Neuronal Control of Steering Maneuver in *Drosophila*

A thesis submitted in partial fulfilment of the requirements for the award of the degree of

Master of Science

by

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under the supervision of

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June, 2020

Declaration

I hereby declare that this thesis is my own work and to the best of my knowledge, it contains no materials previously published or written by any other person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at IISER Kolkata or any other educational institution, except where due acknowledgement is made in the thesis.

Certificate

To whom it may concern

This is to certify that Mr. Naman Agrawal completed his BS-MS final year thesis under my supervision. The title of his thesis is "Neuronal control of steering maneuver in *Drosophila*". His work is original and has not been previously published in any journal or conference proceedings.

I wish him all the best for future research endeavours.

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Acknowledgments

First of all, I would like to acknowledge my parents for providing me with all the opportunities, motivation and freedom to pursue sciences. It is due to their hard work and sacrifices that I am able to present this thesis today.

I thank Center for Advanced European Studies and Research (CAESAR) and Max Planck Foundation for providing me with the infrastructure and resources to carry out my research. I thank my teachers in Indian Institute of Science Education and Research Kolkata for nurturing my interest for Life Sciences through some of the coursework. I thank Innovation in Science Pursuit for Inspired Research (INSPIRE) for providing me fellowship during my study in IISER Kolkata.

I would like to thank my MS project guide, Dr. Bettina Schnell for providing opportunity and liberty to work in her lab. Her necessary inputs and guidance not only made this whole project possible, my work smoother but also taught me important lessons in ways of daily scientific research. I would like to thank Dr. Bidisha Sinha, my co-supervisor in IISER for handling all the logistics and giving me her inputs on how I can improve upon the project.

I thank my lab members - Philippe Fischer, Elhanan Ben Yishay and Cemre Kizilarmut for their support in the lab. I thank my friends Ezgi Bulca, Ivan Vishniakou, Andres Flores Valles and Giacomo Basseto for their warm friendship and support outside the lab. I would also like to thank Dr. Bjoern Brembs, Dr. Krushnamegh Kunte and Dr. Tina Mukherji, the internships in whose labs gave me the necessary skills needed to work on this project.

I want to thank my friends - NSCB first floor - for making these 5 years in IISER perhaps the best years of my life. I want to thank Aarshi - the Dramatics club of IISER Kolkata, for being the one place I could call home. I also want to thank my Improv Theatre group in Bonn - strangers in a strange country, making it feel like home again. I want to thank some of the strongest women I know - Simli Mishra, Meghana Oak, Anchita Addhya, Avani Koparkar, Gaia Bianchini, Stefanie Steffan - for being the beacon of inspiration for me, and carrying my boat through stormy days. Thank you Skies of IISER, for being an outlet of creative spirit. Thank you BoJack, for making me a better person.

It was nice while it lasted.

O genotype! O phenotype!
the kiss had better last her
He is off to see his first love
Drosophila melanogaster

Abstract

Flying is a complex computational process, which involves path stabilization and object avoidance. *Drosophila melanogaster* is an amiable model organism to study neural underpinnings of flight control maneuvers due to its genetic amenability and small, well characterized nervous system. Previous studies have shown a particular descending neuron, termed **AX neuron**, to be an important controller of turning maneuvers in flies. However, the study had inadvertently activated some other neurons alongwith the AX neuron while studying the behavior. In this project, I confirmed that the AX neuron is indeed a controller of the left-right steering response by using a split-GAL4 approach that specifically labelled AX neuron only. I expressed P_2X_2, a cation channel, in the AX neuron. P_2X_2 opens up upon binding to extracellular ATP and depolarizes the cells. I observed the steering response of flies by looking at difference in Wing Stroke Amplitude between the Left and the Right Wing (L-R WSA) and I found that activation of the AX neuron by injecting ATP on one hemisphere of the brain triggers an ipsilateral turn by the fly. This project lays down the groundwork for similar studies to be carried on other descending neurons, which have similar anatomical characteristics as the AX neuron and might also play a role in controlling mid-flight changes of direction.

HS cells (Horizontal system cells) in flies have been known to play a role in path stabilization by responding to translational and rotational visual stimuli along the vertical axis of the fly. There have been studies investigating the role of HS cells in stabilizing rapid left-right steering maneuvers, however their exact functionality in directional changes has not yet been unequivocally confirmed. Following a similar approach as in AX neurons, I expressed P_2X_2 in HS cells and activated them for different time lengths in one hemisphere of the brain. I did not observe any change in the behavior of the fly upon activation, and hence I concluded that activation of HS cells by P_2X_2 might not be enough to elicit a turning response. This results differs from other studies where HS cells were activated optogenetically and flies elicited a turning response.

Contents

Chapter 1

Introduction

If your subject has ever moved his/her head while you are pressing the shutter on your camera, you probably know how motion blur can ruin a beautiful image. Yet, we as humans, are rarely aware of this motion blur. The camera we carry in our heads all the time, our eyes, experience and successfully perceive moving subjects. This seemingly straightforward but computationally complicated feat is achieved by quick punctuating jumps in eye movements, also known as saccades. (Robinson, 1964)(Theobald, 2017).

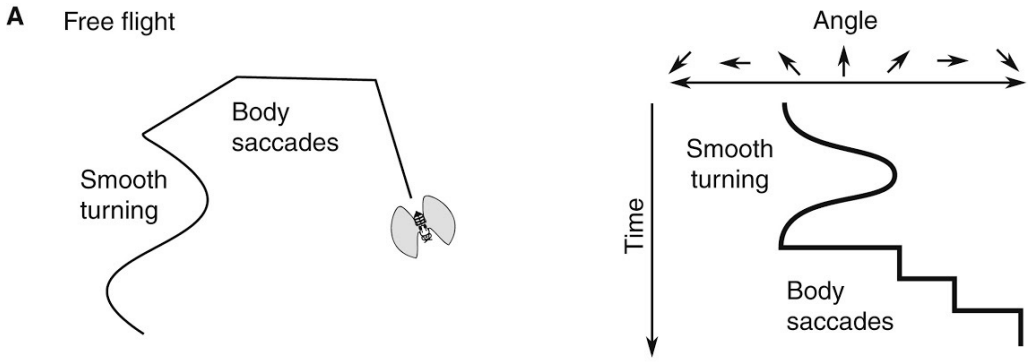


Figure 1.1: Illustration of a Drosophila exhibiting a smooth turning maneuver followed by a body saccade (Theobald, 2017)

These rapid jumps, or saccades, allow you to fixate a small region of the visual landscape on the fovea (a tiny spot on the macula lutea region of the retina, which has densely packed cone cells, and hence gives the clearest vision (Iwasaki & Inomata, 1986). This allows the brain to take in a stable and unblurred image, and then immediately switch to the other interesting spot. With a speed of about 500 degrees per second, these rapid eye movements are easy to miss by the eye. They are constantly shifting from one scene to another without you consciously noticing it. Eye saccades occur at a frequency of about three to four times a second, and so occupy fully about 10 percent of your day (Land & Tatler, 2009) while you navigate around the world, meet your friends, and even read this document. Insects, however, have immovable fixed eyes. Thus most of their gaze shifting occurs through whole body turning.

Insect flight is characterized by long straight flight segments interspersed with whole

body saccades (van Breugel & Dickinson, 2012). The straight flight segments are a result for gaze stabilization strategy known as optomotor response, which is seen in most organisms. A common reflex in most organisms is to follow the angular motion of visual stimuli in their visual field, which is also known as optomotor response. However, whenever an animal deviates from a straight path, the surrounding visual objects seem to to rotate in opposite direction. The tendency to turn with the surroundings due to the optomotor reflex compensates for the deviations from the previous course and hence stabilizes the path in a straight direction (Goetz, 1968).

The body saccades in *Drosophila* account for at least 80% of total net change in heading during free flight in *Drosophila* (van Breugel & Dickinson, 2012). Further, several studies have suggested that saccades in Drosophila are triggered by a visual looming stimulus, thus suggesting that saccades might serve as collision-avoidance reflex (Tammero & Dickinson, 2002; Stewart et al., 2010; Reiser & Dickinson, 2013; Censi et al., 2013). However, a few studies have also shown that saccades are also triggered spontaneously by some internal stochastic mechanism which might have a function in improving food foraging efficiency (Maye et al., 2007; Reynolds & Frye, 2007).

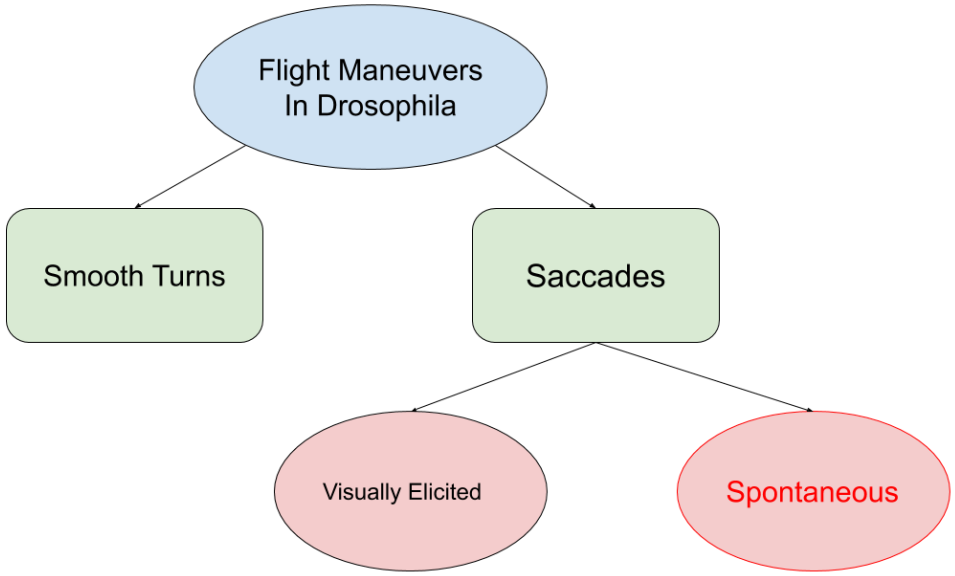


Figure 1.2: Illustration of different types of flight maneuvers in *Drosophila*

Without going into the functional utility of **spontaneous saccades, the neural circuitry underlying them is of interest to us in this project.**

Chapter 2

Theory

In this section, we will discuss the theoretical basis of the genetic tools used in the project.

