Optogenetic activation of neuronal circuits and its effect on naïve gustatory behavior in Drosophila larvae

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Abstract

The mushroom bodies located in insects' central nervous system serve a significant function, as they are the main center for olfactory learning and memory formation. Despite this, the connection between mushroom bodies and innate gustatory behavior remains unclear. Larvae from the fruit fly *Drosophila melanogaster* were used to study how optogenetic activation of different neurons connected to the mushroom bodies affect naïve gustatory behavior. By utilizing the optogenetic tool Channelrhodopsin-2-XXL, genetically modified larvae were tested in gustatory preference tests to see how their behavior changes based on different tastants and their changing concentration. This study shows that activating Kenyon cells and specific dopaminergic neurons increases the larvae's aversive response to salt but does not affect their natural preference towards sugar. These findings indicate that KCs and DANs in association with MBs do play a role in affecting larval *Drosophila's* innate behavior.

Zusammenfassung

Die Pilzkörper im zentralen Nervensystem von Insekten erfüllen eine bedeutende Funktion, da sie das Hauptzentrum für olfaktorisches Lernen und Gedächtnisbildung darstellen. Die Verbindung zwischen den Pilzkörpern und dem angeborenen gustatorischen Verhalten ist jedoch unklar. Larven der Fruchtfliege *Drosophila melanogaster* wurden verwendet, um herauszufinden, wie die optogenetische Aktivierung verschiedenster Neurone in Verbindung mit den Pilzkörpern das naive gustatorische Verhalten beeinflusst. Durch Verwendung des optogenetischen Werkzeugs Channelrhodopsin-2-XXL wurden genetisch veränderte Larven in gustatorischen Präferenztests getestet, um zu sehen, wie sich ihr Verhalten basierend auf unterschiedlichen Geschmacksstoffen und sich derer ändernder Konzentration ändert. Diese Studie zeigt, dass die Aktivierung von Kenyon Zellen und spezifischen dopaminergen Neuronen die aversive Reaktion der Larven auf Salz verstärkt, jedoch ihre Präferenz für Zucker nicht beeinflusst. Diese Ergebnisse deuten darauf hin, dass Kenyon Zellen und dopaminerge Neuronen in Verbindung mit den Pilzkörpern eine Rolle bei der Beeinflussung des angeborenen Verhaltens der *Drosophila* Larven spielen.

1 Introduction

The reason of any organism effectively navigating through its environment lays in its ability to distinguish between good and bad feeding decisions. The perception and response to different chemicals is therefore crucial (Schipanski et al., 2008). Two different systems in the organism help with differentiating: olfactory and gustatory. This is especially important in larval *Drosophila,* as they spent most of their life feeding (Sewell et al., 1974). Based on experience, larvae learn how to respond to changings in their nutritional environment accordingly (Aso et al., 2014). The region playing the most important role in *Drosophila* with remembering such changes is the mushroom body (MB). It receives olfactory and gustatory inputs, through which a memory trace can be formed in adult (Heisenberg, 2003), as well as in larval animals (Gerber, et al., 2009). In contrast to memory formation, animals with no prior experience exhibit their naïve behavior which leads to the idea that there are specific innate genetic neuronal circuits (Aso et al., 2014). Numerous studies have shown the connection between mushroom bodies and olfactory learning and learning in general (reviewed by Boto et al., 2020). Yet there are no studies so far on how the mushroom bodies stand in correlation with larvae's naïve gustatory behavior.

The mushroom body in the larval *Drosophila's* central nervous system (CNS) is a paired structure which is made up of intrinsic cells called Kenyon cells (KCs) (Ito and Hotta, 1992). With the help of EM-reconstructions it has been show that first instar larvae have 223 KCs (Eichler et al., 2017), while third star larvae have around 800 KCs (Ito and Hotta, 1992). The Kenyon cells in the MB receive local inputs through their innervation with MBINs, which are either dopaminergic (DANs) or octopaminergic neurons (OANs) (Eichler et al., 2017). DANs can be divided into two clusters, depending on their location. One cluster is called the primary protocerebral anterior medial cluster (pPAM), and the other one dorsolateral 1 cluster (DL1) (Weber et al., 2023). The larval MB is organized into 11 compartments that are innervated by mushroom body input and output neurons (MBINs/MBONs) (Saumweber et al., 2018). Eight out of these 11 are defined by the input of dopaminergic neurons (DANs) (Weber et al., 2023). Previous studies have shown that while optogenetic activation of OANs and pPAM DANs signal appetitive stimuli towards the mushroom body, artificially activating DL1 neurons from the DANs cluster is perceived as a form of punishment (Schroll et al., 2006; Rohwedder et al., 2016; Saumweber et al., 2018). Regarding the organisms' neurons, only 120 out of 10.000 neurons existing in *Drosophila* larvae are of dopaminergic nature (Selcho et al., 2009; Rohwedder et al., 2016). Thus, dopaminergic neurons mediate the appetitive/aversive signals (DANs/OANs) received from Kenyon cells which further project onto MBONs to modulate

output for managing behavior (Aso et al., 2014; Eichler et al., 2017). As mentioned, it is crucial for the larvae's survival to distinguish between nutritional, non-nutritional and even toxic foods (Apostolopoulou et al., 2015). With focus on the gustatory system, it is consistent of three major sense organs located in the larval head region, namely dorsal organ (DO), terminal organ (TO) and ventral organ (VO) (Vosshall and Stocker, 2007). Additionally leading to three more chemosensory organs located along the pharynx (Colomb et al., 2007). All gustatory inputs received through sensory organs get projected via distinct nerves straight to the brain of the larvae (Rohwedder et al., 2012). All neurons lead to the major taste center, the subesophageal ganglion/zone (SOG/SEZ) (Kwon et al., 2011). Previous studies have shown, that according to the nature and concentration of the tastant, different receptors are activated in the larvae's brain and therefore different behavioral responses can be observed (Niewalda et al., 2008; Mishra et al., 2013; Apostolopoulou et al., 2015).

Considering this genetical background of larval *Drosophila*, gustatory preference tests were performed. With the help of a blue light activated channel named Channelrhodopsin-2-XXL, neurons of transgene flies were artificially activated. ChR2-XXL is an optogenetic tool with which neurons can be depolarized with light and a change in behavior might be observed (Dawydow et al., 2014). To achieve targeted gene expression in the wanted organisms, the GAL4-UAS and the LexA/LexAop systems were used, which work similar (Fischer et al., 1988; Lai and Lee, 2006). As previously mentioned, many studies focused on the mushroom bodies function in context with olfactory memory and the behavioral changes following, with little regard to naïve gustatory (reviewed by Boto et al., 2020). This thesis aims to further understand what role the mushroom bodies play in larvae's naïve gustatory preference, as it has been recently discovered, that mushroom bodies affect naïve olfactory behavior (Vogt et al., 2021, Radostina Lyutova, doctoral thesis). Furthermore, I intended to find out how optogenetic activation of different cell clusters in close connection to the MB affects the innate gustatory behavior of *Drosophila* larvae.

2 Material and Methods

2.1 Fly Stocks and maintenance

All fly strains were raised in a 12h/12h light-dark cycle at 25°C and 60 % humidity in glass vials containing food with yeast. Driver and effector lines used for crossing are listed in Table 1. Newly hatched female virgin flies were collected each morning and up to every two hours and stored at 18°C for 3 to 4 days before being used for experiments.

Table 1: List of fly lines used for crossing.

