DOPAMINERGIC MODULATION OF ATTENTIONAL GATING BETWEEN SENSORY MODALITIES IN *DROSOPHILA MELANOGASTER*

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submitted by **BARIŞ SERHAN** in partial fulfillment of the requirements for the degree of **Master of Science in Cognitive Science Department, Middle East Technical University** by,

Prof. Dr. Nazife Baykal _____
Director, **Graduate School of Informatics Institute**

Assist. Prof. Dr. Cengiz Acartürk _____
Head of Department, **Cognitive Science**

Assoc. Prof. Dr. Annette Hohenberger _____
Supervisor, **Cognitive Science**

Assoc. Prof. Dr. Münire Özlem Çevik _____
Co-supervisor, **Psychology Department,TOBB UET**

Examining Committee Members:

Assist. Prof. Dr. Murat Perit Çakır
Cognitive Science, METU _____

Assoc. Prof. Dr. Annette Hohenberger
Cognitive Science, METU _____

Assoc. Prof. Dr. Münire Özlem Çevik
Psychology Department, TOBB UET _____

Assist. Prof. Dr. Didem Kadıhasanoğlu
Psychology Department, TOBB UET _____

Assist. Prof. Dr. Umut Özge
Cognitive Science, METU _____

Date: **05 September 2016**

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: BARIŞ SERHAN

Signature :

ABSTRACT

DOPAMINERGIC MODULATION OF ATTENTIONAL GATING
BETWEEN SENSORY MODALITIES IN *DROSOPHILA MELANOGASTER*

Serhan, Barış
M.S., Department of Cognitive Science
Supervisor : Assoc. Prof. Dr. Annette Hohenberger
Co-Supervisor : Assoc. Prof. Dr. Münire Özlem Çevik

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The fruit fly -*Drosophila melanogaster*- is one of the most popular model organisms for translational neuroscience and medicine. The monoamines like dopamine, norepinephrine and serotonin that modulate cognitive processes have homologs in the fly brain. The availability of genetic tools that enable selective manipulation of aminergic neurons in the fly brain provide an enormous advantage to study the neural circuits underlying cognitive processes. In our experiments, the role of dopaminergic modulation in the allocation of attention between two sensory modalities -vision and taste- was addressed by using a cross-modal attention protocol. First, the effects of food deprivation and stimulus parameters on the probability of responding to a looming stimulus versus feeding were assayed. Then, the flies that were mutants for different types of dopaminergic or octopaminergic receptors/transporters were tested to understand the involvement of amine modulators in attentional allocation. Finally, a particular mutation on a dopamine (i.e. *Dop1R1*) receptor gene was examined in order to pinpoint the state-dependent responsiveness and the cross-modal suppression in the *Drosophila* model, which might underlie preliminary characteristics of attention in any attention-like mechanism.

Keywords: Attention, *Drosophila melanogaster*, Dopamine, Neuromodulation

ÖZ

DROSOPHİLA MELANOGASTER'DA, DİKKATİN DOPAMİNEJİK MODÜLASYONLA DUYUMSAL MODALİTELER ARASINDA KAPILANMASI

Serhan, Barış

Yüksek Lisans, Bilişsel Bilimler Bölümü

Tez Yöneticisi : Doç. Dr. Annette Hohenberger

Ortak Tez Yöneticisi : Doç. Dr. Münire Özlem Çevik

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Meyve sineği -*Drosophila melanogaster*- translasyonel sinirbilim ve tıpta en popüler model organizmalardan biridir. Dopamin, norepinefrin ve serotonin gibi, bilişsel süreçleri modüle eden monoaminlerin homologları sinek beyninde mevcuttur. Sinek beynindeki aminerjik nöronları seçici olarak manipüle edebilen genetik araçların mevcudiyeti, bilişsel süreçlerin altında yatan nöral devreleri araştırmak için büyük avantaj sağlamaktadır. Deneylerimizde, bir modaliteler-arası dikkat protokolü kullanarak, dikkatin iki duyumsal modalite -görme ve tat- arasında tahsisatında dopaminerjik modulasyonun rolü üzerinde duruldu. Öncelikle, açlığın ve uyaran parametrelerinin, sukroza ya da görsel uyarana tepki yaratma olasılığına etkileri ölçüldü. Ardından amin modülatörlerin dikkatin tahsisatındaki dahiliyetini anlamak için, farklı tipte dopaminerjik ve oktopaminerjik reseptör/taşıyıcı mutantı olan sinekler test edildi. Son olarak, araştırmanın ilgi odağı belirli bir dopamin (i.e. *Dop1R1*) reseptör geni mutasyonuna çevirilerek, herhangi bir dikkatbenzeri mekanizmanın öncülü sayılabilecek modaliteler-arası baskılama ve duruma göre yanıt verebilme kavramlarını *Drosophila* modelinde tanımlamak için, toplanan veriler çözümlendi.

Anahtar Kelimeler: Dikkat, *Drosophila melanogaster*, Dopamin, Nöromodülasyon

to my father, Salih Murat Serhan, RIP

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LIST OF ABBREVIATIONS

5906	The wild-type background genes of TM2/TM6C line (Bloomington Stock no: 5906 or BL #5906)
CAR	Collision Avoidance Reflex (or Response, inter changeably)
CSS	Wildtype Canton-S flies that were provided by Dr. Scott Waddell from University of Oxford
CX	Central Complex
DA	Dopamine
DAT	Dopamine Transporter
Dop1R1	Dopamine 1-like receptor 1 (refers to a *gene* if it was written in Italic)
EB	Ellipsoid Body
hfd	Hours of Food Deprivation
ITI	Inter-trial Interval
NE	Norephinephrine
OCT	Octopamine
PER	Proboscis Extension Reflex (or Response, inter changeably)
TR	Trial interval
Ubx	Ultrabithorax
wt	wild-type

CHAPTER 1

INTRODUCTION

When the nature of a research subject was subjective because of its nature, the research question can be portrayed as a chicken-egg question. Thus, the general tendency is to eliminate subjectivity with observable measures. However, if the target system is too complex, idiosyncratic or multidimensional, it may be hard to find an accountable predictor to explain the intended high level cognitive process. Therefore, reducing the problem space and addressing the same processes on a relatively simple model may help to explain underlying mechanisms.

In this study, in an attempt to decipher the mechanisms that may underlie the cross-modal attention or attention-like processes, the common fruit fly, *Drosophila melanogaster*, was used as a model organism. The genetic tractability of *Drosophila* provides a stable platform where its genes, neurons or neural circuits can be marked or manipulated very precisely at a particular time. Furthermore, some of these target genes and tissues were evolutionary preserved both structurally and functionally across many other species, including even humans.

The first cross-modal attention protocol for fruit flies was used to address the potential mechanisms that drive fly's attention between sensory cues. In this protocol, a gustatory stimulus together with a visual looming stimulus are presented to an individual fruit fly and its behavioral responses are registered accordingly. As the fruit flies exhibit two mutually exclusive responses that are directly linked with these modalities, this protocol makes it possible to indicate the selection and cross-modal suppression mechanisms.

Furthermore, in an attempt to understand underlying mechanisms of attention-like processes in fruit flies, dopaminergic modulation examined by comparing behavioral characteristics of wild-type flies with certain mutants lines that carry a defective copy of a dopamine receptor gene. A particular mutant line (i.e. Dop1R1) was also proposed as a candidate model for attention deficit hyperactivity desorder (ADHD).

The general organization of the thesis as is the following;

In the next chapter, fundamental background knowledge that is necessary to keep track of this study and its arguments was elucidated in an explicit way

considering that the interdisciplinary nature of the study may attract readers from different fields of research.

The materials and methods chapter focused on the details of the cross-modal attention protocol. In this chapter, fruit fly strains that were used in the experiments, as well as, technical materials and the experimental setups were introduced.

In the following chapter, results of the parametric experiments are first presented. Then, pilot experiments on particular monoaminergic receptor or transporter mutants are shown in comparison to the wild-type flies. Later on, *Dop1R1* receptor mutants were particularly examined considering balancer and background effects. Finally, potential underlying attentional mechanisms that were modulated by dopamine were highlighted.

The last chapter is the conclusion chapter that consists of an overview of the findings that supported the hypothesis. Possible directions for further studies were also discussed in this chapter.

CHAPTER 2

BACKGROUND

2.1 Attention

Humans, like all other animals, perceive their environment through multiple sensory modalities. Animals process sensory inputs together with the information that was already acquired and once this procedure is done, the most appropriate behavior from their repertoire for the current situation is exhibited (Shettleworth, 2001). The amount of the current input that is going to be processed is a crucial constraint for the system, since the input quantity rises up, the processing costs will also increase. The other crucial constraint is the significance of this incoming stimulus (e.g. a nearby predator). Thus a biological system requires a mechanism to filter the competing incoming information while retaining its essence in order to adapt its environment and maintain its homeostasis. This process may also known as attention.

An information selection or reduction mechanism may emerge at different levels in different forms. For example, the number of the photoreceptors in the retinal ganglion is much higher than the number of axons in the optic nerve, which might indicate that the photonic information is filtered out at the retinal level even before arriving to the visual cortex (Purves et al., 2004), (Jonas, Schmidt, Müller-Bergh, Schlötzer-Schrehardt, & Naumann, 1992). On the other hand, in order to determine whether this low level mechanism is sufficient to be counted as an attention-like process or not, a solid definition is necessary.

William James' definition is still capturing the essence of attention after a century later and potentially there might never be a better definition. He described the attention as the follows:

> *"Everyone knows what attention is. It is the taking possession of the mind, in clear and vivid form, of one out of what may seem several simultaneously possible objects or trains of thought. Focalization, concentration, of consciousness are of its essence. It implies withdrawal from some things in order to deal effectively with others."* (James, 1910).

Even if everyone knows intuitively what attention is, as James said, once it is

approached as an object of a scientific study, compartmentalizations or clarifications are required. Many different approaches, taxonomies and manifestations on attention emerged in the last century, although some of them gained wider acceptance. A common dichotomy is the "bottom-up" and "top-down" processes of attention (Treisman & Gelade, 1980), (Desimone & Duncan, 1995). Bottom-up mechanisms can be thought as an involuntary attentional shift to the most salient object in the visual field or to an unexpected boom captured by the ears. In other words, the physical properties of the stimulus is in charge to drive these processes. Top-down processes, on the other hand, are involved endogenous cognitive strategies and they address the goal driven selective attention tasks (Treue, 2003). Accordingly, the attention is controlled by the internal state of the subject, an old memory, a thought or an emotional state may drive the attention on a particular event such as searching for food when hungry.

An important figure for the cognitive neuroscience of attention, Michael Posner, proposed a framework for attention which is broadly accepted today (Posner & Petersen, 1989), (Petersen & Posner, 2012). His framework suggested three distinct brain networks that are responsible for controlling attention: Alerting, orienting and executive networks. This distinction provided a solid base for the studies on the neural correlates and mechanisms underlying top-down and bottom-up attention. Alerting network was related to the function of obtaining and sustaining the alert state of the system within which the fronto-parietal regions as well as the locus coruleus are the major active areas. This alert state regulates the speed of the neural processing by modulating the general arousal and sensitivity of the incoming stimuli with the help of norepinephrine (NE) (Pfaff, Kieffer, et al., 2008). Orienting network involved directing the attention between different sensory events in which Frontal eye fields (FEF), Superior colliculus (SC) and some parietal areas are the main active brain regions. Unlike alerting network, it is modulated by another neurotransmitter called Acetylcholine (Ach). Finally, the executive network was in charge of regulating thoughts, behaviors and emotions especially when there was a conflict between sensory cues and the attentional task concerned. The dopamine was an essential neurotransmitter for this network, therefore dopaminergic centers such as nucleus accumbens (NA), substantia nigra pars compacta (SNPC) in basal ganglia become active, as well as anterior cingulate cortex since it was frequently considered with the processes like decision making, rewarding and emotion (Gökçay, 2010).

The studies that follows Posner's approach involved identifying the large scale neural networks in mammalian brain. The main aim of this approach is to correlate the activity of cortical or sub-cortical areas with cognitive processes in order to discover distributed domain specific brain regions related to memory, decision making or attention, using electrophysiological and magnetic imaging techniques (Barrett & Satpute, 2013). On the other hand, there are alternative approaches on attention that focus on how dopaminergic or norephinephrinergic transmissions lead the orientation or maintenance of attention, or on how these mechanisms cause attentional deficits such as hyperactivity or hypoactivity symptoms by using pharmacological and neuroge-

netic techniques (Barnes, Dean, Nandam, O'Connell, & Bellgrove, 2011), (del Campo, Chamberlain, Sahakian, & Robbins, 2011).

Considering many different approaches, experimental techniques, levels of analysis, it is crucial to draw a pathway for an attention research. Going back to William James' quotation to highlight some critical details that was going to be necessary to form a common basis for an attention research. If we penetrate into his statement phrase by phrase;

> *"It is the taking possession of the mind, in clear and vivid form, of one out of what may seem several simultaneously possible objects or trains of thought."*

The first thing that can be deduced is that the attention involves **"acquiring"** of a (mental) **"state"**. As also mentioned earlier, attention has **"bottom-up"** (*objects*) or **"top-down"** (*trains of thought*) constituents. Furthermore, the attentional process involves **"selection"** (*"one out of what ..."*). However, what is not clear in James' statement is where those *several simultaneously possible objects* were situated. They may both exist as visual objects in the mind (**"unimodal"**, i.e. selected among stimuli of the same modality), or one may be a visual object while the other is, for example, an auditory object (**"cross-modal"** or multimodal, i.e. selected among stimuli of separate modalities).

> *"Focalization, concentration, of consciousness are of its essence."*

The attention is a **"high-level"** process that requires some other high-level processes attributed usually to **"human"** beings or occasionally to their mammal cousins. Furthermore, it involves **"reduction"** of the currently available information into a small (*Focalization*) yet intense (*concentration*) piece.

> *"It implies withdrawal from some things in order to deal effectively with others."*

Finally, and maybe the most important, the attention involves **"suppression"** of the other available stimuli (*withdrawal from some things*) in the current environment, since they may act as distractors for the selected task.

In sum, attention is a high-level state acquiring function involves selecting one stimulus coming from a single modality while suppressing the others from the same and/or other modalities to reduce the distraction from competing information of bottom-up or top-down networks.

Recently, de Bivort and van Swinderen highlighted 4 practical characteristics of attention that were commonly targeted in animal studies and their counterparts in insect studies (de Bivort & van Swinderen, 2016). Their criteria are also coherent with some of the highlighted concepts above. In a nutshell, first

one, the responsiveness to the incoming input is enhanced by attention to select a subset of stimuli (i.e. **Unity**), second one, attentional processing source are limited (i.e. **Resource limitation**), third one, attention is a serial process and shifts between its objects in particular time-span (i.e. **Alternation**), and the last one, the neural correspondences of these characteristics can be identified in flies (i.e. **Neural correlates**).

The scaffolding of this research was built upon 3 main themes by taking these crucial points together into consideration; **Unity**, **Cross-modal suppression** and **neural mechanism**. Suppression can be thought as a combination of de Bivort and van Swinderen's "resource limitation" and "alternation criteria" in which top-down and bottom-up interactions, as well as cross-modal compulsion between distractor and rewarding stimuli were targeted. The details can be found in the following sections.

2.2 *Drosophila Melanogaster* as a Model Organism

In cognitive science, there are two broadly accepted suppositions. First, the primary aim of the field is to understand mechanisms underlying cognitive processes. Second, the cognitive modeling is the core tool to grasp the essence of these mechanisms (Bechtel & Abrahamsen, 2010).

According to McClelland, the cognitive modeling is needed since it allows us to examine the projections of the ideas that human thinking might fail to explore itself (McClelland, 2009). Although he refers most of the time to the connectionist models while characterizing cognitive modeling, there are also other approaches. For example, Bechtel emphasized the importance of the dynamical view in the cognitive modeling in detail by reviewing the studies on circadian timing mechanism of *Drosophila* (Bechtel & Abrahamsen, 2010). In this review, He also extended his former characterization of mechanistic explanation (Bechtel & Abrahamsen, 2005) by the following quote:

> *"A mechanism is a structure performing a function in virtue of its component parts, component operations, and their organization. The orchestrated functioning of the mechanism, manifested in patterns of change over time in properties of its parts and operations, is responsible for one or more phenomena."*

Bechtel suggested that the experimental methodologies involve defining differential relations between decomposed parts and operations, used in chronobiology studies on fruit flies, were very useful for cognitive scientists. Apparently, the same experimental practices can be adapted to cognitive science to discover the non linear interactions between the parts and operations within the mechanisms of the intended cognitive phenomena.