2.1 GAL4/UAS system

Drosophila Melanogaster has been widely used as a model organism due to the wide range of tools that are available for genetic manipulation. One such tool is the famous GAL4/UAS system. It is a system for in-vivo targeted gene expression in a spatially controlled fashion, and is used by fly geneticists all around the globe. For example, it has led to the identification of conserved genes involved in apoptosis (Wing et al., 2002), tyrosine kinase signaling(Guichard et al., 2002) and neurodegenerative diseases.

The GAL4/UAS system, described by Brand and Perrimon in their seminal 1993 paper, is a bipartite system (Brand & Perrimon, 1993). This bipartite system is based on the properties of the yeast Saccharomyces cerevisiae GAL4 transcription factor, which activates transcription of its target genes by binding to specific cis-regulatory sites called UAS (Upstream Activation Sequence).

The bipartite system functions by generating two seperate genetic fly lines **(see fig. 2.1)**. The Driver line contains the regulatory element-GAL4 construct, providing precise stage, cell or tissue specificity. The responder line contains the target gene placed under the control of a basal promoter associated with a UAS site optimized for GAL4 binding. When the two lines are crossed, the resultant progeny contain both the RE-GAL4 and UAS-Target Gene construct, thereby making the system functional. The GAL4 binds to and activates the UAS, and subsequently the target gene, in the tissue specific manner that reflects the expression pattern of the regulatory element. This has led to the production of an astounding array of GAL4 drivers for targeting expression to almost every major tissue type.

2.2 split-GAL4/UAS system

Though the binary GAL4/UAS system by itself is a fantastic tool for genetic manipulation, it can be made even more specific by using intersectional strategies. GAL4

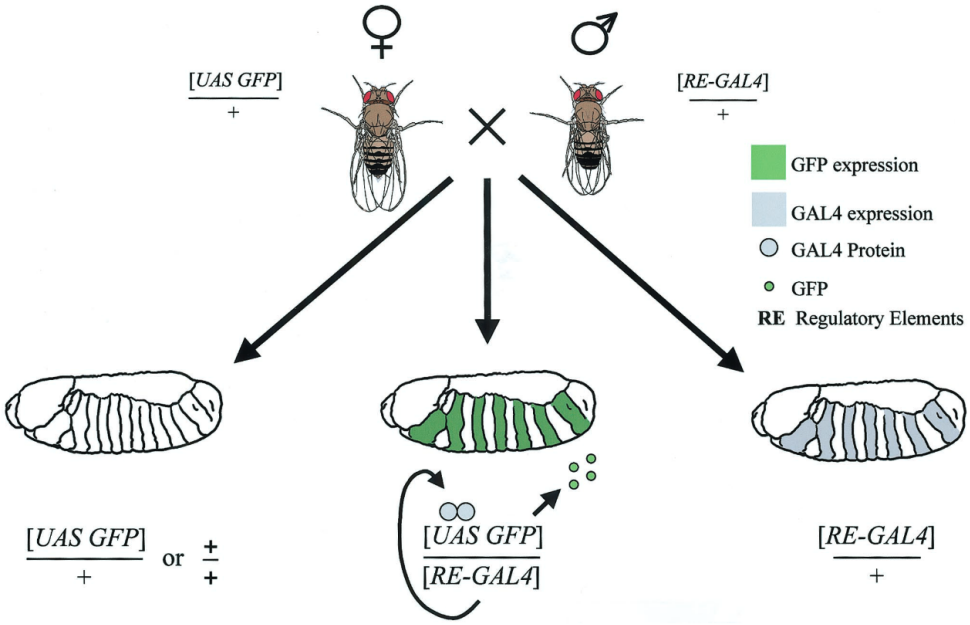


Figure 2.1: The bipartite UAS/GAL4 system in Drosophila. When females carrying a UAS responder(UAS-GFP) are mated to males carrying a GAL4 driver progeny containing both elements of the system are produced. The presence of GAL4 in an alternating segmental pattern in the depicted embryos then drives expression of the UAS responder gene in a corresponding pattern(Duffy, 2002)

consists of two separable functional domains for site specific DNA binding (DBD) and Activation Domain (AD), but neither of them can activate transcription on their own. However, when joined either by covalent or non-covalent bonds, they can restart gene expression (at the appropriate promoter sites). As depicted in **figure 2.2**, the split GAL4 system makes use of this modularity, where GAL4 is divided into DBD and AD, which can be independently expressed using different promoters (P1 and P2). Each domain is fused to a heterodimer Leucine motif (Zip+ or Zip-), so that the two domains bind when expressed in the same cell to become transcriptionally active. When these constructs ("hemidrivers") are brought together in crosses to flies bearing a UAS-transgene, the transgene is expressed in progeny only at the intersection of the expression patterns of P1 and P2 **(see fig.2.2)**. Thus, the split-GAL4 system can be used to achieve almost single cell specificity (Luan et al., 2006).

2.3 P_2X_2

We have so far identified methods of genetically targeting a neuron for expressing our protein of interest in it. Naturally, the question arises of the choice of protein that should be expressed in the neuronal surface to activate them artificially. The current external activation methods encompass - (1) activation using optogenetics,(2) Activation using temperature shifts by TRP channels, and (3) activation using external ligands. We will consider each of these methods one by one.

Optogenetic techniques enable one to target specific neurons with light-sensitive

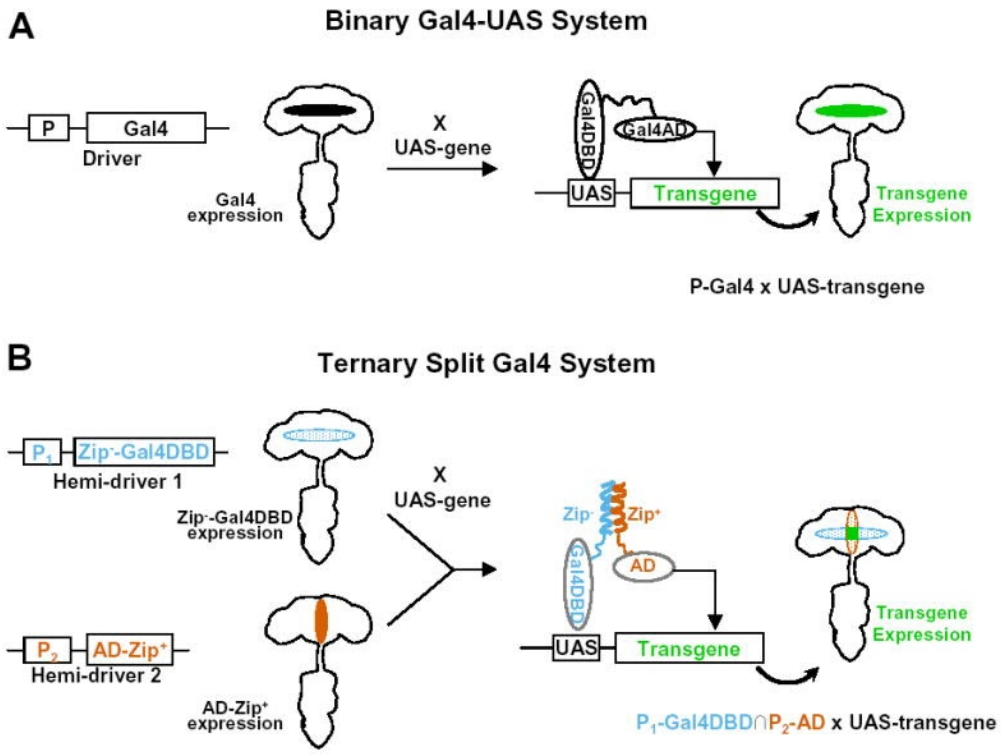


Figure 2.2: Comparitive figure between GAL4/UAS system and split-GAL4 system. The split-GAL4 system splits the GAL4 domain in AD domain and DBD domain which bind later with a Leucine motif to activate transcription of UAS-responder in a highly specific stage, cell or tissue (Luan et al., 2006)

proteins. Currently, preferred light sensitive proteins include *ChR2*, a green light (470 nm)-sensitive nonselective cation channel derived from the green alga *Chlamydomonas reinhardtii*, and *CsChrimson*, an artificial red light (590 nm)-activated channel with the extracellular N-terminus of the *Chlamydomonas noctigama* CnChR1 'Chrimson' gene has been replaced with the corresponding sequence from the *Chloromonas subdivisa* CsChR gene, which are both light gated cation channels (Klapoetke et al., 2014). The thermal activation approach involves using dTRPA1 (an ortholog of the mammalian TRPA1 channel), which is a temperature and voltage-gated cation channel that regulates Drosophila thermotactic behavior. Previous work has shown that neurons expressing dTRPA1 begin firing action potentials when ambient temperatures rise above 25°C(Hamada et al., 2008). By ectopically expressing TRPA1 (using the GAL4-UAS system), and then delivering modest heat pulses, sets of neurons can be remotely activated in freely behaving animals (Hamada et al., 2008). However, using either of these methods is not a viable solution for us. It is because, in this project, we are trying to investigate the turning behavior of flies towards one direction. Therefore, naturally, we need to activate the neurons responsible for turning only in one hemisphere of the brain. Wing descending neurons (see 3.1), the neurons that we will be targeting in this project, are bilaterally present. Therefore, activating them bilaterally would result in cancelling out of the left and right turn, and therefore the result will be nullified. At the moment there exists few methods of unilateral expression of ectopic receptors, and hence for the sake of simplicity, we will use the third approach of unilateral activation using external ligands.