Stock	Genotype	Source
W1118	w[1118]	
H24-Gal4	P{w[+mW.hs]=GawB}H24	Andreas Thum
TH-Gal4	w[*]; P{w[+mC]=pleGAL4.F}3	
R58E02-GAL4	w[1118]; P{y[+t7.7] w[+mC]=GMR58E02-GAL4}attP2	Andreas Thum
MB054B		Andreas Thum
UAS-ChR2-XXL	y[1] w[w1118]; PBac{y[+mDint2] w[+mC]=UAS-ChR2.XXL}VK00018	Robert Kittel, Tobias Langenhan
$\frac{R58E02-LexA}{Cyo}$; $\frac{H24-Gal4}{TM2}$		Radostina Lyutova
$\frac{UAS-ChR2-XXL}{Cyo}$; $\frac{LexAop-rpr}{TM6b}$		Radostina Lyutova

2.2 Agar Plates

For all experiments, Petri dishes were divided straight down the middle to obtain two equally big sides. Agarose solution was brought to a boil in a microwave and thinly poured into Petri dishes. After drying, one half was removed with a small spatula and instead filled with 1,5% agarose containing either 2M fructose, 1,5M sodium chloride or 2,5M sodium chloride (**Figure 1**). It is crucial that both sides are evenly filled to prevent the formation of a slope. After ensuring the agarose is fully dried, experiments should be conducted shortly after to avoid diffusion of salt into the pure agarose side and thereby falsifying the experiment. **Table 2** provides the quantities for about 20 agar plates.

Table 2: Ingredient list for about 20 agarose plates

	Water	**Agarose**	**Salt**	**Fructose**
Standard Agarose	250 ml	3,75 g	-	-
Agarose with salt (1,5M)	150 ml	2,25 g	13,05 g	-
Agarose with salt (2,5M)	150 ml	2,25 g	21,90 g	-
Agarose with sugar (2M)	100 ml	1,5 g	-	36 g

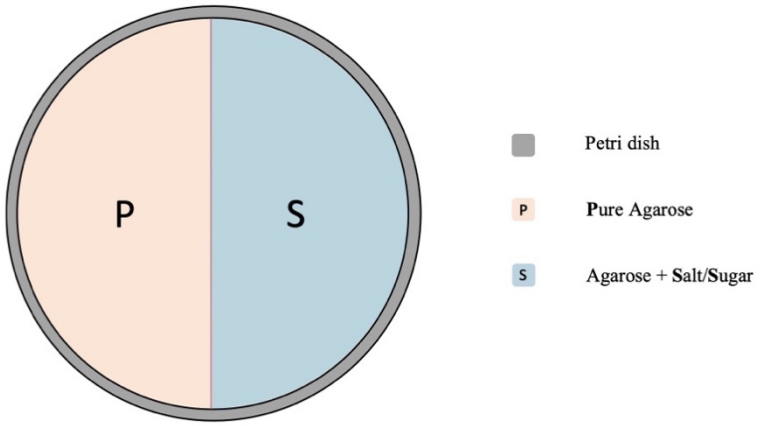

Figure 1: Experimental setup: Prepared agar plate.

2.3 Crossing schemes

Table 3: Combined lines created for experiments

H24-Gal4/UAS-ChR2-XXL
R58E02-Gal4/UAS-ChR2-XXL
TH-Gal4/UAS-ChR2-XXL
H24-Gal4;R58E02-LexA/UAS-ChR2-XXL;LexAop-rpr
MB054B/UAS-ChR2-XXL

In cases where there is a potential impact on the larvae's naïve gustatory preference through optogenetic activation, the respective driver and effector lines were crossed with w^{1118} as negative genetic controls.
Alternatively, positive controls were created with lines lacking the LexA/LexAop construct, as the use of the GAL4/UAS system already implies a phenotypic expression.

2.4 Gustatory preference tests

To start the experiments and guarantee a high larval density, 20 female virgin flies were crossed with 10 males and allowed to lay eggs for 24 hours. After crossing, the vials containing the *Drosophila* larvae were covered with aluminum foil to ensure complete darkness and prevent premature and constant activation of the manipulated neurons. For optogenetic stimulation of ChR2-XXL, 470nm LEDs with an intensity of 14W were used. Daily experiments were possible by flipping the flies into fresh vials every day, while making sure they are fully covered. This way, a continuous cycle was obtained. 6 days after crossing, third instar larvae were ready to be tested.
Using a spatula, a small amount of food paste with larvae was taken from each genotype to an empty Petri dish and carefully washed. After washing, 30 larvae were picked up with a small brush, collected in a small drop of water and afterwards collectively placed in the middle of the Petri dish with the no tastant on one side and salt/sugar on the other side. Tiny holes were added into the lid of the Petri dish to avoid condensation and therefore minimizing the number of larvae crawling to the top. The animals were then exposed to blue light to activate different cell

clusters, depending on which driver line was used. After 3 minutes of the larvae being allowed to move around freely, the number of larvae on each side and on the lid were counted and evaluated. All other steps of the experiment were conducted under red light.

2.5 Evaluations

To evaluate the larvae's naïve preference, a preference index (PREF) was calculated:

$$\boldsymbol{PREF = \frac{\#Tastant - \#NT}{\#Total}}$$

The number of larvae on the tastant side (**#tastant**), on the no tastant side (**#NT**) and on the lid of the Petri dish were counted and put into the equation.

#Total = #tastant + #NT + #lid
#: number of larvae

Positive preference indices indicate approach towards the tastant (appetitive behavior), whereas negative PI suggests avoidance (aversive behavior).

2.6 Statistical Analysis

Using R studio (Version 2023.06.2+561), all data was analyzed for normal distribution with the Shapiro-Wilk Normality test. In case of any non-normally distributed data, a Wilcoxon Signed Rank test was performed. To test for statistical significance, ($p < 0,05$) a one-sided t-test was performed for normal distributed data, otherwise a Wilcoxon Signed Rank was conducted. For comparing the genotypes with one another, a pairwise t-test was carried out to look for significant effects. All results are presented as boxplots. Boxplots divide the data into different sections, with the box containing 50% of the values and whiskers showing the entirety of the data. Outliers are presented as white circles. The median of the preference indices (PI) is shown as a thick black line within the box. Significance levels of and between the genotypes are shown above the boxplots, and represent the p-value, with one star (*) indicating a p-value $< 0,05$, two stars (**) indicating $p < 0,01$ and three stars (***) indicating $p < 0,001$. N.s. indicates a non-significant result ($p > 0,05$).

2.7 Chemicals

Table 4: Chemicals used for behavioral experiments

Chemicals	Manufacturer	CAS
Agarose Standard	Carl Roth GmbH	9012-36-6
D(-)-Fructose	Carl Roth GmbH	57-48-7
Sodium chloride	Carl Roth GmbH	7647-14-5

3 Results

Having outlined the material and methods used for my experiments, the effect of artificially activating different neuronal clusters on the naïve gustatory behavior of *Drosophila* larvae was now investigated.