The brain is the principal actor for cognitive processes and the emergence of behaviors. Despite many existing brain imaging techniques (e.g. fMRI, PET,

EEG etc.) and even non-invasive induction devices such as transcranial magnetic stimulation (TMS) (Orrison Jr, Lewine, Sanders, & Hartshorne, 2015), the resolution of the existing technology is not sufficient today to study low level processes such as the action potentials of single neurons, neural circuit formations or aminergic neuromodulation at receptor level. Low level approaches require invasive monitoring or manipulation techniques which are not suitable usually for human subjects. As we shared many structural and functional similarities with animals through evolution in terms of the neural mechanisms as well as the genes (Morley & Montgomery, 2001), (Berridge & Kringelbach, 2008), animal experiments have a crucial role on the study of neural mechanisms of cognition.

2.2.1 *Drosophila melanogaster*

For more than 100 years, the common fruit fly, *Drosophila melanogaster*, has been one of the best studied system for genetics, biology, neuroscience, or translational medicine (Bellen, Tong, & Tsuda, 2010). The scientific popularity of the *Drosophila* emerged from many advantages that it provides as a model animal. First of all, despite the seemingly big differences between humans and fruit flies, they are very similar in terms of genetics. Mammals and fruit flies share around 90% homology in the functional domains of some proteins (Pandey & Nichols, 2011). Furthermore, the homologs of 75% of the human disease genes were found on the *Drosophila* genome (Reiter, Potocki, Chien, Gribskov, & Bier, 2001).

Figure 2.1: *Drosophila melanogaster*. (photograph by Muhammad Mahdi Karim, distributed under a GNU Free Documentation License, Version 1.2)

The fruit fly has a short reproduction period and life cycle which is a timesaving advantage for the studies that monitor the effects of changes on particular gene over generations. The generation occurs around 10 days and they became sexually mature within 42 to 72 hours after their emergence from the pupal. The aging starts around 20-day-old with the degeneration of the cell bodies with decreasing dopamine levels (Neckameyer, Woodrome, Holt, & Mayer, 2000) and they arrive to the end of their life span around at the 40-day

of age (Tatar et al., 2001).

Table 2.1 represents the approximate numbers of genes and neurons in 4 different model organisms of the Human Genome Project. The number of neurons of these animals increase by thousandfold in each step, where the number of genes increase only twofold between fruit fly and mammals. Consequently, the relatively simple systems like fruit fly or the roundworm may offer advantages over mammalian models depending on the level analysis of the study. For example, to study the involvement of particular genes in specific neural circuits, choosing *Drosophila* or *C. elegans* would reduce the number of neurons while keeping constant the range of genes.

Table 2.1: Approximate number of genes and neurons of certain species

Specie	**Number of Genes**	**Number of Neurons**
C. elegans (Roundworm)	$\sim$ 20,000	302
D. melanogaster (Fruit fly)	$\sim$ 13,600	$\sim$ 100,000
Mus musculus (House mouse)	$\sim$ 25,000	$\sim$ 100,000,000
Homo sapiens (Human)	$\sim$ 25,000	$\sim$ 100,000,000,000

When the whole genome was sequenced within the scope of the Human Genome Project (HGP) in 2000, *Drosophila melanogaster* turned into one of the most powerful animal model (Adams et al., 2000). However, the first whole genome sequence came two years earlier from *C. elegans* in 1998 (Consortium et al., 1998) despite few absent sequences which were going to be discovered in 2002 (Blumenthal et al., 2002). Furthermore, the connectome of the *C. elegans* including all 6393 synaptic connections between its 302 neurons were identified in 2011 (Varshney, Chen, Paniagua, Hall, & Chklovskii, 2011), thus revealing new perspectives for computational studies. Few years later, an international collaboration called OpenWorm developed a simulation engine to run computational models of *C. elegans* in silico (Szigeti et al., 2014). On the other part, the *Drosophila* connetome is still in progress (Alivisatos et al., 2012). Currently 16,000 of all 135,000 neurons have been identified with a mesoscopic map of their synaptic wiring network and the database is open to public via FlyCircuit (Chiang et al., 2011).

Another important reason for the popularity of the models like *D. melanogaster* and *C.elegans* in neuroscience was the selectivity. This notion involves the opportunities for selective manipulation of genes, molecules, neurons or subset of neurons controlled over time and space by using genetic tools. For example, one can activate or deactivate a specific neuron that innervates structurally and functionally known subset of neurons in vivo, without any invasion, just by triggering that specific neuron using optogenetics (Lima & Miesenböck, 2005), (Wu et al., 2014).

2.2.2 Investigating high level processes on a simple organism

Higher level cognitive processes of human cognition such as memory, learning, attention, or decision making involve central brain regions and their co-

ordinated dynamic interaction (Haberkern & Jayaraman, 2016). To have a better understanding of cognition, these processes can be decomposed into their sub-mechanisms to have a new level of research. For example, the underlying circuit motifs of a connectivity map may aid in understanding the computations of the network. Many sub-mechanisms that underlay high level cognition might be conserved also in other animals through the course of evolution (Fiore, Dolan, Strausfeld, & Hirth, 2015). Furthermore, the similarities between the mechanisms of different animals increase as the level of analyses decreases. A list of different levels of organization of the brain can be found in Table 2.2 which was adapted from the Human Brain Project report (Markram, 2012). In general, experimental studies might involve manipulations and measurements at different levels of analysis, such as lesioning a particular brain region of a rodent to determine the functional outcomes on the behavior of that region (i.e. Brain regions and Body levels in Table 2.2), or manipulating an aminergic receptor gene of a round worm to capture the involvement of a particular neural network. Thus, the number of uncontrolled variables increase, since the distance between levels in which the independent and dependent variables were picked, as well as the spatial scale, increased.

Considering high level mechanisms, the first learning and memory mutant *"dunce"* had been isolated in *Drosophila* at the Benzer laboratory (Dudai, Jan, Byers, Quinn, & Benzer, 1976) in 1976, and later on, the homolog counter parts of this gene had found in rats and humans (R. L. Davis, Takayasu, Eberwine, & Myres, 1989). These results paved the way for the new studies within which the first molecular mechanisms of short term and long term memory, as well as the synaptic modulation mechanisms regulated by neurotransmitters such as serotonin, were founded together with the electrophysiological recordings on the mammals (Kandel, 2001).

Furthermore, these results led the way for translational research that involves using data acquired from the animal studies to the human based studies to improve global health care system (e.g. by finding new drugs). Today, there are many *Drosophila* disease models that involve structural or functional deficits of the nervous system such as Down syndrome (Flight, 2013), Parkinson's disease (Martin et al., 2014), schizophrenia (Furukubo-Tokunaga, 2009), or epilepsy (Parker, Howlett, Rusan, & Tanouye, 2011) etc.

Table 2.2: Spatial scales for different levels of organization of the human brain

Levels Of Organization	**Numbers**	**Spatial Scale**
Body	1	Meters 10^0
Whole Brain	1	Centimeters 10^{-2}
Brain Regions	n.a.	Millimeters 10^{-3}
Neural Circuits	n.a	Millimeters / Micrometers $10^{-3}/10^{-6}$
Neurons	$\sim 10 \times 10^{11}$	Micrometers 10^{-6}
Synapses	$\sim 10 \times 10^{15}$	Micrometers 10^{-6}
Chromosomes	$\sim$ 23 pairs	Micrometers 10^{-6}
Genes	$\sim$ 25,000	Nanometers 10^{-9}

Alongside the translational research, connectome projects in the last 15 years showed that the similar neural mechanisms were responsible for the similar sensory or motor functions both in vertebrates and fruit flies (Wilson, 2013). Recently, high quality research indicated that the main neural computing modules of the invertebrate brain had many similarities with more complex brains in molecular, in system as well as in behavioral levels. For example, the research on the neural basis of the odor perception that indicated the analogy of odor receptors both in fruit fly and mammal brain, brought the Nobel Prize in Physiology or Medicine 2014 jointly to Richard Axel and Linda Buck (Vosshall, Wong, & Axel, 2000).

2.3 Attention in *Drosophila melanogaster*

In this section, the literature on the fruit fly attention or closely correlated studies were taken into consideration in term of 3 themes: unity, suppression and neural mechanism (de Bivort & van Swinderen, 2016).

2.3.1 Unity

Unity concept can be thought as the ability of the fly brain to focus on particular objects or subsets of stimuli while there are distractor stimuli in the current environment. Modulating the synaptic gain of the circuits that are relevant to the current internal needs and the external opportunities may be used to ignore distractor.

The brain of the *Drosophila melanogaster* was organized in a more distributed fashion with respect to mammalian brains, which was probably evolutionarily more economic considering the 100,000 of neurons in a tiny space. Sensory processing regions are situated near, or even within, the sensory receptors (i.e. sensilla) and transmit information to the multi-modal areas localized more deeply in the mid brain. For example, flies have two optical lobes instead of one and they are located just behind the compound eyes, starting with photoreceptors in eye facets, then the following processing units, respectively as, lamina, medulla and lobula plate (Paulk, Millard, & van Swinderen, 2013). While the input was arriving to the central brain structures through these neuropils, in each step it became less retinatopic, in other words, the preservation of spatial relations at the retina decrease gradually (Rister et al., 2007). In this way, the photonic information coming to each eye were processed distinctively before they were integrated in the central regions.

Furthermore, as the head and the neck of *Drosophila* is fixed, the experimenter can have absolute control on what the fly sees separately on the right and the left visual fields. First studies on visual selective attention based on this property of the fly body and conducted on tethered flies in flight arenas (Heisenberg & Wolf, 1984). In these studies, an individual fly is tethered from its dorsal thorax to the top of a pin and is exposed certain visual stimuli to each visual

field while being able to fly. Tethered flies is attached to a torque meter in order to measure the orientation behavior (i.e. yaw torque) (Wolf & Heisenberg, 1991). Thus the flight direction indicates flies' choice. Flight arenas provide high level in vivo selectivity with today's technology. A pioneer figure on flying mechanisms of *Drosophila*, Dr. M. Dickinson, and his colegue Dr. P. Wier recently pointed out that the all central complex (CX) structures were reacted certain visual stimuli during tethered flight in contrast to non-active state during quiescence supposing that the CX was involved visual flight navigation with a context-dependent manner (Weir & Dickinson, 2015). More importantly, after he and his colleagues determined that the gain of certain motion processing neurons (i.e. vertical-system visual neurons (VS cells)) was chanced accordingly to the locomotor state (Maimon, Straw, & Dickinson, 2010), they detected primary proofs that the state-dependent changes on the membrane potential of these VS cells were modulated by the octopaminergic neurons that were projected to optic lobes (Suver, Mamiya, & Dickinson, 2012). Beside these studies, the state-dependent modulation of walking was also revealed (Chiappe, Seelig, Reiser, & Jayaraman, 2010).

In the last decade, studies showed that the CX was taking part on memory (G. Liu et al., 2006) along with the MB neuropil which was the principal target for memory studies(McGuire, Le, & Davis, 2001). Furthermore, the underlying circuitry and the sub components of the CX which might motivate memory formation for different visual patterns were clarified day by day (Pan et al., 2009), considering also the effects of this visual pattern memory on the optomotor responses of the fly in orientation behavior (Seelig & Jayaraman, 2013), (Seelig & Jayaraman, 2015)

The concept of information reduction is based on the limitations of the attentional resources, therefore, if the information existıng in the current environment was numerously and/or complex, the reduction process will more likely to take more time and/or to cause higher error-rates on the selection task. This **reduction** attribute for insects was shown on *Apis mellifera* and *Bombus terrestris* (i.e. honeybee and bumblebee) by applying this idea to the experimental setup (Morawetz & Spaethe, 2012). Morawetz et al. measured the effects of a distracting visual stimuli on a visual searching task and their results indicated a rise both on the processing time and the error-rates for this task when the attentional load was increased. A recent study of Hiesenberg et al. (Koenig, Wolf, & Heisenberg, 2016) can be counted as an indication for the **alternation** feature of attention (de Bivort & van Swinderen, 2016). They used two visual stripes which can displace front to back simultaneously and were located left and right visual fields of an individual fly in a flight arena. Their focus of attention was captured by using a torque meter, following 3 different conditions which where there were whether one single stimulus moving, two stimuli but only one moving, or two simultaneously moving stimuli. Their results demonstrated that fruit flies attend to one particular stimulus at a particular time instead of behaving accordingly to the average of two stimuli. Furthermore, they shifted their attention in a particular time-span, which was 4 seconds for the wild type flies (Koenig et al., 2016).

2.3.2 Cross-modal suppression

Suppression is one of the fundamental mechanism of attention as some things should not be attended in order to attend some other things. Similarly, in order to select some things, there should be some other things in the current situation which may also be electable. Suppression is basically selecting a thing over an other thing, however inverse is not valid. Therefore, at least two different stimuli must exist in the experimental paradigm, more importantly, there needs to also be at least two different measures that distinguish between inhibited stimulus and selected stimulus in order to argue about an inhibition mechanism. As explained above, it is possible to present two different visual object through one single modality within the tethered flight studies in the current literature (Van Swinderen, 2011). However, opto-motor responses may indicate only the intended flight direction but not any inhibition for the unintended one. Furthermore, even if there are some experimental design in which different stimuli different modalities (Chow & Frye, 2008), because of the same reason they are not eligible to show any suppression mechanisms yet. First cross-modal attention design for fruit flies was used in this study. The conceptual details of the protocol can be found in the next section, technical details can be found in the methods chapter.

2.3.3 Neural mechanism

"Neural correlates" is the last criterion of de Bivort and van Swinderen's categorization and it is crucial that a study can identify neural mechanisms or activity patterns related to attentional processing (de Bivort & van Swinderen, 2016). Most of the behavioral studies on fly's attention are commonly based on simultaneous electrophysiological recordings during the experimental protocols and they try to identify local field potentials (LFP) related to the behavioral observation (Van Swinderen, 2011). On the other hand, in this study, a neuromodulation mechanism was taken into consideration as a neural correlate of attention.

The current literature of cognitive neuroscience has already addressed the role of neuromodulation in attentional processing in humans. For example, all three attention networks of Posner mentioned earlier in this chapter, depend on specific neuromodulators (Petersen & Posner, 2012). Neuromodulation is a biochemical process of controlling the gain of the synaptic transmission by secreting the chemicals messengers known as neuromodulators. These chemicals are usually amine modulators (e.g. DA, 5-HT or NE) or neuropeptides (e.g. endorphins, substances P). Beside the traditional synaptic transmission between a pre-synaptic and a post-synaptic sites of two intercommunicating neurons, the neuromodulation involves axo-axonic or axo-synaptic connections. By the virtue of these connections, a small number of neuromodulator neurons can rule out a large neuron population by controlling the gain of synaptic transmissions of their network (Katz & Calin-Jageman, 2008).

Dopaminergic modulation

Mammalian monoamine neuromodulators include norepinephrine, dopamine or serotonin (5-HT). The modulation of the attention in mammals is mostly attributed to the monoamine neurotrasmitter called dopamine and its dopaminergic pathways (Nieoullon, 2002). In humans, dopamine is the essential neuromodulator of Posner's executive network (Petersen & Posner, 2012). The human brain involves 5 types of DA receptors (i.e D_1 - D_5), which can be classified into 2 major categories depending on whether they inhibit (i.e. D_2-like receptors: D_2, D_3 and D_4) or activate (i.e. D_1-like receptors: D_1 and D_5) adenylyl cyclase (AC). Activation of AC might in turn promote the depolarization of the neuron, and vice-versa.

The 5 distinct types of dopamine receptors were identified in humans (i.e D_1 - D_5) and they divided into two subcategories depending on their role on the enzyme called adenylyl cyclase (AC), such that D_1-like receptors (i.e D_1 and D_5) stimulate adenylyl cyclase whereas the D_2-like receptors (i.e D_2, D_3 and D_4) inhibit AC (Missale, Nash, Robinson, Jaber, & Caron, 1998). Since this AC enzyme takes part in processes that change membrane potential by activating or deactivating Ca^{2+} and K^+ channels, D_1-like receptors promote the depolarization of neurons' cell body, while the D_2-like receptors inhibit the action potentials.