P2X receptors are cation-specific membrane channels that open on binding to external ATP. Homomeric P_2X_1, P_2X_2, P_2X_3, P_2X_4, P_2X_5, and P_2X_7 channels and heteromeric $P_2X_{2/3}$ and $P_2X_{1/5}$ channels have been most fully characterized following heterologous expression. They are multimeric with each subunit consisting of two transmembrane domains, with one extracellular domain connecting the two (North, 2002)**(see fig. 2.3)**. Though there are several interesting characteristic properties of P2X receptors, the feature that is of most use to us is the fact that P2X receptors are absent in fruit flies (Littleton & Ganetzky, 2000). This is of huge importance, since now we can be sure that the ectopic expression of P_2X_2 in the target neurons will give us the cellular level of specificity upon external application of ATP. In a previous study, this system has been used for targeting and activating the **giant fibre neurons** in *Drosophila*. Upon ATP application and subsequent activation of the giant fibre neurons, the flies elicited the typical GF-mediated escape movements, leg extension, jumping, wing opening, and high-frequency wing flapping (Lima & Miesenböck, 2005).

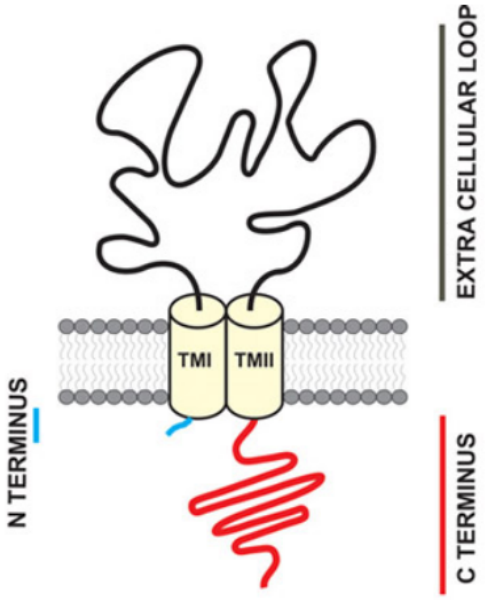


Figure 2.3: Illustration of the topology of P2X receptor subunit(Singh et al., 2009)

Thus we chose P_2X_2 as the receptor for neuronal activation to be used in our investigation.

Chapter 3

AX neuron

3.1 Introduction

We know that in most animals the brain integrates sensory information from central and peripheral sensory systems, as well as contextual information and the internal state of the animal. The computation that follows determines the appropriate behavioral response. While the brain controls the neural circuits responsible for this computation, the motor neural circuits directly responsible for behavior are located near the muscles they control - in the ventral nerve cord.
What then is the connecting link between the brain and the muscles?

In flies, as in most invertebrates, the commands from the brain are sent to the motor neuropils by a set of neurons known as **descending neurons**. The descending neurons occur in about 250-550 pairs, which is approximately about 0.5% of the total number of neurons (Hsu & Bhandawat, 2016). Thus, the descending neurons represent a crucial bottleneck of the information flow, and hence are of interest to this study. Descending neurons arborize (extend dendrites) in 20 highly conserved clusters in the brain involved in sensory processing and motor behavior, and each descending neuron extends a single axon from these clusters to the VNC, where it synapses onto separate interneurons (associated with controlling various muscles - leg, neck and wings (Namiki et al., 2018)), or directly onto the motor neurons. Using state-of-the-art genetic tools, Namiki et al. anatomically characterized 190 pairs of descending neurons divided into 54 distinct cell types.
Previous studies have shown that activating individual DNs is often enough to elicit a specific behavior such as leg movements and walking directions (Bidaye et al., 2014; Kien & Altman, 1984), courtship behavior and singing (von Philipsborn et al., 2011; Kohatsu et al., 2011) or escape behavior (King & Wyman, 1980). Thus DNs are a strategic target for studying neural circuitry underlying saccades.

Indeed, a study by Schnell et al. identified a particular descending neuron, tentatively labelled as **AX neuron**, whose activity strongly correlates with both visually elicited and spontaneous saccades. Through *calcium imaging* and *patch clamp recording*, Schnell et al. confirmed that whenever the fly elicits a rapid turn, there was activity in the AX neuron. To test whether activating an AX neuron is enough to elicit a saccade, the GAL4-UAS system was used to express P_2X_2, an ATP-gated ion channel, and GFP in the AX neuron. It was observed that upon injecting ATP

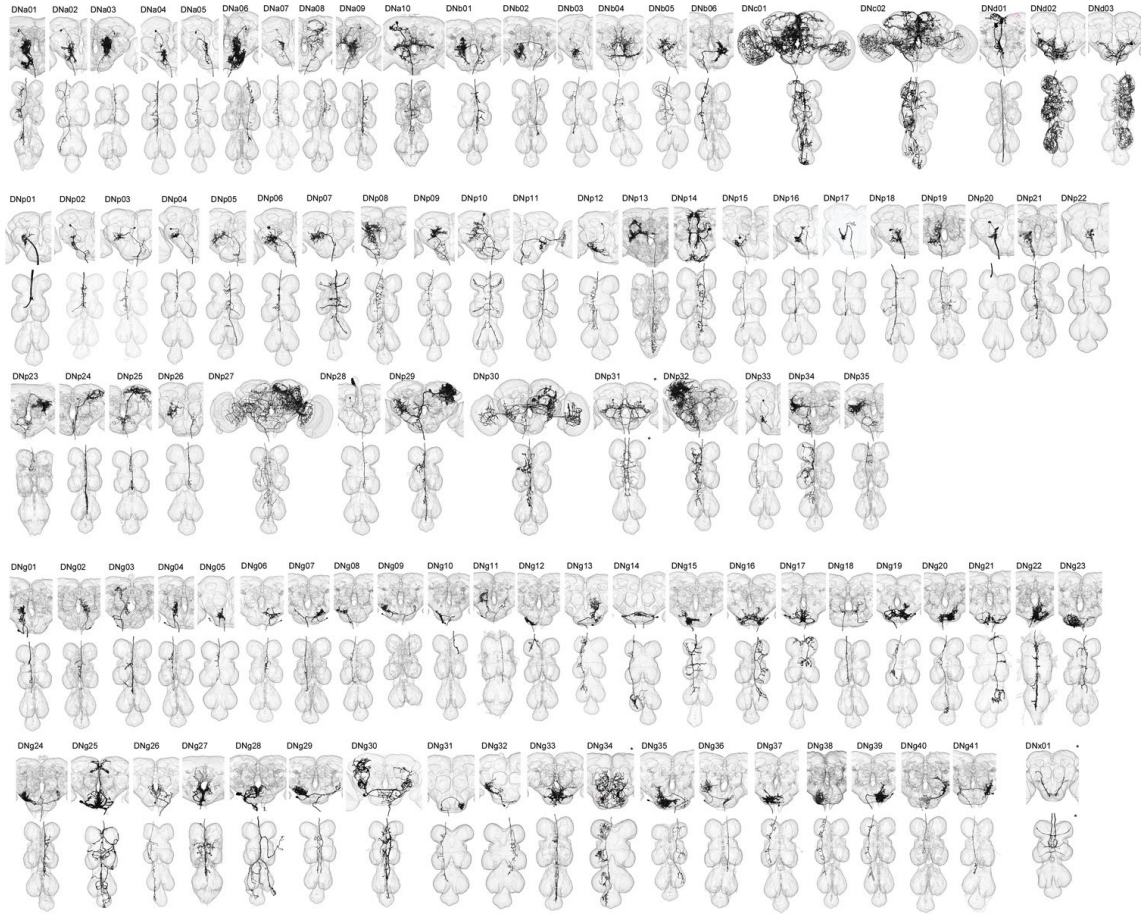
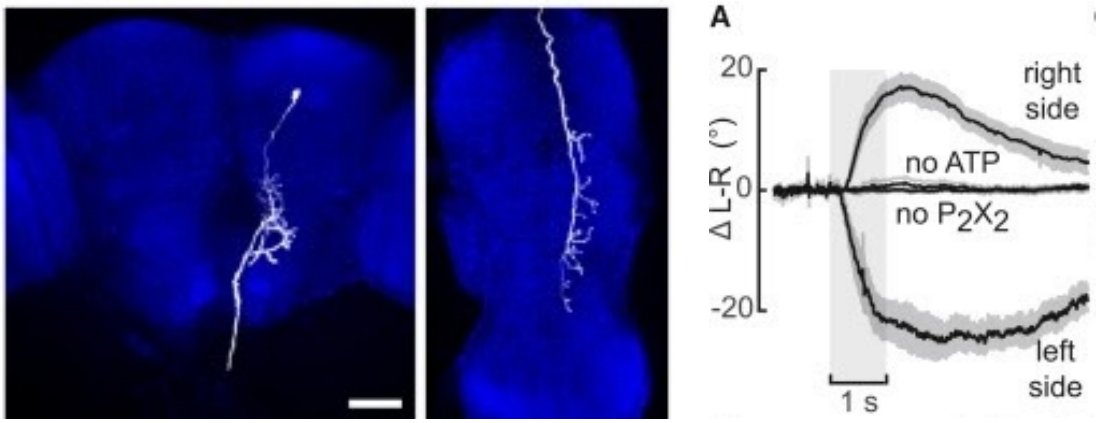


Figure 3.1: Morphology of descending neurons identified in the present study. Neurons (black) and neuropil regions of the brain and VNC are shown (transparent). A total of 98 different cell types are shown (Namiki et al., 2018)

near the dendrites of the AX neuron expressing GFP and P_2X_2 on only one side of the brain, the flies exhibited a turn towards the ipsilateral side (Schnell et al., 2017)**(see fig. 3.2)**.

