Bachelor Thesis

Testing the PPM3 cluster on valence under optogenetic activation

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February 2025

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Abstract

Dopamine plays a key role in reinforcement learning in Drosophila melanogaster. This study investigated whether a subset of dopaminergic neurons in the PPM3 cluster mediated approach or avoidance behavior using an operant optogenetic paradigm. The Joystick paradigm, a single-fly behavioral assay where flies control neuronal activation through their movement was used to assess the behavioral responses. However, no consistent shift of behavior was observed in the experimental group TH-FLP-PI0;64H06-Gal4. Moreover confocal imaging revealed a lack of neuronal labeling. This suggests that the targeted neurons were not successfully activated. Moreover, the effectiveness of dopamine depletion via 3-iodo-tyrosine (3IY) was tested in the Buridan paradigm. This experiment served exclusively as a control to test whether 3IY is successfully metabolized by wild-type Berlin (WTB). After adjusting the protocol the results showed a reduction in total distance traveled indicating successful dopamine depletion.

Zusammenfassung

Dopamin spielt eine zentrale Rolle im „Reinforcement“ von Drosophila melanogaster. In dieser Arbeit wurde mit Hilfe eines operanten optogenetischen Paradigma untersucht, ob eine Untergruppe dopaminerger Neuronen im PPM3-Cluster Annäherungs- oder Vermeidungsverhalten steuert. Hierfür wurde das Joystick-Paradigma, ein Verhaltenstest einer einzelnen Fliege, welche durch ihre Bewegung die neuronale Aktivierung selbst steuert, verwendet. Die experimentelle Gruppe TH-FLP-PI0;64H06-Gal4 zeigte allerdings keine konstante Verhaltensänderung. Konfokale Bildgebung ergab eine fehlende neuronale Markierung, was darauf hindeutet, dass die zielgerichtete Aktivierung nicht funktioniert hat. Zusätzlich wurde die Effektivität der Dopamin-Depletion durch 3-Iodo-Tyrosin (3IY) im Buridan-Paradigma getestet. Der Versuch diente ausschließlich als Kontrolle, um zu prüfen, ob 3IY vom Wildtyp Berlin (WTB) erfolgreich aufgenommen und metabolisiert wird. Eine Reduktion der zurückgelegten Distanz konnte beobachtet werden, nachdem das Protokoll angepasst wurde. Dies deutet auf eine wirksame Aufnahme des 3IY hin.

1. Introduction

Organisms constantly adapt their behavior to optimize survival and reproduction in dynamic environments. Neural circuits that process reward and punishment play a critical role in this adaptation. Dopaminergic neurons (DANs) are key components in assigning value to stimuli and guiding decision-making processes (Kim et al., 2007).

Previous studies have demonstrated that DANs play a crucial role in classical conditioning and reinforcement learning in Drosophila (Rajagopalan et al., 2023). It was initially assumed that specific DAN populations encode either reward or punishment, consistently mediating either approach or avoidance behavior. However, Rohrsen investigated different DAN subpopulations in four operant conditioning paradigms (Rohrsen et al., 2021). They found out that flies exhibited context-dependent behaviors, without a consistent pattern of approach or avoidance across different experimental conditions.

This thesis focuses on the specific dopaminergic driver line TH-FLP-PI0;64H06-Gal4 that selectively targets DANs of the PPM3 cluster (Xie et al., 2018) which projects to the medial Fan-shaped-Body (mFB), the Ellipsoid-Body (EB), and ventral Fan-shaped-Body (vFB). The PPM3 cluster itself consists of 8 dopaminergic neurons per hemisphere (8.02 ± 0.19) (Mao & Davis, 2009) and has been associated with a raised level of aggression when activated (Alekseyenko et al., 2013). The FB in general is involved in avoidance behavior (Hu et al., 2018), locomotion control (Strauss, 2002), visual processing and pattern learning (Liu et al., 2006; Ernst & Heisenberg, 1999). The EB is indicated to play a key role in navigation and motor control by processing sensory inputs to locomotor circuits (Wolff et al., 2015).

To determine whether the targeted subset of dopaminergic neurons in the PPM3 cluster (Xie et al., 2018) consistently mediate approach or avoidance behavior in an operant learning task, the Joystick paradigm is used. It investigates whether activation of these neurons influences behavioral preferences and describes how this response evolves over multiple training phases. Additionally, the effect of the wavelength of optogenetic stimulation on the strength or dynamics of the observed behavior is examined by using red and yellow light. Additionally, confocal microscopy was used to validate the anatomical expression of targeted dopaminergic populations, ensuring that the intended neurons were correctly labeled.

A Buridan experiment was conducted with the Wild-Type Berlin (WTB) strain to verify whether 3-iodo-tyrosine (3IY) was effectively metabolized and could serve as a functional dopamine-depleting agent. This served as a positive control to determine if dopamine depletion affected general locomotion before testing neuronal subsets in the Joystick paradigm. After adjusting the feeding protocol, a reduction in the total distance traveled was observed, indicating a potential effect of 3IY on locomotion.

2. Material and Methods

2.1 Fly stocks and maintenance

All flies used in this experiment were raised in plastic vials on a specific medium (Table 5). Vials were stored in a temperature-controlled room at 25°C and 60% humidity. The day-night cycle was maintained on a 12-hour schedule, with light on from 8:00 AM to 8:00 PM and darkness for the remaining 12 hours. Flies were transferred into new vials every day to control the larval density and create consistent conditions. Virgin females were collected each morning and stored at 18°C for a minimum of five days until they were used for experiments.

For the experimental crossings, ten males were used which contained the dopaminergic driver line $\frac{\mathrm{TH-FLP-PIo}}{\mathrm{Sco}};\frac{\mathrm{64H06-Gal4}}{\mathrm{Tm3}}$ with sco and tm3 as balancer. This line labels specifically for DANs in the PPM3 cluster which projects into the medial Fan-shaped body (mfB), the Ellipsoid Body (EB) and ventral Fan-shaped Body (vFB) (Xie et al., 2018). They were crossed with twenty female virgin flies of the effector line HS-FLP -PEST; 20xUAS>STOP>CsChrimson. This enables the expression of CsChrimson in the targeted dopaminergic neurons and allows optogenetic activation of these neurons. All-trans-retinal (ATR) is required to activate the CsChrimson channel (Yu et al., 2015).

For the control groups, w;Gr28bd-Gal4;TrpA1-Gal4 males were crossed with the effector line norpAP24;20xUAS-CsChrimson, resulting in flies that express CsChrimson in heat-sensitive neurons (Mishra et al., 2018), including TrpA1 channels (Tang et al., 2013). The positive control group was treated with ATR solved in ethanol and the negative control group was treated only with ethanol.

For the Buridan experiment (2.4), the Wild-Type Berlin (WTB) strain was used and handled as described.

To perform the anatomy experiment where the flies brain was dissected, TH-FLP-PI0;64H06-Gal4 female virgins were crossed with FRT-STOP-FRT::GFP male flies. As a positive control for the staining, the TH-GAL4 > UAS-GFP line was used to ensure proper visualization and validation of GFP expression.

2.2 Joystick

The Joystick enables the examination of Drosophila's decision-making processes through operant conditioning. By tracking platform movements in real time, behavioral decisions can be recorded. This experimental setup enables each fly to select the activation or inactivation of specific neurons using optogenetic stimulation. There is no interference from external sensory inputs.