The mesolimbic dopamine system consists of ventral tegmental area (VTA) and the nucleus accumbens (NA) at the ventral straitum is responsible of rewarding processes in the cases like food, sex or the abuse of alcohol or drugs (Nestler & Carlezon, 2006). The dopamine system is implicated in several processes including reward, attention and addiction (Wise, 1998). Accordingly, it is related to attention deficit hyperactivity disorder (ADHD) (LaHoste et al., 1996), as well as, to disorders such as schizophrenia (K. Davis, Kahn, Ko, & Davidson, 1991) and Parkinson's disease (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973).

Dopaminergic modulation in the fly brain

Fruit flies, like humans, have multi-sensation. The functions of their sensory organs and receptors are also similar to those of humans. They have senses such as vision, olfaction, gustation, nociception (i.e. detecting harmful stimuli) (Dubnau, 2014) or mechanosensation that involve capturing air vibrations (Jarman, 2002). Sensory inputs are first processed in unimodal areas, then this information is combined in multimodal areas (such as the human heretomodal cortex) and finally, behavioral outputs transmit to the motor control centers (Ohyama et al., 2015), (Yagi, Mabuchi, Mizunami, & Tanaka, 2016), (Zhang, Guo, Peng, Xi, & Guo, 2007). A fly should consider the current environmental factors and its internal needs so that it may choose an appropriate response from its behavioral repertoire that was necessary for the current situation. This decision mechanism would be succeeded if and only if the gain of transmission at its sensory and motor networks was capable to be transient,

reversible and in coordination at the time of the selection.

The aminergic modulation in the fly brain is achieved by a small number of neurons as the mammal brains. The monoamin modulators such as serotonin (5-HT), octopamine (OA) - which is the homolog of mammalian norepinephrine - and dopamine (DA) arborize excessively in multimodal areas within which they are synthesized by many neurons that reach to sensory and motor areas (Sinakevitch & Strausfeld, 2006), (Mao & Davis, 2009). The fly brain has around 200 dopaminergic neurons, thus, merely 1 over 5000 of all of its neurons was capable to produce DA. In humans, this ratio is around 1/35,000 with respect to around 600,000 dopaminergic neurons against 20 x 10^9 neocortical neurons (Chinta & Andersen, 2005).

Drosophila melanogaster has 4 types of dopamine receptors. Two of them (i.e. DopR and DopR2) are from the D_1-like receptor family, one (i.e. Dop2R, also known as D2R or DD2R) has a homology with D_2-like receptor family (Hearn et al., 2002), and the last one (i.e. DopEcr) binds both dopamine and ecdysone which is an insect sex hormone, however, a mammalian homolog of this DopEcr receptor does not exist. All of these 4 receptors have a relation with the AC and the AC has a critical role on the cAMP pathway. In the fly brain, the dopamine regulates the processes such as rewarding (Burke et al., 2012), learning and memory formation (Schwaerzel et al., 2003), (Kim, Lee, & Han, 2007), sleep and arousal (Ueno et al., 2012), stress (Neckameyer & Weinstein, 2005), aggression related activation (Alekseyenko, Chan, Li, & Kravitz, 2013), and decision making (Zhang et al., 2007) congruent with the human brain.

2.4 *Drosophila* Model of Attention in this Research

The main aim of this research was to identify the modulatory mechanisms that might take part on gating the fly's attention between different sensory modalities. The first cross-modal attention protocol was used for the experiments. The protocol involved presenting a visual looming stimulus to an individual tethered fly, simultaneously with a gustatory stimulus. Tethered fruit flies can hold a styrofoam ball that has almost the same size of their body and they can engage in regular activities such as walking, grooming or feeding on the ball (Figures 3.7, 3.9 and 3.8 in Section 3.3).

In addition to that, the fruit flies were engaged two mutually exclusive behaviors against the visually approaching stimulus and the gustatory stimulus in this protocol. These behaviors are collision avoidance reflex (CAR) and proboscis extension reflex (PER) (see chapter Material and Methods). These behaviorally distinguishable outcomes provided an experimental platform to capture **cross-modal suppression** mechanisms that can be related to the fly attention.

Furthermore, the bottom-up reactions can be adjusted by changing the stimulus parameters both for vision and the taste, as well as, top-down processes

such as the internal state of the individual fruit fly can be regulated by food depriving them for particular periods of time. Therefore, the interaction between the bottom-up and top-down counter parts of attentional processes might also be speculated with the provided platform. The details of this protocol can be found in Section 3.3.

Even though there are some other protocols in which simultaneous stimuli could be presented to vision and odor modalities, they are not suitable to study cross-modal attention, as the behavioral responses of the animal was recorded as a single outcome, either as an optomotor response on tethered flying (Chow & Frye, 2008), (Duistermars & Frye, 2010), or by recording its flying path on free flight (Frye, Tarsitano, & Dickinson, 2003). That is, the flies do not made a choice between two distinct behaviors. Since these registered data of the behavioral outcomes were not distinguishable for a particular modality, they cannot indicate cross-modal suppression mechanisms necessary to argue about attentional processes.

Using this cross-modal attention protocol, first, gustatory and visual stimulus parameters were titrated using wildtype flies. Then, mutant flies that carry defective copies of monoamine receptors or transporters were screened. Finally, a particular mutation on *Dop1R1* receptor gene was examined in detail in order to understand its involvement of the attentional selection mechanisms.

The examination was done by focusing on unity, cross-modal suppression and neural mechanism. Late onset habituation/sensitization responses of the wild-type flies were compared with mutants for unity. Complementariness of the feeding and CAR responses, in other words, reciprocity of their trends were examined for cross-modal suppression. Other behavioral choices (i.e. not CAR or PER) were also considered for this attentional mechanism. Furthermore, dopaminergic modulation was taken into focus as a neural mechanism that controls the attentional gating to understand whether mutants that carry a defective copy of the dopamine type-1 receptor 1 gene can be used as a model for ADHD.

Current literature of fly's attention is commonly based on the determination of attention or attention-like processes in fruit flies. However, despite the great scientific value of these high-end researches, none of these aforementioned studies, declares anything about the underlying mechanisms of the attention in the fruit fly. The main purpose of this study is to detect an underlying mechanism of the Drosophila's attention such as the dopaminergic modulation. Therefore, our study is an attempt to contribute to current literature as being the first study on the mechanisms of cross-modal attention the fruit fly.

CHAPTER 3

MATERIALS AND METHODS

In this chapter, the materials that were used in the different stages of our experimental design and the main structure of the experimental protocol are explained.

3.1 The Fruit Fly

This section is related to the maintenance phase of the experiments. One can find details of the fly strains that were used in this study. These highlight the conditions of the experiment as well as how the flies were maintained

3.1.1 Fly strains

- Wildtype Canton-S flies were provided by Dr. Scott Waddell from University of Oxford.
- Balancer line :
  - **TM2**, Ubx**/TM6C**, Sb – $w^{1118}/Dp(1;Y)y^{+};TM2/TM6C,Sb^{1}$ – The first and the second chromosomes are isogenic with BL # 5905 wild-type stock – BL # 5906
- Loss of function mutants:
  - ***Dop1R1*/TM6B**, Tb – Dopamine 1-like receptor 1 – $PBac\{WH\}Dop1R1^{f02676}$ – obtained from Exelixis Collection at Harvard Medical School – Stock no: f02676
  - ***Dop2R*** – Dopamine 2-like receptor – $PBac\{WH\}Dop2R^{f06521}$ – obtained from Exelixis Collection – Stock no: f06521
  - ***fmn;UAS-dat*** – Dopamine transporter – Background : 2202U – provided by Dr. Kazuhiko Kume, Nagoya City University, Japan.
  - ***Oct*β*2R*** – Octopamine β2 receptor – $PBac\{WH\}Oct\beta2R^{f05679}$ – obtained from Exelixis Collection – Stock no: f05679 - (BL # 18896)
  - ***Oct*β*1R*** – Octopamine β1 receptor – $PBac\{WH\}Oct\beta1R^{f02819}$ – obtained from Exelixis Collection – Stock no: f02819 - (BL # 18596)

3.1.2 Maintenance

Incubation
Our fly stocks were kept in a Memmert ICH 260L Climate Chamber which controls both for humidity and light. The incubator was adjusted to 12 h day light and 12 h dark cycle starting from 7:00 AM. The temperature and the relative humidity inside the climate chamber were set at $+25°\mathrm{C}$ and $50\%\ rh$. Due to a technical problem in our incubator, some of the stocks were kept in room conditions in the lab ($24 \pm 6°\mathrm{C}$, $30 \pm 10\%$). We kept the fly strains inside of 50 ml plastic vials with 10 ml fly food each.

Flies were collected on the day they emerged from the pupae, and tested when they were 4-5 days old.

Figure 3.1: Memmert ICH 260L Climate Chamber with our stocks

Nutrition
Since how you fed flies directly effects the behavioral responses during exper-

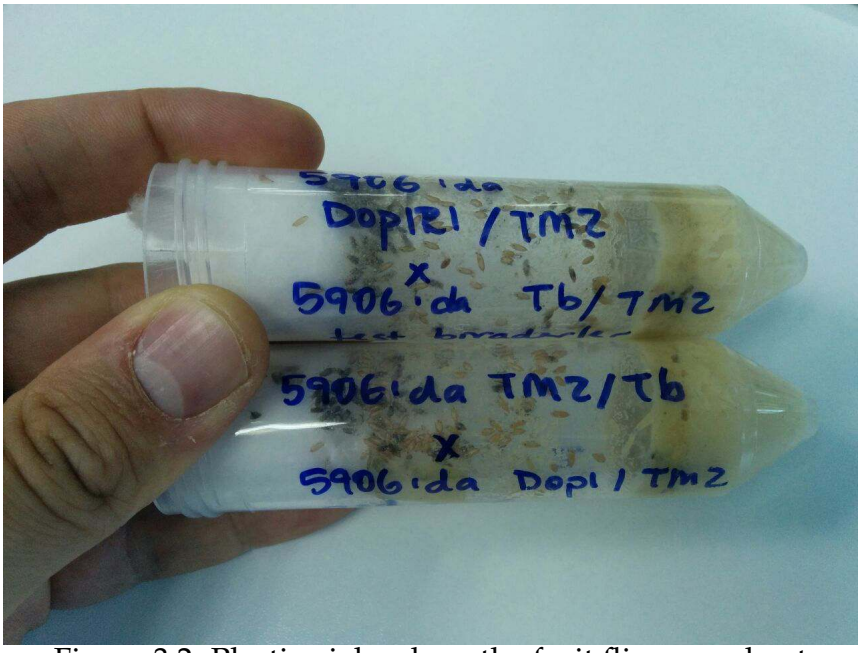


Figure 3.2: Plastic vials where the fruit flies were kept

iments in the presence of an appetitive stimulus, it is very crucial what to give them to eat and using the exact same recipe for all flies in order to avoid possible differences in their response thresholds. In our lab, we feed flies based on the formula provided by the Bloomington Drosophila Stock Center in Indiana University. According to that formula, 1.1 L of fly food contains: 73.07 gr Cornmeal (Bağdat), 10 gr soy flour (Doğalsan), 17.4 gr yeast (Dr. Oetker), 80 ml high fructose corn syrup (Cargill, Fructose Concentration: 55%), 4.82 ml propionic acid (Sigma, Concentration:99%) and finally 5.5 gr of agar (Roth) (Çevik & Erden, 2012).

3.2 Experimental Materials

Experiment Computer

It had 3.5 Ghz Intel Core i3-4150 processor, 64 GB memory, 1 GB Nvidia GeForce 210 graphic card (64 bit, DDR3),as well as, an onboard Intel HD Graphics 4400 display adapter and 120 GB SSD hard drive. Two monitors were plugged to this computer, one of which was for monitoring the experiment sessions (connected to the onboard adapter), and the other one was for presenting the visual looming stimulus to the fly. Stimulus presentation monitor was a 21-inch LED-backlit LCD monitor (i.e. Philips 223V5L) with 60 Hz screen refresh rate, 1920 x 1080 resolution, 200 $cd/m(^2)$ brightness and 5 ms response time. – This computer system was used for collecting data with Matlab during experiments and for recording the experiment sessions in video.

Software

Psychophysics Toolbox Version 3 (Psychtoolbox-3) on Matlab 2014a **–** This is a free toolbox (Kleiner et al., 2007) which is compatible with both Matlab and GNU Octave and contains many functions for neuroscience and vision research. Its libraries were used for coding the stimulus presentation Matlab script. First versions of these scripts were developed and adapted from the tutorial demos of Dr. Peter Scarfe, the Vision and Haptics Lab, University of Reading, by Dr. Didem Kadıhasanoğlu, TOBB ETU, then improved with certain additional functionalities by myself. The codes of a particular version of this script can be found in Annex as an example.

EOS Utility 3.4 – This is the official software of Canon EOS DSLR cameras. It was used for video recordings of experiment sessions.

IBM SPSS Statistics 23– Most of the statistical analysis were done in SPSS.

Recording system

Each experiment session were recorded with a Canon EOS 5D Mark III full frame DSLR Camera and Canon MP-E 65 mm macro photo lens. This video recording device was fixed to the experiment environment with an articulating arm and a table clamp (Figure 3.3).

The SLR lens has the aperture range f2.8 to f16 and 1:1 to 5:1 magnification ratio with a manual focus system. We adjusted the manual focus around the midpoint of 4X and 5X magnification markers in almost all of our experiments since the behavior of the fly is very clearly observable within this setting. Figure 3.7 can be seen to have an opinion of the video sizes.

Particular light conditions for the experiment room and appropriate camera settings for them were considered including conditions such as no indoor lighting, only red indoor lighting (fruit flies are blind to the red light), indoor fluorescent lighting and indoor fluorescent lighting together with LED video spot. The optimal solution was using the simple indoor fluorescent lighting as we did not observe any particular effect on the fly behavior. On the other hand, macro photography and macro video shooting require extra illumination for clearness and sharpness of the records and when we used a LED video spot, the visual responsiveness of flies decreased observably. Therefore, we had to set the camera to high ISO rate such as ISO 8000 by accepting the cost of more grainy recordings. We set most of the time the shutter speed to 1/30 and the aperture to f/5.6 for the same reason.

Other tools

Beside the materials introduced above, we also needed many other equipment to conduct our experiments. In this subsection, these equipment and their use were tried to summarized.

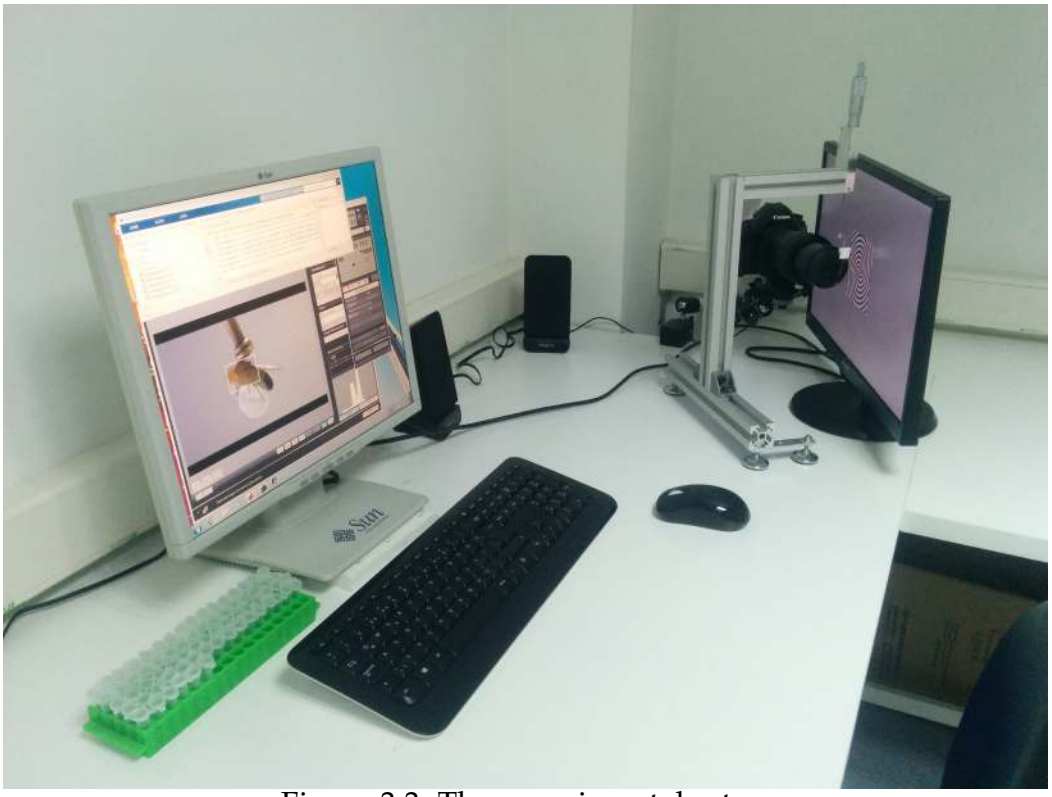


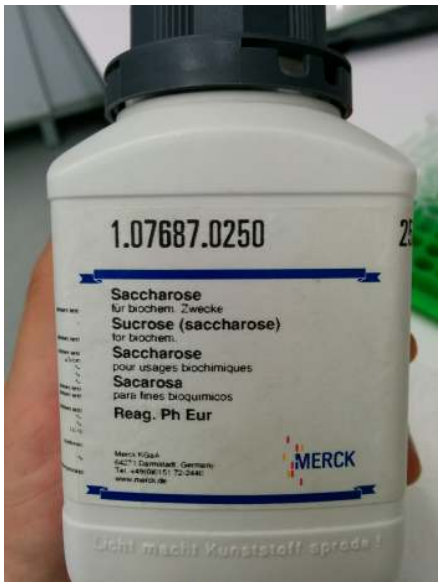
Figure 3.3: The experimental setup

Because of the sucrose concentration and the quality is crucial for feeding experiments, we used a precision balance branded Denver Instruments which can weigh up to 310 g with 0.001 g precision to weigh the sucrose out (Figure 3.4b). The sucrose stocks were provided by Merck Millipore (Figure 3.4a). We used simple styrofoam balls in order to introduce the sucrose as a gustatory stimulus to the fly. We basically pulled them off from various styrofoam packages or boxes by paying all of our attention to not contaminate them. For example, we did not use any styrofoam ball detached from any external surface of the package. We also chose them very carefully to have them in similar sizes.

