for the difference between acute and chronic manipulations. However, because the visual responsiveness increased upon chronic activation of the serotonergic neurons, we suggest that the serotonergic neurons of interest might be modulating sensory integration areas rather than hunger *per se*.

Keywords:Serotonin; *D. melanogaster* ; visual; gustatory; behavioural choice

ÖZ

SEROTONERJİK MODÜLASYONUN *DROSOPHILA MELANOGASTER'DE* DAVRANIŞSAL SEÇİME ETKİSİ

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Bir monoamin modülatör olan serotoninin (5-HT), Drosophila'da uyku, beslenme, öğrenme, hafıza ve kaygı güdümlü davranışlar gibi birçok fonksiyonda rolü vardır. Biz bu çalışmada, sineklerin tat alma ile ilgili iştah açıcı ve tehlikeli bir görsel uyaran arasında seçim yapmalarını gerektiren bir davranışsal yaklaşım kullandık. İştah açıcı/beslenme ve uzaklaştırıcı/kaçma davranış sistemleri arasındaki rekabetli baskılama mekanizmasını anlamak amacıyla GAL4-UAS ikili sistemini kullanarak, sinek beynindeki serotonin nöronlarını seçici olarak aktive veya deaktive ettik. Serotonerjik nöronlarının akut manipülasyonlarını içeren önceki deneylerin sonuçlarının aksine, bizim sonuçlarımız Gal4-R50H05>UAS-Kir2.1 ile aynı serotonerjik nöronları kronik olarak inaktive ettiğimizde yeme davranışında bir düşüş göstermedi. Benzer şekilde, aynı serotonerjik nöronları Gal4-R50H05>UAS-NaChBac ile kronik olarak aktive ettiğimizde de aşırı beslenme davranışı gözlemlemedik. Bu uyuşmazlık akut ve kronik manipülasyonlar arasında fark ile açıklanabilir. Ancak, serotonerjik nöronların kronik aktivasyonu sonucunda görsel uyarıcıya cevap verme olasılığının da arttığını fark ettik. Dolayısıyla, ilgilendiğimiz serotonerjik nöronların tek başına açlığı kontrol etmek yerine duyusal entegrasyon alanlarını modüle ediyor olabileceğini öne sürüyoruz.

Anahtar Sözcükler: Serotonin; *D. melanogaster* ; görsel; tatma; davranışsal seçi

To My Loving and Lovely Family

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

CAR	Collision Avoidance Reflex
PER	Proboscis Extension Reflex
Kir2.1	Inwardly Rectifying Potassium Channels
NaChBac	Voltage Gated Sodium Channels
TRPA1	Transient Receptor Potential Cation Channel A1
5-HT	Serotonin
UAS	Upstream Activating Sequence
TRPA1	Transient Receptor Potential Cation Channel A1
Shi	Shibire / Temperature sensitive mutation in *Drosophila*
SOP	Subesophageal ganglion

CHAPTER 1

INTRODUCTION

Drosophila melanogaster, the fruit fly, is one the most popular model organisms used in neuroscience research (Stephenson &Metcalfe, 2013). It is convenient to use them for studies of cognition and brain functions due to their similarity to humans in terms of certain processes and mechanisms underlying cognitive mechanisms such as learning, memory and attention. It is also beneficial to use them for genetic studies since their genome is considerably small and the role of their genes have been mostly identified. 80 % of their genome has homologs in humans (Aquadro, Bauer DuMont &Reed, 2001). Therefore, comparable genetic or neuroscientific studies could be performed by using fruit flies. Moreover, their connectome is well studied (Chiang et al.,2011). Thereby, connection between neuronal and behavioural levels can be established. This makes *Drosophila* even more favourable for experimental studies investigating behaviour and the underlying processes. Further, in recent years, neurogenetic technologies and experimental tools enable tissue and time selective manipulation of genes and even neural circuits. Performing such manipulations and observing the results in *Drosophila* is considerably easier due to their short lifetime and ability to produce many progenies at once. This helps in a way that the physiological, behavioural and genetic changes can be traced through the lifetime of flies and fly strains can also be observed through generations. That also makes them a suitable model for translational science.

The current study focuses on the role of serotonergic neurons on attention and decision making. Serotonin is a monoamine modulator involved in sleep,

learning, memory and anxiety in fruit flies. Thus, a behavioural paradigm was used to understand the role of some specific serotonergic neurons in the fruit fly.

Two different receptor types expressed on certain serotonergic neurons in the fly brain were used in the current study. One of them was the Kir2.1 (inwardly rectifying potassium channels) channels (Döring, Wischmeyer, Kühnlein, Jackle & Karschin, 2002). These channels control the potassium flow through the neuron membrane and the neurons of the flies whose serotonergic neurons are inactivated cannot depolarize so that action potentials cannot be transmitted. This leads to a chronic inactivation of the serotonergic neurons where these potassium channels are expressed. Another type of receptor examined in this study is NaChBac (Yue, Navarro, Ren, Ramos & Clapham, 2002) which are voltage gated sodium channels. In this case, the change

is again persistent and chronic activation of the serotonergic neurons expressing these sodium channels was achieved.

The serotonergic neurons expressing these channels were tagged by the Gal4-UAS system so that the gene expression was modulated (Brand & Perrimon, 1993). By using this system, flies with needed genotypes were obtained by doing the appropriate crosses by using parent strains.

The modulatory roles of the serotonergic neurons expressing these receptors were observed. The flies choose between two different stimuli, visual and gustatory. The visual stimulus creates a looming. This triggers the collision avoidance reflex (CAR) and flies raise their front legs to protect themselves. The appetitive gustatory stimulus was presented by handing them small balls dipped in 0.5M sucrose (a valuable, nutritious sugar for flies) or water and they responded to by extending their proboscis (PER) and feeding. They either showed an avoidance reflex as a response to the aversive visual stimulus or they fed on the appetitive stimulus they were given. The interest of this study was to see whether the selective activation or deactivation of serotonergic neurons modulate these behaviours or suppress/ favour either one of them.

CHAPTER 2

BACKGROUND

2.1 *Drosophila melanogaster* as the model organism

Drosophila melanogaster, the fruit fly, has been one of the most popular model organisms to work with and the studies with fruit flies also led to other advances in biological sciences (Roberts, 2006). As stated by Jennings (2011), conducting genetic studies with the fruit fly is easier compared to the vertebrate models and the techniques used are also rather inexpensive. There are also other reasons to prefer *D. melanogaster* over other invertebrates as the model organism. They have relatively small genome with only four chromosomes, whose functions have mostly been identified. Since the genes of interest can easily be located on the genome, chemical or genetic manipulations could be done more precisely. The other advantage of using them for genetic studies is that the alterations in their genome can easily be detected just by observing their phenotypes i.e. their physical appearance. Moreover, neurons can be pinpointed and be switched on or off via recently developed technologies (Owald et. al., 2015).

There are also basic genetic and biological mechanisms that are shared by many animals since they have been conserved through the evolutionary course (Lessing & Bonini, 2009). Thus, rather than examining them such features on humans, which is more expensive, time consuming and mostly impractical, fruit fly can be studied. Experimenting with them also makes it possible to conduct invasive studies that cannot be implemented on humans.

Drosophila is also a valuable model to conduct comparable studies with humans. Many cognitive mechanisms such as learning and attention are similar in humans and fruit flies. Therefore, these mechanisms both behaviourally and genetically could be studied in flies and then inferences about the humans could be done (Swinderen, 2005; van Swinderen et. al, 2009).

As a result of these advantages to study fruit flies, new behavioural techniques and genetic tools have been discovered which make *D. melanogaster* even more preferable. Mutant *D. melanogaster* strains for particular genes can be produced or the expression of genes or receptors of interest could be manipulated (Jennings, 2011). Thereby cognitive mechanisms are not the only focus of *Drosophila* studies but they may be used to study other physiological functions as well.

Van Swinderen (2011) proposes that studying attention in small animals like Drosophila can be difficult since there is no way to test their attention like it is done

 for humans or non-human primates. To illustrate; attentional protocols can be created by using psychophysics setups but the attention of flies can only be measured by observing their behavioural and physiological responses. However attentional processes of flies are analogous to that of humans so that they are still precious models to study attention and its neural correlates. Other studies also revealed that *Drosophila* has selective attention, which according to van Swinderen is an experience dependent top-down process (van Swinderen, 2011). This may also lead us into using *Drosophila* as a model for learning and memory studies since both of these mechanisms are influenced by experiences.

2.2 Attention in *D. melanogaster*

Attentional processes have four main features as described by de Bivort and van Swinderen (2016). First, attention is a selective process such that responses are given only to a certain amount of incoming data. Second, attention has limited resources and there is always a competition between explorative and exploitative behaviours (Berger-Tal, Nathan, Meron & Saltz, 2014). To illustrate, while foraging is an explorative behaviour, feeding is an exploitative one. Thus, an animal needs to decide between these two behaviours such that it may continue foraging for new and more nutritious food or it may stay and feed on the readily available one. Third, serial alternation of attention occurs between different entities. If an animal is not deliberately directing sustained attention to something that it is not focusing, it serially attends to either one of the entities for a typical attention span. Thus, attention is rather a momentary commitment although persistence may be observed if there is a malfunctioning of the system (de Bivort & van Swinderen, 2016). Last, de Bivort and van Swinderen (2016) also proposes that there are neural correlates for attentional processes.

We used a behavioural test to study attention and behavioural choice in *D. melanogaster*. In this protocol we varied the duration of food deprivation period or the physical characteristics of the stimuli. By using different experimental manipulations that may make the visual or gustatory stimulus more proponent, we observed how the decision-making process of the flies varied. Thereby, we sought to reveal whether either one of the stimuli (visual or gustatory) are more attention grabbing or favourable for the flies. Moreover, with the variation of food deprivation period, we attempted to reveal whether hunger influences the allocation of attention between different stimuli and also the decision making between them. This experimental protocol is suitable to study attention considering the criteria described by de Bivort and van Swinderen (2016). First, attentional selectivity can be observed with our protocol. Flies may choose between avoidance and approach behaviours when they encounter with stimuli in our experiment. They may either select to stop whatever they are doing and show a response

that would decrease the effect of stimuli. Alternatively, they may show a behavioural response to increase and sustain the effect of stimulus and exploit it. The first behaviour in our case is proboscis extension reflex, which they use to feed on gustatory stimulus and the second is the collision avoidance reflex that they show in response to the looming visual stimulus. So, since the attention has limited resources, a fly may attend to either one of the presented stimuli at a time.