Preparation of flies

Small vials without yeast were prepared with either 15 µl of 200mM ATR (**Table 4**) dissolved in ethanol or just ethanol. They were wrapped in aluminum to block all the light and prevent optogenetic activation before the experiment started. One day old flies were anaesthetized on a cooling station and potential mutants (balancer) were sorted out. Then 20-25 males were selected and moved into the small vials by using a brush. The positive control group was fed with ATR. The negative control group was fed with the same amount of ethanol but without the ATR. All vials got stored at 25°C for 48 hours before the flies were used for testing, so they were able to metabolize the ATR (Yu et al., 2015).

Gluing

On the same day the flies were tested, they were attached to a fishing line approximately 2 cm long and 0.6 mm thick. This procedure was carried out under blue light instead of white light to avoid unintentional optogenetic stimulation. UV-sensitive glue (**Table 4**) was used, which was hardened with UV light. The fishing line was attached to the dorsal side of the flies thorax to create a right angle. All prepared flies were kept in completely darkened boxes for about one and a half hours at 25°C before being tested.

Test setup

The experimental test setup of the "Joystick" is illustrated in (**Figure 1**). The testing procedure started by feeding the flies a sugar solution for 5–10 minutes, using a dark chamber where the flies were placed on damp filter paper sprinkled with sugar crystals. After feeding, the fishing line attached to each fly was trimmed to approximately 5 mm in length with scissors or clippers. For each test session, three flies were tested simultaneously. The flies were positioned under

the light guide and secured in a clamp, with the Joystick platform adjusted to ensure a neutral hanging position, avoiding excessive stretching or compression of their legs. To minimize external light exposure, a cardboard cover was placed over each Joystick apparatus, ensuring that the flies interacted exclusively with the light emitted by the setup. The power of emitted light is strong enough to illuminate the whole brain which leads to the activation of neurons containing the CsChrimson channel. The red light was emitted at 6 V, which corresponds to approximately 450–500 lux in this experimental setup, with a wavelength of 660 nm. The yellow light was emitted at 3.8 V, corresponding to approximately 750–850 lux, with a wavelength of 590 nm. Light was delivered as a 20 Hz pulse with a 50 ms pulse width and no cycle delay.

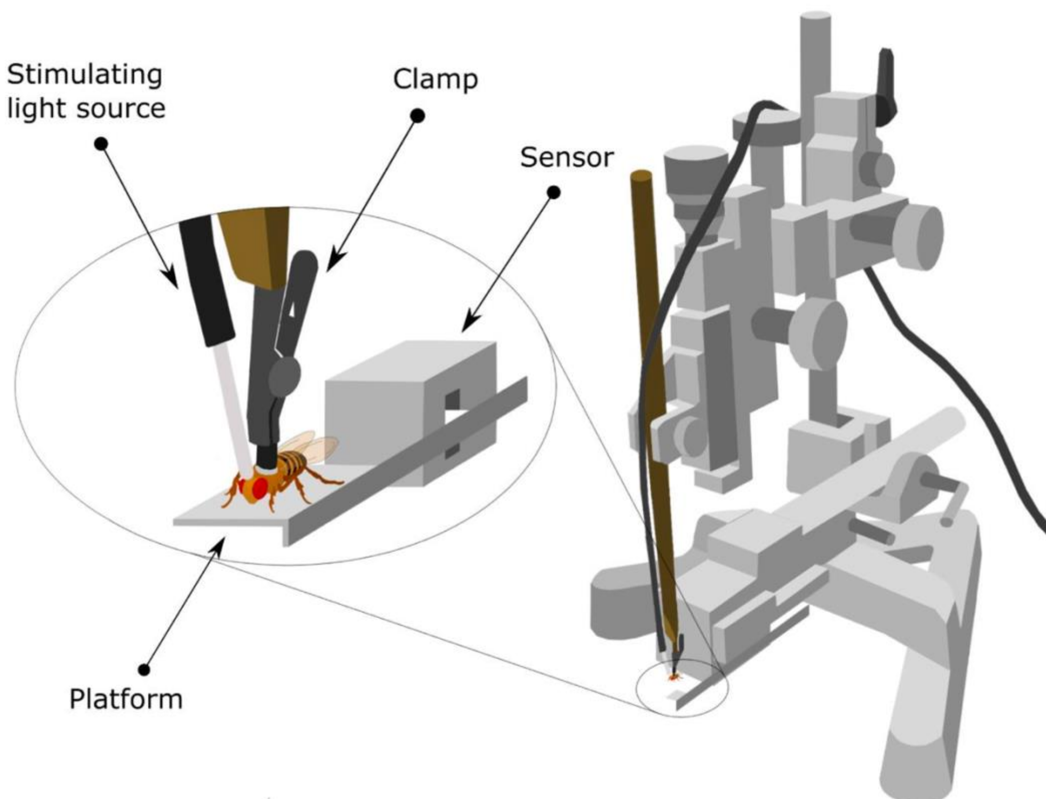

Figure 1: Joystick mechanism: *The fly is positioned on the platform allowing it to move the platform with its legs. The head is placed as close as possible to the light guide. When the fly moves the platform, a sensor detects this movement, and the computer program turns the light on or off accordingly. (Guyton, 2023.)*

Test Protocol

The test protocol consisted of 10 periods which were 60 seconds long (**Table 1**). Period 1 was a pre-test to see if flies show an initial preference for one or another side with no optogenetic activation. Period 10 was a post-test where also no optogenetic activation was performed to analyze potential learning behavior. Period 2 to 9 were training periods where the light activating side stayed the same throughout the whole experimental cycle. The light activating side was changed randomly between tests to balance the results for both sides.

Table 1: Joystick Test Protocol

Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Period 7	Period 8	Period 9	Period 10
Pre-test	Training	Training	Training	Training	Training	Training	Training	Training	Post-test
60 sec.	60 sec.	60 sec.	60 sec.	60 sec.	60 sec.	60 sec.	60 sec.	60 sec.	60 sec.

Evaluation

The computer calculated a preference index (PI) for every period.

$$PI = \frac{(x - y)}{(x + Y)}$$

The PI represents a value ranging from +1 to -1, with positive values indicating a preference for the activation of the targeted neurons and negative values reflecting avoidance or preference for inactivation. X corresponds to data points collected from the area where the targeted neurons were optogenetically activated, while Y refers to data points from the area where the neurons remained inactive due to the flies behavior.

The raw data was analyzed using a script written in R which can be accessed on https://github.com/brembslab/Platform-Drosophila/tree/master/Platform-Optogenetics’’. For data analysis RStudio was used.

2.3 Data collection and pooling of Joystick

The data used in this study was pooled with already collected data. For the red-light experiments (**3.1.1**), as part of this bachelor thesis data for 29 negative control flies, 26 positive control flies and 39 flies from the experimental FLP line were collected. These datasets were supplemented with additional data to reach a final sample sizes of N=49 for the negative control group, N=50 for the positive control group, and N=50 for the FLP line. Similarly, for the yellow light experiments (**3.1.2**), data for 26 negative control flies, 26 positive control flies, and 33 flies from the FLP line was collected. These were then pooled with additional data to achieve final sample sizes of N=50 for the negative control group, N=49 for the positive control group, and N=49 for the FLP line. All experiments were conducted using identical setups and protocols to ensure consistency and the datasets were carefully examined for compatibility before merging them.