It is very important to note that all those materials were carefully kept in specific hygiene conditions. When there is a high possibility of contamination disposable products were chosen and they directly threw away, on the other hand, certain equipment such as tweezers, medical hand tools etc. were washed by machine every day regularly.

3.3 Experimental Protocol

In our experiments, we addressed the role of dopaminergic modulation in the allocation of attention between two sensory modalities, namely -vision and taste-, using a cross-modal attention protocol developed by Dr. Münire Özlem Çevik (Çevik & Serhan, 2015). In this section, our main methodology is tried to be elucidated in detail with respect to the scaffolding of our study



(a) Sucrose Stock

(b) Precision Balance

Figure 3.4: Various materials for feeding experiments

that was conceptualized in Background Chapter.

3.3.1 Preparation of the flies

In this protocol, the first step is to take apart 4 - 5 days old male flies from their original vials to a separate vial where there is just a wet paper tissue instead of food. We do this in order maintain the flies in proper humidity conditions while they are deprived of food in this separate vial for a target period of time before the experiments. The preparation phase starts 1 hours before the experiments therefore a fly is deprived of food for a target time plus 1 hour at the beginning of an experiment which we called as *hours of food deprivation (hfd)*. The details of the hunger state of the flies can be found in the following sections.

The preparation starts with anesthetizing the flies with cold shock. The flies are basically put inside of a frozen plastic vial (around $-4°\mathrm{C}$). The anesthetized flies then are put on a cold platform for the further processes. The flies are stuck together to the pinhead of a simple pin from their dorsal thorax under a 8 diopter magnifying lamp. We used simple wax as a fastener for that step of preparation. After that step, we keep these flies in styrofoam cooler boxes to tranquilize them until the experiments. The temperature and the humidity of the boxes are monitored and tried to keep in $20(\pm2)°\mathrm{C}$ and $80(\pm10)\%$.

3.3.2 Visual stimulus

After a target period of time is passed, a prepared fly is carefully positioned in front of a computer monitor for the test phase of the experiment. In our exper-

iments, we used a spiral image that turns clock-wise at a rate of 5^o per frame on the screen as a visual stimulus (Figure 3.5). This stimulus creates a visual looming effect for the fly which can be deduced from the classical landing response of the insects (Goodman, 1960). When there is an expanding object aproaching to the fruit fly, it can whether choose to land on the object or it can engage the collision-avoidance manuever (Tammero & Dickinson, 2002). On the other hand, in both of these cases, the flies first extend their forelegs to the expanding object. Similar visual mechanism were first used on *Musca Domestica* by Braitenberg and Ferretti in 1966 (Braitenberg & Ferretti, 1966). They were using a physical rotating disk with a black spiral on white background and they showed that the landing response is only observed within unique rotational direction (clock-wise) where there is an expansion of the image. They also showed that this landing reaction is closely related to a threshold function which has parameters such as the angular velocity of the expansion, luminosity conditions, thickness of the spiral and the distance between the fly and the spiral disk.

We also conducted parametric experiments on wildtype fruit flies towards finding the optimal visual parameters for our experiments such as the angular velocity for the spiral. The details can be found in the parametric tests section of Results and Discussions. Considering these experiments conducted with different angular velocity rates (i.e. 1^o 5^o and 9^o), our final decision was using the angular velocity at a rate of 5^o per frame (60Hz) on the screen. At the final setup of the experiments, the distance between the tethered fly and the screen was fixed to 1.5 cm. It should be also noted that the fly must be very well positioned to the center of the spiral by paying attention that its thorax is perpendicular to the screen. Otherwise, small dislocations or wrong body angles can cause big differences in the behavior.

3.3.3 Gustatory stimulus

The second sensory modality in our cross modal attention study is the taste modality. We presented gustatory stimuli to fruit flies while they were exposed also a visual stimulus described in the subsection above. The essential substance that we used as a gustatory stimulus was the sucrose. Fruit flies have many taste receptors which are very well distributed on different locations of their body such as the proboscis, the pharynx, wings, female genitalia and -maybe the most important location for our protocol- legs (Stocker, 1994). Each gustatory sensillum located at those cites has four gustatory neurons and each of these neurons associated with different taste modalities.

We presented the gustatory stimuli to the sucrose receptors of their legs. In order to do that, we basically dipped simple styrofoam balls into sucrose solutions that has different concentrations and we passed these balls to flies at the test phase by using a tweezers. You can find a picture of a fruit fly while it was feeding on a styrofoam ball in Figure 3.7 in coding behaviors section.

Sucrose solutions were carefully prepared in each experiment day and threw

Figure 3.5: The visual looming stimulus

away at the end of the day in order to prevent the potential contamination that might be occurred with continuous use of the same solution. The sucrose quantity and the distilled water were also attentively measured for this process (Figure 3.4b). Three different sucrose concentration were used in the experiments: wet (solely distilled water) , 0.1 M Suc (100 mmol/L Sucrose) and 0.5 M Suc. The details of concentration selection criteria can be found in the next Chapter.

3.3.4 Food deprivation period

One of the crucial parameter of our experiments is the hunger or satiety state of the fruit fly. Unlike visual stimuli and sucrose presentation, this parameter is highly correlated with the state of the observer. As also mentioned earlier in Background, these hunger or satiety states might be taken as an elementary models for internal states (Inagaki et al., 2012), and the internal state of the observer has a part in the top-down modulation of the attention whereas stimulus parameters such as the visual properties of the spiral or sucrose concentration parameter of the gustatory stimulus have a role in the bottom-up modulation of the attention. It can be thought that the hunger state drives the attention to the possible food sources currently available in the environment in an endogenous way, on the other hand, visual and gustatory stimulus parameters control the attentional shifting more reflexively in an exogenous manner.

We modulated the hunger state by removing fruit flies from the food source. We quantified this modulation according to the duration between the time when flies were removed from food source and the start time of the test phase of the experiments. We called that parameter as the *hours of food deprivation (hfd)* and we used 3 different intervals: 2-hfd, 6-hfd and 16-hfd.

After around 15 hfd, there is a sudden decrease in the sugar levels in the hemolyph, as a reason for that, 16-hfd was initially selected before the parametric experiments as a pilot parameter to capture the effects of that drop (Inagaki et al., 2012). It is also important to note that the time passed in the preparation phase which includes tethering flies and tranquilizing them in humidified boxes until the test phase is also included to *hfd*. An other important point is that each *hfd* represents the mid point of two hours time intervals (e.g. for 6-hfd, flies were food deprived for 5 to 7 hours before the test phase of the experiment), which is more plausible, since we tested more then one fly in each experiment.

3.3.5 Stimulus duration

It is very critical to choose the appropriate time intervals for both ITI and TR. The main reason is that the fruit flies that were exposed different duration of visual stimuli or different waiting periods between those stimuli would most likely be inclined to have different habituation and sensitization responses.

Therefore it is also crucial to compare the experiment results that were obtained under the exact same duration parameters. Otherwise, we cannot have a reliable comparison baseline for the experiments that were conducted with different fly strains. In order to find the best ITI or TR duration for our study, we conducted parametric tests on different wildtype fruit flies (i.e. CSS, CSB and CSW) where we changed systematically the ITI and TR duration (e.g. 3 s, 6 s or 9 s ITI with 3 s TR, or 3 s ITI with 3 s, 6 s or 9 s TR.). The detail of these suitable stimulus duration detection experiments can be found in next Chapter. Considering the results coming from these parametric tests, what we thought that the optimal duration for ITI was 6 seconds whereas it was 2 seconds for TR.

3.3.6 Coding Behaviors

During the test phase of the experiments, we recorded the behaviors of fruit flies according to a predefined coding system. We transcribed one sole behavior according to our coding system for each trial interval (TR) and one for each inter-trial interval (ITI), this means that even if the fly engaged in different behaviors during TR or ITI, we wrote down the most appropriate one for this short time period. This process depends on the observer's judgment, on the other hand, we also have several conventions for choosing the appropriate code when more than one behavior were occurred during an interval and this conventions will be addressed later on in this subsection.

The following list shows the codes of the system:

- **0** $\rightarrow$ Missing behavior
- **1** $\rightarrow$ Proboscis Extension Reflex (PER)
- **2** $\rightarrow$ Proboscis Extension Reflex for feeding (Figure 3.7)
- **3** $\rightarrow$ Standing still (no observable behavior)
- **4** $\rightarrow$ Walking
- **5** $\rightarrow$ Front grooming
- **6** $\rightarrow$ Back grooming (Figure 3.8)
- **7** $\rightarrow$ Tethered flying (Figure 3.9)
- **8** $\rightarrow$ Preparation for the Collision Avoidance Reflex (CAR)
- **9** $\rightarrow$ Collision Avoidance Reflex (CAR) (Figure 3.6)

Those codes were basically typed into the experiment computer during the test phase. The running Matlab script captured these key strokes while it was concurrently dealing also with the turning spiral. At the end of the session, the program produced a comma separated value (.csv) file contains collected

data, as well as some other parameters such the current time or stimulus parameters. Even if we recorded all those behaviors with our coding system, what we especially interested was two mutually exclusive behavoirs: Collision Avoidance Reflex and Proboscis Extension Reflex.

When a fly engaged with two or more behaviors in a unique ITI or TR, as mentioned earlier, we had several conventions to code them. First of all, if a fly was showing a CAR response (beside several cases, CAR occurs during TR) even it engaged other behaviors during the same TR, the behavior should be recorded as **9**. It can be noted that if an other behavior occurs in the same TR with CAR, it occurs almost all the time before the CAR response, in other words, once a CAR response was triggered, it continues until the next ITI by suppressing all the other behaviors.

When several behaviors occurred in the interval, we recorded the most long acting behavior according to a personal judgement unless an exceptional case showed up. An exceptional case can be;

a) The behavior occurs at the last moments of the ITI and the behavior is changed because the effect of the visual stimulus in the next TR. (e.g. Lets consider a fly was consecutively back grooming for 4 s and front grooming for 2 s in an 6 s ITI and when the spiral was started to turn, it engages again back grooming. In this case, the behavior that should be recorded for ITI was the front grooming (i.e. 5) since this would help us to capture the change of the behavior due to visual stimulus.)

b) The occurrence of a feeding behavior (i.e. 2) can be short lasting (or instantaneous) against the other behaviors therefore PER for feeding can have a superiority upon other behaviors (meanwhile upmost behavoir in the coding hierarchy is always CAR) in some cases. (e.g. Even 3 instantaneous PER for feeding took less than 2 s in total over a 6 s interval in a feeding experiment session, this behavior must recorded as PER for feeding).

c) Fruit flies sometimes drop the styrofoam ball and start flying during sessions. In these cases, a new ball is given to the fly as fast as possible. If the fly engaged tethered flying during an entire interval, the behavior is coded as tethered flying (i.e. 7). Although, for example, if the fly had the new ball at the 4th second of an ITI and engaged an other behavior for the remaining 2 seconds, this other behavior is coded for the ITI instead of tethered flying.

Almost all the behaviors at the list above have mutually exclusive motor programs. The exception is the back grooming which can be occurred with PER (i.e. 1 or 2) and if it was the case in an interval, the codes of these two behaviors must be recorded together (e.g. 26 for simultaneous feeding and back-grooming in an ITI.).

If an unusual situation was observed in an interval, it was written down on both the physical experiment record and the excel file, then whether this in-

terval was marked as tethered flying or the entire session was canceled. If the tethered flying was registered for more than 3 TR per session, that session was canceled and excluded from any further analysis.

3.3.6.1 Collision Avoidance Reflex (CAR)

The collision avoidance reflex is our main behavior of concern in the presence of the visual stimulus. CAR involves the extension of the front legs against the visual looming stimulus while stretching out the central and hind legs to the opposite direction (Figure 3.6). As mentioned earlier, a fly can choose whether landing on the approaching object or collision avoidance maneuver when it is exposed to the looming stimulus (Tammero & Dickinson, 2002). On the other hand, we do not concern whether the fly is escaping or landing, since we observe a single behavior in both cases. It is very important to note that the current literature on landing and collision avoidance responses were based on the experiments that are done on tethered flying (an example of tethered flying can be found in Figure 3.9). Our study is the first study that measures those behaviors on freely walking tethered flies.

Figure 3.6: CAR - Collision Avoidance Reflex

3.3.6.2 Proboscis Extension Reflex (PER)

The proboscis extension reflex is produced when the gustatory receptors of a fly were introduced to an appetitive solution (Dethier, 1976). As can be seen in Figure 3.7, it involves the extension of the proboscis - most of the time - for feeding. One of the most essential side of this experimental protocol is that the first time in the literature, a protocol allows to monitor PERs of fruit flies while they were tethered, thus, it is possible to study cross-modal sensory interactions of taste and vision modalities considering their effects on various other motor programs for walking, grooming etc. rather than CAR or PER.

Figure 3.7: PER - Proboscis Extension Reflex (feeding)

When we consider both of these responses (CAR and PER) in terms of the similarities of their triggering mechanisms to the human attentional processes, it is hard to say whether one of them corresponds to a top-down mechanism whereas the other one corresponds to a bottom-up or not. Because both CAR and PER carry sub-components of these approaches. For example, at first glance, the triggering mechanism of feeding behavior that was quantified with PER, can be taken into account as a top-down system because it seems to be endogenous and somehow voluntary due to its very closed relation with the satiety state of the animal, on the other hand, the proboscis extension might be considered also as a "reflex" and the density of the sucrose concentration can also trigger this reflex even if the fly's hunger state does not send any feeding requirement signal. Similarly, CAR can be controlled both stimulus-driven or state-dependent ways, thus we cannot only count the number of the behaviors occurring during trials, the responsiveness state of the fly should be also included to the equation, for that reason, we also analyzed our data to understand the state-dependency.

3.3.6.3 Other behaviors

Beside these two crucial responses, some other behaviors were also registered during experiment sessions as mentioned earlier in the coding system section.

Grooming is a very common behavior in flies. It involves cleaning their body and sensory receptors distributed all around it. In our study, we did not regard all differences between the grooming types or sequences since we did not particularly interested with that behavior. Therefore, whether a fly grooms its abdomen, thorax or wings, we considered a grooming sequence as a backgrooming if it involves using hind legs (Figure 3.8). In the same manner, if a fly was grooming with its forelegs, we registered the behavior as front grooming regardless where it was cleaning (e.g. the proboscis, compound eyes, antenna etc.).

Backgrooming was the most observed behavior in our experiments. Uninterrupted long backgrooming sequences might indicate being indifferent to the presented stimuli, therefore, deficiency on directing the attention to the environmentally important objects or current internal needs.