3.1 Blue light does not affect the larvae's naïve behavior

Before starting all optogenetic experiments, wild type *Canton S* larvae were used to conduct a control experiment. As previous studies already conducted various preference tests under room light (Schipansky et al., 2008), I wanted to determine whether blue light affects the larva's innate gustatory preference, since blue light itself acts as a negative stimulus for the larvae (Luna et al., 2013). The control experiment is important to ensure that any effect observed can be specifically attributed to the optogenetic manipulation due to Channelrhodopsin and not due to the larvae's photonegative response towards the blue light. **Figure 2** shows that blue light did not affect the larvae's naïve behavior towards salt (**Figure 2A**) or sugar (**Figure 2B**). Also, when testing sugar against salt in a preference test, no difference in behavior can be observed under blue light (**Figure 2C**). Worth mentioning is, that when testing larvae on a salt/sugar plate, larvae showed a higher approach towards sugar, than on a pure/sugar plate. After establishing that blue light itself does not affect larvae's natural response to any tastant, preference tests with different genotypes could be conducted.

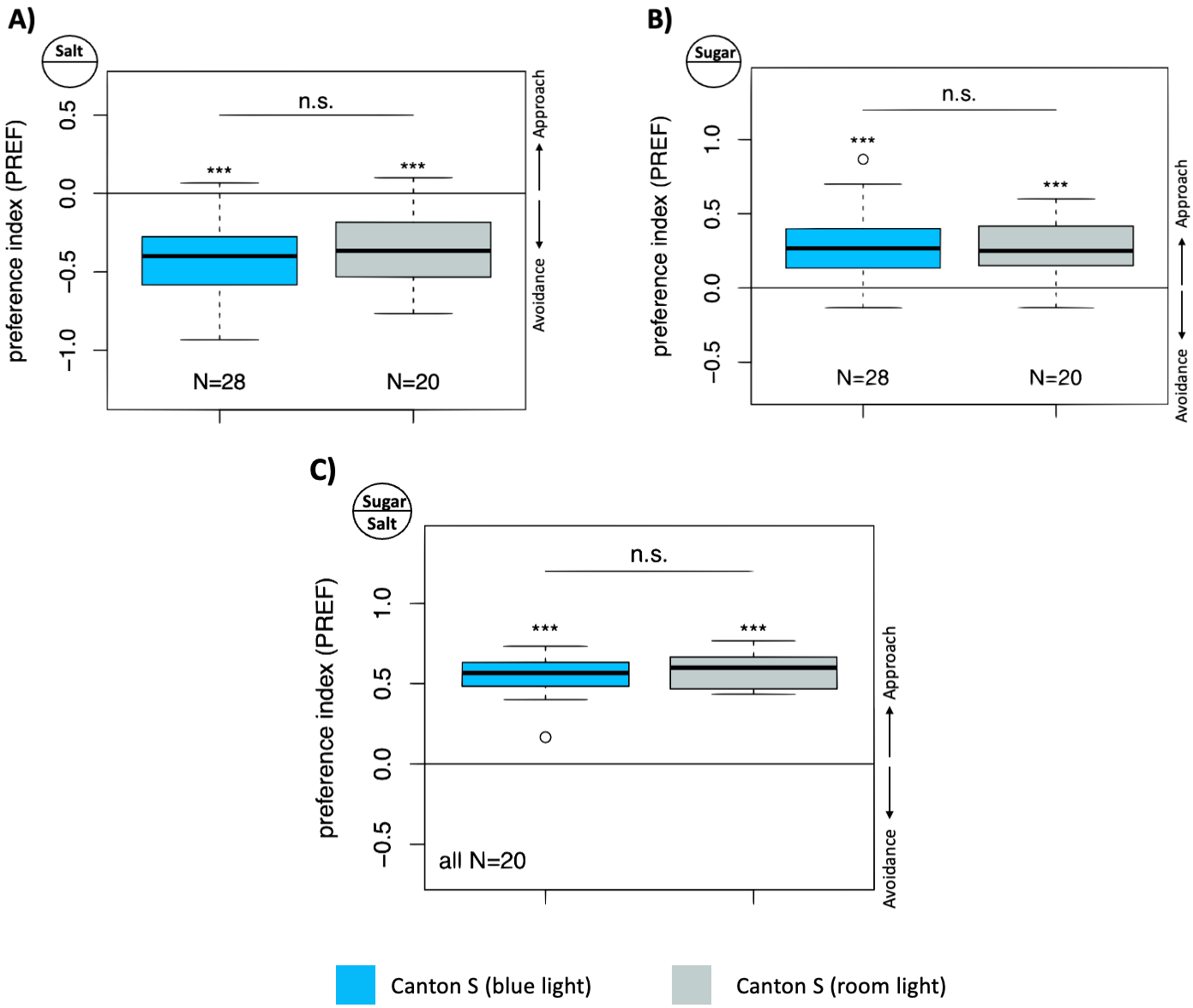

Figure 2: Blue light does not affect the larvae's innate behavior: 1) Wild type *Canton S* larvae tested for their salt preference under blue and room light. Larvae show significant avoidance of salt ($p < 0{,}001$) independent of light conditions. **2)** Wild type *Canton S* larvae were tested for their sugar preference under blue and room light. Larvae show significant approach towards sugar ($p < 0{,}001$) independent of light conditions. **3)** Wild type *Canton S* larvae were tested for their preference on a sugar-salt plate. Larvae show significant approach towards sugar ($p<0{,}001$) independent of light conditions. Each boxplot shows the data for *Canton S* under blue/room light, corresponding to the color in the legend beneath. Lines above boxplots indicate if there is a significant difference between the light conditions. White circles demonstrate outliers. Sample sizes are shown on the bottom. 1N equals 30 larvae. Stars above each boxplots represent p-value (n.s. $p > 0{,}05$, *$p < 0{,}05$, **$p < 0{,}01$, ***$p < 0{,}001$).

3.2 Is naïve sugar preference affected by optogenetic activation of Kenyon cells?

Numerous studies have investigated the involvement of the mushroom bodies in different aspects of behavior in both adult and larval *Drosophila*. It has been established that mushroom bodies play a critical role in associative learning and for memory in general (Roman and Davis, 2001; Heisenberg, 2003). Yet, there are no studies so far on the role of mushroom bodies in the naïve gustatory behavior of *Drosophila* larvae. I wanted to determine whether the activation of Kenyon cells in the MBs of *Drosophila* larvae had any effect on their naïve gustatory behavior. First, naïve sugar preference was tested. A Gustatory preference test (*Material and Methods,* **2.4**) was conducted under red and blue light. *H24-Gal4>UAS-ChR2-XXL* and negative control groups for driver and effector line crossed with w^{1118} were tested. Results show that all three genotypes display a significant preference for sugar in both test scenarios. However, the experimental larvae under blue light showed no significant difference in the naïve response in comparison to the genetic controls (**Figure 3**).