2.4 Buridan

Buridan Paradigm

The Buridan paradigm was used to assess the general locomotion of Drosophila melanogaster under the influence of 3-iodo-tyrosine (3IY), a dopamine synthesis inhibitor. Unlike the classic Buridan setup, no visual landmarks were provided, ensuring that locomotor activity was not influenced by external spatial cues. The primary objective of this experiment was to assess whether Drosophila melanogaster of the Wild-Type Berlin (WTB) strain successfully metabolized 3-iodo-tyrosine (3IY) under the given feeding protocol. In future experiments, this method can serve as a valuable tool to verify whether the results from the joystick experiment can be attributed to dopamin.

Fly Preparation

The Wild-Type Berlin (WT Berlin) strain was used for this experiment. WTB flies were held under standard conditions as described (**2.1**). Dopamine levels of the experimental groups were manipulated by feeding them with 180 µL of 3IY (10 mg/mL) dissolved in a 5% sucrose solution on a filter paper for 48 hours prior to testing (**Figure 5**) as well as 96 hours (**Figure 6**). Flies of the negative control group were also provided with 180 µL of a 5% sucrose solution but without

3IY. Flies were transferred onto new filter paper (**Table 4**) with a fresh 3IY or sucrose solution every 24 hours. After the feeding period, flies were anesthetized on a cooling plate, and their wings were clipped to one third of their original length using fine micro scissors. This ensured that only walking behavior was analyzed. After the wing clipping, flies were individually placed into small vials to recover at least five hours before testing.

Experimental Setup

The experiment was conducted in a circular arena, surrounded by a moat filled with distilled water to prevent flies from escaping. The arena was evenly illuminated under standardized conditions, and a camera positioned above the setup recorded the flies movements for further analysis.

Testing Procedure

The surface of the arena was cleaned with 70% ethanol before each trial to eliminate potential olfactory cues that could influence locomotion. Flies were carefully transferred to the center of the arena. The locomotor activity of each fly was recorded for 15 minutes using “Buritrack”. Since no visual landmarks were present, locomotion was driven solely by the flies intrinsic activity.

Data Analysis

All behavioral data was analyzed using “CeTrAn”.

2.5 Dissection of adult Drosophila brains with antibody staining

To examine neuronal labeling, adult Drosophila brains were dissected and stained with antibodies to visualize the targeted neuronal populations. The TH-FLP-PI0;64H06-Gal4 female virgins were crossed with FRT-STOP-FRT::GFP male flies to perform the experimental stainings. The positive control staining's were performed with TH-GAL4 > UAS-GFP flies.

A 4% PFA-TX100 solution was prepared by mixing frozen 8% PFA with 1x PBST containing 0.1% TX-100. Flies were anesthetized on a cooling station and transferred into the 4% PFA solution and fixed for two hours at 4°C degrees under rotation. Brains were dissected in PBS, and samples were washed five times for five minutes with PBST containing 0.1% TX-100. Brains were then blocked for 1 hour at room temperature in a solution of 10% NGS in PBST containing 0.1% TX-100. After blocking, the solution was removed and the brains were incubated overnight at 4°C in a primary antibody mixture (**Table 2, Table 4**) prepared in PBST containing 0.1% TX-100 and 3% NGS. The containers were covered with a glass plate and placed in a dark box on a moist cloth to prevent dehydration overnight. The following day, the samples were adjusted to room temperature for 30 minutes. The primary antibody solution was removed. Samples were then washed five times for five minutes with PBST containing 0.05% TX-100. Secondary antibody (**Table 3, Table 4**) incubation was performed for two hours at room temperature in the dark, using a solution prepared in PBST containing 0.1% TX-100 and 3% NGS. The samples were washed five times for five minutes with PBST containing 0.1% TX-100 and mounted with VECTASHIELD® Antifade Mounting Medium (**Table 4**) for imaging. For visualization a confocal microscope (Leica SP8, RRID: SCR_018169) was used and the pictures were edited in LAS X (Version: 1.0.0.12269).

Table 2: Primary Antibody Solution

Volume (µl)	Name
459	PBST 0.1%TX-100%
15	NGS (3%)
25	Mouse-anti-Bruchpilot (1:20)
1	Rabbit-anti-GFP

Table 3: Secondary Antibody Solution

Volume (µl)	Name
480	PBST 0.1% TX-100%
15	NGS (3%)
2.5	Goat-anti-Rabbit AF488
2.5	Goat-anti-Mouse AF555

3.Results

3.1 Optogenetic testing with Joystick

This experiment was conducted to explore fly behavior and determine which specific dopaminergic neurons were activated using optogenetic activation. The thesis focused on how preference indices (PI) changed over the course of the training sessions and whether differences between the experimental and control groups could be observed. It was evaluated whether the behavior of the flies remained consistent throughout the training phases or adapted to the conditions. Red and yellow light was used in this experiment to investigate potential differences in behavioral responses to optogenetic activation at different wavelengths.

3.1.1 Red light

Optogenetic activation with red light resulted in observable differences in the behavioral patterns across the groups. The negative control group showed no significant preference for or against the activated side during the training periods. The PI of this group remained nearly neutral throughout all phases, with a slight negative tendency (-0.1) (**Figure 3A**). The positive control group exhibited avoidance behavior toward the side activated by red light (**Figure 3B**). The PI values started close to zero in the pre-test and decreased during the initial training periods. During the training phases, the median PI values stabilized at -0.6 . In the post-test, the median PI was approximately -0.7. The experimental group displayed variations in behavior over the course of the training periods (**Figure 3C**). The PI values started at approximately -0.1 during the pre-test and fluctuated throughout the training. By the final training period, the median PI throughout all training phases was at -0.1, and in the post-test, the median PI was approximately +0.1.