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We can unequivocally determine which of the stimuli the fly is attending or if it was attending neither one of them. This is because the behaviours triggered by two stimuli are mutually exclusive that is they inhibit each other. Thus, it is a winner take all competition. When one of the reflexes (CAR or PER) is observed, we can certainly conclude that this behaviour is not controlled by the other stimulus. The third criteria of de Bivort and van Swinderen also applies in our protocol. Neither collision avoidance reflex (CAR) nor proboscis extension reflex (PER) can be seen throughout a session in wild type animals. Flies display adaptive switches between these two. If they were too hungry they feed on the gustatory stimulus (PER) at the beginning of the session and then show avoidance reflex (CAR) when they are sated. If they were not food deprived, they show CAR from the beginning of the session. In the latter case, they feed on the gustatory stimulus during the inter trial intervals. This means that attention is not constantly allocated to either of these stimuli, i.e., gustatory and visual. Thus, we were able to observe the serial alternation between behaviours clearly. Finally, they claim that neural basis of all these mechanisms should be studied. The purpose of our study is study the involvement of serotonergic transmission in the behavioural choice between the visual and the gustatory stimuli.

2.3 Serotonergic modulation of attention and behavioural control

In Drosophila, serotonergic transmission has previously been shown to modulate diverse behavioural functions including memory (Sitaraman et al., 2008; Sitaraman, LaFerriere, Birman & Zars, 2012), anxiety (Mohammad et al., 2016), feeding (Eriksson et al., 2017), aggression (Zwarts, Versteven & Callaerts, 2012; Alekseyenko, Lee & Kravitz, 2010) and sleep (Yuan, Joiner & Sehgal, 2006). For example, the intensity of aggressive behaviours in flies escalate progressively. There is a decision-making process happening at each level such that flies may decide to continue fighting or they may stop and retreat. There are some studies claiming that serotonin has no effect on aggression (Baier, Wittek & Brembs, 2002). However, the method mentioned in this study of Baier and others adopts a general technique that affects the serotonergic system as a whole.

More recent studies show otherwise such that aggression in fruit flies may be modulated by the serotonergic receptors. Alekseyenko and others (2014) claimed, serotonergic PLP neurons are involved in the regulation of the intensity and escalation of aggressive activity in flies. The technique used in the latter is more selective since they target single or a group of neurons. Further, there are other works focusing on the roles of single serotonergic receptors that supported the claim that serotonergic framework has modulatory functions on different aspects of aggression (Johnson, Becnel & Nichols, 2009).

Another study targeting specific serotonergic neurons also showed that serotonin has an inhibitory role on certain behaviours such as feeding and mating (Pooryasin & Fiala, 2015). Thus, they concluded that serotonin selectively modulates states of arousal. It has also been proposed that Drosophila has some states comparable to the mammalian sleep stages (Greenspan, Tononi, Cirelli & Shaw, 2001). Other than serotonin's role of triggering quiescence, it is also involved in the regulation of circadian rhythm and sleep in Drosophila (Nall & Sehgal, 2014).

Serotonin is also involved in the regulation of appetite and feeding (Neckameyer, 2010). Altering the amount of serotonin synthesis during the development of fly

larvae influences the development of fibers branching to the gut and also the feeding behaviour of the mature flies.

Albin and others (2015) also work on feeding decisions in flies. They used R50H05-Gal4 line which target a group of neurons producing serotonin and tagged 25 serotonergic and 15 non-serotonergic neurons. They activated or deactivated these serotonergic neurons with different UAS lines. To illustrate; UAS-TRPA1 was used to temporarily activate the neurons with heat and acute deactivation was achieved by using UAS-Shi^{ts1}, i.e., a temperature sensitive dynamin mutant used to inactivate the neurons by inhibiting the synaptic transmission. Normally, proboscis extension reflex (PER) and feeding are observed when the flies are hungry. However, when they activated the serotonergic neurons with UAS-TRPA1, the flies expressed PER and feeding as if they were food deprived. On the other hand, when the acute deactivation of serotonergic neurons was done with UAS-Shi^{ts1}, which is also temperature sensitive, they behaved as if they were sated and probability of observing PER and feeding decreased. According to the interpretation of the writers, the serotonergic neurons targeted by R50H05-Gal4 line modulate hunger. These neurons are found in SE1, ALP, LP2, PLP and PMP clusters in both hemispheres of the brain. Although they suggest that these serotonergic neurons modulate hunger, they do not innervate the primary taste or motor areas in the subesophageal ganglion (SOP) that regulate taste or feeding. There are still areas in the fly brain that can be considered as terra incognita meaning that the functions of those locations are not well known. The serotonergic neurons targeted by the R50H05-Gal4 line are thought be located in those indeterminate areas. They also propose that these neurons do not regulate taste but hunger. That is why, activation or inactivation of these 25 serotonergic neurons does not induce indiscriminate feeding. Flies still does not prefer feeding on water or bitter food implicating that there is still taste discrimination. Further, activation or inactivation of the neurons in interest do not lead to a persistent change in terms of feeding behaviour.

Feeding is triggered when the flies whose serotonergic neurons were activated via UAS-TRPA1 are heated. However, they stop feeding when the temperature drops back to room temperature (Albin et al., 2015).

This last study by Albin and others was our starting point to choose serotonergic neurons to work with. The areas that R50H05-Gal4 targets are terra incognita, i.e., it is not known whether these parts of the brain are sensory integration areas affecting the decision making between gustatory and visual stimuli. If so, these areas may be modulating attention but not hunger alone. We thought that if these serotonergic neurons were modulating only hunger, the change in the behaviours of the flies would be more persistent. Hunger is a more persistent state rather than a behaviour that lasts for brief periods. When an animal becomes hungry, even though it is fed, there needs to be some time for it to be sated and its hunger to be suppressed. Moreover, there are other modulators and mechanisms modulating the appetite, food selection and hunger of *Drosophila* (Zhang, Branch & Shen, 2013). Thus, we came up with the idea that these neurons may be located at the sensory motor or sensory integration areas. In order to examine this, we used Gal4-UAS binary system to selectively activate or deactivate specific serotonergic neurons.

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2.4 Gal4-UAS System

Gal4-UAS binary system is used for selective targeting and manipulation of genes and neurons in D. melanogaster (Duffy, 2002). UAS (upstream regulation sequence) is a promoter i.e. specific sequence in DNA involving in the transcription process

and it was obtained from yeast. Gal4 is a transcription factor that was extracted from yeast (Fischer, Giniger, Maniatis & Ptashne, 1988). Using Gal4-UAS binary system in *Drosophila* provides specificity and control both temporally and spatially (Venken, Simpson &Bellen, 2011).

This transcriptional control gives the ability to manipulate which proteins and genes are expressed in a cell. During a transcription process, a signal indicating which protein is needed in the cell and thus which sequence on the DNA will be transcribed comes to the nucleus. When the signal reaches the promoter sequences located near or in the gene of interest, the matching transcription factor comes and binds to the promoter and the transcription process begins. This shows that there is a selective relationship between the promoters and transcription factors and therefore only certain transcription factors can bind to specific promoters. Gal4 and UAS have this kind of relationship as well and they are not functional when they present in a cell alone. There are also other binary systems like this but Gal4-UAS system is considered as the best since it has the least leakage in the fly brain. This means that the affinity of flies' own promoters to Gal4 is low or none. The same is true for flies' own transcription factors such that they cannot bind to UAS as well. This makes Gal4-UAS is a highly specialized system for flies. It is also sensitive since it works well in the fly genome (Pfeiffer et. al., 2010; de Valle Rodriguez, Didiano &Desplan, 2011).

We used Gal4-UAS system in our study to selectively activate or deactivate serotonergic neurons. The Gal4 line we used (R50H05-Gal4 also called 38764) targets 25 serotonergic and 15 non-serotonergic neurons in the fly brain (Albin et. Al., 2015). To activate or deactivate serotonergic neurons, R50H05-Gal4 line was crossed to UAS-NaChBac and UAS-Kir2.1, respectively.

2.5 UAS-NaChBac

Activation experiments were carried out by using flies carrying UAS-NaChBac. NaChBac are voltage gated sodium channels. Neurons that were targeted by the R50H05-Gal4 line were activated during development as well as during the experiments. Therefore, this was a chronic activation protocol. Thus, persistent activation of those sodium channels was achieved via Gal4-UAS binary system. Since R50H05-Gal4 line targets only 25 serotonergic neurons in the fly brain, UAS-NaChBac was expressed in those neurons. Thereby, the neurons' excitability level was near depolarization all the time and could depolarize with a small inward current of sodium without any special signal (Nitabach, 2006).

R50H05>UAS-NaChBac flies were tested to see if these group of neurons regulate the feeding and decision-making processes. If the serotonergic neurons tagged with 38764 Gal4 line are sufficient to induce the state of hunger, sated flies should be feeding as if they were food deprived upon their activation. Thus, in this experiment the flies were tested without any food deprivation.

2.6 UAS-Kir2.1

Kir2.1 are potassium channels that play a role in the modulation of excitability of the cells and in the maintenance of the resting membrane potential (Hodge, 2009). They are also involved in the regulation of the pH and volume of the cells (Dahal, 2013). This class of potassium channels are called inwardly rectifying since they

support inward currents more relative to the outward currents. Thus, when the persistent deactivation of these channels is achieved with Gal4-UAS system, potassium goes out of the membrane all the time and no action potential is produced in the neurons expressing Gal4.

In our study, serotonergic neurons carrying Kir2.1 channels were inactivated by using Gal4-UAS system. Thus, 25 serotonergic neurons (Albin et. al., 2015) that were targeted by R50H05-Gal4 line could not trigger action potentials because the membrane voltage did not increase beyond the threshold potential. This was a chronic manipulation that lasts throughout the lifetime of the flies. To understand if this group of serotonergic neurons are involved in feeding regulation and whether they have a role in the decision-making process, we tested R50H05 > UAS-Kir2.1 flies when they were food deprived for 20 hours. If these serotonergic neurons that were tagged by the Gal4 line are necessary for hunger, then R50H05 > UAS-Kir2.1 flies should not be feeding even if they are food deprived.

2.7 Activation and inactivation experiments

Previous experiments with fruit flies have been focusing on single modality to examine serotonergic system. However, the serotonergic neurons targeted by R50H05-Gal4 line are not located in primary taste or primary motor areas modulating hunger (SOP). Instead, they may be located at a multimodal sensory integration area. So, we thought cross modal sensory integration might be playing a role in this system. To test this hypothesis, we focused on the neurobiological basis of attention and sought to identify neural circuits enabling the shifting of attention between sensory modalities. Therefore, chronic activation and inactivation experiments were conducted by using UAS-NaChBac and UAS-Kir2.1 respectively. Gal4-UAS binary system was used as the tool to be able to specifically select neurons to be activated/inactivated. Then, each group of flies were included in experiments, where they needed to decide between visual and gustatory stimuli and the behavioural shift they showed has been examined.