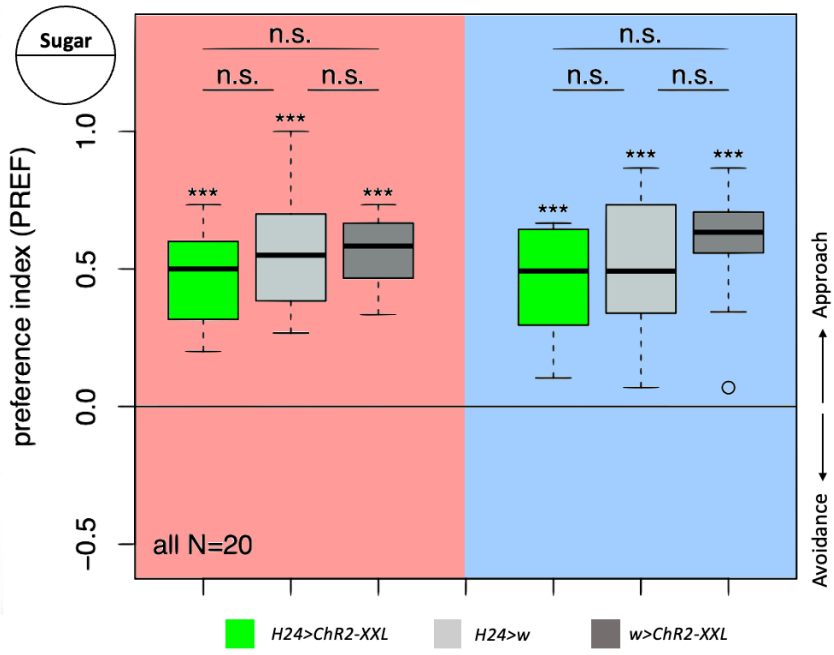

Figure 3: Optogenetic activation of Kenyon cells <u>does not</u> affect naïve sugar preference: Larvae were tested for their preference towards sugar under red and blue light. Positive preference index shows approach towards sugar. Both control groups and the experimental group showed a strong preference towards sugar regardless of lighting conditions ($p < 0{,}001$). Comparing the experimental larvae with control lines, no significant difference was observed when activating KCs under blue light ($p > 0{,}05$). Each boxplot shows the data for one genotype. The legend beneath the figure indicate which genotype was tested based on the corresponding color. Lines above boxplots indicate if there is a significant difference between genotypes. White circles demonstrate outliers. Sample size for all genotypes shown on the bottom left. 1N equals 30 larvae (N=20). Stars above each boxplots represent p-value (n.s. $p > 0{,}05$, *$p < 0{,}05$, **$p < 0{,}01$, ***$p < 0{,}001$).

3.3 Is naïve salt preference affected by optogenetic activation of Kenyon cells?

Following the evaluation of the larvae's naïve sugar preference, the question was raised whether optogenetic activation of Kenyon cells in the mushroom bodies has any effect on the larvae's naïve response to salt. The same setup, experimental group and negative controls were used as described in **3.2**. First, a concentration of 1,5M Salt was used and evaluated. Larvae from all genotypes showed significant aversion to salt. Under red light, no difference in the experimental larvae's gustatory response was observed. However, when tested under blue light, the experimental genotype exhibited a significant higher avoidance of salt than the control groups (pairwise t.test, p = 0.019) (**Figure 4A**).

On base of these findings, the salt concentration was raised to 2,5M and tested whether the effect would be amplified. Comparing the medians to the experiment with lower concentration, the aversion towards salt increased in all genotypes. However, our results showed, that using a higher salt concentration abolished our effect and a significant difference can no longer be seen between the experimental and control groups under blue light (**Figure 4B**).

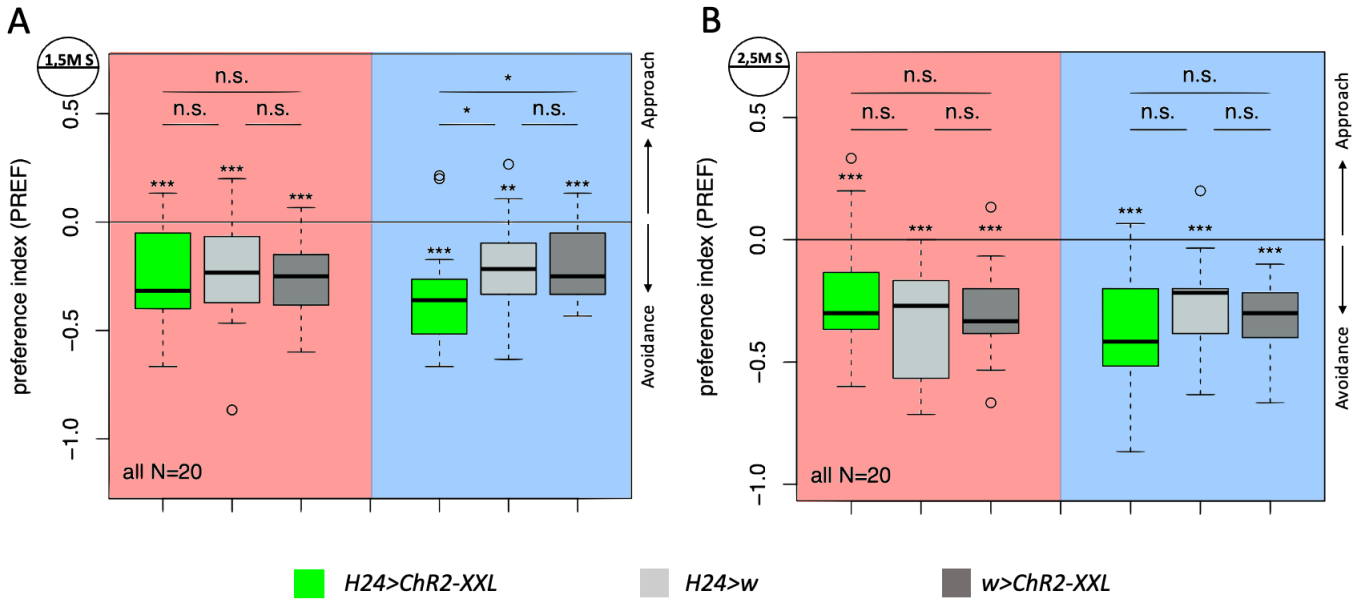

Figure 4: Naïve salt preference upon optogenetic activation dependent on salt concentration: (A) Larvae were tested for their salt preference under red and blue light with a salt concentration of 1,5M. Experimental larvae tested under blue light showed a significant higher avoidance of salt than the control groups (pairwise.t.test, p = 0.0019). **(B)** Experimental larvae were tested for their salt preference under red and blue light with a raised salt concentration of 2,5M. With a raised salt concentration, no significant difference in avoidance under blue light can be observed (p > 0.05). Negative preference index shows avoidance of salt. Each boxplot shows data for one genotype. The legend beneath the figure indicate which genotype was tested based on the corresponding color. Lines above boxplots indicate if there is a significant difference between genotypes. White circles demonstrate outliers. Sample size for all genotypes shown on the bottom left. 1N equals 30 larvae (N=20). Stars above each boxplots represent p-value (n.s. p > 0,05, *p < 0,05, **p < 0,01, ***p < 0,001).

3.4 Significance of DANs for gustatory preference

Dopaminergic neurons (DANs) have been shown to play an important role in MBs as their primary role is transmitting information about both aversive and appetitive stimuli (Mao and Davis, 2009; Selcho et al., 2009; Eichler et al., 2017). To determine how the larvae's preference is affected, two different subsets of dopaminergic neurons were tested. For both experiments, negative controls were achieved by crossing driver and effector line with w^{1118}. First *R58E02-Gal4* was used as a driver line. *R58E02-Gal4* activates three out of four pPAM DANs which are necessary for appetitive signals (Liu et al., 2012; Yamagata et al., 2015; Rohwedder et al. 2016). With this background in mind, a gustatory preference test was performed. Genotypes tested for control purposes under red light displayed aversion, with no significant distinctions between each other. Upon optogenetic activation under blue light, *R58E02-Gal4>UAS-ChR2-XXL* larvae showed no significantly different avoidance of salt than the control groups. (**Figure 5A**).