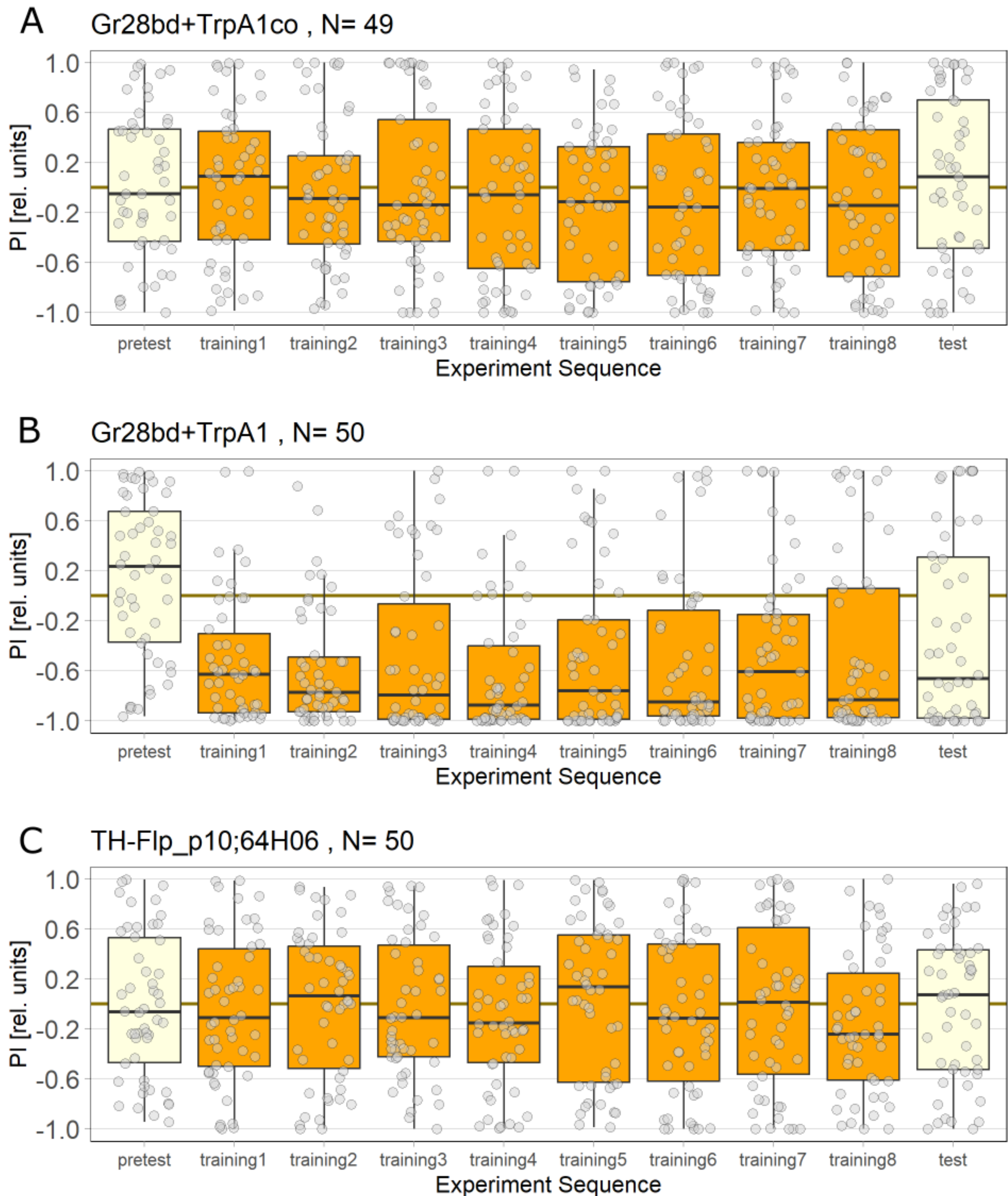

*Figure 2: **Behavioral responses during Joystick experiments with red light:** (**A**) Negative control flies expressing CsChrimson in heat-sensing neurons with no optogenetic activation through ATR show no consistent preference for or against the activated light side throughout the training phases (orange). Their behavior remains neutral in the post-test (white). (**B**) Positive control flies expressing CsChrimson in heat-sensing neurons avoid the light-activated side during all training periods (orange). This avoidance persists into the post-test where the previously light-associated side continues to be avoided (white). (**C**) Experimental flies expressing the optogenetic channel in TH-FIp_p10;64H06 neurons show an alternating pattern of behavior throughout the training periods, shifting between slightly negative and slightly positive PIs. The box plots represent medians (solid lines), quartiles (boxes), and non-outlier ranges (whiskers), with individual data points shown in grey.*

3.1.2 Yellow light

Similar results were observed under yellow light. The negative control group (**Figure 3A**) started at approximately zero in the pre-test and showed a slightly negative trend with a median training PI of -0.1. The positive control group (**Figure 3B**) also started very close to zero in the pre-test, however switching to strong avoidance over the training periods, with values ranging between -0.5 and -0.8 during the training phases and a post-test value of approximately -0.6. This equals the median training PI of -0.6. The experimental group (**Figure 3C**) started exactly at zero in the pre-test and showed slight variations in behavior throughout the training phases. In period 2, the PI was slightly negative, before becoming slightly positive by period 4. In period 5, the PI dropped slightly negative again but increased steadily up to period 9. The post-test PI returned to a value close to zero and the overall median training PI was close to zero.

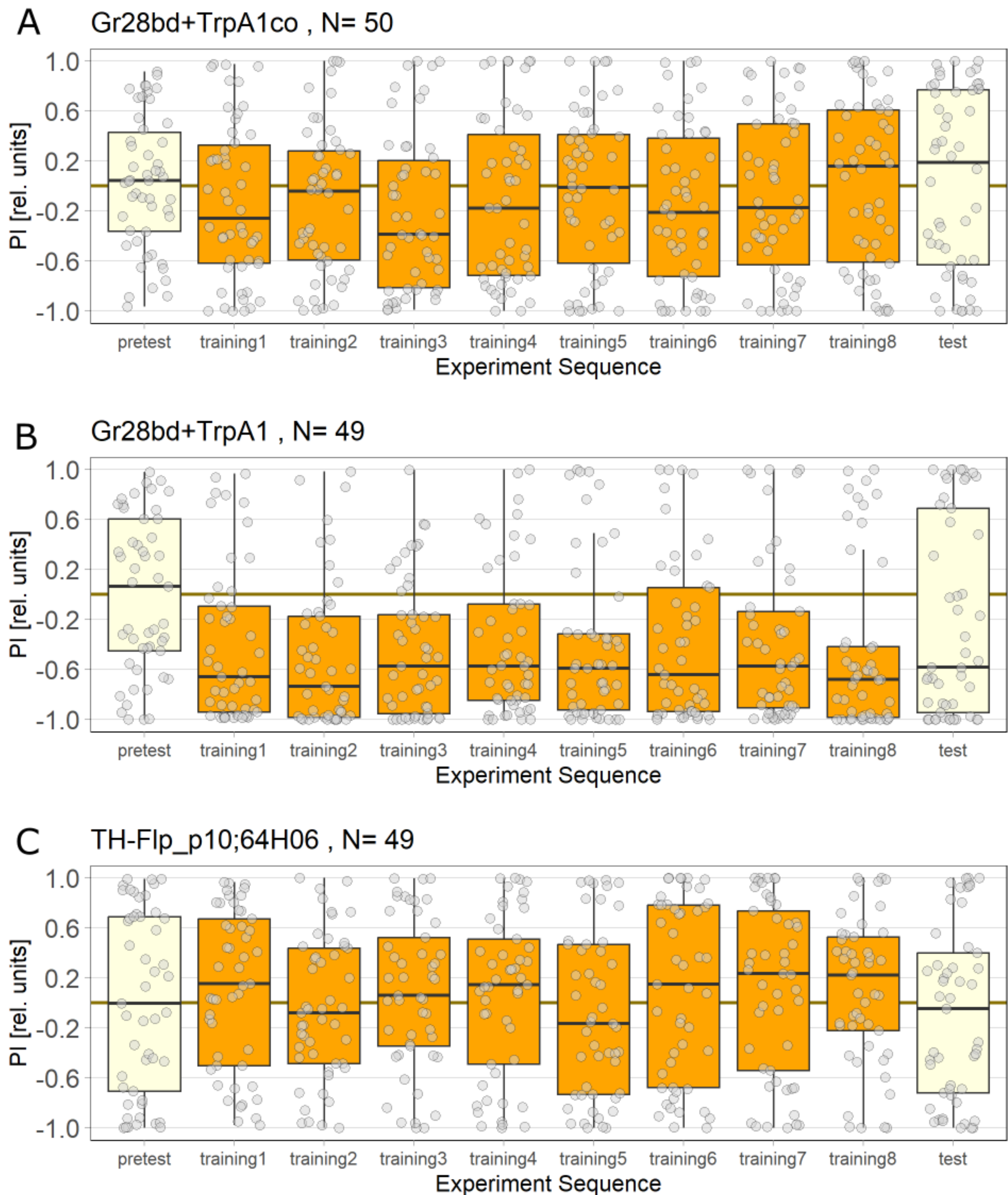

*Figure 3: **Behavioral responses during Joystick experiments with yellow light:** (**A**) Negative control flies expressing CsChrimson in heat-sensing neurons show no consistent preference for or against the light activated side throughout the training phases (orange). Their behavior remains neutral in the post-test (white). (**B**) Positive control flies expressing CsChrimson in heat-sensing neurons avoid the light-activated side during all training periods (orange). This avoidance persists into the post-test, where the previously light-associated side continues to be avoided (white). (**C**) TH-Flp_p10;64H06 flies exhibit an alternating pattern of behavior throughout the training periods, finishing with a PI close to zero in the post test.*