In our model, where we activated the serotonergic neurons with UAS-NaChBac, action potentials are triggered in these neurons all the time. Thus, if these neurons are regulating hunger as suggested by Albin (2015), hunger and feeding behaviours should be triggered as well. So, to test if the activation of these neurons also increases the probability of feeding, we tested these flies when they were sated. The hypothesis here is that the flies should be feeding on the gustatory stimulus as if they were food deprived. In this way, we would be able to see if these serotonergic neurons are sufficient to induce hunger and feeding. On the other hand, inactivation flies' serotonergic neurons targeted by R50H05-Gal4 line were inactivated chronically. Thus, no action potential will be triggered in these neurons. So, we hypothesize that these flies would be behaving as if they were sated and not feeding on the gustatory

stimulus more than normal flies. To find out if this hypothesis is true, we tested these flies when they were food deprived for 20 hours. If these neurons are necessary for triggering hunger and feeding, the flies should not be feeding in this condition as well.

CHAPTER 3

METHOD

3.1 Flies

The following fly stocks were obtained from the Bloomington Drosophila Stock Center. R50H05, BL# 38764 (w[1118]; P{y[+t7.7] w[+mc]=GMR50H05-GAL4}attP2), UAS-Kir2.1, BL# 6595 (w[*]; P{w[+mC]=UAS-Hsap\KCNJ2.EGFP}7), UAS-NaChBac, BL# 9469 (y[1] w[*]; P{+mC]=UAS-NaChBac}2). They have been outcrossed to Canton-S background. The flies carrying the transgene on the third chromosome (BL# 38764, BL# 6595, BL# 9469) were crossed to BL# 5906 (w[1118]/Dp(1;Y)y[+]; TM2/TM6C, Sb[1]).

3.2 Fly breeding and maintenance

The fly food was prepared following the recipe of Bloomington Drosophila Stock Center, Indiana University. The ingredients of this recipe for 1.1 L of food include 73.07 grams Cornmeal (Bağdat), 10 grams soy flour (Doğalsan), 80 ml high fructose corn syrup (Cargill with 55% fructose concentration), 4.82 ml propionic acid (Sigma with 99% concentration), 5.5 grams agar (Roth) and 17.4 grams yeast (Dr. Oetker) (Çevik & Erden, 2012).

Stocks were maintained in controlled environmental conditions so that the results of the experiments were not influenced by any other variable such as the developmental temperature. To accomplish this, Memmert ICL 260L was used as the climate chamber (Figure 1A), in which the temperature and humidity were kept constant at 25 °C and at 50% relative humidity level (Figure 1B). Besides, the circadian daylight was mimicked by adjusting 12-hour day and 12-hour light cycles with dark cycle starting at 7:00 PM and lasts until 7:00 AM.

Flies were kept inside 50 ml falcon tubes with 7.5 ml of food in each and these tubes were closed with cotton plugs. Parents were transferred to new food bottles before the offspring emerged (6 days after the preparation of the bottle) to prevent mating between parent and offspring generations. These transfers were done only three times before the parents were disposed. By this means, use of offspring from old parents was prevented.

Figure 1. A. Climate chamber/ incubator with the parent stocks inside. B. Conditions inside the climate chamber.

3.3 Experimental Setup

The room where the experiments were conducted can be seen in Figure 2. During the experiments room temperature was held constant at 23 +/- 1.5 °C and the relative humidity was 33 +/- 2 %. The temperature and the relative humidity of the box where the flies were kept inside were 23 +/- 1.5 °C and 79 +/- 6 % respectively. The experiments were performed under the standard room illumination.

Two setups were used for the experiments. One of these setups is demonstrated in the Figure 3.

Computer used for one of these setups had 3.5 Ghz Intel Core i3-4150 processor with 64 GB memory and 120 GB SSD hard drive, 1 GB Nvidia GeForce 210 Graphic Card (64 bit, DDR3) and onboard Intel HD Graphics 4400 display adapter. The other computer (Fujitsu Lifebook

AH531) had 2.30 GHz Intel Core i5 with 2410M CPU, 4 GB installed memory and 64 bit operating system with dual channel DDR3 memory controller.
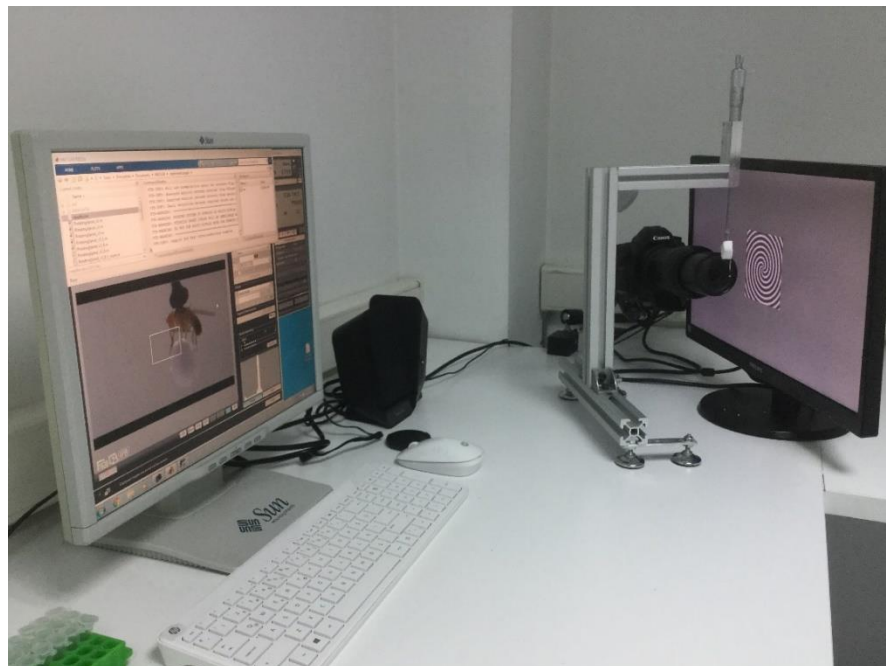

Figure 2. Experiment room.

Figure 3. Experimental setup.

Two identical LED-blacklit 21inch LCD monitors (Philips 223V5L) with 60 Hz screen refresh rate, 200 cd/m^2 brightness and 1920 x 1080 resolution and 5ms response time were used to present the visual stimulus to the flies. The experimenter used another monitor, which is connected to the interface of the camera, to observe the behaviours of the fly and to use MATLAB.

The sessions were monitored and recorded with Canon EOS 5D DSLR camera with Canon MP-E 65 mm macro lens.

Flies were stabilised in front of the monitor using a specially built suspension device made up of aluminium (Figure 3).

3.4 Experimental Protocol

Male flies were tested when they were 4 and/or 5 days old. They were collected on the day they emerged, transferred to fresh vials and tested 4-5 days later.

3.4.1 Preparation of the flies for the experiment

The flies that were tested when sated were removed from the food and directly given cold anaesthesia. Cold anaesthesia was used so that they would not be exposed to any chemicals such as ether, esther and carbon dioxide. These chemicals may dehydrate the flies and/or alter their behaviour. After the cold anaesthesia was applied, the flies were pinned to the tips of needles from the back of their thorax by using melted candles (Figure 4). Care was taken not to harm the flies physically and unhealthy flies were not used in the experiments. Then, the pins were attached on their sharp end to the walls of a Styrofoam box (30 x 20 cm) that was filled with some water. The lid of the box was covered and flies were kept there for half an hour at 79 +/- 6% relative humidity. This recovery period lasted only half an hour, long enough for the flies to stabilise their metabolism but short enough to prevent them from getting hungry.

Figure 4. Pinning area and pinned flies.

If the flies were tested when they were food deprived, they were separated from the food the day before the experiment and kept in 100 ml vials containing wet wipes. These vials were plugged with cottons and kept in the incubator for 18 hours. Wet wipes were placed inside these vials so that the flies remain hydrated during the food deprivation period. Keeping the flies hydrated is important because thirst can also trigger proboscis extension reflex (PER) and liquid ingestion even when the flies are not hungry. After 18 hours, the flies were given cold anaesthesia and pinned to the tips of needles from their thorax. Then, pins were attached to a Styrofoam box that was filled with water and kept there for an hour. At the 19th hour of food deprivation the experiment started and it lasted 2 hours at most. By this way, we intended to achieve 20 hours of food deprivation on average.

3.4.2 Targeted activation with UAS-NaChBac

R50H05 > UAS-NaChBac flies were tested when they were sated. Therefore, these experiments could be conducted both in the morning and in the afternoon. When the flies were tested in the morning, they were removed from the incubator at 2.5 hours and tested between 3-5 hours of their circadian time. For the experiments performed in the afternoon, the flies were removed from the incubator at 6.5 hours and tested between 7-9 hours into their circadian time.

3.4.3 Targeted inactivation with UAS-Kir2.1

The experiments with R50H05 > Kir2.1 flies were performed when they were food deprived for 20 hours. In order to equalize the conditions in both activation and deactivation experiments, inactivation flies were also tested both in the morning and in the afternoon. To test them in the morning, they were separated from the food the day before the experiment, at 8.5 hours of their circadian time and kept into the incubator overnight. They were removed from the incubator the next day at 2.5 hours and tested between 3.5-5.5 hours into their circadian time. Alternatively, the flies were separated from the food at 11.5 hours into their circadian time and placed into the incubator for 18 hours. Then, they were removed from the climate chamber the next day at 5.5 hours and tested at 6.5-8.5 hours of their circadian time.

3.4.4 Crosses

In the experiments where the serotonergic neurons were activated, R50H05 Gal4 virgin females were crossed to males carrying UAS-NaChBac.

Virgin females that were taken from R50H05 Gal4 line were crossed to males carrying UAS-Kir2.1 for the experiments where serotonergic neurons were inactivated.

There are other balancers that can be used in fly studies but we only used TM6C. We used TM6C for practical reasons. First, TM6C balancer is easily observable through phenotypes i.e. the physical appearance of the flies. The body hairs of the flies carrying TM6C balancer are thicker and stubby compared to the wild type flies. Further, the phenotype for other balancers such as TM2 are more variable and depend on the genetic background.

3.5 Behavioural assays

The experiment was written in MATLAB 2014 by using Psychophysics Toolbox (Psychtoolbox 3) extension. Psychtoolbox was used to specify the features of visual stimulus (Brainard, 1997; Pelli, 1997; Kleiner et al, 2007).

3.5.1 Visual looming stimulus

The visual stimulus and a fly facing it can be seen in the Figure 5. The looming effect was created by using a spiral image (10 x 10 cm) that was turning clockwise at a rate of 5°.