For the next part of the experiment a tyrosine hydroxylase *GAL4*-transgene was used. *TH-Gal4* for once is used to label four DANs of the DL1 cluster, which are important for aversive signaling. However, *TH-Gal4* also labels most other DANs, except for pPAM DANs and neurons in the SEZ (Selcho et al., 2009; Weber et al., 2023). All larvae tested under red light exhibited an avoidance to salt, yet there was no significant difference between the genotypes. Upon artificial activation, experimental larvae (*TH-Gal4>UAS-ChR2-XXL*) showed a significant higher aversion to salt compared to the genetic negative controls (pairwise.t.test, $p = 0.0349$; $p = 0.0085$) (**Figure 5B**).

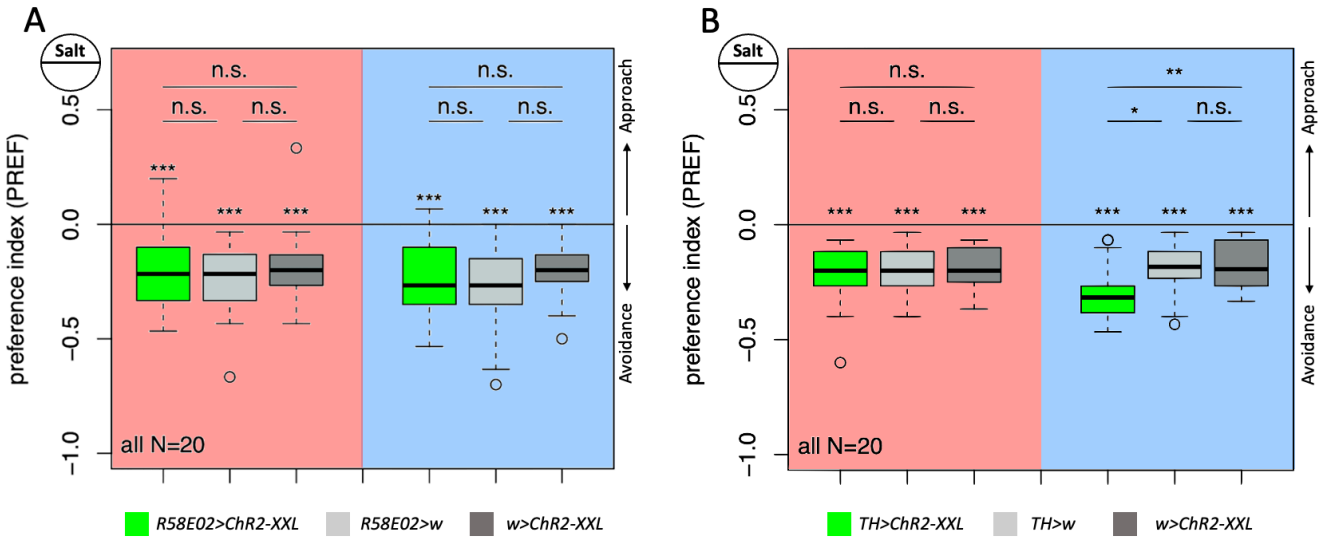

Figure 5: Optogenetic activation of different dopaminergic neurons: (A) *R58E02-Gal4* larvae were tested for their salt preference under red and blue light. Experimental larvae under blue light exhibited no significant difference in their preference compared to control groups or under red light ($p > 0.05$). **(B)** *TH-Gal4* larvae were tested for their salt preference under red and blue light. Experimental larvae under blue light showed a significant higher avoidance than under red light or both control groups (pairwise.t.test, $p = 0.0349$; $p = 0.0085$) Negative preference index shows avoidance of salt. Each boxplot shows data for one genotype. The legend beneath the figure indicate which genotype was tested based on the corresponding color. Lines above boxplots indicate if there is a significant difference between genotypes. White circles demonstrate outliers. Sample size for all genotypes shown on the bottom left. 1N equals 30 larvae (N=20). Stars above each boxplots represent p-value (n.s. $p > 0,05$, *$p < 0,05$, **$p < 0,01$, ***$p < 0,001$).

3.5 Activating specific DANs

After concluding that artificial activation of *TH-Gal4>UAS-ChR2-XXL* has an impact on larvae's naïve response to salt (**Figure 5B**), the question arose which specific neurons labeled with the *TH-Gal4* driver are responsible for these results. Since approximately 120 cells get activated with the driver line, it is complicated to narrow down specific cells (Weber et al., 2023). Since previous studies conducted that the DL1 cluster plays a role in sending aversive teaching signals, (Selcho et al., 2009; Eschbach et al., 2020) the next experiment focused on the activation of these specific neurons. DAN-c1, DAN-d1, DAN-f1, and DAN-g1 are the four DANs in the DL1 cluster which all innervate different compartments of the mushroom body (Eichler et al., 2017; Weber et al., 2023). To further investigate if the activation of individual DL1 neurons is responsible for boosting salt avoidance behavior in naïve *Drosophila* larvae, a split-Gal4 line (MB054B) was used. Through screening of lots of Gal4 lines, MB054B has been found to show strong expression in DAN-f1 and DAN-g1 (Weber et al., 2023). A gustatory preference test was conducted with negative controls for driver and effector lines. Larvae tested under red light exhibited significant avoidance of salt, but with no significant differences

between the genotypes. Testing the experimental line (*MB054B>UAS-ChR2-XXL*) under blue light conditions also showed a strong aversion to salt, however not significantly lower than both negative control groups (**Figure 6**).

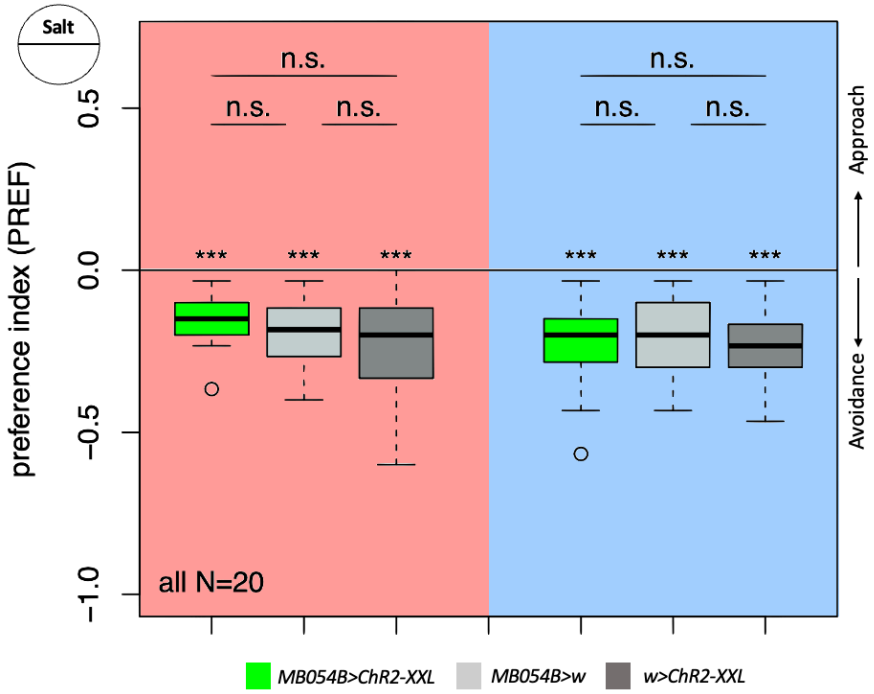

Figure 6: Activating DAN-f1 and DAN-g1 shows no significant effect: Larvae tested for their salt preference under red and blue light. Experimental larvae show no significant higher avoidance of salt under blue light than the controls ($p > 0.05$). Negative preference index shows avoidance of salt. Each boxplot shows the data for one genotype. The legend beneath the figure indicate which genotype was tested based on the corresponding color. Lines above boxplots indicate if there is a significant difference between genotypes. White circles demonstrate outliers. Sample size for all genotypes shown on the bottom left. 1N equals 30 larvae (N=20). Stars above each boxplots represent p-value (n.s. $p > 0,05$, *$p < 0,05$, **$p < 0,01$, ***$p < 0,001$).