3.1.3 Effect of pre-test feeding on learning behavior in positive controls

In previous experiments, flies actively avoided the light in the positive control but ultimately did not show strong learning (Rohrsen et al., 2021). However, it is now clear that the positive control clearly avoids the light during the post-test, even though the light is no longer active. The purpose of this experiment is to determine whether this learning is potentially linked to feeding before training and whether the flies might still learn if they are not fed prior to the training. The experiment was conducted using yellow light. There was almost no difference between the two groups in the post test.

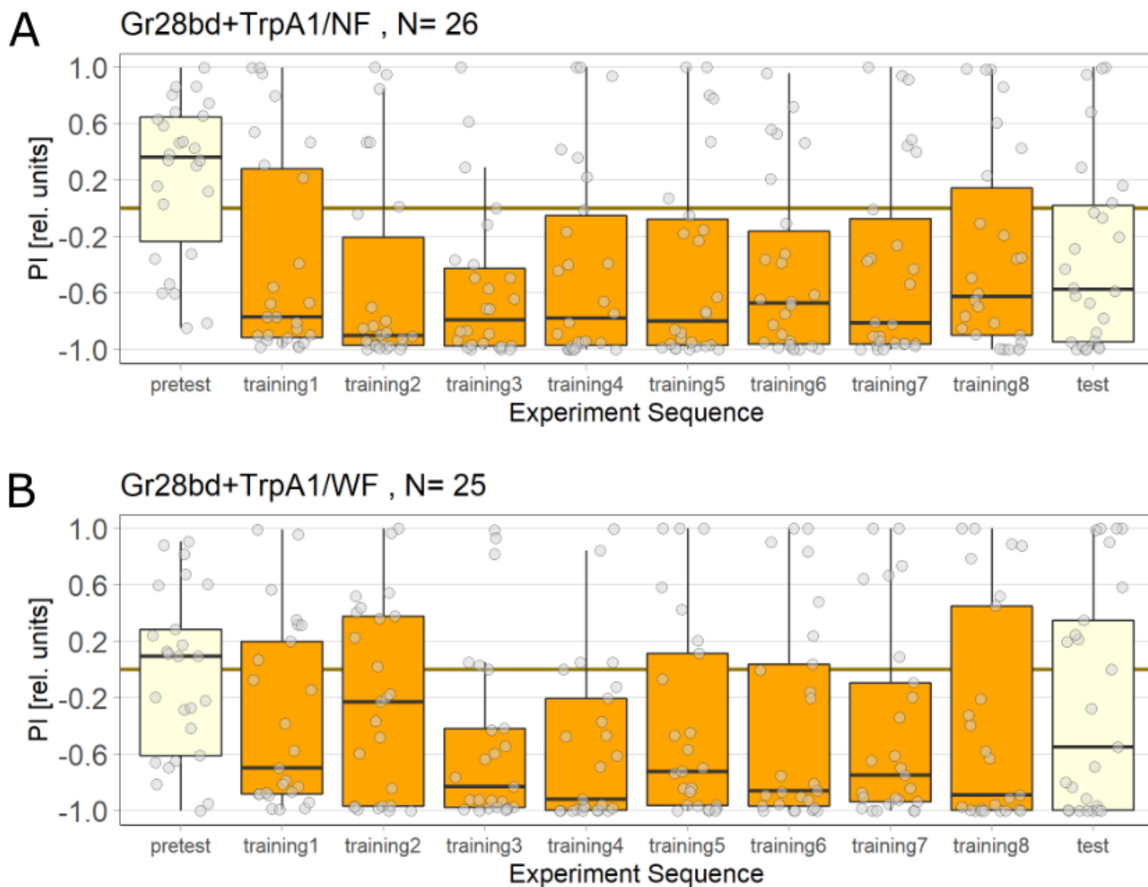

*Figure 4: Time course of Joystick experiments without and with prior feeding: (A) Flies that were not fed prior to testing show a slightly positive PI during the pre-test. Throughout the training periods, the PI becomes progressively negative. During the post-test, the flies show strong learning by continuing to avoid the previously light associated side, even when the light is not activated anymore. (**B**) Flies that were fed prior to testing start with a pre-test PI close to zero. During training, the flies exhibit strong light avoidance across most training periods. A slight exception is observed in the second training period. This avoidance continues into the post-test, where the flies consistently avoid the previously light-associated side.*

3.2 Buridan

The Buridan experiment was conducted to evaluate the overall locomotion of the flies under the influence of 3-iodo-tyrosine (3IY), a dopamine synthesis inhibitor. In this setup no visual landmarks were provided to ensure that the locomotor activity remained uninfluenced by external spatial cues. The primary goal was to determine whether the Wild-Type Berlin (WTB) strain successfully metabolized 3IY under the given feeding protocol (**2.4**) before applying it to a different experimental group. To assess the differences in 3IY metabolism, flies were tested at two different time points, after 48 hours (**Figure 5**) and 96 hours (**Figure 6**).

3.2.1 Feeding window of 48 hours

After 48 hours there was no significant difference when median speed and traveled distance was compared.