3.5.2 Appetitive gustatory stimulus

The other stimulus was an appetitive gustatory stimulus, which was provided to the flies by handing them Styrofoam balls dipped in 0.5 M sucrose (Merck (1.07651.1000)) or water.
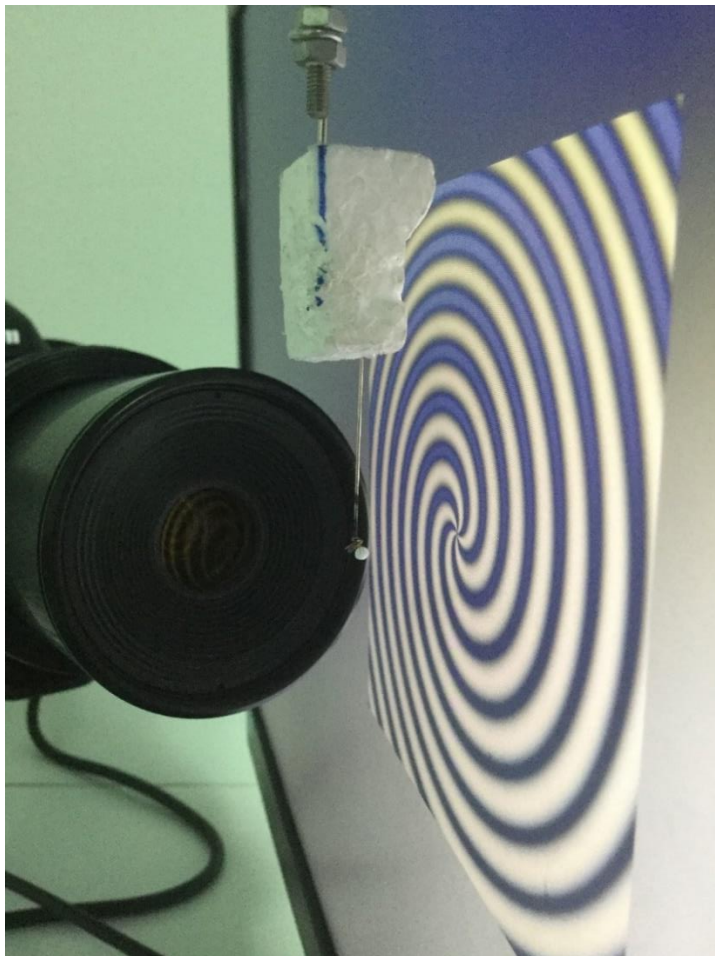
Figure 5. Visual stimulus and a fly at the tip of the needle facing the center of the spiral image.

3.5.3 CAR, PER/feeding and other behaviours

The flies were placed 1.5 cm away from the monitor and aligned with the centre of the spiral image. Then, they were handed Styrofoam balls. When the experiment was started, the spiral turns clockwise after the 6 seconds of first inter trial interval and it creates a looming effect for the flies. This leads flies to feel if they were landing to somewhere and they instinctively raise their front legs while drawing the rest of the body towards the back. This is called the collision avoidance reflex (CAR) and a demonstration of a fly performing this reflex can be seen in the Figure 6.

Figure 6. Collision Avoidance Reflex (CAR)

Figure 7. Proboscis extension reflex (PER) with feeding

Sucrose is a nutritious sugar for the fruit flies and when they are handed balls dipped in sucrose, it may trigger feeding response with the probability depending on their hunger states. Figure 7 shows the proboscis extension reflex (PER) with feeding.

While the flies were presented with these stimuli the experimenter was observing their behaviour from another monitor and coding these behaviours through MATLAB. We especially focused on the flies' choice between two main stimuli; CAR and PER/feeding although they may show some other behaviours as well. All the behaviours that the flies performed were coded to the MATLAB program.

3.5.4 Trials and behavioural coding

One experiment included 25 trial-inter trial interval cycles each of which was 8 seconds long. Each cycle consists of 2 seconds of visual stimulus presentation, and 6 seconds of inter trial interval,

during which the spiral stays still. Thus, an experiment for a single fly lasts about three and a half minutes. During this time, the behaviours of the flies were observed and different behavioural codes were entered to the MATLAB program for each. Flies' behaviour was coded as follows:

0—>The code MATLAB gives when there is no entry

1—>Proboscis extension reflex (PER) without feeding

2—>Proboscis extension reflex (PER) with feeding

3—>Staying still on the ball without any observable movement of body parts

4—>Walking on the ball, the ball turns around

5—>Front grooming

6—>Back grooming

7—>The fly holding no ball (Ball may fell off)

8—>Collision avoidance reflex (CAR) with one of the front legs (can be considered as the preparation to the actual CAR)

9—>Collision avoidance reflex (CAR) with both front legs

One of these behavioural codes were entered to the MATLAB-run program for trial and inter trial interval respectively. Thus, codes with two numbers were entered for each trial. If, flies exhibited more than one behaviour, response transition upon stimulus presentation was entered. Thus, behaviour just before the looming stimulus was presented and the one just when the looming effect was created were coded.

PER has two categories in this behavioural coding since flies may extend their proboscis (feeding organ) when their taste receptors, which are also present

in many body parts other than their proboscis, encounter with a gustatory signal. In this case they may either extend their proboscis or extend it and feed on the food. Further, PER without feeding can also be observed when they are given the Styrofoam ball dipped in water. Normally, if they are not hungry, they hold the ball but do not show any feeding reflex. However, if they are hungry, they may either extend their proboscis but do not feed on water or literally feed on water depending on their dehydration level.

CHAPTER 4

RESULTS AND DISCUSSION

The two stimuli presented in this paradigm trigger different behavioural responses. Collision avoidance reflex(CAR) and proboscis extension reflex(PER)/feeding are two key behaviours given in response to the visual looming stimulus or the appetitive tastant respectively. Since these behaviours are mutually exclusive, flies can express only one at a time or they may display another behaviour such as grooming. In order to test our paradigm, the flies were tested in the presence of both stimuli. The temporal pattern of decision making and CAR and PER/feeding responses were analyzed. Primary analysis was done with Microsoft Excel 2016 and then IBM SPSS Statistics Version 24 was used for statistical computations.

4.1 A selective activation of serotonergic neurons by UAS-NaChBac failed to increase feeding

The serotonergic neurons of R50H05-Gal4>UAS-NaChBac flies were activated chronically. So, if these serotonergic neurons are sufficient to induce hunger, R50H05-Gal4>UAS-NaChBac flies should behave as if they were food deprived even though they were sated.

Figure 9 shows that feeding was driven by the appetitive quality of sucrose in sated flies because they did not feed on water. (Figure 9, compare blue and orange lines, $F(1,275)=82.949$, $p<0.001$, $\eta_p{}^2=0.232$)

Contrary to what has previously been observed upon acute activation of R50H05 serotonergic neurons by TRPA1 (Albin et.al., 2015), chronic activation using NaChBac caused a reduction in feeding (Figures 8 and 9). The genotype main effect was significant ($F(2,275)=11.584$, $p<0.001$, $\eta_p{}^2=0.078$) and Scheffe analysis confirmed that ingestion was lower for R50H05-Gal4> UAS-NaChBac activation group relative to both controls ($p<0.001$; Figure 8, compare rightmost bars with middle and left; Figure 9, compare right panel with middle and left). Accordingly, the homogenous subsets analysis revealed the two control groups form a subset distinct from the activation group.
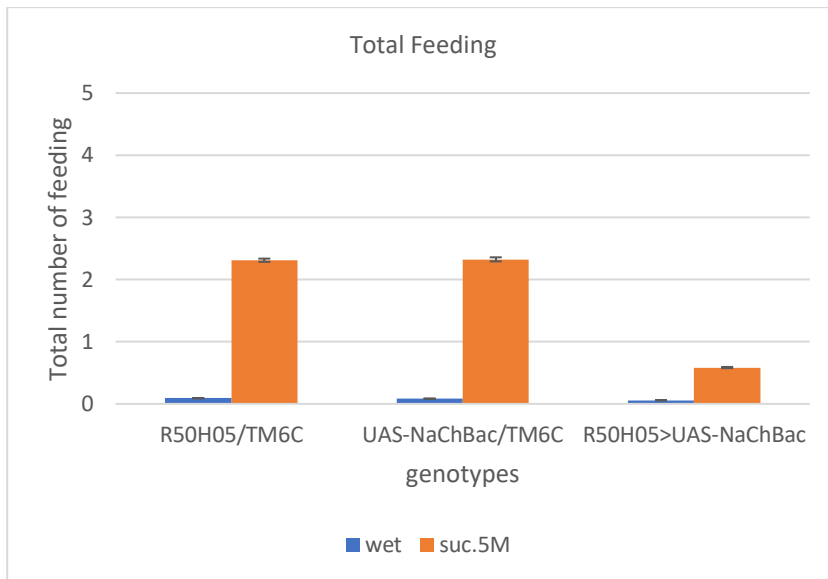
Figure 8: Activation flies. Total number of feeding during visual stimulus. (Error bars indicate Standard Error (*SE*))

Because the reduction of ingestion upon the selective activation of serotonergic neurons was unexpected, we wanted to know if it could be accounted for by the presence of competition from the visual stimulus. To that end, we analyzed feeding in the absence of visual stimulus, i.e., during the inter trial intervals.