This particular experiment forms the basis of this project. The shortcoming of this experiment was that though the GAL4 line labelled the AX neuron, it was not very specific. Thus there were some other neurons that were marked along with the AX neuron, and subsequently expressed P_2X_2. Thus we may suspect that they too were activated along with AX neuron when ATP was injected. To solve this problem, in this project, I used the split GAL4/UAS system(see chapter 2, section 2) to mark the AX neuron with close to 100% specificity, expressed P_2X_2 in these cells, and hence when we apply ATP near its dendrites, I can be sure that the phenotype observed will be due to the standalone activity of the AX neuron.
In the first step, to test for the sufficiency of AX neuron to elicit a saccade, we first check whether the AX neuron is sufficient to elicit a turning maneuver in the fly. We do this by measuring the Left-Right Wing Stroke Amplitude (L-R WSA). An increase in the L-R WSA upon activation of the AX neuron in the right hemisphere of the brain would confirm that the fly is trying to turn right.



(a) (b)

Figure 3.2: **(a)** Reconstruction of Biocytin filled AX neuron in the brain (left) and in the Ventral Nerve cord (right).
(b) Changes in the L-R WSA upon stimulation with ATP/P_2X_2 driven by R56G08- GAL4 in either the right(N=11 flies) or the left hemisphere (N=9) or of control flies(no ATP: N=14, no P_2X_2 :N=10). Shaded areas represent SEM (Schnell et al., 2017)

3.2 Methodology

Fly strains

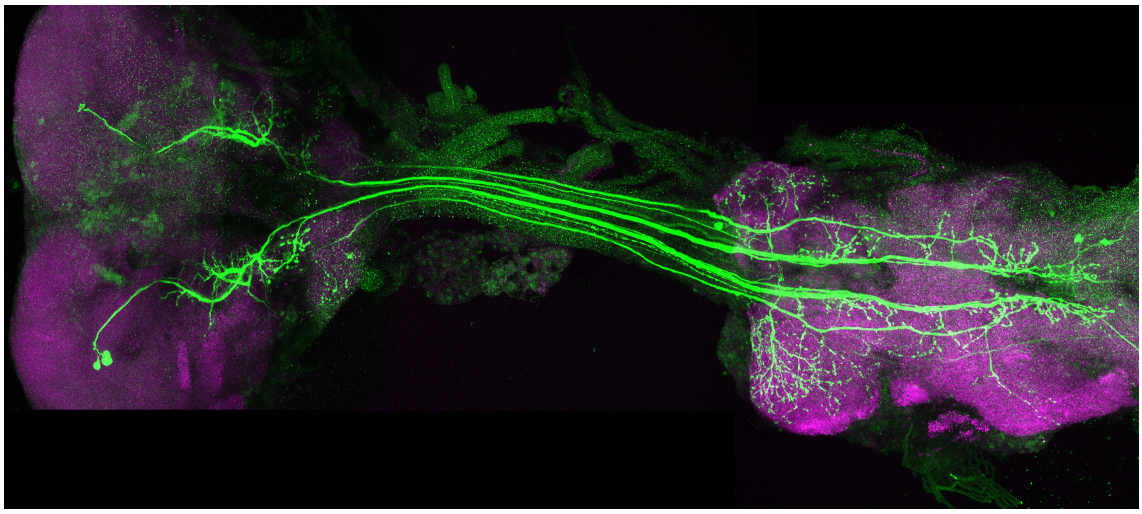


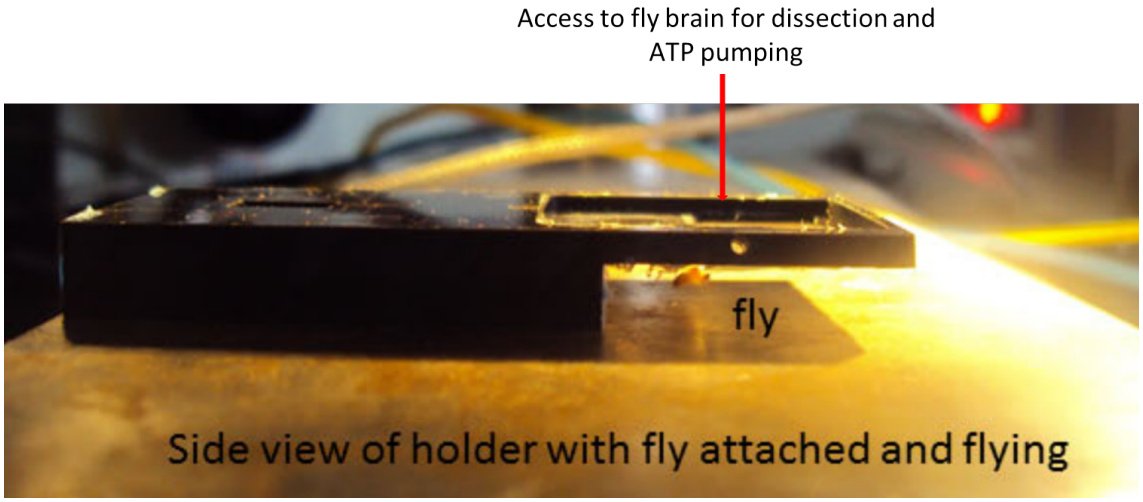
Figure 3.3: Maximum intensity projection of a confocal stack of AX-Split GAL4 line expressing GFP. Violet in the background is the brain and VNC tissue (staining- α-NC82)

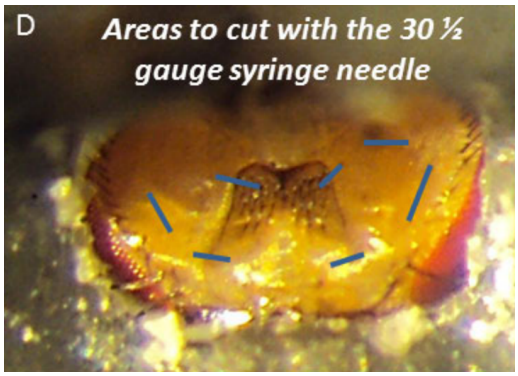
We used the following parental lines in our experiments.

Ax-split GAL4 (DRIVER LINE) corresponds to ? ; Py[+t7.7]w[+mC]=VT025718-p65.ADattP40/CyO ; Py[+t7.7]w[+mC] =R56G08-GAL4.DBDattP2,

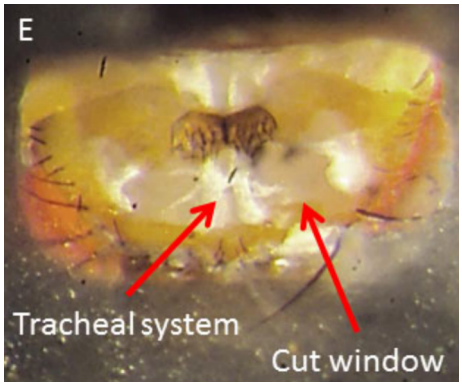
UAS-P_2X_2 (RESPONDER LINE) corresponds to w[*]/w+ ; w[+mC], UAS-P_2X_2/cyo ; w[+mC],UAS-eGFP/TM6B

Crosses were made and flies were raised on the standard Drosophila medium at





(a) Dorsal view of the fly head. The area of the cuticle to be removed to access the brain



(b) Fly head when the cuticle is removed. The brain can now be accessed by using a micropipette

Figure 3.4: Fly tethering and preparation steps

25°C with a 14h/10h light dark cycle. Female F1 progeny with genotype -
1st chromosome - Wild type
2nd Chromosome - w[+mC], UAS-P_2X_2/Py[+t7.7]w[+mC]=VT025718-p65.ADattP40
3rd Chromosome - UAS-eGFP/Py[+t7.7]w[+mC]=R56G08-GAL4.DBDattP2
- were selected by filtering Cyo mutants (identifiable by their curled wings) and TM6B mutants (identifiable by extra hair bristles on the thorax).