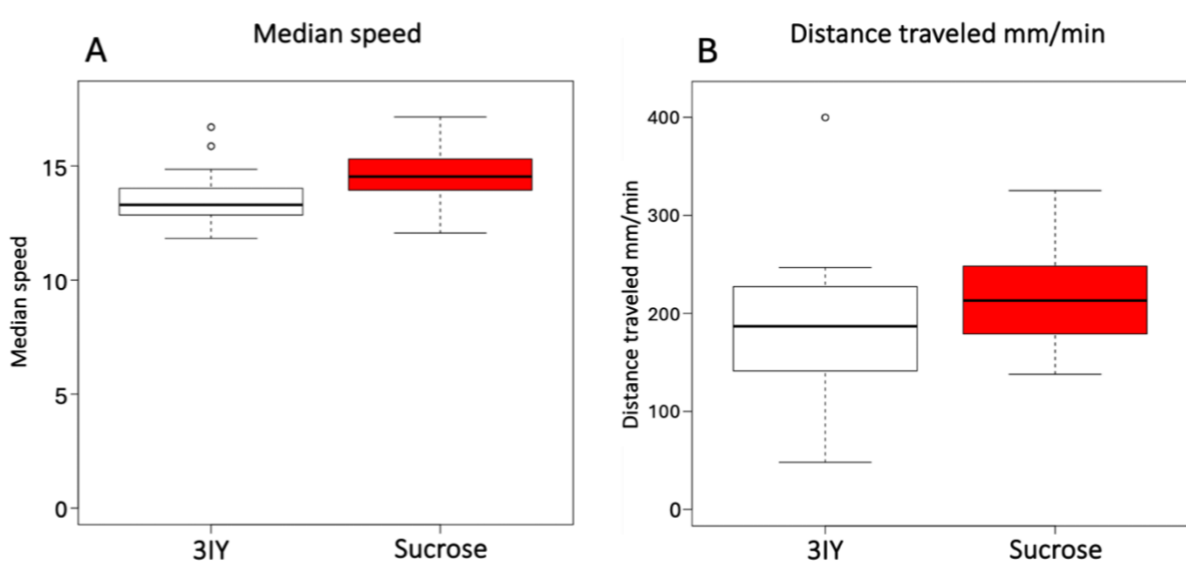

Figure 5: Locomotion in the Buridan Paradigm under 3IY and Sucrose Treatment for 48 Hours: This *figure presents two box plots. They illustrate locomotion metrics of WTB in the Buridan paradigm after 48 hours of treatment with 3-iodo-tyrosine (3IY) or sucrose (N = 16 per group). (**A**) Median speed: Flies treated with 3IY exhibit a median speed of approximately 13.5 mm/s, with an interquartile range (IQR) from 13 to 14 and a few outliers above 15. Flies treated with sucrose show a slightly higher median speed of around 14.8 mm/s, with an IQR from 14 to 16. (**B**) Distance traveled per minute: The 3IY-treated flies show a median distance traveled of approximately 190 mm/min, with an IQR from 140 to 230 mm/min and one outlier above 400 mm/min. Sucrose treated flies display a higher median distance of around 220 mm/min, with an IQR from 160 to 260 mm/min. The bold line in the box plots represents the median, while the box indicates the interquartile range (IQR), and the whiskers show the non-outlier range. Individual outliers are displayed as grey circles.*

3.2.2 Feeding window of 96 hours

After 96 hours of feeding the median speed stayed the same but the traveled distance of 3IY treated flies was reduced.

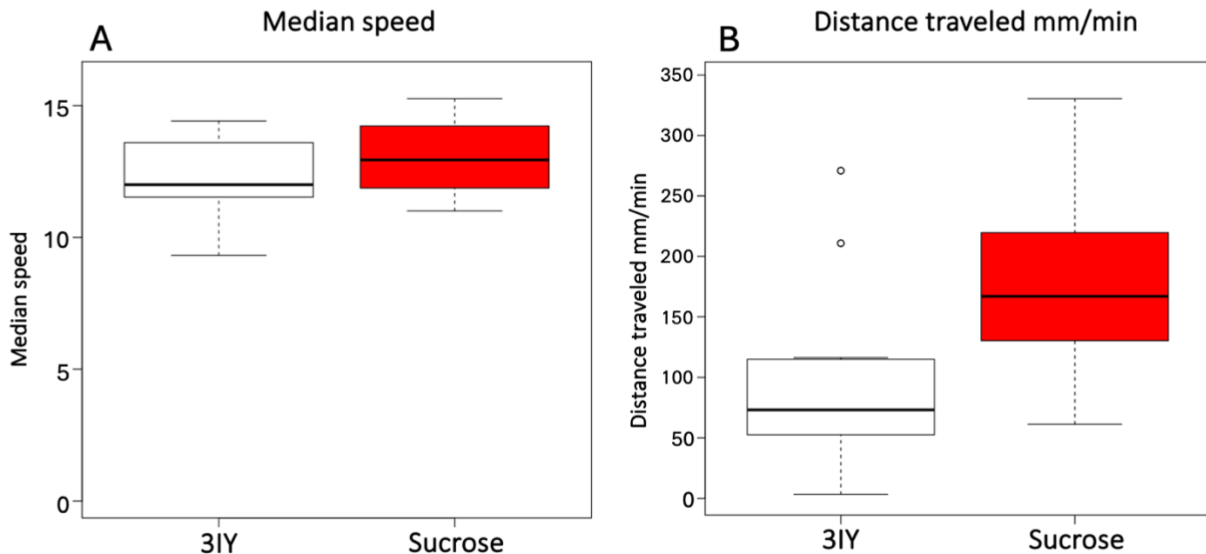

Figure 6: Locomotion in the Buridan Paradigm under 3IY and Sucrose Treatment for 96 Hours: (A) *Median speed:* *Flies treated with 3-iodo-tyrosine (3IY) (N = 11, left, white) and flies treated with sucrose (N = 10, right, red) were analyzed. The median speed for the 3IY group is approximately 12 mm/s, with an interquartile range (IQR) from 11 to 13. The median speed for the sucrose group is higher, around 13 mm/s, with an IQR from 12 to 14. (**B**) Distance traveled per minute: The median distance traveled for the 3IY group is approximately 75 mm/min, with an interquartile range (IQR) from 50 to 110 mm/min and a few outliers above 250 mm/min. The median distance for the sucrose group is higher, around 175 mm/min, with an IQR from 120 to 230 mm/min. Box plots display the median (bold line), interquartile range (box), and non-outlier range (whiskers), with individual outliers represented as circles.*

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3.3 Brain dissection with antibody staining

To prove whether the correct neurons were targeted TH-FLP-PI0;64H06-Gal4 female virgins were crossed with FRT-STOP-FRT::GFP male flies (**Figure.7**). The driver line specifically targets neurons in the PPM3 cluster. The control stainings were performed with TH-GAL4 > UAS-GFP (**Figure.8**) which targets dopaminergic cells (Friggi-Grelin et al., 2003).

3.3.1 Brain visualization of TH-FLP-P10;64H06

The convocal imaging showed no significant GFP signalling of dopaminergic neurons in the PPM3 cluster.