When we compared feeding during the trials to the feeding during the inter trial intervals, sucrose feeding was higher in the absence of the visual stimulus relative to its presence yielding a significant difference ($F(1,147)=11,022$, $p<0.001$, $\eta_p^2=0.07$). This means that there is a visually driven suppression of feeding during the trials. However, suppression effect is not differential across phenotypes. In other words, suppression of feeding by the visual stimulus is not higher for R50H05-Gal4>UAS-NaChBac activation group relative to the controls, yielding a non-significant interaction ($p<0.140$; Figures 10 and 11). So, our failure to observe increased amounts of feeding upon the activation of serotonergic neurons in R50H05-Gal4>UAS-NaChBac flies cannot be accounted for a differentially higher suppression of feeding by the visual stimulus for this group.
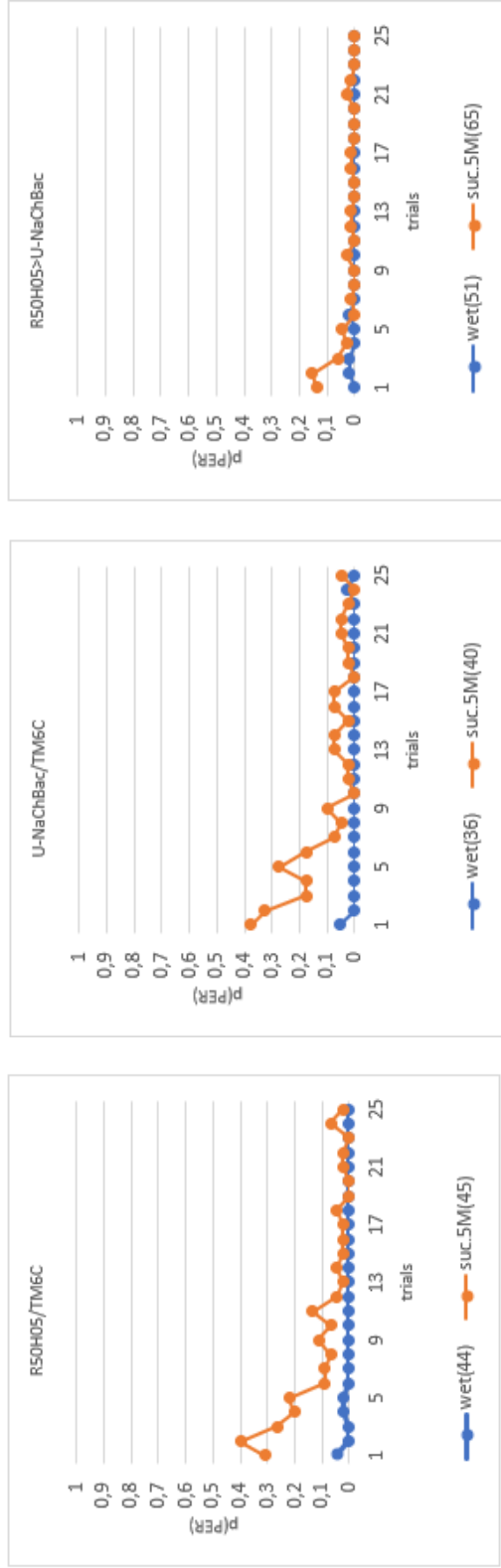
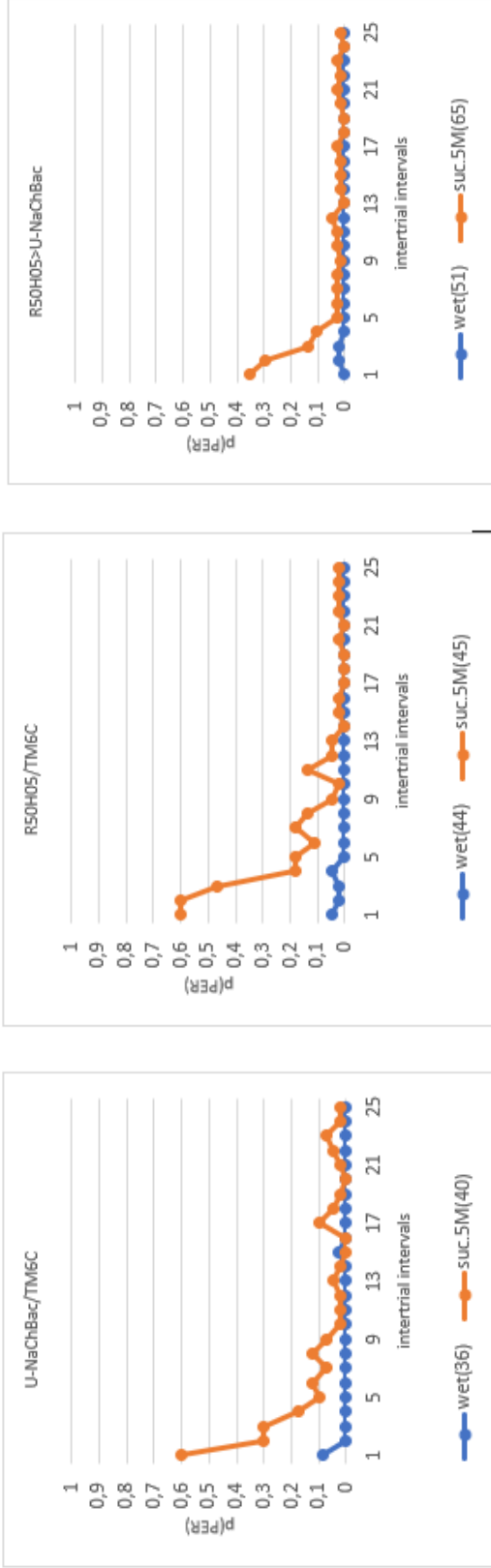Figure 9: Activation flies. Feeding during the visual stimulus.

Figure 10: Activation flies. Feeding during intertrial intervals i.e. between the visual stimulus presentations.
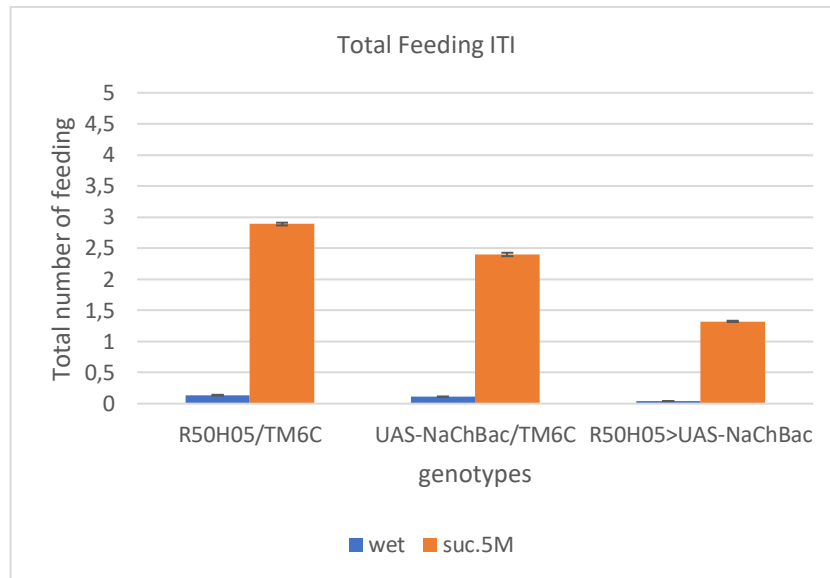
Figure 11: Activation flies. Total number of feeding during intertrial intervals. (Error bars indicate Standard Error (*SE*))

Nevertheless, we also wanted to test whether feeding during visual stimulus for the R50H05-Gal4>UAS-NaChBac activation group could be lower due to an increased cumulative sensitization for the visual stimulus for this group. Therefore, we tested R50H05-Gal4>UAS-NaChBac flies under an additional condition where the visual stimulus was removed but the temporal parameters (e.g. trial duration, inter trial interval duration, number of trials) of the experiment were otherwise the same.

Feeding during the trial period was significantly different among groups (with the visual stimulus for UAS-NaChBac control, R50H05-Gal4 control, R50H05-Gal4>UAS-NaChBac activation group; without the visual stimulus for the late added R50H05-Gal4>UAS-NaChBac activation group) (F(3,190)=9.837, p<0.001, η_p^2=0.134).

For the R50H05-Gal4>UAS-NaChBac group, difference between the feeding during the trial period when there was no visual stimulus and feeding during the visual stimulus presentation failed to reach significance(p<0.299). So, it can be concluded that feeding was not suppressed by the visual stimulus in activation flies. However, a differential cumulative sensitization for the visual stimulus fails to account for the lower levels of feeding for the activation group (R50H05-Gal4>UAS-NaChBac) because their feeding in the absence of the visual stimulus was not higher than that of the control groups (UAS-NaChBac control and R50H05-Gal4 control) in the presence of the visual stimulus (p<0.116 and p<0.105 respectively; compare Figure 12 left panel and Figure 9 middle and left panels orange lines).
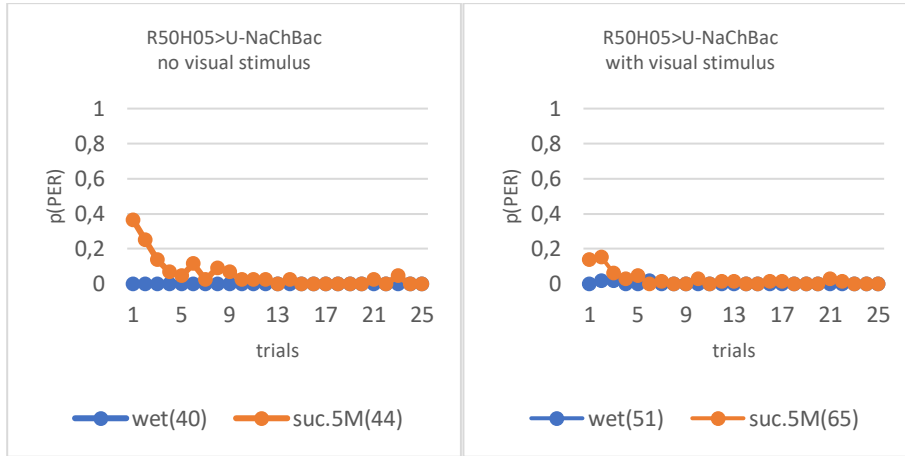
25

Figure 12: Activation flies. Probabilities of proboscis extension reflex (PER)/feeding with (right) and without (left) the visual stimulus.

4.2 Selective activation of serotonergic neurons by UAS-NaChBac increased visually driven responsiveness

R50H05/TM6C and UAS-NaChBac/TM6C control groups showed lower visual responsiveness compared to R50H05>UAS-NaChBac activation group (Figure 13 right panel with middle and left panels). This might be due to a visually enhancing effect of R50H05-Gal4>UAS-NaChBac activation or a possible diminishing effect of the TM6C balancer. Flies exhibited a habituation of CARs, which is evident as a reduction in visual responsivity during the first 10 trials. After the 10th trial, CAR probability stabilizes across a few trials before it starts to increase again towards the end of the session. This increment suggests the presence of a late onset sensitization upon repeated administaration of the visual stimulus. Yet, CAR probability at the asymptote was lower relative to the beginning.

The appetitive quality of the gustatory stimulus did not influence the visual responsiveness of sated flies. The probability of collision avoidance reflexes (visual responsiveness) was not different when they fed on sucrose or water ($F_{(1,275)}=4.712$, $p<0.031$, $\eta_p^2=0.017$; Figure 13, compare orange and blue bars; Figure 14, compare orange and blue lines).

The genotype main effect for the groups (R50H05/TM6C control, UAS-NaChBac/TM6C control, R50H05>UAS-NaChBac activation group) was significant ($F_{(2,275)}=15.884$, $p<0.001$, $\eta_p^2=0.104$; Figure 13, compare rightmost, middle and leftmost bars; Figure 14, compare right, middle and left panels). A Scheffe analysis confirmed that R50H05-Gal4>UAS-NaChBac activation group was significantly different than both control groups ($p<0.001$).