3.6 Activation of KCs with simultaneous ablation of pPAM

To confirm that Kenyon cells in the mushroom bodies influence naïve salt behavior (**Figure 4A**) and that pPAM neurons can be disregarded (**Figure 5A**), the following experiment was conducted. For crossing, both the LexA/LexAop system and the Gal4/UAS system were utilized to activate KCs (*H24-Gal4 > UAS-ChR2-XXL*) while simultaneously ablating pPAM neurons (*R58E02-LexA > LexAop-reaper*) (Lyutova et al., 2019). To create positive genetic controls, lines lacking either the LexA or LexAop construct were used, as a behavioral expression was anticipated due to the usage of Gal4/UAS.

Under red light conditions, all genotypes displayed a significant aversion of salt, yet no considerable difference among each other. The positive controls tested under blue light showed a significant higher aversion in comparison to the control groups tested under red light (*see* **Attachment A, Figure 8**). Upon optogenetic activation, *H24>ChR2;R58E02>rpr* larvae with ablated pPAM neurons show a significantly higher avoidance than both positive genetic controls under blue light (pairwise.t.test p = 0.048; p = 0.043) (**Figure 7**).

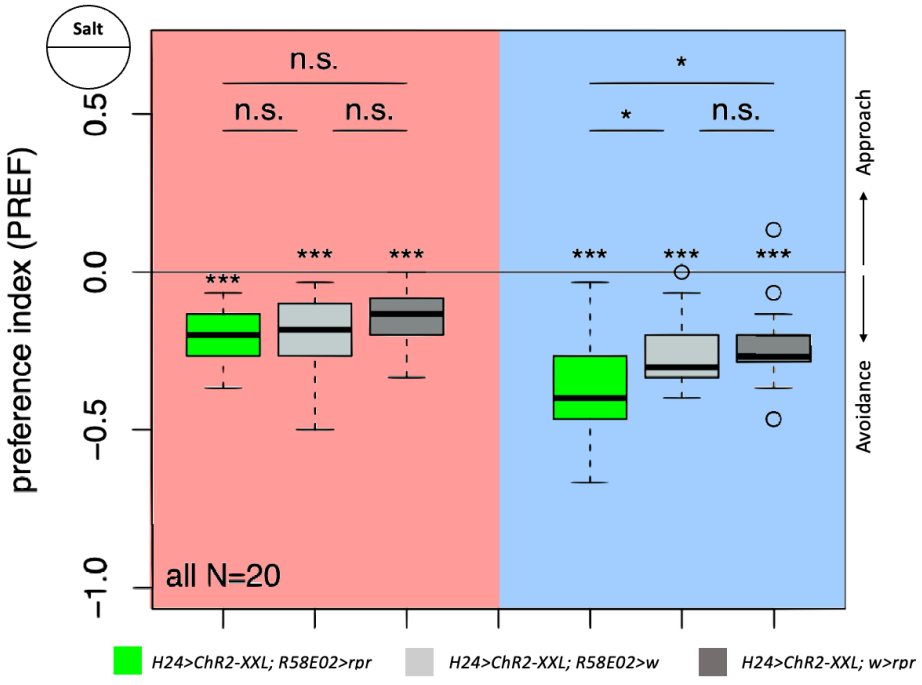

Figure 7: Activation of KCs with ablation of pPAM neurons show significant effect: Larvae tested for their salt preference under red and blue light. Experimental larvae show a significant higher avoidance of salt under blue light than the positive controls ($p < 0.05$). Negative preference index shows avoidance of salt. Each boxplot shows the data or one genotype. The legend beneath the figure indicate which genotype was tested based on the corresponding color. Lines above boxplots indicate if there is a significant difference between genotypes. White circles demonstrate outliers. Sample size for all genotypes shown on the bottom left. 1N equals 30 larvae (N=20). Stars above each boxplots represent p-value (n.s. $p > 0,05$, *$p < 0,05$, **$p < 0,01$, ***$p < 0,001$).

4 Discussion

After conducting all experiments, effects on the larvae's naïve gustatory preference were observed, depending on the type of tastant used and which specific neuronal regions were activated with optogenetic tools. As mentioned in **3.1**, experiments conducted under red light were purely done for control purposes, as Channelrhodpsin is not activated. Additionally, non-experimental larvae tested under blue light demonstrated that the blue light itself has no impact on the larvae's naïve gustatory preference (**Figure 2**). Instead, the significant effects exhibited in the following experiments were solely due to optogenetic activation with the blue light sensitive Channelrhodopsin (ChR2-XXL). The higher appetitive responses of larvae towards sugar on a salt/sugar plate (**Figure 2C**), can be attributed to the additive effect of salt avoidance and the simultaneous attraction to sugar.

Initially, it was investigated whether there were any differences in response to different tastants. When testing larvae on a sugar plate, no effect was observed in the evaluation (**Figure 3**). However, testing the experimental larvae on a salt plate showed, that optogenetic activation of Kenyon cells in the mushroom bodies does influence larvae's behavior towards salt (**Figure 4A**). It is important to note that the aversion to salt was about half as great as the animal's attraction to sugar. Larvae's appetitive responses to 2M sugar concentration shows median values of approximately -0.5 for all genotypes. These findings are consistent with previous studies where the animal's preference to different sugars, including fructose, was assessed (Schipanski et al., 2008). However, larvae did not react as avoidant of salt as expected. Interestingly, Niewalda et al. showed an average aversion of around -0.8 with a similar salt concentration as in this experiment (approximately 1.5M) (Niewalda et al., 2008). This contradicts the findings of this thesis, as any average results for the larvae's aversion to salt was around four times lower. However, there are possible reasons explaining these discrepancies. Firstly, Niewalda et al. used wild-type *Canton S* larvae in comparison to the larvae modified with a UAS/Gal4 construct used in this thesis (Niewalda et al., 2008). Another factor is all larvae tested in my experiments were raised in complete darkness to prevent premature and constant activation of the neuronal clusters. In contrast, the larvae from Niewalda et al.'s experiment were raised under normal room light conditions (Niewalda et al., 2008). To achieve similar results, like in the reference, gustatory preference tests should be conducted firstly with the same genotype, same salt concentrations and under normal light conditions.

Having established that MB KCs affect naïve gustatory behavior concerning salt, the concentration was raised to 2,5M for another preference test (*Material and Methods*, **2.4**).

Increasing the concentration revealed, that the significant effect previously observed in the experimental group had disappeared and no difference to the negative control groups could be noted (**Figure 4B**). The results showed all genotypes tested at a higher concentration exhibited a stronger aversion to salt, reaching a point where no significant difference can be observed in comparison to the experimental larvae. This aligns with previous studies confirming higher salt concentration induces a higher aversion of salt (Miyakawa, 1981). However, when focusing on the average median of aversion, these findings are contradictory with the results published by Niewalda et al., where the maximum aversion reached almost -1 (Niewalda et al., 2008). In comparison to results from this thesis, the maximum aversion only reached a median of -0.36 with a salt concentration of 2.5M (**Figure 4B**). One potential explanation for these findings could be that the maximum aversion is reached at around -0.36, therefore abolishing the effect observed with the lower salt concentration. Through comparing the medians, a difference can be seen in the experimental group compared to the control groups. Another reason therefore could be that the big dispersion of data in the experimental group (**Figure 4B**) abolishes the effect observed at 1.5M. However, keeping the differences of the experimental design with Niewalda et al. in mind, previously mentioned reasons could again be an explanation for these differences (Niewalda et al., 2008).