Figure 3.8: Backgrooming

The tethered flying can be seen in Figure 3.9. As mentioned in Background, the tethered flying were monitored frequently in virtual flight arenas to focus on various research subjects such as optomotor responses, visual place learning, flying dynamics, selective visual attention etc. In our study, it is desired that a subject fruit fly that was tethered from its dorsal thorax can smoothly and easily fly, in this way, we know that tethering process was done without causing a functional problem. In contrast with that, it is not desired that a subject fly engaged tethered flying during the experiment sessions as they will drop the styrofoam ball for flying.

Figure 3.9: Tethered flying

The fruit flies can also walk on the styrofoam balls. From our data set, at first glance, it can be thought that fruit flies do not walk often in this proto-

col, although, most of the when they walked in an interval, there might be some other behavior that we registered. For example, it was common that a fly walked just before showing a CAR, therefore, the CAR was registered for that particular interval. Similarly, they were usually walking while they were feeding, it should be noted again that the feeding and walking are also mutually exclusive, although, between several proboscis extension responses flies were often walking (grooming the proboscis was also common with feeding behavior) especially when they became a bit saturated. This behavior might be related to the foraging behavior in the nature, unfortunately, the trails of this behavior cannot be tracked in our data as they were most of the time recorded as feeding.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Parametric Tests

Standardize experimental parameters are crucial to provide repeatability in experiments carried out. First of all, it is hard to argue about results coming from various experiments that have different parameters. Besides that, more importantly, if the appropriate parameters cannot be found before the principal experiments, there is a risk of missing significant difference in the behavior of interest. Even if there is a meaningful difference between different subjects, since relevant behaviors cannot be emerged (or they can be over emerged), it is impossible to capture those meaningful differences. For example, we can change various parameters of the visual looming stimulus such as the angular velocity of the spiral so that it can cause an increase in the probability of response to stimulus with a collision avoidance reflex in wild-type fruit flies. If we set the angular speed parameter to a value where each wild-type fly responds with CAR, for example, around in 18 TR out of 20 TR, then we can have a trouble detecting a hyperactive mutant. On the other hand, this inference can be misleading, because, if the stimulus parameters were set to a value where we can observe around 10 CAR responses in 20 TR in wild-type flies and then the same mutation screen were done, mutant flies might have continued to behave very responsive as before (e.g. 18 CAR in 20 TR), in this new case, even if the same group of flies were tested, one can easily capture the difference and argue about the effects of this specific mutation on the attentional processing.

For these reasons, we conducted many parametric tests around a period of 8 months and on around 800 individual wild-type fruit flies. In this section, the details of these parametric tests can be found. We try to clarify why we have chosen a specific parameter and our final experimental parameters for further experiments were declared.

4.1.1 Rotational velocity of the visual stimulus

The rotational velocity of the visual stimulus characterizes how much the spiral image on the screen will turn in each iteration. We manipulated the degree

of the rotation per frame change just by changing the value of "rolvel" variable. For example, if this variable is set for 6, as we used a 60 Hz monitor (each frame is around 17 ms), the spiral will turn one full cycle in each consecutive second (i.e. $6 * 60 = 360^o$).

We tested Canton-S flies (CSS) after 1 to 4 hours and 5 to 7 hours food deprivation periods on wet styrofoam balls with different rotational velocity parameters to see how the speed of the spiral effects the probability of CAR responses. We first essayed wild type flies on a short period of food deprivation by setting the rotvel variable to 1^o, 5^o and 9^o degrees per frame. As can be seen in the graph in Figure 4.1, when the speed was set to 1^o per frame (slow stimulus), the probability of CAR was almost 0, on the other hand, when this value was set to 5^o or 9^o per frame, there was an increase on flies' responsiveness but there is no significant difference between moderate (i.e. 5^o per frame) and fast (i.e. 9^o per frame) looming stimuli, $p < .059$.

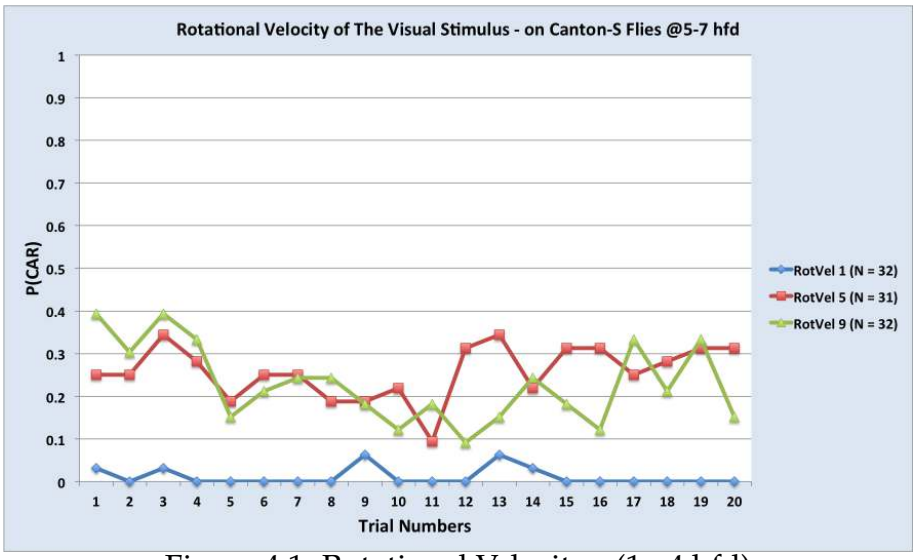


Figure 4.1: Rotational Velocity - (1 - 4 hfd)

On the other hand, when we tested the same strain of flies in the same conditions except the food deprivation period which was longer, we observed that the relation between moderate and fast stimuli changed (Figure 4.2). It can be seen that irrespective of whether the flies were famished or not, slow looming stimuli did not give rise to a CAR in most of the time. The reason for that the looming stimulus might not be able to pass the looming perception threshold of fruit flies. When flies satiety state was 6 hfd, the disintegration between moderate and fast stimuli was much more clear. Strangely, moderate stimuli caused more CAR responses than the faster stimuli.

The total number of CAR responses in 20 trials was analyzed by factorial ANOVA where hours of food deprivation (hfd) and rotational velocity were used as fixed factors while number of CAR responses was the dependent variable. There was a significant main effect of the rotational velocity parameter and the production of the CAR, $F(2, 131) = 32.39, p < .001, \eta_p^2 = .499$. On the other hand, there was no significant effect of hours of food deprivation by itself alone, $F(1, 131) = 2.06, p = .154, \eta_p^2 = .015$. However, once we analyzed

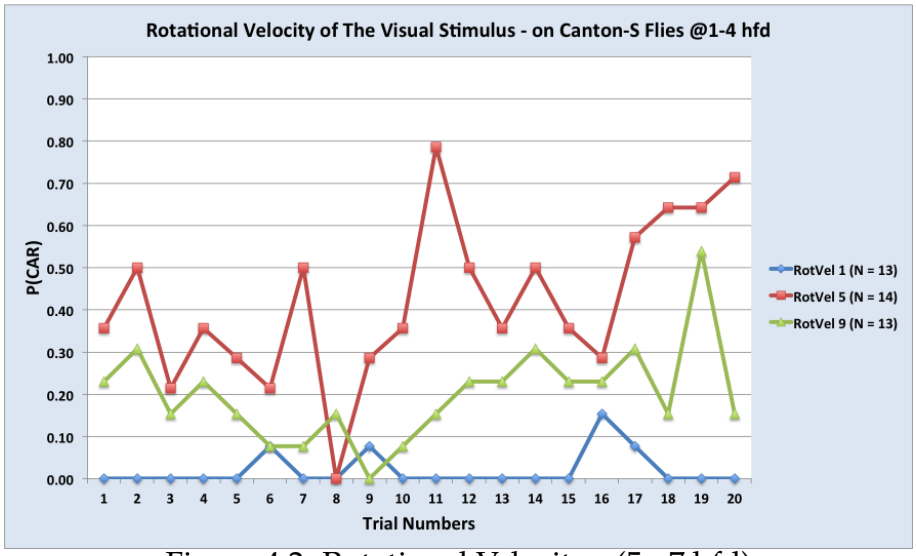


Figure 4.2: Rotational Velocity - (5 - 7 hfd)

the interaction between hfd and rotational velocity, we observed a significant interaction between those variables, $F(2, 131) = 3.193, p < .05, \eta_p^2 = .046$.

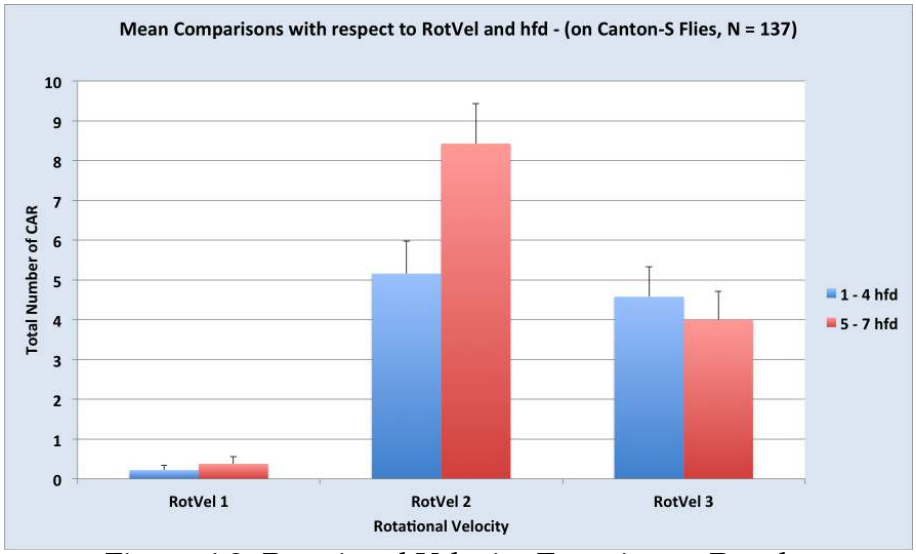


Figure 4.3: Rotational Velocity Experiment Results

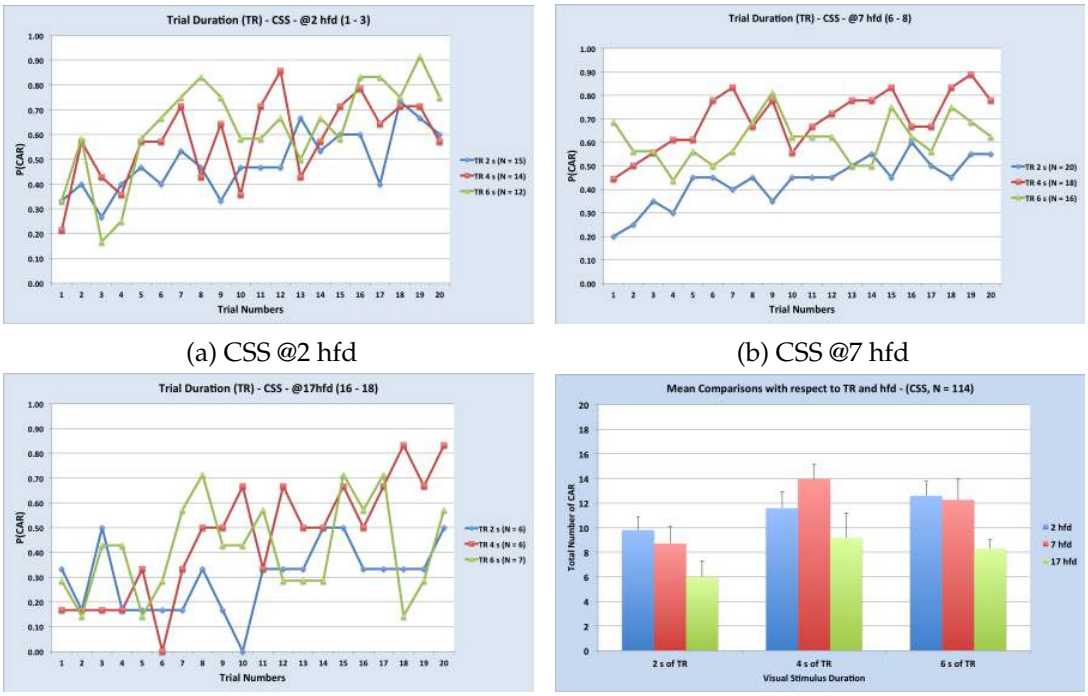
Based on these results, we decided to use a rotational velocity of 5^o per frame for our subsequent experiments.

4.1.2 Duration of the visual stimulus

The trial duration (TR) that flies were exposed to a looming stimulus is important for affecting habituation and sensitization. We conducted experiments on wild type flies after 2 hfd, 7 hfd or 17 hfd on a wet styrofoam ball in order to find best TR for our experiments. We tested these flies in 3 different TR conditions: 2 s, 4 s and 6 s. We used a fixed duration of 6 s for every ITI.

Figure 4.4 represents the probability of responding the looming stimulus with

a CAR when we food deprived Canton-S flies for 2, 7 or 17 hours on average. As can be seen in the figure 4.4a, when the stimulus duration increased, there was a slight increase on probability of CAR in CSS for 4 s and 6 s TR after 2 hfd. The segregation between curves were more clear at 7 hfd for the same 3 trial duration (Figure 4.4b). When we considered the total number of CAR responses of CSS flies at 7 hfd as a dependent variable in ANOVA, there was a significant effect of 3 different trial duration in which the turning spiral were presented to those flies, $F(2, 51) = 3.747, p < .05, \eta_p^2 = .128$.



(a) CSS @2 hfd

(b) CSS @7 hfd

(c) CSS @17 hfd

(d) Mean comparisons

Figure 4.4: Trial durations (2 s, 4 s or 6 s) for certain food deprivation periods (2 hfd, 7 hfd, or 17 hfd)

Overall, there was a significant effect of stimulus duration, $F(2, 105) = 3.943, p < .05, \eta_p^2 = .07$, and hours of food deprivation, $F(2, 105) = 4.044, p < .05, \eta_p^2 = .072$, on the probability of responding to the visual looming stimulus with a CAR. However, the interaction of these independent variables was not significant, $F(4, 105) = 0.524, p = .719$ (Figure 4.4).

As it can be seen in figure 4.4d, after 7 and 17 hours of food deprivation periods, the responsiveness of the wild-type flies increased by 50% from 2 s to 4 s presentation of the looming stimulus. In contrast, responsiveness declined when the stimulus duration increased to 6 s. On the other hand, the responsiveness of wild-type flies that were food deprived for 2 hours slightly rose while the trial duration was increased from 2 s to 6 s.

Based on these results, the stimulus presentation was set to an interval of 2 seconds. This took into consideration the possibility of receptor desensitization which can occur as a result of long repetitive stimulus exposure. The receptor desensitization might be a reason for the decline in the probability of CAR from 4 s to 6 s after 7 or 17 hfd and the effects of these kind of processes

should be eliminated so far as one can for the sake of the reliability of the results, otherwise it may be hard to conclude whether an outcome arises out of an experimental manipulation or a process such as the receptor desensitization. Beside that, 2 s stimulus interval causes also a moderate level of CAR response. Since there may be an increase or decrease in the analysis of the responses of the mutant flies, it was more desirable to set the trial duration parameter with which a moderate reactiveness can be observed in wildtype flies.

4.1.3 Sucrose concentration

We tested the effects of sucrose concentration and the food deprivation period on the probability of CAR and feeding in a 2 x 3 between-group experimental design. Within that design, we conducted experiments on wildtype Canton-S fruit flies after 6 hfd or 16 hfd on styrofoam balls which were either dipped in 0.1 M or 0.5 M sucrose concentrations, or water (i.e. 0 M sucrose).

The occurrence probabilities of CAR and feeding during stimulus presentation can be found in figure 4.5. In this figure, upper panels belong to 6 hfd groups whereas lower panels to the 16 hfd groups. The panels at the right side show the probability of CAR whereas the panels at the left side indicate the feeding responses of the same group of flies. It is very important to emphasize again that the fruit flies at the right and left panels were the same flies. Since the proboscis extension reflex (PER) and the collision avoidance reflex (CAR) were mutually exclusive, flies cannot engage these behaviors at the same time. In other words, the sum of the probabilities of feeding and CAR of any of these three groups at any particular trial will not exceed "1". For example, if we considered the .5 M sucrose group (i.e. green curve, N = 85, @16 hfd) in the lower panels, when the flies were exposed to the visual stimulus on the first trial, 60% of the group decided to feed (lower right panel) whereas the 33% of this group showed a CAR (lower left panel) and the remaining flies which was around 7%, engaged any other behavior such as back grooming, walking etc.