Preparation

Each fly was anesthetized by cooling it to 4°C in a non-condensing cooling plate and then the pro- and mesothoracic legs at the coxa/trochanter joint as well as the tibia and tarsi of the metathoracic legs were removed. This was done because leg movements interfere with wing tracking. Removing the legs also helps in flight initiation and duration, because the flies are not able groom themselves midflight. We then glued the head to a custom holder with UV-cured Bondic glue, and also fixed the proboscis with some additional glue **(see fig 3.4)**. To access the neurons in the brain using a micropipette, we used a hypodermic needle to remove a small area of the cuticle in the head region **(see fig. 3.4)**. Trachea lying above the neuron was

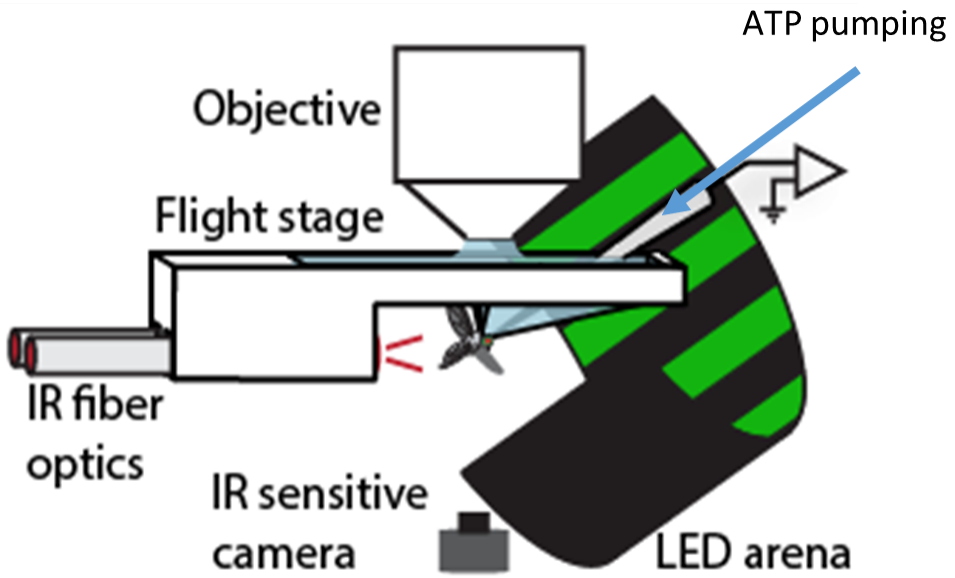
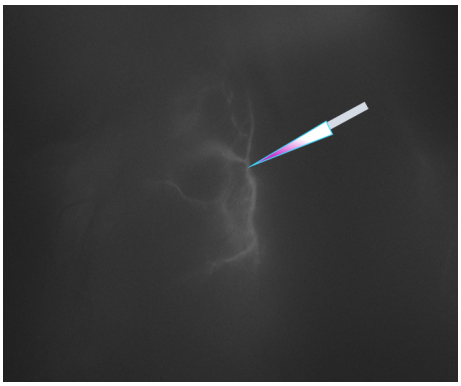
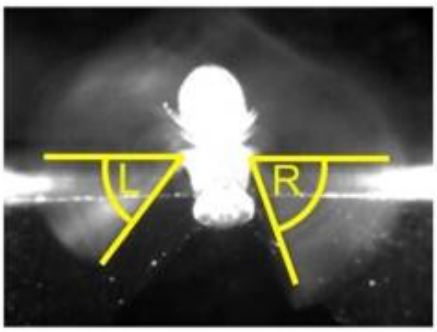


Figure 3.5: Illustration of the tracking apparatus used. Fly wings are illuminated from the rear using IR-lights and the wing movement is tracked from below using an IR-sensitive camera.The objective of the microscope is used to guide the micropipette to the neuron of interest to apply ATP. The LED arena is not used in our experiment because we monitored internally generated saccades without any external stimulus.



(a) Image of an Ax neuron as captured during a real experiment using GFP filter. The needle denotes the approximate area of ATP release.



(b) Tracking of the wings by IR sensitive camera and Kinefly

Figure 3.6: Activation of AX Neuron and tracking of Wing Stroke Amplitude of tethered flies

removed using the needle. The holder was filled with oxygenated saline, thus the brain was perfused with saline and hence remained functional.

Activation Experiments

We pressure injected 1 mM Mg-ATP (A9187, SigmaAldrich, St. Louis, MO) using a PDES-01DE-2 (NPI) and thin-walled micropipettes with a tip of about 3 µm diameter positioned next to the targeted dendrites (the neuron expressed GFP, and

was identified subsequently) in either the right or left brain hemisphere. We added 0.03 mM Alexa568 (A-10437, Invitrogen) to the solution to visualize the ejected solution under the fluorescence microscope (Scientifica Electrophysiology rigs) during a few control pulses before each experiment. The pressure was adjusted such that a visible, but locally defined, amount of solution was ejected (pressures ranged from 1-30 psi).

Behavior Measurements

Flies was lightly blown upon to initiate flight. Infrared LEDs(Thorlabs) (850nm peak wavelength) illuminated the fly from the posterior direction. An infrared-sensitive camera recorded images of the fly from below at 50 frames/s, and custom software (Kinefly) calculated the amplitude of the left and right wing stroke envelopes in each frame**(see fig. 3.6b)**.

Data Analysis

I applied 0.1s pulses every 10 seconds and for a trial to be included in the analysis, the fly had to fly continuously from 1 s before the start of the pulse through the end of the pulse. I only included flies that were able to complete 5 such trials. Left-Right Wing stroke Amplitude(L-R WSA), which would give me an idea of the turning tendency of the fly, during each pulse was plotted against time. All analysis was done on Matlab (Mathworks).

3.3 Results and Discussion

I expressed P_2X_2 and GFP in AX neuron of flies. Then by using a fine micropipette, I applied ATP near the dendrites of the neuron. ATP binds to P_2X_2 and opens ion channels, which depolarizes the cell. By this protocol, I was able to activate the AX neuron in 3 flying flies. The turning response (L-R WSA) response of one fly after the ATP pulse is shown in **Figure 3.7**. Each curve represents the response after one ATP pulse over the course of the whole experiment. The response was delayed, i.e. flies attempted a turn a few milliseconds after the ATP is pumped. **Figure 3.8** represents the mean across the 3 flies after calculating the mean of all flight responses for individual flies. The shaded area represents SEM.

From **Figure 3.8**, we can clearly see that flight response of flies increases after ATP is applied, and then gradually decreases. This indicates that flies attempt a turn towards the right side when ATP is applied on the AX neuron in the right side of the brain. This bolsters our hypothesis that AX neuron is indeed sufficient for eliciting a turning maneuver, by increasing the L-R WSA in flies. The turning maneuver is not as quick as observed in Schnell et al., 2017, but that might just be because the leftover ATP from the pulse is not removed immediately and hence causes a prolonged activation of the AX neuron.
Schnell et al. had already done control experiments **(as seen in fig 3.2b)**, (1) With flies carrying P_2X_2 but saline without ATP was injected, and (2) ATP was injected but P_2X_2 was not expressed. In both these controls, the flies showed no response.

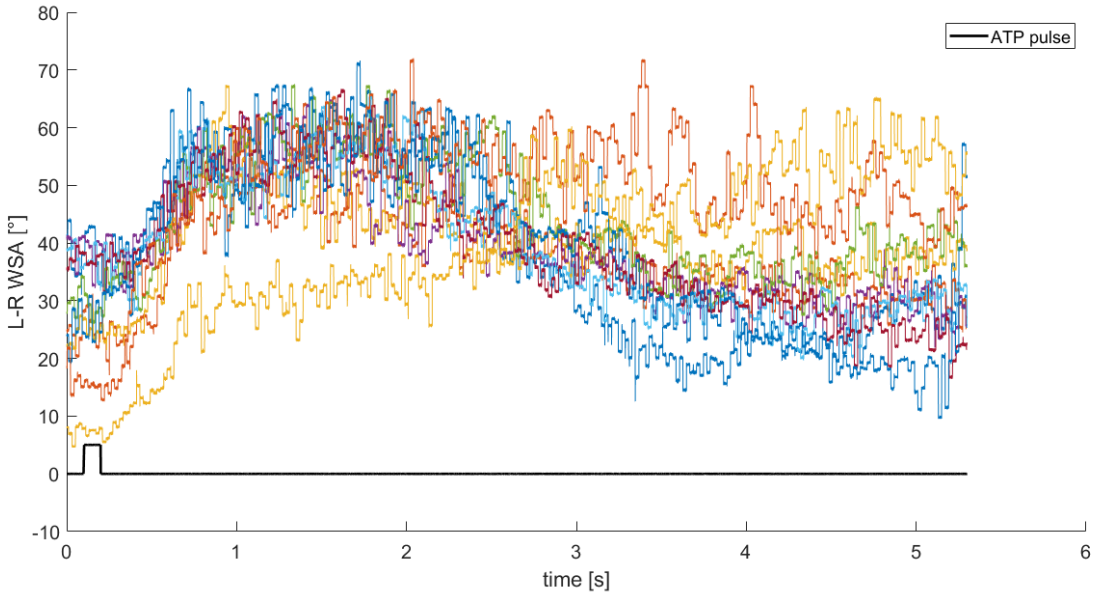


Figure 3.7: Plot of the flight response of a single fly after activation of the AX neuron on the right hemisphere of the brain by a 0.1 seconds long ATP pulse. Each curve represents the flight response (L-R WSA) of the fly after one ATP pulse.