Although the suppression of visual responsiveness during sucrose is more visible for R50H05/TM6C control group relative to UAS-NaChBac/TM6C control group and R50H05>UAS-NaChBac activation group, the genotype by sucrose interaction failed to reach significance ($p<0.508$).
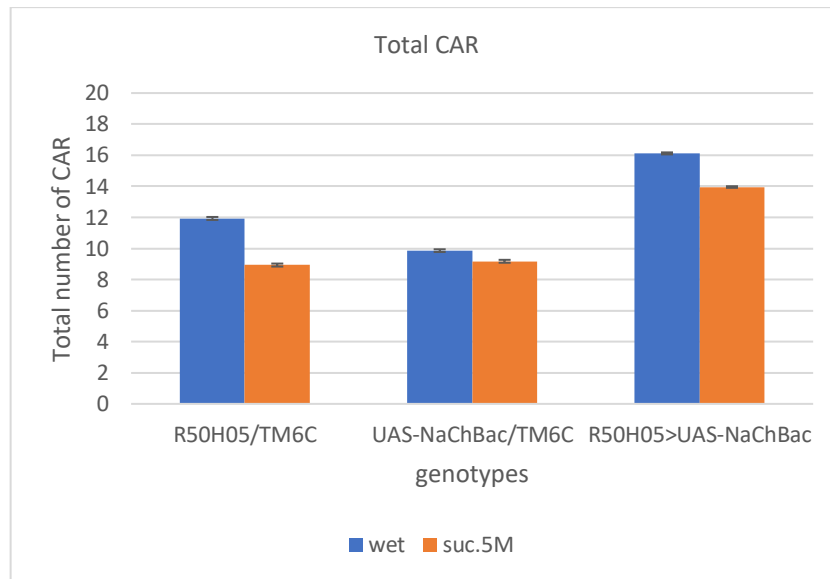
26

Figure 13: Activation flies. Total number of Collision Avoidance Reflex(CAR). (Error bars indicate Standard Error (*SE*))

4.3 Overall discussion of activation experiments

The behavioural pattern of flies during the intertrial intervals were examined in order to reveal whether the low responsivity in terms of PER/feeding was due to the suppression from visual stimulus presentation. Yet, the results showed that the probability of PER/feeding was not too high as expected even in the absence of visual stimulus i.e. during intertrial intervals. Then, we assumed that this suppression of feeding even through the intertrial interval might be due to the ongoing excitability caused by visual stimulus. However, this hypothesis might be disproved when the results of feeding scores during intertrial intervals (ITI) and the ones when there was no visual stimulus throughout the session i.e. no visual stimulus case are compared. The feeding score of R50H05-Gal4>UAS-NaChBac activation flies in no visual stimulus case is not higher than that of controls in the intertrial intervals.

R50H05-Gal4>UAS-NaChBac activation flies' visual responsiveness was higher compared to both controls but the activation flies ingested less sucrose relative to the controls. When the same serotonergic neurons were activated acutely (Albin et.al., 2015), the activation flies started to feed on the food as if they were food deprived. However, we failed to observe such a behaviour when we activated the same serotonergic neurons chronically. Thus, the reason of this conflict might be the difference between the acute and chronic manipulations. The activation flies should have been showing different feeding behaviours prior the experiment. In terms of acute manipulations, the serotonergic neurons of the flies were activated during the experiment and hunger and feeding were triggered only during the activation process. On the other hand, since the serotonergic neurons of R50H05-Gal4>UAS-
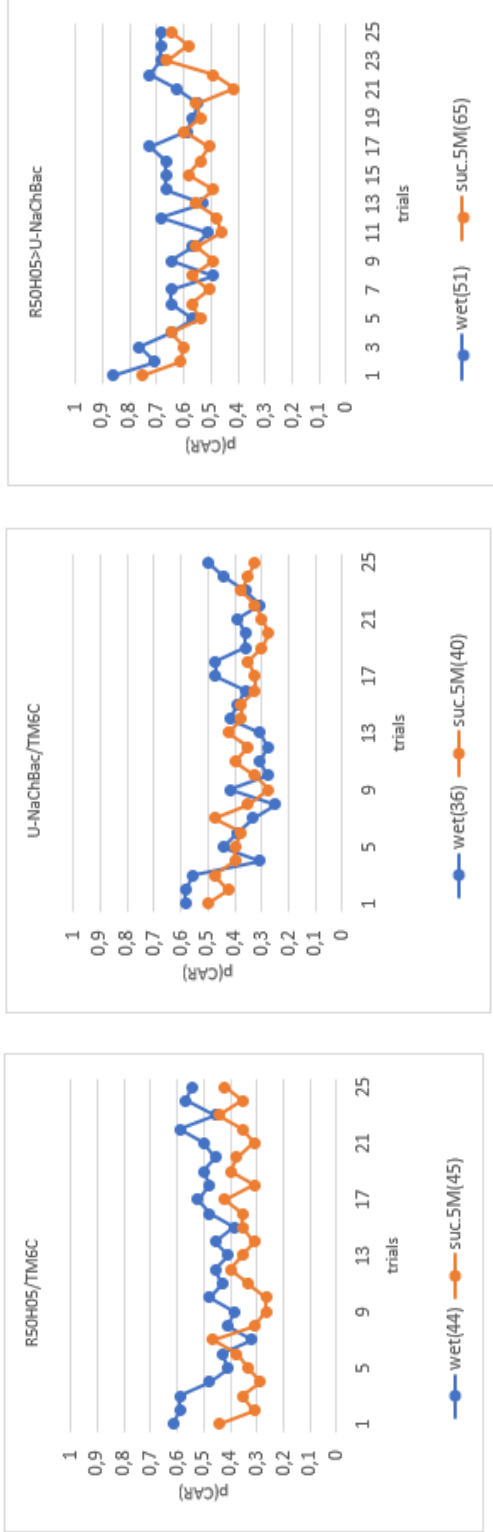
27

Figure 14: Activation flies. Probabilities of collision avoidance reflex(CAR).

NaChBac flies were activated chronically in our experiment, their serotonergic neurons were active throughout their lifes. Thus, whenever the activation of these neurons of the activation flies were triggered, they may have fed on the food more than normal. As a result, they may have been more sated during the experiments relative to the flies used in acute activation.

4.4 Feeding still increased for the hungry flies in spite of the selective deactivation of the serotonergic neurons

A subset of serotonergic neurons were chronically inactivated in R50H05-Gal4>UAS-Kir2.1 flies. So, if these serotonergic neurons are necessary to induce hunger and feeding, these flies should behave as if they were sated even though they were food deprived.
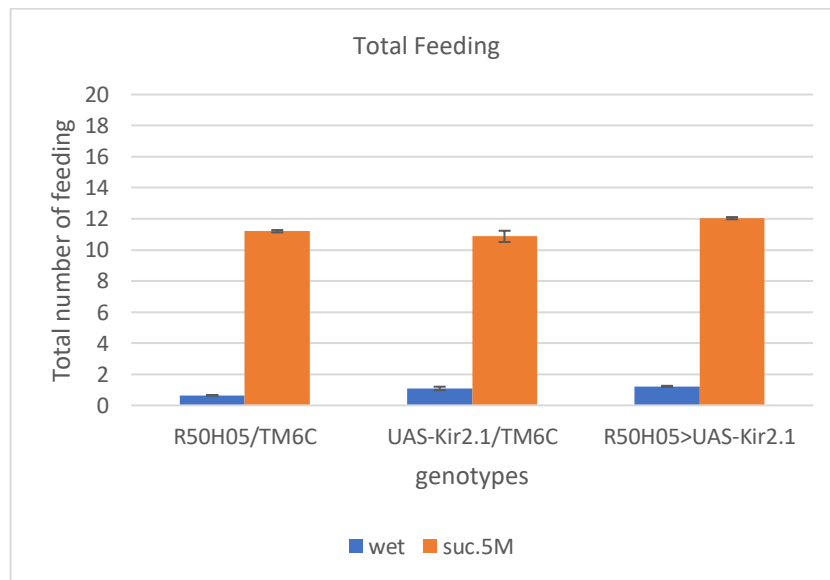
Figure 15: Inactivation flies. Total number of feeding during visual stimulus. (Error bars indicate Standard Error (*SE*))

The feeding score of all three groups (R50H05/TM6C, UAS-Kir2.1/TM6C, R50H05>UAS-Kir2.1) are quite high as demonstrated by Figure 16. The flies fed on sucrose at almost every trial at the beginning of the session. They also fed on water for several trials since they had been food deprived for 20 hours. The feeding scores decreased gradually as satiation started around the 10th trial. However, flies continued to feed in roughly 30% of the trials until the end of the session.

Although the flies also fed on water at the beginning of the sessions, feeding induced by the appetitive quality of sucrose was significantly higher (Figure 15, compare blue and orange bars; Figure 16, compare orange and blue lines; $F_{(1,169)}=146.351$, $p<0.001$, $\eta_p^2=0.464$).

Pattern of feeding for all groups (R50H05-Gal4>UAS-Kir2.1, R50H05/TM6C, UAS-Kir2.1/TM6C) was similar and the genotype main effect was not significant (Figure 15, compare right, middle and left bars, Figure 16, compare right, middle and left panels; $F_{(2,169)}=0.503$, $p<0.606$, $\eta_p^2=0.006$).

The feeding scores of the flies during the inter trial intervals were examined as well to see whether the presence of visual stimulus had any effect on feeding behaviour. Probability of feeding was higher for all groups during the inter trial intervals (compare Figure 15 and Figure 17; compare Figure 16 and Figure 18). However, the genotype main effect for the feeding during inter trial intervals was not significant ($F_{(2,169)}=1.974$, $p<0.142$, $\eta_p^2=0.023$), suggesting that feeding was not differentially suppressed across genotypes.
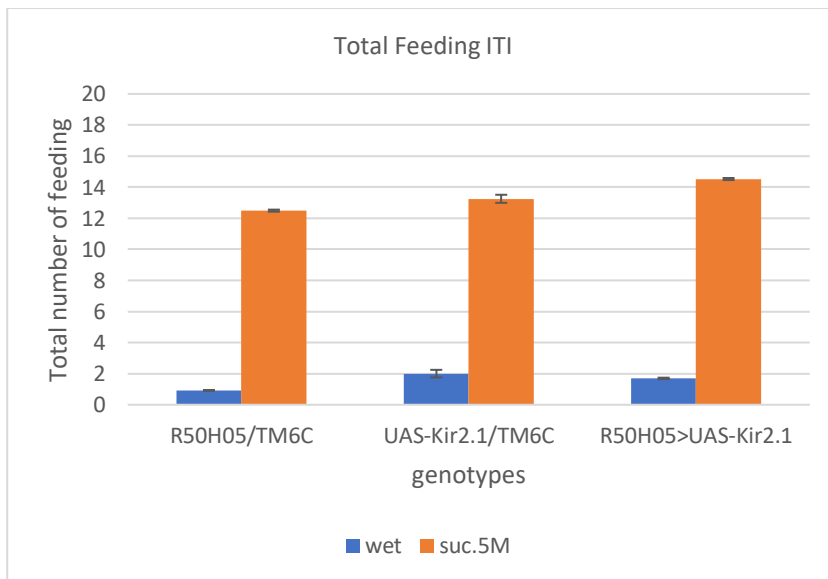