Comparing these panels of feeding and CAR also helped us to observe how they changed over time while one was increasing and the other was decreasing. For example, the blue curves in upper panels represent a group of 97 flies that were assayed on wet balls after 6 hfd. We do not observe a PER for feeding while they were on wet balls. Thus can be seen at right panels, the blue curves stayed steady at "O" PER level for feeding as there was no sucrose on the ball. The probability of observing a CAR from this group was higher with respect to the other two groups (i.e. the flies that were on sucrose) at the upper right panel (Figure 4.5), as there was not any suppression effect of the feeding.

There was a significant effect of the food deprivation periods on feeding, $F(1, 485) = 58.974, p < .001, \eta_p^2 = .108$, the effect of sucrose concentration on feeding probability was even higher than food deprivation period, $F(2, 485) =$

$145.806, p < .001, \eta_p^2 = .375$. Pairwise comparisons indicated that .1 M and .5 M sucrose concentrations were not significantly different than each other $(p = .141)$.

The left panels illustrate the probability of CAR against the visual looming stimulus after 6 hfd and 16 hfd again for three different sucrose conditions. Even if the effect sizes were smaller with respect to feeding, there was a significant effect of food deprivation, $F(1, 485) = 16.619, p < .001, \eta_p^2 = .033$, and sucrose concentration, $F(2, 485) = 10.188, p < .001, \eta_p^2 = .040$ on the probability of CAR. Despite its slight effect size, the interaction between these two independent variables was also significant, $F(1, 485) = 5.358, p < .005, \eta_p^2 = .022$.

Since the collision avoidance reflex was suppressed by sucrose regardless of its concentration (red and green curves), and as the amount of this suppression rose with hunger, the difference between sucrose condition and the control (i.e. red and green curves vs. blue curves) increased when flies were food deprived for 16 hours.

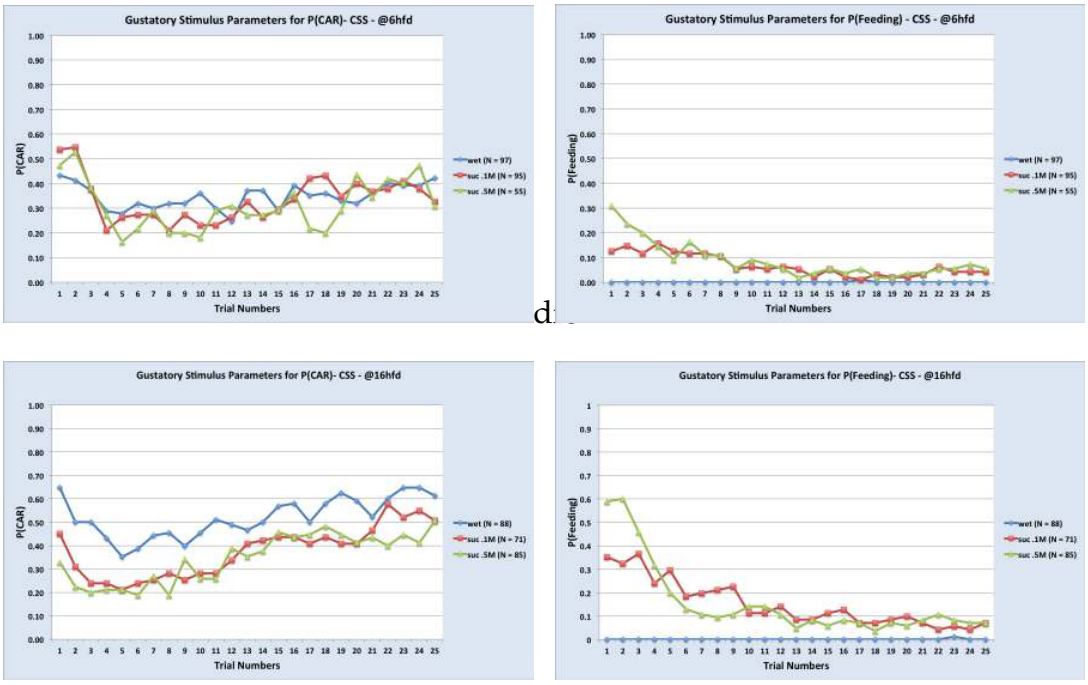


Figure 4.5: Gustatory Stimulus Parameters on CSS

The bar chart in Figure 4.6 shows the mean values of the behaviors occurred during the stimulus presentation of all fruit flies that were subjected to our 2 x 3 between-group experiments. As explained above, our results indicated that the existence of sucrose was far more important than its concentration in terms of feeding behavior. Furthermore, the suppression of the collision avoidance behavior against looming stimulus increase with the longer food deprivation period.

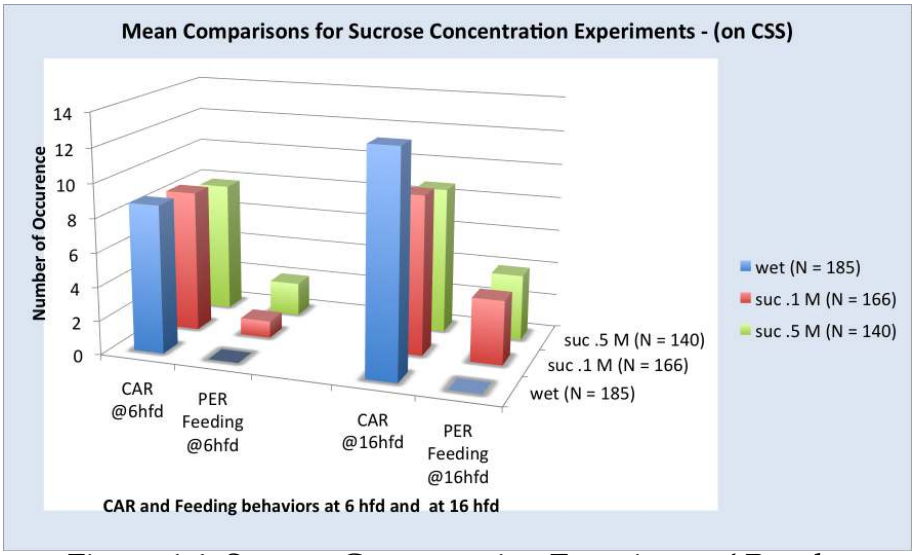


Figure 4.6: Sucrose Concentration Experiments' Results

4.1.4 Final decision of the experimental parameters

Considering all these test results, we decided to set the experimental parameters for the mutant screens and rescue experiments as the following;

- Rotational velocity of the spiral : **5º per frame**
- Duration of the visual stimulus (TR) : **2 Seconds**
- Duration between visual stimuli (ITI) : **6 Seconds**
- Hours of food deprivation : **6 hfd** or **16 hfd**
- Sucrose concentration : **0.5 M** (500 mmol/L) or **Wet** (i.e. 0 M)
- Number of trials per session : **25 Trials**

4.2 Pilot Mutant Screens

Next, we screened the fruit flies that were mutant for various dopamine and octopamine receptors or transporters. Our first findings showed the collision avoidance responses (CAR) of several octopamine and dopamine receptors/-transpoters mutant strains can be found in this subsection. We particularly tested 5 mutant strains: *Dop1R1*/TM6B, *Dop2R*, *fumin;UAS-dat*, *Octβ2R* and *Octβ1R*.

All of these pilot mutant screens were assayed after 6 hours of food deprivation. The other parameters were as described above (i.e. rotational speed:5º, TR: 2 s, ITI: 6, Sucrose condition: wet or 0.5M, @6hfd) except the number of trials which was 20 trials per session. Wild type flies were also tested in the same conditions (e.g. the first 20 trials of the recent wild-type data did not merge with these data). In all of the following figures in this subsection, the

blue curves represented the probability of CAR on wet balls whereas the red ones represented the same behavioral outcome on 0.5 M sucrose concentration. All of these mutant lines except *fumin;UAS-dat* were from The Exelixis Collection at the Harvard Medical School. The dopamine transporter mutant fumin;UAS-dat was provided by Dr Kazuhiko Kume, Nagoya City University, Japan.

Before continue, it is important to note that none of these strains had been outcrossed to Canton-S background at the time when these pilot experiments were conducted, therefore the effects presented in this section could stem both from the mutation or the background genes (e.g. wild type or balancer).These first results were presented as a poster (Çevik & Serhan, 2015).

4.2.1 *Dop1R1* mutants

The mammalian D1 dopamine receptors shared high homology with dopamine 1-like receptor familly of *Drosophila* through the course of evolution (Gotzes, Balfanz, & Baumann, 1993). Therefore, understanding how a mutation of these receptors governs behavioral outcomes of our model may provide important clues about the similar mechanisms that humans have.

Dopamine 1-like receptor 1 mutants ($Dop1R1^{f02676}$) from Exelixis stock were tested according to the experimental protocol and compared with wild type (i.e. CSS) flies to capture the consequences of this loss of function mutation. The homozygous *Dop1R1* mutants cannot survive most of the time, therefore they are maintained as a balanced stock (i.e. *Dop1R1*/TM6B,Tb). This receptor mutation occurs in the 3rd chromosome, for that reason a dominant marker (e.g. Tb) must also be present on the 3rd chromosome to distinguish their offspring.

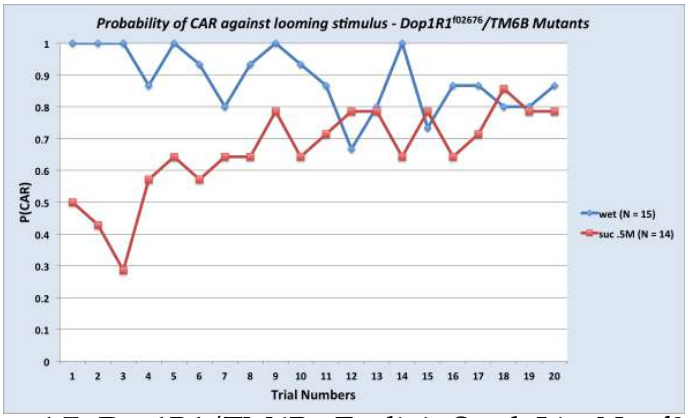


Figure 4.7: *Dop1R1*/TM6B - Exelixis Stock List No: f02676

Dop1R1 mutants showed an hyperactive phenotype during these screens especially when the sucrose was not presented (Figure 4.7). Even if when sucrose was presented, their sensitivity increased gradually towards the end of the sessions and reached a similar response level of the non-sucrose group. An initial suppression of CAR by feeding can be observed in the first half.

4.2.2 *Dop2R* mutants

Drosophila Dopamine 2-like receptor gene is located in X chromosome and it is also homologous to the genes that express mammalian Dopamine 2-like receptor family as Dop1R1 (Hearn et al., 2002). This mutation is mostly abbreviated also as "D2R" (Marella, Mann, & Scott, 2012), (Hearn et al., 2002). *D2R* is also a loss of function mutation as *Dop1R1*. However, in contrast with dopamine type-1 receptors, dopamine type-2 receptors have an inhibitory role both in mammals and flies.

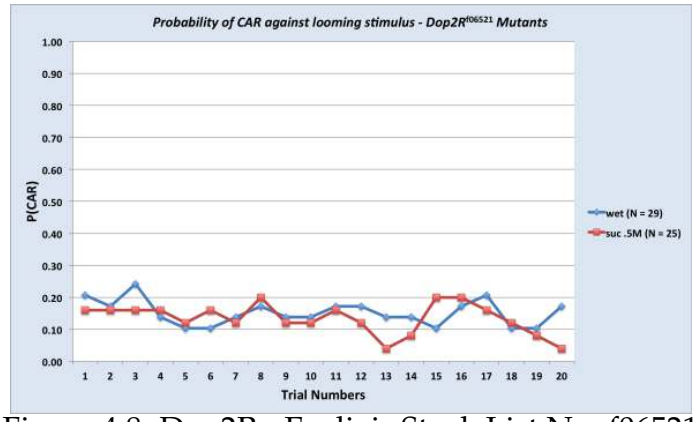


Figure 4.8: Dop2R - Exelixis Stock List No: f06521

Dop2R^{f06521} mutant flies showed an hypoactive phenotype which was coherent with literature. As can be seen in the Figure 4.8, probability of CAR stayed steady around 0.15 which makes around 3 CAR per session, regardless of being on a wet or sucrose dipped styrofoam ball.

4.2.3 *fumin;UAS-dat* mutants

The *fumin (fmn)* mutants have been reported to express a highly active phenotype as a result of a mutation on a *dopamine transporter* gene of *Drosophila* (Kume, Kume, Park, Hirsh, & Jackson, 2005). Dopaminenergic modulation regulates the state of arousal in fruit flies and *fumin* is a prototypical example for that.

In contrast to expectations, the *fumin* mutants did not show high activation (Figure 4.9). Even if the probability of response was similar to D2R mutants, the pattern of behavior was changed.

4.2.4 *Octβ2R* mutants

Octopamine is the insect homolog of mammalian norepinephrine (Maqueira, Chatwin, & Evans, 2005). This neuromodulator regulates the states of certain vision neurons (i.e VS cells) by increasing their activity during flight (Suver et al., 2012).

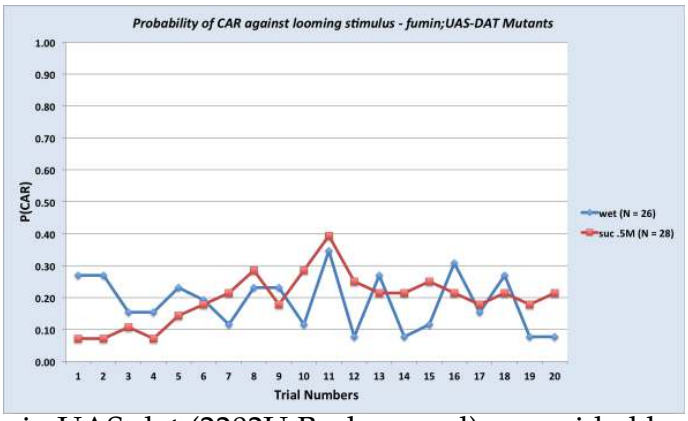


Figure 4.9: fumin;UAS-dat (2202U Background) - provided by Dr. Kazuhiko Kume

Octβ2R is a beta adrenergic-like octapamine receptor has a role on the modulation of the sleep by inducing the wakefulness , although this modulation was observed only in day-time (Crocker, Shahidullah, Levitan, & Sehgal, 2010).

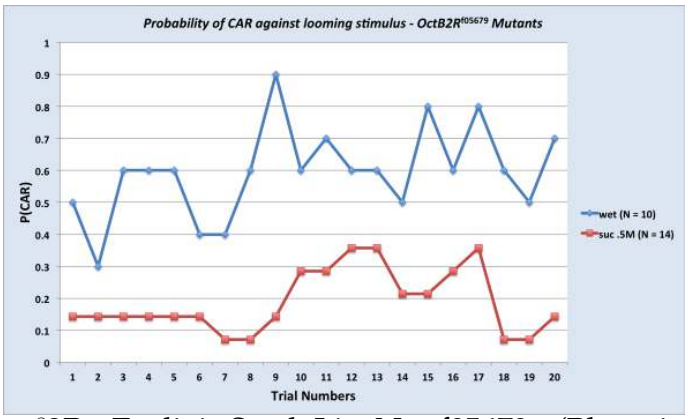


Figure 4.10: Octβ2R - Exelixis Stock List No: f05679 - (Bloomington stock no: 18896)

As can be seen in Figure 4.10, the presentation of the sucrose diminished the probability of collision avoidance response. The difference between sucrose and wet condition was significant $F(1, 22) = 13.645, p < .001, \eta_p^2 = .38$.

4.2.5 Octβ1R mutants

Although there were no clear distinction in probability of CAR between *Octβ2R* and *Octβ1R* mutants on sucrose solution (Figures 4.10 and 4.11), they showed an opposite phenotype on non-sucrose condition, similar to relation between the *Dop1R1* and *D2R* mutations.