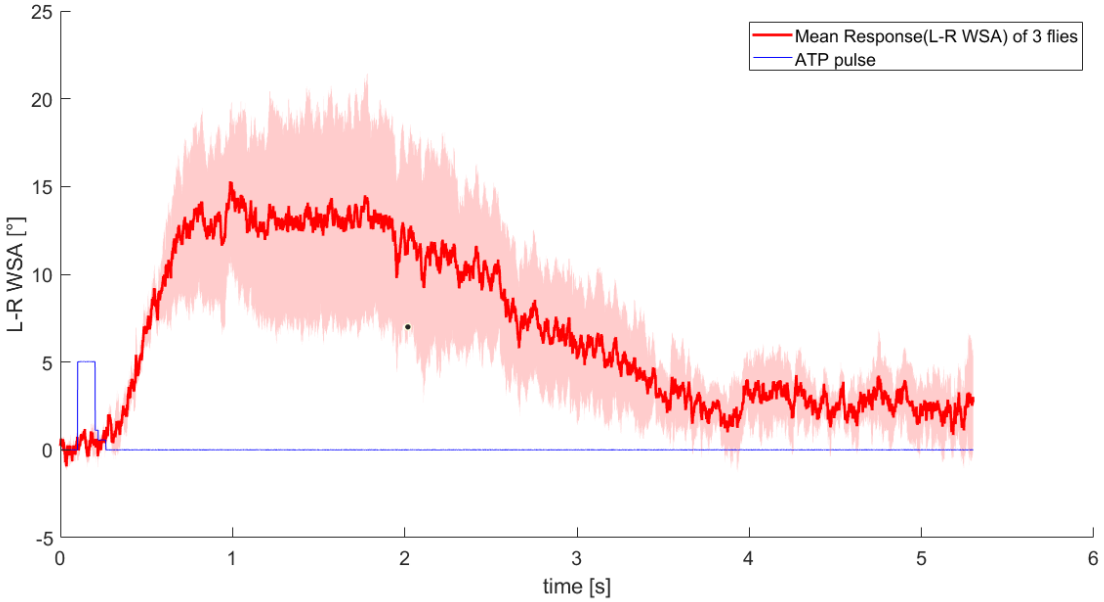


Figure 3.8: Plot of the mean flight response (L-R WSA) of 3 flies after activation of the AX neuron on the left hemisphere of the brain by a 0.1 seconds long ATP pulse. Shaded area represents SEM

3.4 Limitations

We kept a record of 71 flies we tested for the AX neuron line, in an effort to tabulate several external parameters such as age of the flies, pressure of the ATP Pulse, time of the day etc. to take into account which of these factors might be affecting the flight response. This was done mainly because we could not get a lot of the flies to

fly post the cuticle removal. Out of the 71 flies, 51 flies did not fly at all, or flew just for 1-2 seconds when they were blown upon. This can be caused due to accidental damage to certain brain tissue which might be necessary for flight initiation while the cuticle and the air tracts are being removed. AX neuron is anatomically located with the soma in the cervical connective with the soma in the brain. This position marks a very deep coordinate in the brain tissue, and hence the micropipette has to cut through a lot of tissue in order to reach the dendrites of the cell, which increases the probability of a fly to stop flying. Or it can just simply be that the flies were not in the right behavioral state to fly. Flies seemed to fly the best when they are around 4-5 days old, and when post-tethering, a small lump of silver foil is kept beneath their wings to conserve their energy.

In 3 flies, there was a problem in tracking the wings, and by the time that was fixed, the flies stopped flying. In the rest of the flies that did indeed fly, we observed almost no response. This can be due to not enough ATP coming out of the micropipette . Due to all of the brain tissue the micropipette encounters on its way to the AX neuron, more often than not, some tissue enters the pipette and clogs it. Thus, by the time the pipette reaches its target, it gets completely clogged and not enough ATP comes out of it. There is no possible way to check whether the micropipette has clogged or not in the middle of an experiment, unless one turns on the fluorescence microscope, thereby perturbing the behavioral state of the fly. There is another problem associated with the clogging of micropipette. Once the experiment the fly has been placed on the experimental arena and the micropipette is made to reach the neuron and in this process it gets clogged, one has to then restart the process of pulling out a new micropipette, filling it with ATP and micromanipulating it to reach the neuron. This whole process in itself takes around 15 minutes. During this time, the brain is lying open without a cuticle in the saline solution, which of course reduces the probability of the fly flying, which is what was observed as well. The variability in the expression of P_2X_2 can be another cause for the observed absence of response.

Lastly, as we mentioned in the Introduction, this experiment in itself cannot confirm whether the AX neuron controls saccades. All this experiment can confirm is that activating AX neuron induces a turning behavior in the fly by measuring the L-R WSA, not actual saccades. Patch clamp or Calcium signalling studies are required to be done with this genotype to confirm the hypothesis.

3.5 Conclusion

From our experiments on the activation of AX neuron by expressing P_2X_2 and injecting ATP unilaterally and the observed ipsilateral turning behavior corresponding to the activation, we can conclude that activation of AX neuron is sufficient to elicit a turning behavior midflight in a flying *Drosophila*.

In their 1964 paper titled “Interneurons Commanding Swimmeret Movements in the Crayfish”, Wiersma and Ikeda first coined the term “command neuron” to describe how a single activation of any of the four giant fibre neurons running along the crayfish nerve cord caused the crayfish to execute a tail-flip escape response. In

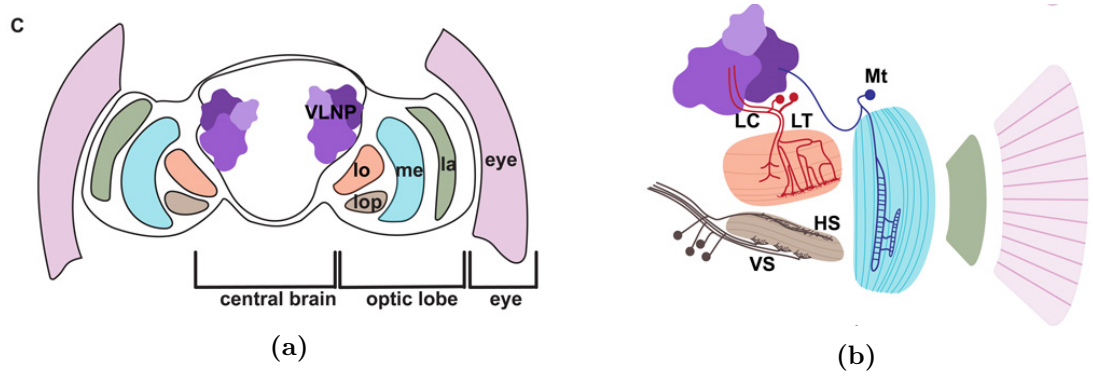
1978, Kupfermann and Weiss suggested a more rigorous definition of the command neuron. For a neuron to be classified as a command neuron by their definition, its activity had to be both necessary and sufficient to initiate the behavior it was purported to command. The modern view of the command neuron concept calls for less stringent definitions, and most neuroscientists seem to agree that animals posses a spectrum of motor control organization. On one end of the spectrum lie command neurons, and on the other end lie the parallel distributed networks, in which the shift in the activity pattern of a population of premotor interneurons causes a corresponding shift in the motor pattern. The position of the control system of particular motor behavior in the spectrum probably reflects flexibility of that behavior.
So far we have found strong evidence that activation of AX neuron is sufficient to elicit an ipsilateral turning response. Investigating a few questions like whether AX neuron elicits a saccade, or whether AX neuron is necessary to elicit a turning response, and which other neurons are activated in conjunction with AX neuron when a turning stimulus is provided, will help us to formulate the exact positioning of the saccade control system in the spectrum of motor control organization.

Chapter 4

HS neuron

4.1 Introduction

In the Drosophila visual system, acquisition of visual information happens in the retina, while visual processing occurs in the optic lobes that comprise more than 60% of the brain's neurons. The optic lobe consists of consecutive neuropil regions called the lamina, medulla, lobula and lobula plate. The optic lobes are the major centers where neuronal computations extract important features from the visual world, such as shape, motion, color, e-vector orientation of polarized light, which are then transmitted to the central brain (Millard & Pecot, 2018).



(a) **(b)**

Figure 4.1: **(a)** Cross section of the fly brain indicating the different parts of the visual system: eye, lamina (la), medulla (me), lobula (lo), lobula plate (lop), and ventrolateral neuropils (VLNPs). **(b)** Interneurons : medulla tangential (Mt, dark blue); lobula columnar (LC, red) and tangential/tree-like (LT, red); lobula plate tangential cells: HS and VS (dark brown).

The lobula plate forms the posterior part of the lobula complex. The Lobula Plate Tangential Cells(LPTCs) found here are one of most extensively studied and characterized set of interneurons. Based on their direction selectivity (firing based on horizontal or vertical direction of image motion), LPTCs are classified into Horizontal System (HS) cells and Vertical System Cells (VS). HS cells are a congregation of 3 cells, HS North (HSN), HS Equatorial (HSE), and HS South. HS neurons are presynaptic to the Descending Neurons of Horizontal Systems (DNHS1), which gives inputs to the neck and haltere motor region (Kim, 2017).

HS cells are responsive to horizontal wide field motion (translation or rotation of the visual stimulus around the vertical body axis) (Krapp et al., 2001; Hausen, 1982), and have been considered to be prime controllers of compensatory head and body movements. A lot of previous studies have built up on this idea. In a study by Heisenberg et al., 1978 the *Drosophila* mutant *optomotorblind H31 (omb ^{H31})* were found to have defective optomotor responses. Geiger and Nässel, 1981, ablated the HS and VS cells in one brain hemisphere of the housefly *Musca domestica* using a laser microbeam and found that responses to moving grating were reduced on the ablated side. Another study by Hausen and Wehrhahn, 1983, performed microsurgical lesions in the HS cells in *Calliphora erythrocephala* to find diminished optomotor responses. In another study on *Calliphora*, Blondeau, 1981, performed electrical stimulation on HS cells and elicited yaw responses in the flies. Schnell et al., 2010, found that the electrical response to rotational and translational field motion in *Drosophila* is similar to large flies **(see Fig 4.2)**.

With the extensive arborization pattern of HS cells, with their anatomy being similar to Descending Neurons, and their known control of the optomotor response, we decided to check whether activation of HS cells is sufficient to elicit a turning behavior.