Figure 16: Inactivation flies. Total number of feeding during inter trial intervals. (Error bars indicate Standard Error (*SE*))
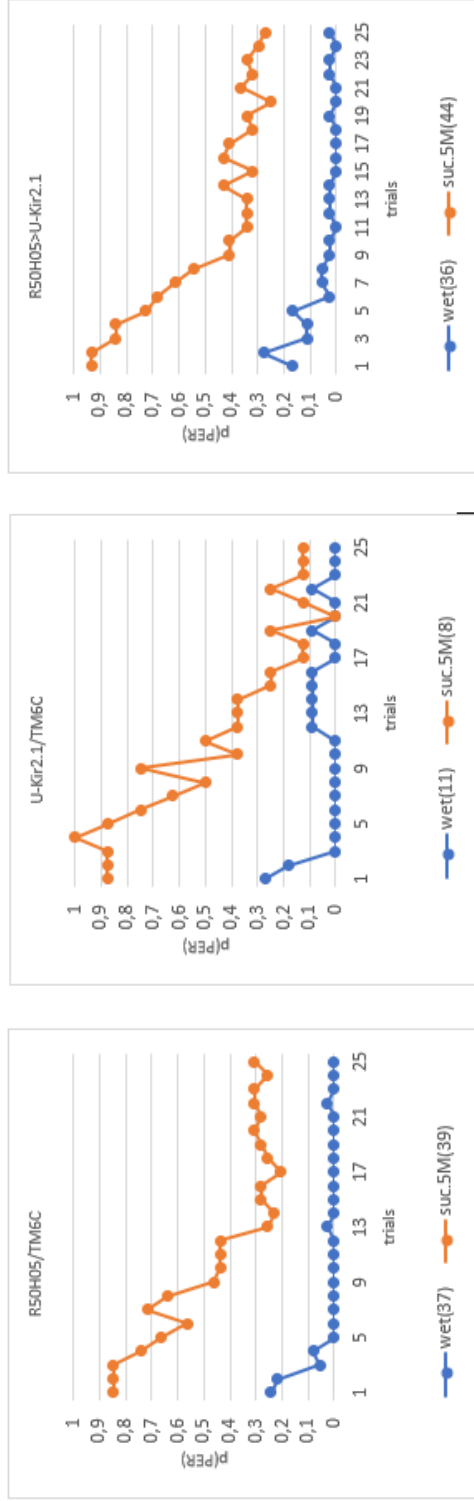
30

Figure 17: Inactivation flies. Proboscis extension reflex (PER)/feeding probabilities during visual stimulus presentations.
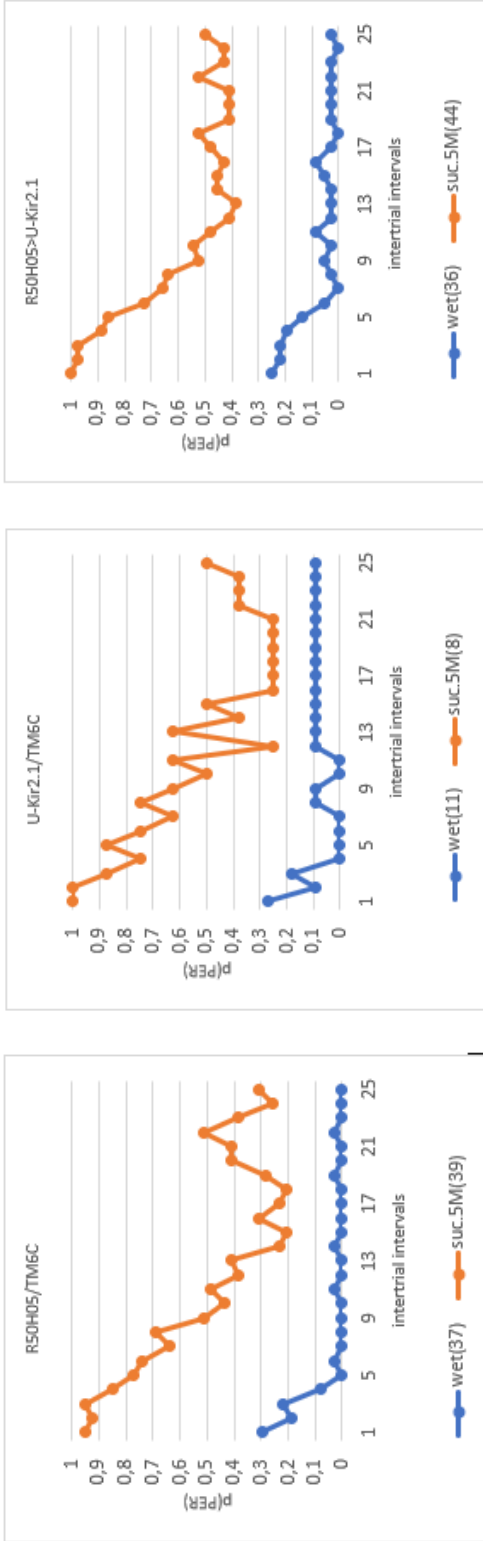
Figure 18: Inactivation flies. Probabilities of proboscis extension reflex (PER)/feeding during intertrial intervals.

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4.5 Feeding suppressed visual responsiveness at the beginning of the session following chronic deactivation of serotonergic neurons

First of all, the suppression of CAR by sucrose was observed following 20 hours of food deprivation. Visual responsiveness of the flies was lower when they were tested on sucrose (Figure 19, compare orange and blue bars, Figure 20, compare orange and blue lines, $F_{(1,169)}=44.454$, $p<0.001$, $\eta_p^2=0.208$). Second, the pattern of change in CAR probability was similar when the flies were tested on sucrose or on water (Figure 20, compare orange and blue lines), suggesting that suppression by sucrose did not change the biphasic pattern of visual responsiveness (Figure 20).
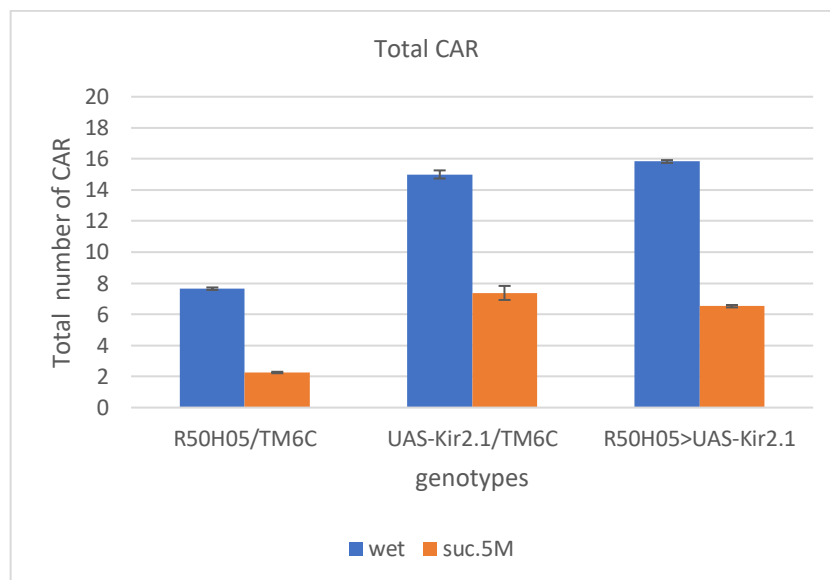
Figure 19: Inactivation flies. Total number of Collision Avoidance Reflex(CAR). (Error bars indicate Standard Error (*SE*))

The visual responsiveness of R50H05/TM6C control group was lower relative to UAS-Kir2.1/TM6C control and R50H05-Gal4>UAS-Kir2.1 activation group (Figure 19, compare left bars with middle and right; Figure 20, compare left panel with middle and right) and the genotype main effect was significant (R50H05/TM6C, UAS-Kir2.1/TM6C, R50H05-Gal4>UAS-Kir2.1) ($F_{(2,169)}=23.577$, $p<0.001$, $\eta_p^2=0.218$). A Scheffe analysis revealed that R50H05/TM6C control and R50H05-Gal4>UAS-Kir2.1 activation groups were significantly different from each other ($p<0.001$). Moreover, the difference between R50H05/TM6C control and UAS-Kir2.1/TM6C control groups was significant as well ($p<0.001$). However, the difference between UAS-Kir2.1/TM6C control and R50H05-Gal4>UAS-Kir2.1 inactivation groups failed to reach significance ($p<0.776$). (Figure 19, compare left, middle and right bars; Figure 20, compare right, middle and left panels).

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If both control groups' (R50H05/TM6C and UAS-Kir2.1/TM6C) visual responsiveness were lower compared to the R50H05-Gal4>UAS-Kir2.1 activation group, we would have inferred that TM6C balancer yielded a diminishing effect. The control groups' behavior in this case were not similar to each other and this might be the result of small number of flies tested for UAS-Kir2.1/TM6C control group.

Even though the visual responsiveness in R50H05/TM6C control group is more evident compared to UAS-Kir2.1/TM6C control and R50H05-Gal4>UAS-Kir2.1 activation groups, the genotype by sucrose interaction failed to reach significance ($p<0.123$).

4.6 Overall discussion of deactivation experiments

Although the feeding probability of R50H05-Gal4>UAS-Kir2.1 inactivation flies was high, their visual responsiveness was high as well. This might be due to the long duration of food deprivation since hen the flies are food deprived for long periods, the hyperactivity and thus the responsiveness to any stimuli elevates (Yang et al., 2015).

The feeding probaility of R50H05-Gal4>UAS-Kir2.1 inactivation flies were high even though their serotonergic neurons were inactivated. The feeding behaviour of these flies during the inter trial intervals was examined as well. The patterns of feeding with or without the visual stimulus were nearly the same. They fed as if there was no visual stimulus. So, the reason for hunger cannot be the activity of these serotonergic neurons alone. Rather, hunger is a more complex process as it is in vertebrates.

4.7 General Discussion

To summarize, activation flies did not fed on the gustatory stimulus as much as we predicted. Moreover, we were expecting inactivation flies to not feed on the gustatory stimulus as if they were food deprived but they did. Although the serotonergic neurons that were targeted by R50H05-Gal4 line are thought to modulate hunger (Albin et al., 2015), there might be other redundant neurons or neural circuits that modulate hunger in parallel. This might be the result of redundant, complementary functioning of different monoamines in the fly brain (Chen et al., 2013). This may explain why food deprived inactivation flies fed on the gustatory sitmulus a lot. So, it may be concluded that the inactivated serotonergic neurons are not necessary to induce hunger and modulate feeding. On the other hand, the sated flies whose serotonergic neurons were activated did not fed on the gustatory stimulus as if they were food deprived, which was the prediction at the beginning. Therefore, it may be suggested that these serotonergic neurons are not sufficient to induce hunger.