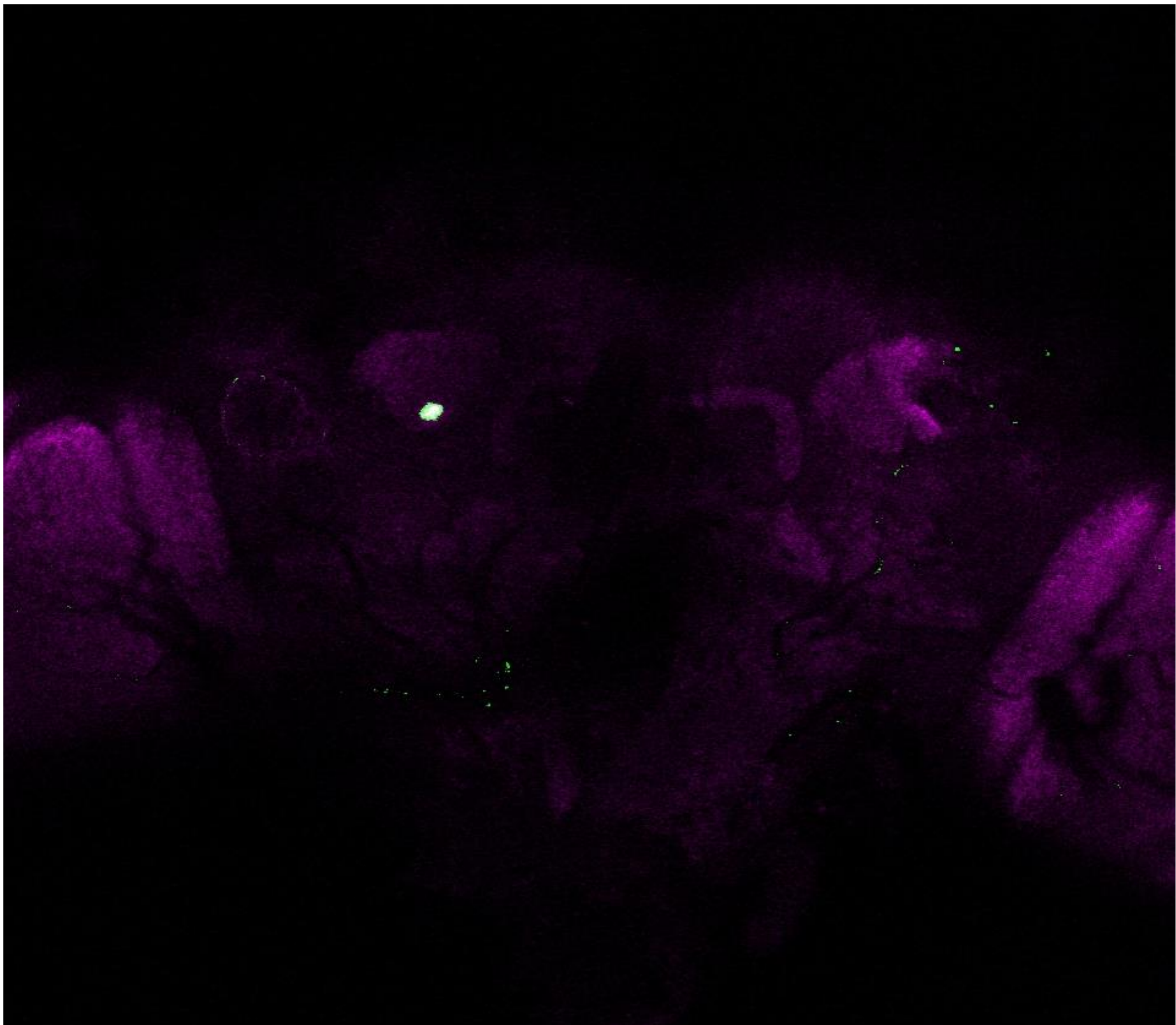

Figure 7:Confocal images with anti-GFP and anti-Brp staining of TH-FLP-PI0;64H06-Gal4 > FRT-STOP-FRT::GFP fly brains: *The image represents a confocal scan of dissected fly brains stained with anti-GFP to visualize targeted dopaminergic neurons and anti-Brp (nc82) to label synaptic structures. No specific GFP signal corresponding to the expected neuronal population was detected and the nc82 staining appeared weak.*

3.3.2 Brain visualization of TH-GAL4 > UAS-GFP

The positive control staining showed significant GFP signals of the dopaminergic neurons after the convocal imaging.

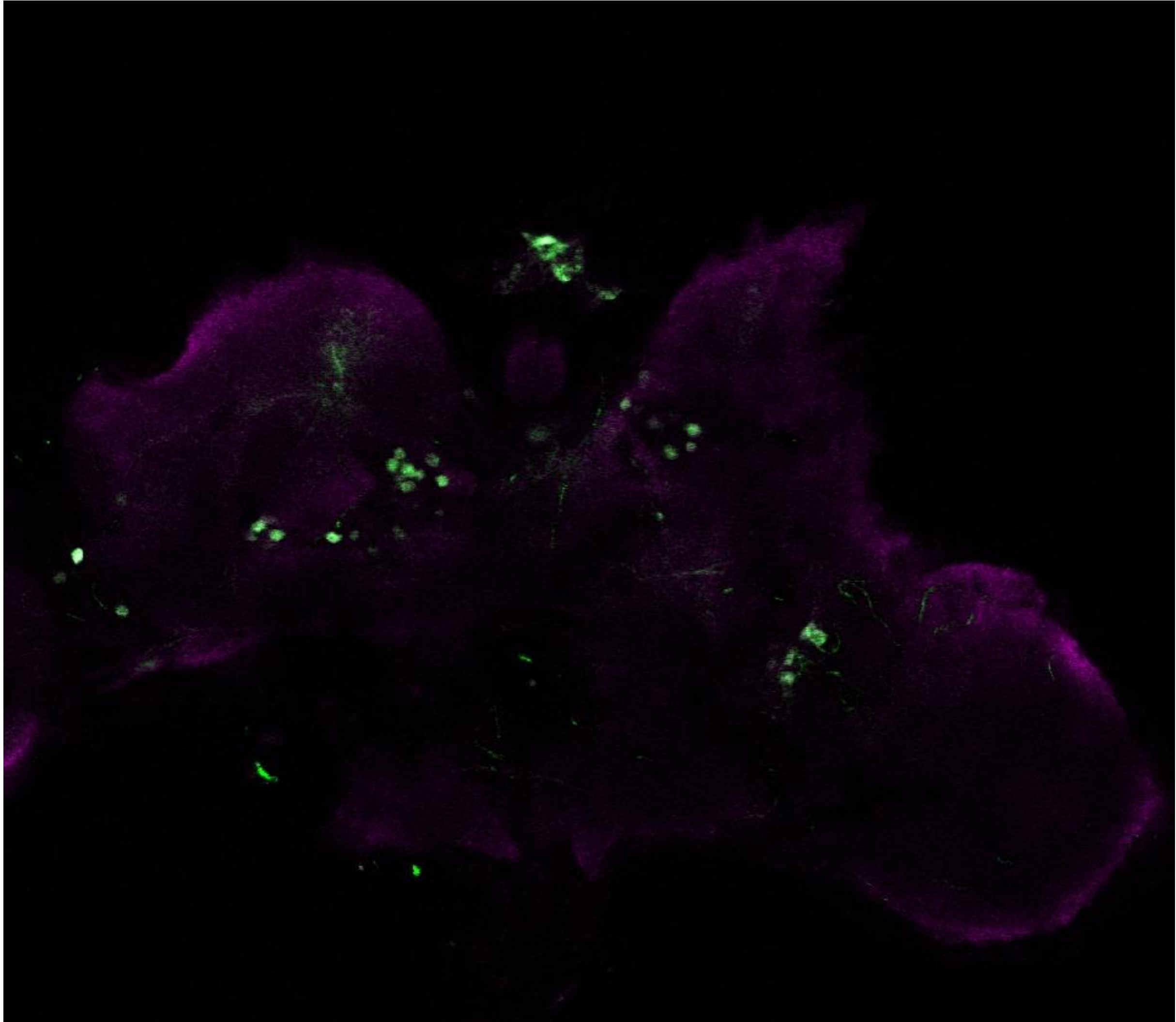

Figure 8: Confocal images with anti-GFP and anti-Brp staining of TH-GAL4 > UAS-GFP: GFP expression (green) marks dopaminergic neurons. They appear in distinct clusters throughout the brain. Anti-Brp (nc82) staining (magenta) weakly highlights synaptic regions. The presence of clear GFP-positive signals confirms successful labeling of dopaminergic neurons.

4. Discussion

This thesis investigated whether dopaminergic neurons in the PPM3 cluster and its projections to the mFB, EB and vFB of the TH-FLP-PI0;64H06-Gal4 line mediate approach or avoidance behavior. Therfore an operant learning paradigm was used, incorporating optogenetic activation (**Figure 1**). A Buridan pradigm was used to assess the effectivness of dopamine depletion via 3-iodo-tyrosine (3IY). Confocal imaging was performed to verify correct neuronal targeting.