Dopaminergic pPAM neurons are necessary for appetitive olfactory learning but have been shown to be dispensable for aversive learning, as well as the larvae's innate behavior. Therefore, artificially activating these neurons can be perceived as an appetitive signal (Rohwedder et al., 2016). Not surprisingly, when testing experimental larvae (*R58E02-Gal4>UAS-ChR2-XXL*) on a salt plate, no significantly different behaviors towards salt than the negative control groups was observed (**Figure 5A**). This result was expected, as salt is not perceived as a reward. Therefore, pPAM neurons can be disregarded and do not play a role in the animal's innate reaction to salt.

In contrast to the appetitive functions of pPAM neurons, DANs are also important for transmitting aversive signals. As mentioned in **3.4**, prior studies have demonstrated that the *TH-Gal4* driver can be used to label four dopaminergic neurons of the DL1 cluster, but also most other DANs in the larvae except for pPAM neurons and neurons in the subesophageal zone (Selcho et al., 2009; Weber et al., 2023). As expected, optogenetic activation of experimental larvae (*TH-Gal4>UAS-ChR2-XXL*) showed significant increase in aversion to salt (**Figure 5B**). It is worth noting, however, that the *TH-Gal4* driver line does not cover the neurons from the subesophageal zone (SEZ), where most sensory neurons in larvae project to. That is why the SEZ is believed to be the central compartment for larvae's naïve gustatory behavior (Scott,

2018). Despite that, *TH-Gal4* does not cover these neurons. Yet, significant changes in the innate response to salt can be noticed under blue light. This raises the idea that another pathway is responsible for these results and not due to the activation of the SEZ neurons. Nevertheless, dopaminergic neurons covered by the *TH-Gal4* driver seem to play a role in affecting the larvae's naïve gustatory preference.

With the focus on neurons from the DL1 cluster, I wanted to determine if activating individual neurons of the DL1 cluster still transmits an aversive signal, like previous studies mentioned (Selcho et al., 2009; Eschbach et al., 2020). For this a split-Gal4 line (MB054B) was used. MB054B is strongly expressed in two out of the four dopaminergic neurons in the DL1 cluster (Weber et al., 2023). Previous studies have shown that optogenetically activating DAN-f1 and DAN-g1 neurons from the DL1 cluster, can substitute for high salt punishment during odor presentation and leads to an aversive olfactory memory (Eschbach et al., 2020; Weiglein et al., 2021; Weber et al., 2023). In contrast, focusing on the larvae's naïve behavior to salt showed, that optogenetically activating DAN-f1 and DAN-g1 did not increase the *MB054B>UAS-ChR2-XXL* larvae's dislike of salt compared to respective control groups (**Figure 6**). The results (**Figure 6**) therefore suggest no impact on naïve behavior to salt. This shows that the role for mediating aversive signals towards the mushroom bodies regarding naïve behaviors could be distributed among other dopaminergic cells (Weber et al., 2023). To gain a better understanding of the connectivity between DL1 neurons and KCs in the mushroom bodies, different subsets of dopaminergic neurons must be tested. This way the neuronal mechanism underlying the effect on naïve gustatory preference can be better understood.

Looking at the previous results, it was already established that artificial activation of Kenyon cells in the mushroom bodies affects naïve salt preference in larval *Drosophila* (**Figure 4A**). Additionally, given the pPAM neurons' role in mediating appetitive signals (Rohwedder et al., 2016), they can be overlooked in regard of aversive signaling. To verify these results, Kenyon cells were activated in a gustatory preference test (*Material and Methods*, **2.4**) with simultaneous ablation of pPAM neurons. Optogenetic activation of the experimental group (*H24>ChR2;R58E02>rpr)* enhanced larvae's salt avoidance significantly compared to both positive genetic controls (**Figure 7**). These findings align with the previous conducted experiments, since artificially activating Kenyon cells in the mushroom bodies enhanced the larval *Drosophila's* dislike of salt (**Figure 4A**). On the other hand, pPAM neurons did not change the larvae's naïve response to salt (**Figure 5A**). Ablating these neurons therefore should make no difference. However, comparing the experimental line to control groups reveals that the significance only reaches p=0.048 and p=0.043, making the results barely significant.

However, the low p-value scores could possibly be attributed to the big dispersion of data. In this experiment, flies carrying balancer chromosomes had to be sorted out to only obtain homozygous flies. It is possible that a fly with a balancer has not been correctly identified and therefore been used for crossing. This mistake could possibly explain the dispersion. In contrast, comparing the results from this thesis with a previously conducted experiment using the same crossings and setup (Radostina Lyutova, doctoral thesis), shows the opposite result (*see* **Attachment B, Figure 9**). Results from that experiment show, that all larvae tested under blue light show higher avoidance of salt than under red light. However, no significant difference in the experimental larvae to the control groups can be observed (**Attachment B, Figure 9**). However, the ablation of pPAM neurons could influence the results by pushing the behavioral balance towards aversion. But due to these contradictory results, experiments should be conducted again to achieve sufficient results and find out more about the underlying process leading to this behavior.

4.1 Outlook

To summarize, it was demonstrated that KCs and dopaminergic neurons play a role in affecting the larvae's innate gustatory behavior to salt. Due to some contrary results and still lots of open questions, more investigation needs to be done on this topic. Future experiments could start by focusing on other split-Gal4 lines specific to the DL1 cluster. Calcium imaging experiments show that using the lines SS02160 (DAN-c1) and MB328B (DAN-d1) showed aversive responses to a 1M salt concentration (Weber et al., 2023). There are only two more split-Gal4 lines available for testing other DL1 DAN combinations, which are MB065B (DAN-c1/DAN-f1) and SS01702 (DAN-c1/MBIN-e1). Gustatory preference tests with these specific neurons activated could possibly enlighten the neurons responsible in behavioral changes. More clarifying results could also be obtained through optogenetic silencing of specific neuronal circuits to study the necessity of neuronal subpopulations and narrowing down the dopaminergic neurons responsible in affecting the naïve gustatory behavior. Furthermore, dopamine receptors could be knocked down via RNAi, to investigate which specific DA receptors in the mushroom bodies are required for the modulation of innate behavior.

The mushroom bodies are known to be involved in olfactory learning, as well as affecting innate naïve olfactory behavior. Mushroom bodies therefore offer a broad field for further investigation and remain an interesting topic to analyze the larvae's behavior not just in context with naïve gustatory behavior.

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…my family and friends for always supporting me.