The serotonergic neurons of interest were activated or inactivated chronically. In other words, the activity of these serotonergic neurons were manipulated genetically and the flies used for this study had either active or inactive serotonergic neurons throughout their lifetime. There may be a possibility that other systems or neurons may have used to compensate for the effects of manipulation.
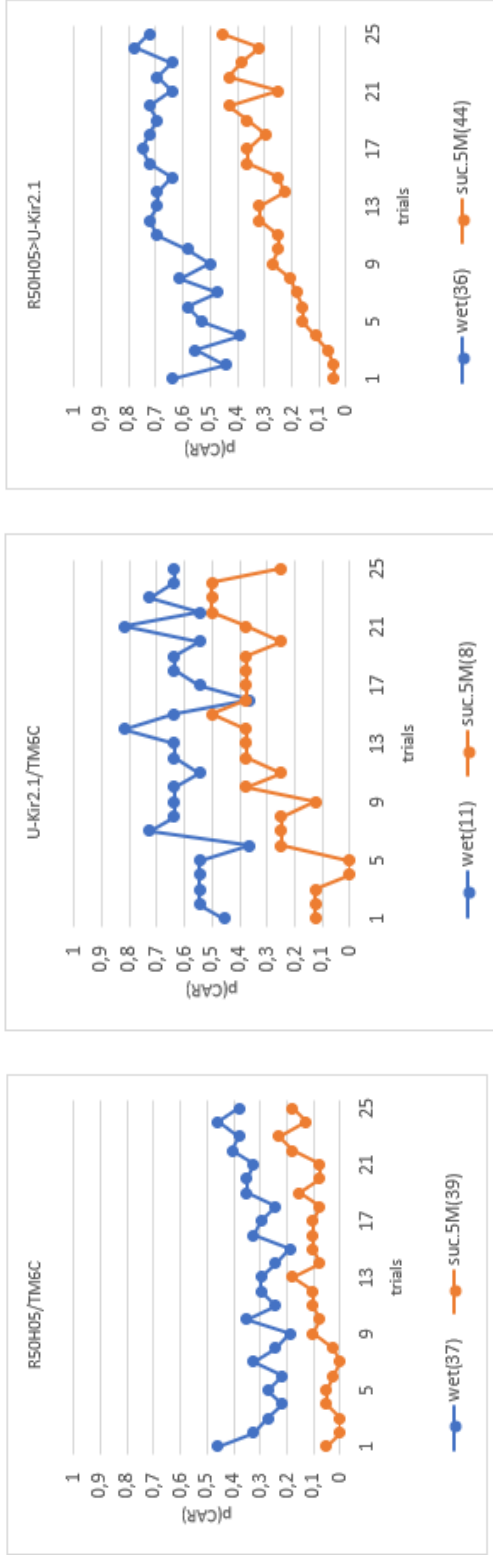
Figure 20: Inactivation flies. Probabilities of collision avoidance reflex (CAR)

There may be another reason for the unexpected results of inactivation flies. When the serotonergic neurons were inactivated with UAS-Kir2.1, the neurons could not trigger action potentials. However, these neurons may have been working with graded potential. Thus, they may have been functioning as well even though the activity level was not as high as normal. Neurons fire when action potentials are reached and this is an all or none phenomenon. If the potential reaches the threshold level of the neurons, they fire. Graded potentials, which are smaller in strength compared to action potentials, can be produced as well. These graded potentials are observed at the non-spiking neurons and by this way, these neurons can transmit these small, graded potentials to the postsynaptic neurons (Simmons, 1999). Thus, their strength may also decrease too much when they travel through the axon such that no action potential is generated. Yet, if more than one graded potential is present at the same point or at the same time, their strength may add up and results in the generation of an action potential. So, even though the neuron is not stimulated enough to trigger a single action potential, it can still fire if small graded potentials sum up (Dharani, 2015).

For inactivation experiments, serotonergic neurons were inactivated chronically. The first prediction was that the flies would not be eating even if they were food deprived for 20 hours and would behave as if they were sated because the feeding scores of the flies whose serotonergic neurons were inactivated by UAS-Shi^{ts1} decreased even though they were starved (Albin et.al., 2015). From this point of view, it may be suggested that they would not be eating at all and this may lead to their death. However, we observed that the probability of feeding for all three groups of flies were quite high. Thus, it can also be concluded that there are other mechanisms controlling the feeding and feeding related behaviours. So, serotonergic neurons of interest do not directly control hunger but they may have more like a modulatory role. Otherwise, flies would stop eating when their serotonergic neurons are inactivated and they would not even survive. Moreover, there may be other mechanisms modulating hunger so that when one of these mechanisms is inactivated, others could compensate the loss.

There is another issue to discuss about using *Drosophila* as the model organism. Fruit flies and humans may be seen as very different organisms but there are many similarities between these two species since they have diverged from common ancestors. As a result, many genes and the elements of protein production mechanisms have been conserved during the evolutionary course (Shih, Hodge & Andrade-Navarro, 2015). Thus, many molecules including neurotransmitters and neuromodulators are common in both of these species. Serotonergic neurons and serotonin receptors located in the human brain also have homologs in *Drosophila*. Further, there are shared characteristics with respect to neural circuits as well. The identification of the fruit fly connectome shed light on the similarities of fly brain and neural circuits to that of humans.

There are also studies to construct the human connectome as well (Sporns, Tononi & Kötter, 2005). So, comparative studies examining both the structural and neuronal roles of the brain of both of these species can be performed.

There might be areas of study where Drosophila is not a good representative of humans. However, studying attention and decision making with them is preferable since a variety of brain circuits and mechanisms are shared among humans and fruit flies. Both of these species have been through similar evolutionary processes which have led them in similar directions during their evolution. Although most would agree to this belief in terms of the biological or anatomical processes, conflicts arise when decision making and consciousness are considered. Humans can make decisions but it might be believed that insects cannot. Still, they choose between stimuli when they encounter more than one. Is it a behavioural choice they perform based on their instincts or is it really a decision-making process involving higher order cognition? The choices fruit flies make are not random but rather based on various features of the situation and the stimulus itself. To illustrate, if fruit fly larvae have more than one food opportunity, they choose what they need or the more nutritious one (Schwarz, Durisko & Dukas, 2013). In our protocol, we have also showed that while some flies responded to the visual stimulus more, others preferred to feed on gustatory stimulus. However, this difference did not occur by chance but the behaviour of the flies was influenced by a variety of factors such as the duration of the food deprivation period, the genotypes they possess, the activity of the serotonergic neurons of interest and the presence of the visual stimulus.

In fact, there are similar structures in the insect brain to the brain areas of humans specialized for action selection. The basal ganglia in the human brain act as the center where multiple excitatory and inhibitory pieces of information come together so that a decision is made at the end. Similarly, lateral protocerebrum of insects have been shown to perform in action selection and decision making with respect to olfactory information (Barron, Gurney, Meah, Vasilaki & Marshall, 2015). Thus, it can be deduced that there is an action selection procedure happening in the insect brain.

Although there are action selection procedures happening in both human and insect brains, whether these selection and decision-making processes occur consciously is another issue. Some may suggest that *Drosophila* makes its decision only based on its instincts. Further, there are studies (Barron & Klein, 2016) showing that insects have subjective experience as humans do. To illustrate, they do not behave in a way just by chance but there are mechanisms in their brain processing incoming information and performing action selection.

Another issue that should be considered here is whether humans make their decisions consciously as well. The free will experiments performed by Libet and colleagues (Libet, Gleason, Wright & Pearl, 1983) showed that the cerebral activity precedes the reporting of the conscious intention of the same action when participants are asked to indicate the

 time when they make a decision about the stimuli they are presented. Thus, it may be said that the brain decides earlier than the humans are conscious about it. The decision has been actually made as the brain activities show before we even know that we have made a decision. However, some other studies (Lavazza, 2016) also claim that the brain activity measured as the onset of the decision making can be obtained due to the neuronal noise. The discussion whether or not non-human animals of various kinds can act

in a goal-directed way similar to humans, based on common brain processes, neuronal mechanisms, and transmitters, is ongoing. Studies like the present one can sharpen our understanding of similarities and differences in this respect.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5.1 Conclusion

This study was conducted to reveal the effect of serotonergic system on attention and behavioural choice of *Drosophila melanogaster*, the fruit fly. There have been many studies focusing on the various roles of serotonin (5-HT) in the fly body and brain. However, all of these studies were working on a single modality such as vision. With the current study, we used a cross modal attention process to study the attentional shifting and the decision making by using fruit fly as the model organism.

We conducted experiments where flies needed to attend to either a visual looming stimulus or an appetitive gustatory stimulus. With this protocol, the behavioural pattern and the attentional shifting of the flies could be examined. To do this, serotonergic neurons that are targeted by R50H05-Gal4 line were activated or inactivated by using UAS-NaChBac or UAS-Kir2.1 respectively.

Activation experiments were run when activation flies were sated and they were expected to be feeding on sucrose as if they were food deprived. The feeding score of flies were not too high but they still fed on sucrose. The responsiveness of these flies was high considering both PER and CAR scores.

Inactivation flies were used for inactivation experiments and the tests were run when they were food deprived for 20 hours. Both the feeding and CAR scores of them were too high such that they were either responding to gustatory or to visual stimulus throughout the session. Further, sensitization was observed without any habituation in terms of CAR scores.

We thought that the feeding pattern might have been influenced from the visual stimulus presentation and CAR might be the reason for observing suppression in feeding behaviour. To

reveal this, both R50H05>UAS-NaChBac and R50H05-Gal4>UAS-Kir2.1 flies were tested in the absence of visual stimulus as well. The number of flies for

inactivation experiments was not enough to draw a conclusion. As expected, the feeding scores were higher for R50H05>UAS-NaChBac flies compared to the condition where the experiment included two modalities. Thus, we concluded that the suppression might have caused by the presentation of visual stimulus.

Although the results we have found contradicts with the ones of Albin et al (2015), it can be concluded that the serotonergic neurons targeted by R50H05-Gal4 line modulates feeding and attention. The protocol we used included two different sensory modalities and this might be the reason why we obtained such results. It seems that the role of the serotonergic neurons of interest is not just the modulation of hunger but they are probably involved in the allocation of attention between different sensory modalities. Moreover, since the location of these neurons can be considered as terra incognita, these serotonergic neurons might be functioning in the sensory integration process as well.

5.2 Limitations of the study and future directions

We tried to test 80 flies for each genotype to be able to get statistically significant results. However, we could not reach to that number for some genotypes. We want to continue experimenting with the same protocol to reach our goal of 80 flies for each genotype. Further, while setup for TRPA1 activation experiments

were prepared and some parameter experiments were run, the actual experiments could not be conducted in time. Therefore, our direction is to test these flies as well.

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