4.2 Methodology

Fly strains

We used the following parental lines in our experiments. **R27B03-GAL4** corresponds to w[1118]; Py[+t7.7] w[+mC]=GMR27B03-GAL4attP20,
(P_2X_2) corresponds to w[*]/w+ ; w[+mC], UAS-P_2X_2/cyo ; w[+mC],UAS-eGFP/TM6B

Flies were raised on the standard Drosophila medium at 25C with a 14h/10h light dark cycle. Female F1 progeny with genotype for

1st chromosome - Wild type
2nd Chromosome - w[+mC], UAS-P_2X_2/Py[+t7.7] w[+mC]=GMR27B03-GAL4attP20;
3rd Chromosome - w[+mC],UAS-eGFP/+

were selected.

Protocols for *Preparation, Activation Experiment, Behavior Measurements, Data analysis* remain the same as in Chapter 3.

4.3 Results and Discussion

We targeted the dendritic area of HS cells and gave a pressure pulse of ATP for 0.1 seconds in 4 behaving flies. **Figure 4.4** depicts the turning response (L-R WSA) of 1 fly. Each curve depicts flight after the ATP pulse. The curves, unlike those of the AX neuron, do not show any significant trend. **Figure 4.5** depicts the mean of all the flight response curves across the 4 flies. There is no clear deviation from the

null response in our system, hence we can conclude that activating HS neurons for 0.1 seconds does not result in a turning maneuver.

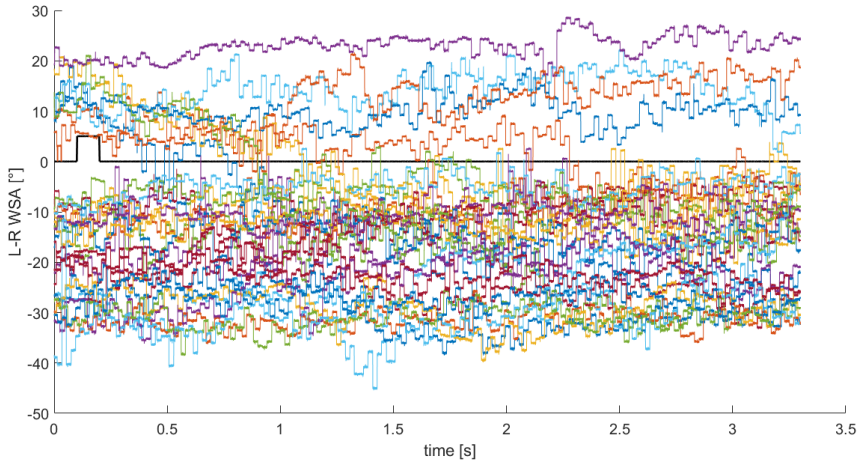


Figure 4.4: Plot depicting flight response (L-R WSA) of a single fly where HS cells were activated in the dendritic area for 0.1 seconds of ATP pulse. Each curve represents the flight response after the ATP is pumped.

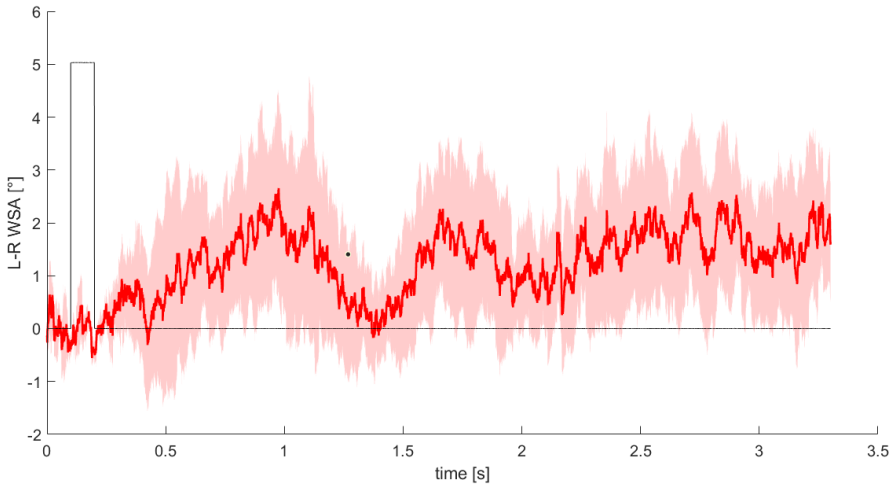


Figure 4.5: Plot depicting the mean of the mean responses (L-R WSA) of 4 flies where HS cells were activated in the dendritic area for 0.1 seconds of ATP pulse. Each curve represents the flight response 3.3 second after the ATP is pumped. Shaded area represents SEM

Next, I decided to activate the cells for a longer period of time to see if the increased activation length could elicit a significant change in L-R WSA. I activated the dendritic area of HS cells of 2 behaving flies (for 0.5 seconds) by applying ATP. The pressure was adjusted accordingly so that an adequate amount of ATP was ejected and it did not flood the whole brain. **Figure 4.6** depicts the flight response (L-R WSA) of 1 fly, with each curve representing the fly's flight response 3.3 seconds after the 0.5 seconds ATP pulse. Similar to 0.1 seconds activation, curves for 0.5 seconds of activation do not show any significant trend. This is also reflected in

the mean across the responses for the 2 flies **(Figure 4.7)**. There is no significant increase or decrease in the WSA after the ATP pulse.
It is possible that the reason we were not seeing any response from HS cells is because HS cells take a longer time to get activated. Therefore, we wanted to check if the HS cells take a longer time to integrate and elicit a response after ATP pulsation. Thus we decided to look at flight response when the interval between the pulses are prolonged from 10 seconds to 20 seconds. Figure **4.8 and 4.9** depict the turning response of 2 flying flies whose dendrites of HS cells were activated by 0.5 second pulse with a duration of 20 seconds between each pulse. As was the case with previous results, we see no significant increase or decrease in the flight response (L-R WSA) in both the plots.
Previously, HS cells have been activated at the terminals by expressing P_2X_2 and applying ATP, and the flies were seen to elicit a turning behavior (Fujiwara et al., 2017). This study however reported the turning behavior of flies on a walking platform rather than a flying one. HS cells have also been activated optogenetically by expressing bi-stable ChR2 variant c128s which can be activated or deactivated by shining blue and yellow light upon them respectively (Haikala et al., 2013). The flies in this experiment showed yaw turning responses upon activation of HS cells. Our results of activation of HS cells using P_2X_2 do not match the results of this study. This can be due to various reasons - (1) Activation by P_2X_2 might not be of the same strength as activation by optogenetics. Further, in our experiments we activate HS cells only in a small dendritic area, whereas the optogenetic activation would shine light on a larger area, hence bolstering the intensity of activation. (2) It might be that in our experiments, the behaving flies might not be in the right internal behavioral state to produce a turning maneuver. In the experiment by (Haikala et al., 2013), close to 30 flies were tested for activation. We were able to test only 2-3 flies, which just by chance, might not have been in a the right internal behavioral state while the experiment was being conducted on them. Some other reasons include HS cells being more important for other body movements like walking or turning head. It can also be that activation of some other neurons along with HS cells is also required for eliciting a robust turning response.

4.4 Limitations

We were not able to get enough flies with HS cells activation on the terminals which would have given us a better insight into the behavioral control of HS cells on flight. Needless to say, it would also have been better if we would have tested more flies for each pulse timing parameter. However, due to the corona pandemic, we did not get the chance to do so. We should have also performed some control experiments by pressure pulsing ATP in wild type flies expressing GFP in the HS cells, but since we can see from the results of our AX neuron activation experiments, the changes in the L-R WSA reach the extent of 15-20 degrees when the flies exhibit a turning maneuver, and in the HS cells experiment, it rarely crosses the 5 degrees barrier, the necessity of a control seems dispensable in this case.

4.5 Conclusion

We activated the HS cells unilaterally in Drosophila melanogaster by expressing P_2X_2 ion channels and then pressure applied ATP near the dendrites for 3 parameters of times-

1. 0.1 seconds of ATP pulse with 10 seconds time interval between each pulse.
2. 0.5 seconds of ATP pulse with 10 seconds time interval between each pulse.
3. 0.5 seconds of ATP pulse with 20 seconds time interval between each pulse.

We did not see any significant change in the L-R WSA in any of the experiments, thus implying that activation of HS cells by ATP does not lead to a turning maneuver, which can either be due to the lower strength of activation as compared to an optogenetic activation, or the lack of the correct behavioral state in our flies.