Attachment

A) Comparison of genotypes under red and blue light (Exp. 3.6)

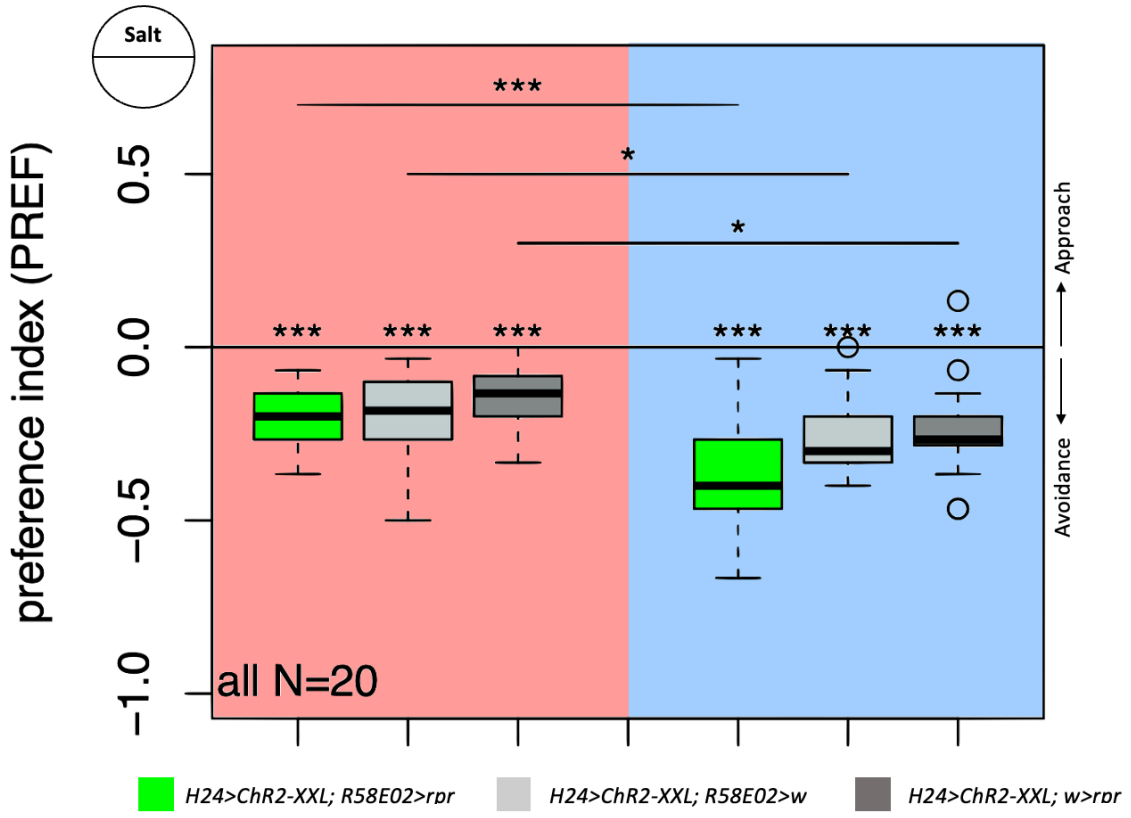

Figure 8: All larvae exhibit higher salt avoidance under blue light: Experimental group (pairwise.t.test, $p < 0{,}001$) and positive controls for driver (pairwise.t.test, $p = 0.03$) and effector line (pairwise.t.test, $p = 0.012$) show significant higher salt aversion upon activation under blue light than in red light. Each boxplot shows the data for one genotype. The legend beneath the figure indicate which genotype was tested based on the corresponding color. Lines above boxplots indicate if there is a significant difference between genotypes. White circles demonstrate outliers. Sample size for all genotypes shown on the bottom left. 1N equals 30 larvae (N=20). Stars above each boxplots represent p-value (n.s. $p > 0{,}05$, *$p < 0{,}05$, **$p < 0{,}01$, ***$p < 0{,}001$).

B) KC activation with pPAM ablation experiment by Radostina Lyutova

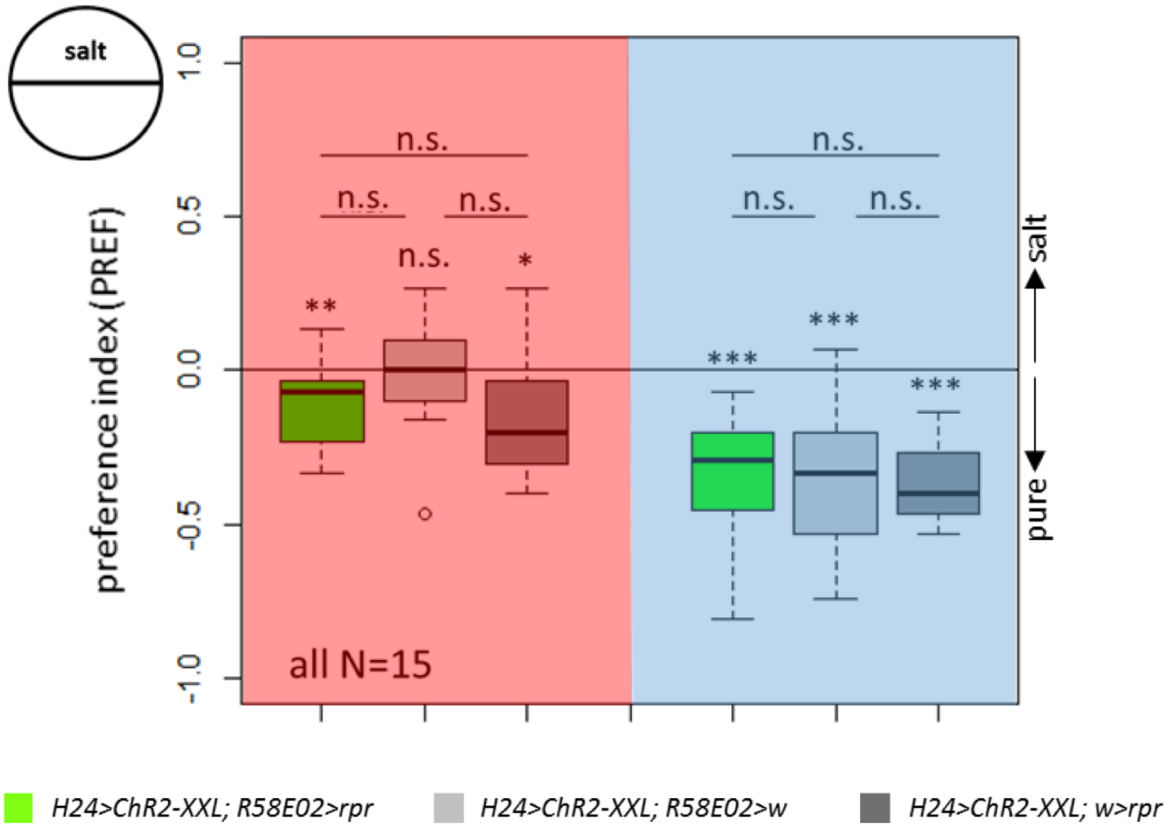

Figure 9: Simultaneous activation of KCs with ablation of pPAM neurons conducted by Radostina Lyutova: Larvae tested for their salt preference under red and blue light. Experimental larvae show no significant higher avoidance of salt under blue light than the controls ($p > 0.05$). Overall, larvae tested under blue light showed an amplified aversion to salt compared to larvae tested under red light. Each boxplot shows the data for one genotype. The legend beneath the figure indicate which genotype was tested based on the corresponding color. Lines above boxplots indicate if there is a significant difference between genotypes. White circles demonstrate outliers. Sample size for all genotypes shown on the bottom left. 1N equals 30 larvae (N=15). Stars above each boxplots represent p-value (n.s. $p > 0{,}05$, *$p < 0{,}05$, **$p < 0{,}01$, ***$p < 0{,}001$).

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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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