The TH-FLP-PI0;64H06-Gal4 line did not show a consistent avoidance or approach response throughout the Joystick. This behavior could be explained by the ineffective neuronal targeting, as confirmed by confocal imaging. If the targeted neurons had been succesfully labeled and activated, there might have been an effect on the behavior. The PPM3 cluster projects to the mFB, vFB and EB which have been conncted to locomotion control, avoidance bahavior and sensory integration (Hu et al., 2018; Strauss, 2002; Liu et al., 2006; Ernst & Heisenberg, 1999). Therefor, activation of these neurons might have led to a noticable modulation of the decision-making process. Since the FB has been linked to avoidance behavior (Hu et al., 2018), targeting the right neurons might have led to a behavioral change.

The positive control group exhibited strong avoidance behavior, with a slightly stronger response under red light (660 nm) stimulation. This does not align with the fact that CsChrimson is more efficiently activated by yellow light (590 nm) compared to red light (Klapoetke et al., 2014). However, previous studies, such as the work by Guyton (*Guyton, 2023*.), have shown that flies do exhibited a stronger behavioral response under yellow light stimulation. It looks like an inconsisten response to these different wavelenghts. This suggests that factors beyond direct opsin activation, such as cuticle light absorption, may play a role. To measure the spectral transmission through the fly cuticle by using a spectrometer could help to determine how much red and yellow light actually reaches the neurons. This would provide further insights into potential wavelength dependent activation differences.

In addition to testing general optogenetic effects, this thesis also tried to investigate whether pre-test feeding influences learning behavior in positive controls. Prevoious studies (Rohrsen et al., 2021) did show strong avoidence of the positive control while the light was activated but

no sustainable learning in the post test where the light is inactivated. Over the course of this thesis it was observed that flies strongly avoided the previously light-associated side on the post test. To determine whether this learning effect might be linked to feeding before training, a separate analysis was conducted. Flies that were fed prior to testing were compared with those that were not fed before the experiment. The results showed that both fed and unfed flies demonstrated learning in the post-test, with avoidance of the previously light-associated side.

The Buridan experiment was conducted to determine whether WTB could serve as a positive control for dopamine depletion using 3IY. If dopamine depletion leads to a reduction in locomotor activity (Riemensperger et al., 2011), this approach could be used as a positive control for other experimental lines. After 48 hours of feeding, no clear difference in locomotion was observed between 3IY-treated and sucrose-fed flies. At first this suggested that dopamine depletion was not sufficient to impact movement. However, extending the feeding duration to 96 hours revealed an effect. While the median locomotion speed remained comparable between both groups, 3IY-treated flies covered significantly less distance overall. This indicates that they paused more frequently or for longer periods. It is possible that the 3IY metabolism requires a longer feeding duration to sufficiently deplete dopamine levels in WTB flies. Also, the effectiveness of 3IY could have declined over time due to degradation during storage. Ordering fresh 3IY could lead to more consistent effects. Moreover, the observed reduction in total distance at 96 hours could indicate that dopamine depletion does not directly impair movement speed. It may affect the regulation of movement, such as the initiation or continuation of locomotion. This might show that dopamine plays a role in changing movement motivation rather than pure motor output.

In future experiments the 3IY can be applied to experimental lines that have previously shown behavioral effects (Rohrsen et al., 2021). This would enable a more detailed investigation into whether the observed behavioral changes are dopamine-dependent and provide further insights into the role of specific dopaminergic neurons in reinforcement learning.

A limitation of this study was the failure to confirm correct neuronal targeting of the TH-FLP-PI0;64H06-Gal4 line. The used driver line was expected to label for dopaminergic neurons in

the PPM3 cluster (Xie et al., 2018), yet no clear GFP expression was detected in the expected brain regions. The genetic crosses might not have worked correctly, leading to an absence of expression in the targeted neurons. To further investigate this, the parental lines can be examined individually to determine whether correct expression is present in these flies. If GFP expression is observed in the parental lines, the issue likely arose during the crossing process. If no GFP expression is detected in the parental lines, this would indicate a potential issue with the original fly stocks. In this case verification with the institution that provided the flies may be necessary to confirm the correct genotype and expression pattern.

4.1 Summary

The optogenetic testing of the TH-FLP-PI0;64H06-Gal4 did not show a significant behavioral change. Confocal imaging revealed that no neurons were labeld, which could explain these results. The positive control of the Joystick showed strong avoidance but also showed slightly different responses at different wavelenghts of the light. Pre-test feeding of the flies before the Joystick experiment does not have an impact on the learning behavior shown in the post-test. WTB flies showed a reduction of the traveled distance after beeing fed 3IY for 96 hours.

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6. Attachment

Table 4: Material List

Name	Origin	Details
3-iodo-tyrosine (3IY)	Merck	10 mg/mL in 5% sucrose
All-Trans-Retinal (ATR)	Cayman Chemical	200 mM in ethanol
Normal Goat Serum (NGS)	PAN^{TM}-Biotech	10% (for blocking in staining)
VECTASHIELD® Mounting Medium	VECTOR Labs	Antifade mounting medium for confocal imaging
Mouse-anti-Bruchpilot (nc82)	Thermo Fischer	Primary antibiody
Rabbit-anti-GFP	Invitrogen	Primary antibody
Goat-anti-Rabbit AF488	Invitrogen	Secondary antibody
Goat-anti-Mouse AF555	Thermo Fischer	Secondary antibody
UV-sensitive glue	3M Deutschland GmbH	For fixing flies in the Joystick setup. (Product is not available anymore)
QIAfilter Midi Plunger 5	QIAGEN	Filter paper, 3IY for Buridan

Table 5: Fly Food Medium

Ingredients	Percentage (%)
Agar-Agar	4
Corn Grits	36
Brewers´Yeast	8
Soy Flour	5
Malt Extract	36
Sugar Beet Syrup	10
Nipagin	1

7. Acknowledgments

I want to thank….

….	my supervisor Prof. Dr. Björn Brembs for giving me the opportunity and the resources to perform my bachelor thesis in his lab.

….	Dr.Radostina Lyutova for her guidance at the beginning of my time in the lab.

….	Daniel Döringer, your guidance and patience throughout the whole time really helped me a lot.

….	Marcela Loza-Hilares for being so kind, always helping and answering questions.

….	Annelise Götz for great help with technical issues.

….	all the lab members for a great time.

….	my girlfriend, friends and family for all the support.

8. Declaration of authorship

The submitted printed copies and the submitted electronic version of the thesis are identical. I have written the thesis independently, have not used any sources or tools other than those indicated and have not already submitted the thesis to another university for the purpose of obtaining an academic degree. Furthermore, I confirm that I am aware of the legal consequences provided in § 27 (5) of the current examination regulations.

Die vorgelegten Druckexemplare und die vorgelegte elektronische Version der Arbeit sind identisch. Ich habe die Arbeit selbstständig verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die Arbeit nicht bereits an einer anderen Hochschule zur Erlangung eines akademischen Grades eingereicht. Weiterhin bestätige ich, dass ich von den in § 27 Abs. 5 der geltenden Prüfungsordnung vorgesehenen Rechtsfolgen Kenntnis habe.

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Date		Signature