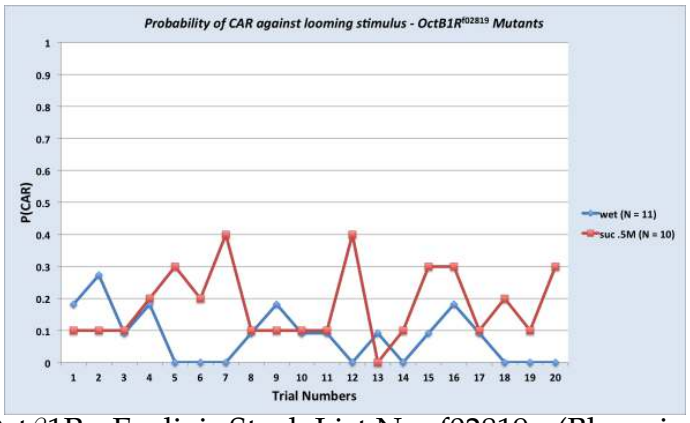


Figure 4.11: Octβ1R - Exelixis Stock List No: f02819 - (Bloomington stock no: 18589)

4.2.6 Summary of the pilot screens

There was a significant effect of the genotype on the probability of collision avoidance response to the visual looming stimulus as might be expected, $F(5, 263) = 31, p < .001, \eta_p^2 = .37$. On the other hand, when wild type flies were compared with these mutant lines on water, as well as sucrose, the sole significant genotype that had affected the measured behavior was *Dop1R1*^{f02676}, $(p < .001)$, when wet condition was evaluated alone by excluding the cases where sucrose was presented, *D2R* was found also significantly different than the wild type strain, $(p < .02)$. On the other hand, this results did not clearly indicated the contribution of neither Exelixis background, heterozygosity nor balancers, thus further studies were conducted.

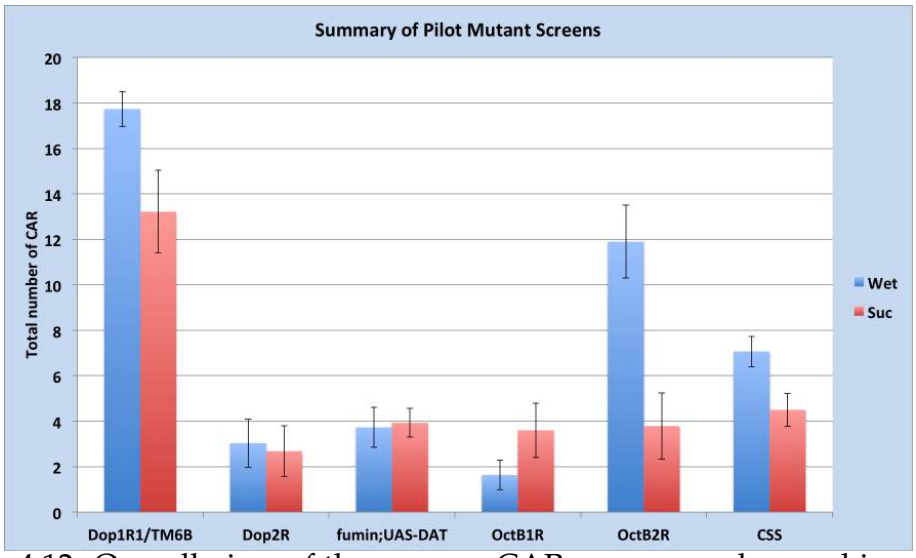


Figure 4.12: Overall view of the average CAR responses observed in mutant screens (the last two bars illustrate behaviors of CSS wildtype strains).

4.3 Dopamine 1-like receptor 1 (*Dop1R1*) mutants

Considering the findings obtained from these pilot mutant screens above, *Dop1R1* receptor mutation was taken into focus for the further analysis as it had evoked the most significant hyperactive phenotype. Dop1R1 flies are maintained as a balanced stock and the balancer can also affect behavior. Furthermore, since these flies had also different background genes than our wild-type stocks, they might also have variant effects on the behavioral responses. Therefore, in order to find and eliminate the potential effects of the balancers and/or the background genes in our analysis, first, *Dop1R1* mutants were outcrossed to the Canton-S background by using # 5906 line from Bloomington *Drosophila* Stock Center that carries TM2, Ubx/TM6C, Sb on a Canton-S background ($w^{1118}/Dp(1;Y)y^{+};TM2/TM6C,Sb^{1}$). Thereafter, the unknown Exelixis background genes were transferred to a strain of the same balancer line, much as the same way yet this time to the opposite direction. The details can be found in the following subsections.

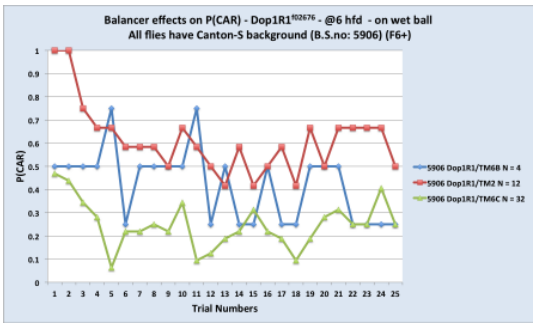
4.3.1 Balacer effects on the observed behavior

The outcrossing was done by crossing the *Dop1R1*^{02676} line to BL # 5906 wild-type background stock 6 or more generations. Thus, Exelixis background genes expected to decrease 50 % on each generation. Therefore, 98.44% of the flies have the intended background genes (i.e. 5906) by the end of 6^{th} cross (i.e. F6).

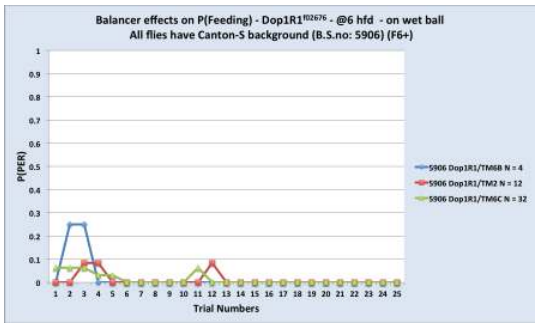
Figures 4.13 and 4.14 show the probabilities of CAR (left panels) and feeding across trials (right panels) when the flies (i.e. *Dop1R1*/Balancer on BL # 5906) were tested on water (upper panels) or 0.5 M sucrose (lower panels) at 6 and 16 hours of food deprivation, respectively.

Similar to the wildtypes, *Dop1R1* heterozygotes on a 5906 background showed lower levels of CAR at 16 hfd ($F(1,268) = 8.16, p < .005$) due to increased levels of feeding at this time ($F(1,268) = 23.3, p < .001$). This suggests that *Dop1R1* function is not necessary for upregulation of feeding by hunger or taste. However, unlike the wild-type flies, *Dop1R1* heterozygotes showed relatively stereotyped CAR patterns that were not affected by the presence of sucrose ($F(1,268) = 1.61, p < .434$), despite the fact that these flies did feed at high levels on sucrose ($F(1,268) = 216.6, p < .001$). In other words, *Dop1R1* heterozygotes did feed on sucrose but that did not change their CARs.

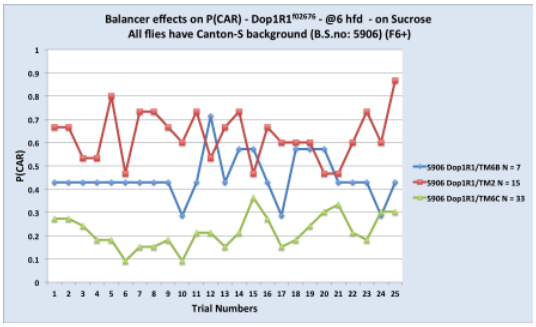
The most prominent result is that, depending on the balancer that it was expressed heterozygously with, *Dop1R1* mutation yielded highly variable levels of CARs yielding a highly significant balancer main effect ($F(2,268) = 53.04, p < .001, \eta_p^2 = .285$). Figures 4.13 and 4.14 show clearly that *Dop1R1* mutation is not sufficient to produce a visually hyperactive phenotype. For example, when expressed on a BL # 5906 background, *Dop1R1*/TM6C, Sb flies (light blue curves) were conspicuously hypo-reactive to the visual stimulus (left panels) irrespective of period of food deprivation (compare figures



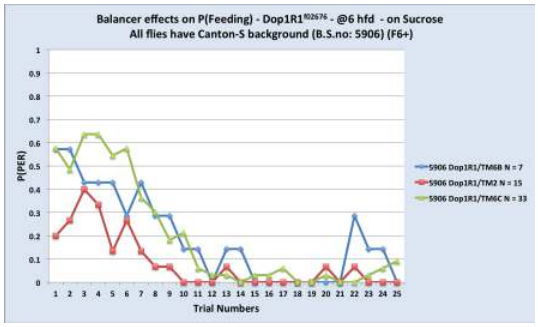
(a) P(CAR) on wet ball



(b) P(Feeding) on wet ball

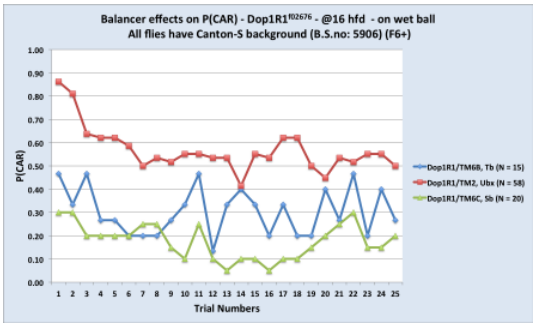


(c) P(CAR) on sucrose

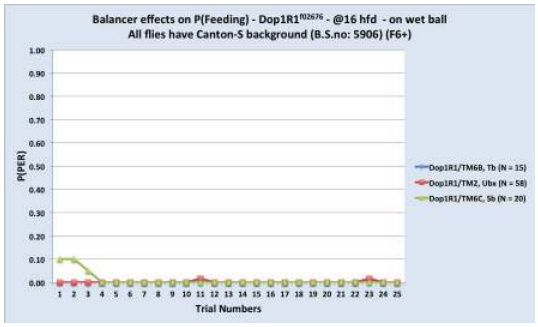


(d) P(Feeding) on sucrose

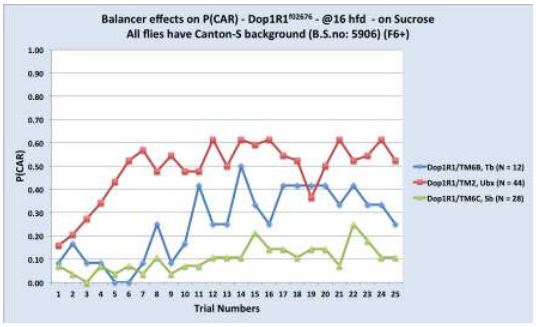
Figure 4.13: Effects of balancers on *Dop1R1*^{02676} - Canton-S background (F6+) (Bloomington # 5906) **@6 hfd**



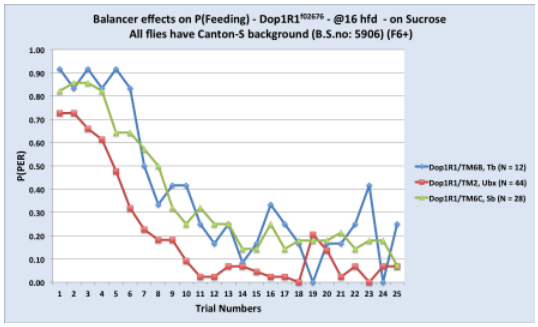
(a) P(CAR) on wet ball



(b) P(Feeding) on wet ball



(c) P(CAR) on sucrose



(d) P(Feeding) on sucrose

Figure 4.14: Effects of balancers on *Dop1R1*^{02676} - Canton-S background (F6+) (Bloomington # 5906) **@16 hfd**

4.13 and 4.14) or sucrose concentration (compare upper and lower panels). In contrast, *Dop1R1*/TM2, Ubx flies (red curves) showed higher levels of CAR. In general, overall levels of CAR decreased in the order of *Dop1R1*/TM2, Ubx > *Dop1R1*/TM6B, Tb > *Dop1R1*,TM6C, Sb, and this order was consistent

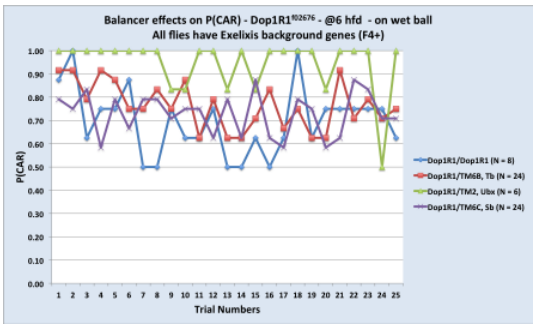
across both the period of food deprivation and sucrose concentration. Further, the relative CAR levels of *Dop1R1*/TM6C, TM2, or TM6B flies cannot be accounted for by suppression by feeding per se, because these three groups of flies fed at similar levels on sucrose (Figures 4.13d and 4.14d).

In summary, *Dop1R1* was not sufficient to cause visually-driven hyperactivity on a 5906 background, suggesting that the background genes of the Exelixis strain contributed to the hyperactivity observed in the *Dop1R1*/TM6B, Tb flies during our pilot mutant screens. Further, the overall levels of CAR (but not feeding) changed with the balancers. Therefore, in an attempt to understand the relative contribution of background genes in the first (X) and the 2nd chromosomes, and the 3rd chromosome balancers to the visually-driven hyperactive phenotype, we outcrossed the TM2, Ubx and TM6c, Sb balancers to the original Exelixis stock that carried *Dop1R1*/TM6B, Tb, and compared the phenotypes of *Dop1R1* heterozygotes of three different balancers on an Exelixis, rather than Canton-S background. The results of these experiments are explained in the next section.

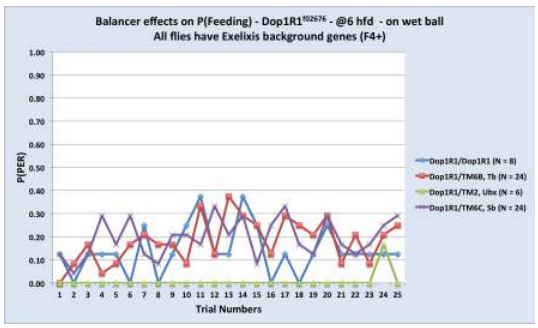
4.3.2 Genetic background effects on the observed behavior

The results given above indicated the balancers affect visual responsivity, although they were not sufficient to argue whether the hyperactive phenotype is caused by *Dop1R1* or background genes. The hyper-responsiveness observed in the pilot experiments (Figure 4.7) decreased in all balancer groups at 6hfd on wet ball (Figure 4.13a), when flies were outcrossed to Canton-S background. To be more clear, in these Figures 4.7 and 4.13a, the blue curves represent the collision avoidance responses of *Dop1R1*/TM6B, Tb flies that had exact same pair of 3^{rd} chromosomes, in the same experimental conditions (i.e. at 6 hfd, on wet ball, TR, ITI and all stimulus parameters), therefore the possible causes for the differences in this behavior was the remaining chromosomes (i.e. first, second and the sex). For that reason, the same outcrossing methodology was applied to TM2, Ubx/TM6C, Sb balancer line, although this time, the wildtype background of this line was replaced with the background genes coming from Exelixis stock. Once the outcrossing was done for 6 and more generation, the flies had the exact 3^{rd} chromosomes.

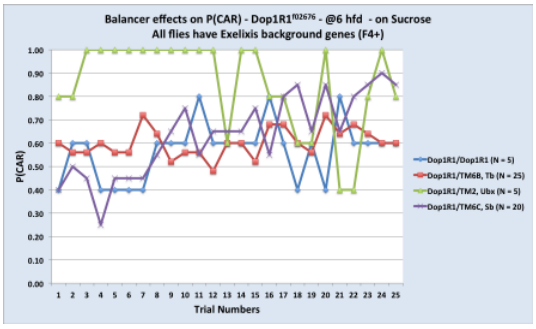
Figures 4.15 and 4.16 represented the effects of the Exelixis background on the observed behaviors. The following ANOVA results showed that both the effect of genotype, $F(3, 187) = 5.828, p < .001, \eta_p^2 = .085$ and gustatory stimulus, $F(1, 187) = 0.069, p < .001, \eta_p^2 = .038$, on the probability of CAR were significant. Scheffe test was used for post hoc analysis, it indicated that the sole different group was *Dop1R1*/TM2 (i.e. $p < .001$ for TM6B, $p < .01$ for TM6C and $p < .053$ for homozygous). When the same analysis steps were done for feeding behavior excluding non-sucrose conditions, the effect of genotype on the probability of PER was significant, $F(3, 93) = 4.858p < .01, \eta_p^2 = .135$, in contrast with that the effect of hfd was again not significant $F(3, 93) = 4.858p = .171$.