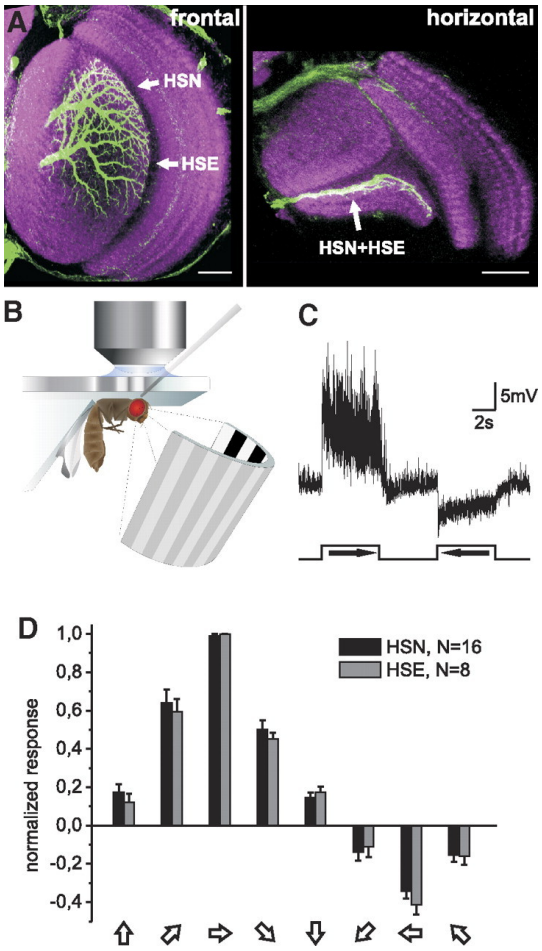


Figure 4.2: **(a)**HSN and HSE cells expressing GFP under the control of the GAL4-line NP0282; in vertical and horizontal cross-section of the fly brain. **(B)** Scheme of the recording setup and preparation of the fly under the fluorescence microscope. In the lower dry half of the preparation the fly is looking at moving patterns presented on a light-emitting diode arena.**(C)** Canonical response of an HSN cell for a moving grating plotted against time. Large-field rotation with an ipsilateral front-to-back component (preferred direction [PD]) elicits a strong depolarization. Motion in the opposite direction (null direction [ND]) elicits a strong hyperpolarization of the membrane potential. **(D)** Directional tuning. Plotted is the mean response amplitude during 5-s grating motion (same stimulus as in C) in 4 different orientations and a total of 8 different directions. HSN and HSE respond strongest to horizontal motion. Error bars indicate SE. (Schnell et al., 2010)

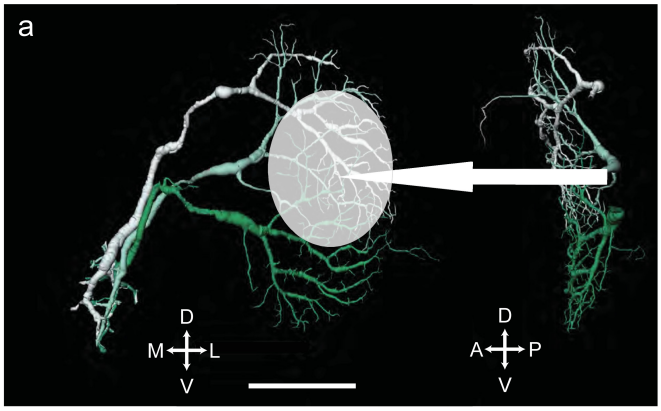


Figure 4.3: Image showing EM reconstruction of HS cells. Arrow and circle depict the approximate area of activation of HS cells by ATP pulse (Boergens et al., 2018)

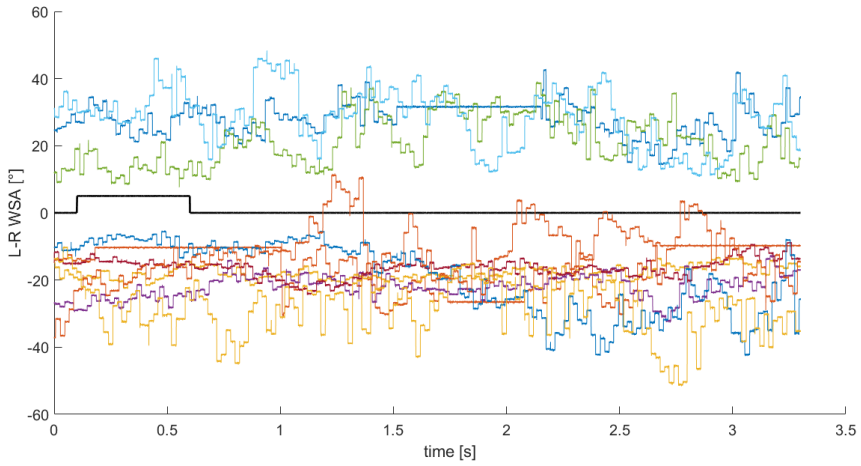


Figure 4.6: Plot depicting flight response (L-R WSA) of a single fly where HS cells were activated in the dendritic area for 0.5 seconds of ATP pulse. Each curve represents the flight response 3.3 second after the ATP is pumped.

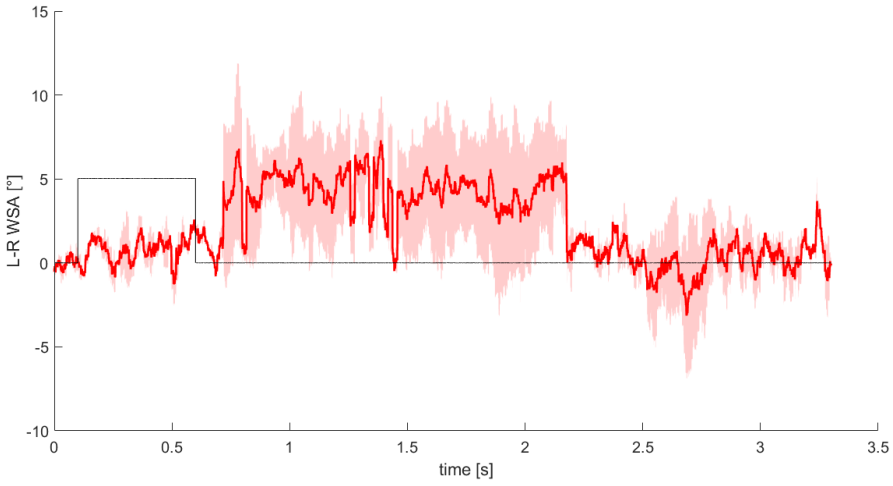


Figure 4.7: Plot depicting mean turning response (L-R WSA) of 2 flies where HS cells were activated in the dendritic area for 0.5 seconds. Shaded area represents SEM

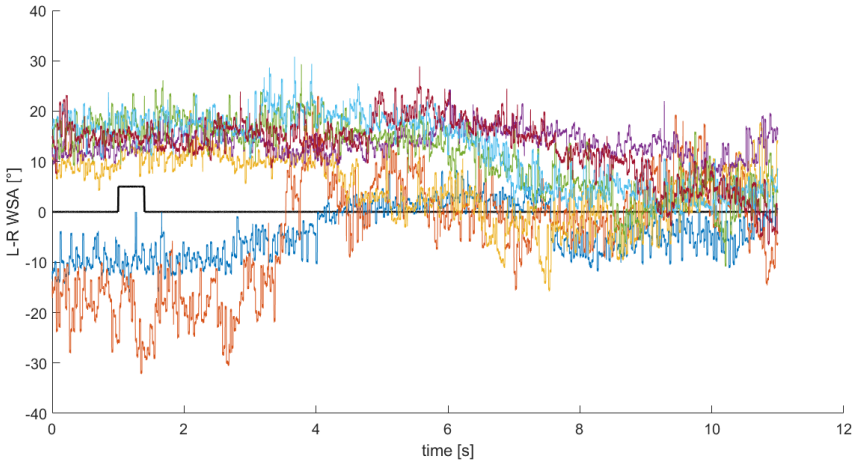


Figure 4.8: Plot depicting turning response (L-R WSA) of a single fly, where HS cells were activated in the dendritic area for 0.5 seconds with 20 seconds of time interval between each pulse.

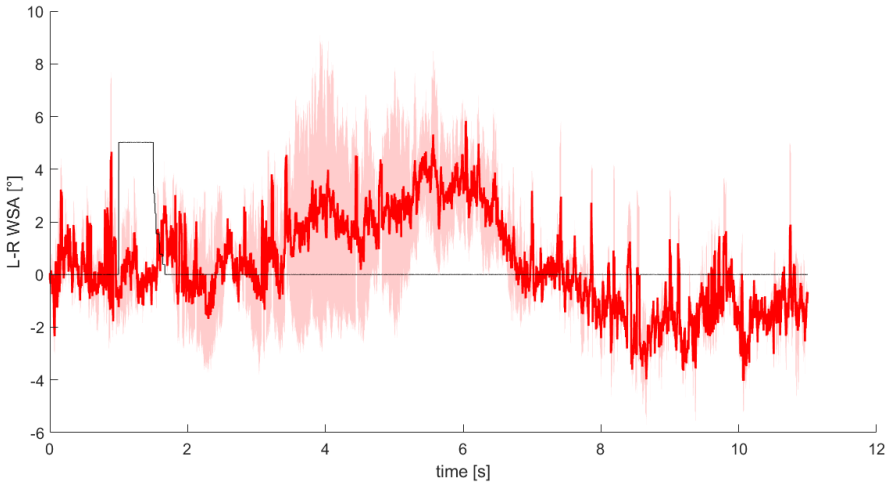


Figure 4.9: Mean turning response (L-R WSA) of 2 flies, where HS cells were activated in the dendritic area for 0.5 seconds of ATP pulse with 20 seconds of time interval between each pulse. Shaded area represents SEM.

Chapter 5

Conclusion

In my first set of experiments, I checked whether activation of AX neuron is sufficient to elicit a turning response. I expressed P_2X_2, which is a cation channel that opens upon binding to extracellular ATP, in the AX neurons by crossing AX-split GAL4 line with the UAS-P_2X_2/GFP line. I used a micropipette to pressure inject ATP in the left hemisphere of the fly brain near the dendrites of the AX neuron. I tracked the wing stroke amplitude during the ATP injection and observed an increase in L-R WSA during the ATP pulsation. Thus my experiments confirm that activation of AX neuron is sufficient to elicit a turning response midflight.

In my second set of experiments, I investigated whether activation of HS cells of the LPTCs is sufficient in causing a turning behavior in *Drosophila*. Similar to the first set of experiments, I expressed P_2X_2 in the HS cells using the GAL4-UAS system. I then pumped ATP in the left hemisphere of the brain near the dendrites of HS cells. I applied ATP for 0.1 and 0.5 seconds but did not see any response (change in L-R WSA) for either of the pulse duration. I conclude that activation of HS cells by ATP is not enough to elicit a turning response in *Drosophila*.

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