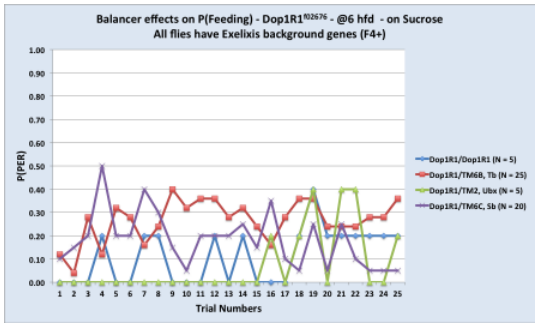
(a) P(CAR) on wet ball



(b) P(Feeding) on wet ball

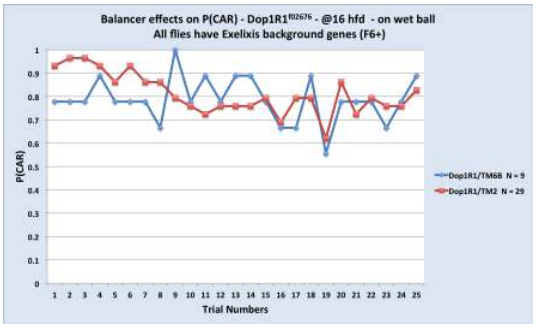


(c) P(CAR) on sucrose

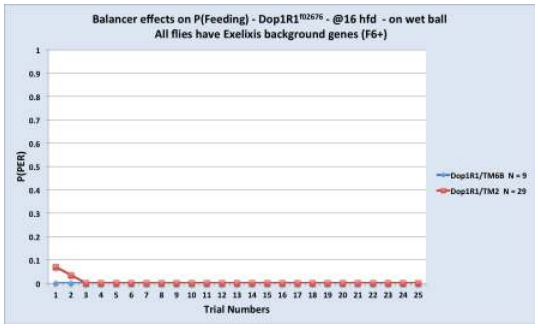


(d) P(Feeding) on sucrose

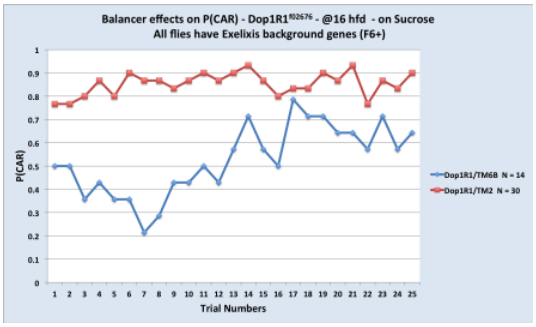
Figure 4.15: Effects of balancers on *Dop1R1*^{02676} - Exelixis background genes (F4+) **@6 hfd**



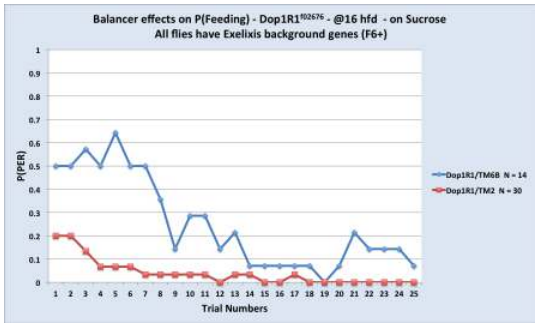
(a) P(CAR) on wet ball



(b) P(Feeding) on wet ball



(c) P(CAR) on sucrose

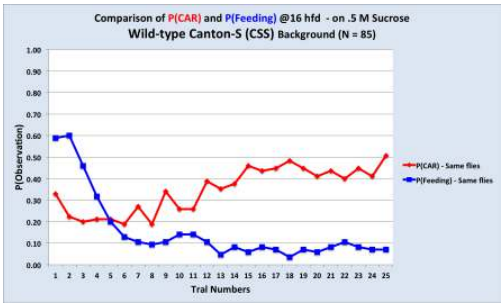


(d) P(Feeding) on sucrose

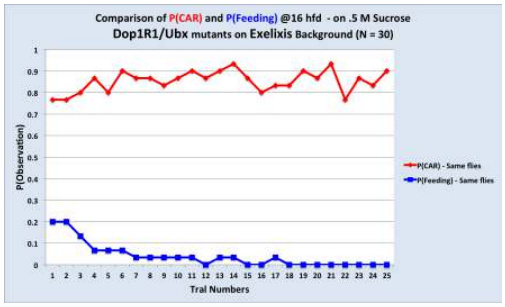
Figure 4.16: Effects of balancers on *Dop1R1*^{02676} - Exelixis background genes (F6+) **@16 hfd**

4.3.3 Discussion on attention-like mechanisms

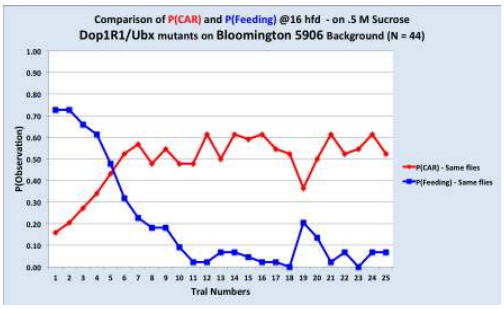
Considering the sample sizes, as well as food deprivation effects, *Dop1R1*/TM2, Ubx flies were particularly analyzed and compared with wild-type flies on .5M sucrose at 16 hfd. Figure 4.17 shows the feeding and CAR responses of 3 different groups: *Dop1R1*/TM2 on Exelixis background (4.17b), Ubx *Dop1R1*/TM2, Ubx on 5906 background (4.17c) and wild-type control group (4.17a). In all of these figures, red curves represent probability of CAR while blue curves represent the probability of feeding of the same group.



(a) Wild-type Canton-S (CSS)



(b) *Dop1R1*/TM2 on Exelixis back.



(c) *Dop1R1*/TM2 on 5906 back.

Figure 4.17: Comparison of 3 different groups in the same experimental conditions (@ 16 hfd - .5M Suc)

A common methodology to study attention is to use distractor stimuli when the task is to attend a particular subset of stimulus (de Bivort & van Swinderen, 2016). Temporal continuity may indicate an underlying mechanism for attention. Since the evaluation of environmental stimuli and internal states change dynamically in time, the resistance to distraction or alternation between stimuli are the indicators of the attentional processes of the particular task.

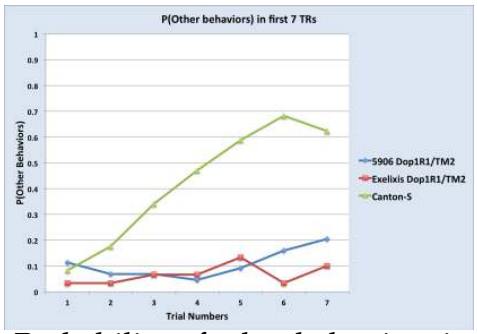
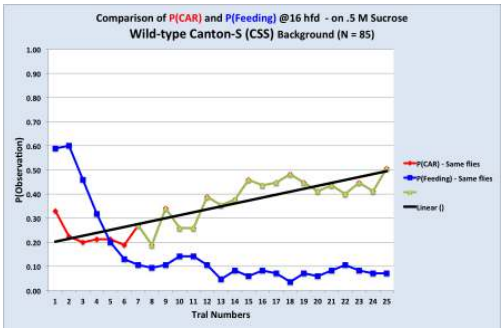


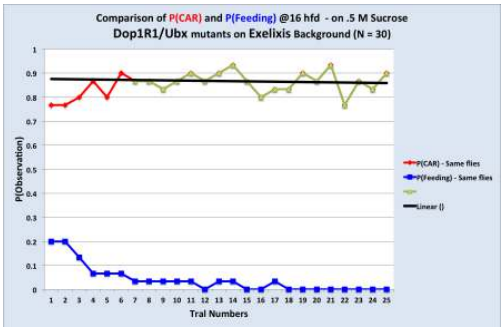
Figure 4.18: Probability of other behaviors in first 7 trials

Dop1R1 mutants showed two consistent differences from wildtype flies, irrespective of their genetic background.

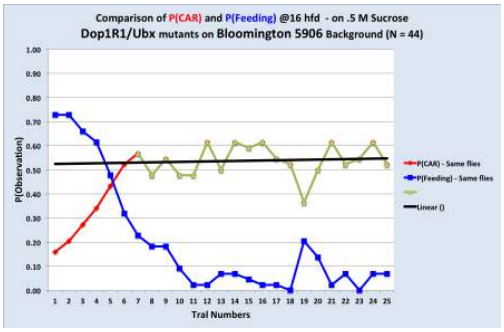
1. Dop1R1 flies fail to disengage from stimulus-driven (bottom-up) responses during the initial part of the session. Figure 4.18 shows the probability of engaging in internally generated behaviors over the first 7 trials for wildtype and Dop1R1/TM2 flies on Canton-S and Exelixis backgrounds. On an Exelixis background, Dop1R1/Tm2 flies get lodged into a state of non-habituating visual responsiveness that cannot be suppressed either by other externally-driven (e.g., feeding) or internally-driven behaviors. In contrast, although they are overall more visually hyperactive than the wildtype controls, Dop1R1/TM2 flies on a 5906 background show an initial feeding-driven suppression of CARs during the first 7 trials. However, their behavior during this period is again controlled either by a visual or by a gustatory stimulus, and their probability of engaging in a stimulus-independent, internally generated behavior is still lower than that of the wildtype flies. This continues until satiation where they return to their high baseline level of visual responsiveness that is observed when the flies are tested on a wet ball. That is, the effects of a gustatory stimulus can only be suppressed by a negative feedback from the feeding system (i.e., satiation) whereby the Dop1R1 flies shift to the state of higher responsiveness to visually-driven behavior. Wildtype flies also engage in higher stimulus-driven behaviors in the beginning of the session, but they display a more rapid disengagement from the effects of either stimulus within the first 10 trials.



(a) Wild-type Canton-S (CSS)



(b) *Dop1R1*/TM2 on Exelixis back.



(c) *Dop1R1*/TM2 on 5906 back.

Figure 4.19: Habituation/Sensitization after trial 7)

2. Dop1R1/TM2 flies fail to show a late-onset sensitization of CAR. Fig-

ure 4.19 shows the regression trends in the probability of CAR after trial 7. Canton-S flies display a steady increase in visual responsiveness until the end of the session. Dop1R1/TM2 flies show an exclusively high responsiveness to the visual stimulus throughout the session, so the failure to sensitize might be the result of a ceiling effect. However, Dop1R1/TM2 flies on 5906 background also show a similar steady-trend despite the fact that there is room for sensitization for these flies. Therefore, irrespective of the variability in the genetic background which modifies the overall level of responsiveness to the visual stimulus, Dop1R1/TM2 flies show a habituation and/or sensitization resistant default responsiveness that is not modulated by the internal state of the fly (e.g., hunger) to the extent observed for the wildtype flies.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

5.1 Conclusion

In this study, the underlying preliminary mechanisms that were necessary for shifting attention between different sensory modalities were taken into the consideration. The parameters of the external stimuli, as well as the endogenous states of the organism can affect these mechanisms. Attention was addressed as a dynamical function embedded on neural networks to produce most suitable motor response for the current situation. Modulating the synaptic gain was considered as the primary mechanism that uses this dynamical function to behave accordingly to the current environmental and internal needs. Moreover, these attentional mechanisms were investigated by taking the fruit fly, *Drosophila melanogaster* as the model organism. The first cross-modal attention protocol for fruit flies was used to conduct experiments on wild type flies to understand the nature of the fly's attention for particular situations. Furthermore, certain genetically engineered mutants were assayed and compared with these wild-type controls to indicate the role of dopaminergic modulation in the attentional allocation.

Our results indicated that attention-like processes likely exist even in a relatively simple animal such as the fruit fly. In our experiments, wild type animals could shift their attention more easily and appropriately for the present situation. Wild-type flies rapidly habituated to the visual looming stimulus in first 5 to 6 trials. After continuous presentation of the visual stimuli, a slight sensitization was observed towards the end of the sessions. The longer hunger periods enhanced the visual responsiveness when the sucrose was not presented. However, the presentation of sucrose decreased the probability of collision avoidance reflex.

Dop1R1/TM2 mutants showed behavioral patterns that can be considered as markers of a cross-modal suppression mechanism. Exelixis background, together with this dopamine receptor mutation promoted a visually-driven hyper-responsive state whereas *Dop1R1*/TM2 mutants on BL # 5906 background were more responsive to the feeding with respect to control group. Both background groups lodged in a particular state and could not show neither habituation nor sensitization to the current situation after initial feeding responses. On the other hand, balancer and background effects cannot

clearly excluded from the mutation effects. However, our findings characterized the dopaminergic modulation as a necessary condition for disengaging from stimulus-driven responses according to the current environmental and internal needs.Therefore, the dopaminergic modulation is suggested as the first cross-modal attention mechanism necessary to control attention in *Drosophila melanogaster*.

The regulation of the human attentional processes by dopamine is a well studied area. Today, the neuropharmacologic agents that targets dopaminergic system were widely used in the treatment of attention disorders such as Attention Deficit Hyperactivity Disorder (ADHD) (Rubia et al., 2009). The patients suffering from ADHD had problems on driving their attention appropriately by being whether in a low responsive (i.e. hypo-active) state within which they cannot attend particular events, or in a hyper-active state within which they lodged in the currently engaged activity with a high arousal level (Konrad, Gauggel, Manz, & Schöll, 2000). Our findings may suggest that the mutation on the *Dop1R1* receptor gene promoted a particular *Drosophila* phenotype which might be correlated with ADHD. Because they both involve lodging in a particular states where they cannot behave according to the current situation, as well as, these mechanisms are both modulated by dopaminergic system. Although there are many *Drosophila* models proposed for various psychological disease, there is not any fly model for ADHD. Considering the stereotypical similarities, this study proposed that the *Drosophila Dop1R1* receptor mutants may be used as an endophenotype for the psychological researches on ADHD.

5.2 Future Directions

Conducted rescue experiments were not included to this study, however, it is crucial to identify particular neurons that take part in the attentional processing by using binary expression systems such as GAL4-UAS. The pilot rescue experiments indicated that the sufficiency of the role of R2/R4m neurons on driving the attention-like processes in the fruit fly brain (Çevik & Serhan, 2015).

Thermogenetic methodologies can be used to understand the involvement of the long and short term memory formation on the attentional selection within this experimental protocol. Two GAL4 lines (i.e. $GAL4^{R15A04}$ for STM and $GAL4^{R48B04}$ for LTM) selectively target different set of dopaminergic neurons in the PAM cluster which were arborized to modulate certain parts of the mushroom body to control distinctively long term and short term memory formation by changing the gain of reward signals for certain appetitive stimuli (Yamagata et al., 2015). These reward signals for the appetitive reinforcement can also distinguish the nutritive value and the sweet taste of the gustatory stimulus. In addition to these drivers, targeting neurons in PAM cluster with the $GAL4^{R58E05}$ might be elucidative within the cross-modal attention protocol, since remotely activating these dopaminergic neurons promoted strongly the long term memory formation when flies were starved (C. Liu

et al., 2012). This GAL4 line might be also useful to corroborate the idea of the internal state, since the induced activation of these dopaminergic neurons did not trigger any observable treat such as the proboscis extension response, while forming apetitive memories.

The clues on the various temporal points of the experimental sessions (e.g. the steep drop observed around 5^{th} trial - 30 s -) might indicate a processing time of the release of a particular neuromodulator at a particular neural cite. In order to pursue this type of clues, first, the inter-trial interval duration can be changed to determine whether there exist a particular pattern occurred on a specific time or not. If an existed regularity might be found, then further investigation can be done to capture both the responsible circuit and underlying temporal structure by using calcium imaging

The stereotypicality that was observed in *Drosophila* Dop1R1 receptor mutants may pave their way for being accepted as an endophenotype of Attention Deficit Hyperactivity Disorder (ADHD). Screening and investigating latent behavioral patterns of various *Drosophila* mutants lines by individuality analyses may reveal other endophenotypes for many other psychological and neurological diseases. In addition to that, such analysis based on the individual differences within groups, contradistinctively to numerous studies in which the individual animals were usually treated as identical members, may uncover the evolutionary roots of the personality.

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