Dopaminergic neuronal populations mediating reward in *Drosophila melanogaster*

Master Thesis

accomplished at the Lehrstuhl für Entwicklungsbiologie

Naturwissenschaftliche Fakultät III

Biologie und Vorklinische Medizin

Universität Regensburg

Submitted by

Christian Rohrsen

from Algeciras (Spain)

25.04.2012

Table of contents

ABSTRACT

Evolution has shaped the amount of information that an individual stores in order to optimize the relation of the benefits of knowing certain information to the costs of having this memory stored. In order to filter the relevant information from the irrelevant one, animals are endowed with a system that attaches a value to environmental events. Dopamine appears to be an important neurotransmitter involved in this value assignment in vertebrates and invertebrates. Dopaminergic neurons are known to play an essential role in the reinforcement in *Drosophila*. The direction of learning depends on the dopaminergic cluster involved: whereas the PAM cluster mediates appetitive learning, the PPL1 cluster mediates aversive learning. Nevertheless, studies in the fruit fly have focused in these two clusters because they are the dopaminergic clusters projecting to the mushroom body, an essential brain structure for learning. In this study, we decided to find dopaminergic neurons, projecting and non-projecting to the mushroom bodies, that confer a positive valence to flies. We believe that although assigning a value to events is important for learning, there could be other neurons involved in value assignment without any relevance to learning. For assessing the role of different dopaminergic clusters in mediating valence, we tested the flies in a T-maze so that if an individual fly approached one arm, a given cluster is activated whereas when approaching the other arm no neurons are activated. The activation of neurons was accomplished with optogenetics by displaying the light that excites the Channelrhodopsin in one arm and the non-excitable light in the other arm. Strikingly, results were different to that observed in olfactory conditioning studies in flies. We were able to show that the technique functions, as well as some insights into its methodology.

INTRODUCTION

Channelrhodopsin and optogenetics

Opsins are seven transmembrane apoproteins which need to be covalently linked to retinal in order to be functional. This functional photoreceptor (with the covalently bound chromophore) is then called rhodopsin (Kato et al., 2012; Ullrich et al., 2013).

Rhodopsins are classified into two different groups according to their primary DNA sequences: microbial (type I), which are light-gated regulators of ion conductance and have all-trans-retinal (ATR) as a chromophore, and animal (type II), which are light-gated GPCRs. Type I rhodopsins comprise bacteriorhodopsins (BR), halorhodopsins (HR) and channelrhodopsins (ChR). Whereas the two former are light-gated ion pumps, the latter are light-gated channels (Kato et al., 2012).

Channelrhodopsin-2 (ChR2) is native to the green alga *Chlamydomonas reinhardtii*. Its aperture produces a cation influx into the cell, and when expressed in neurons, it depolarizes them (Bamann et al., 2010; Ullrich et al., 2013). Due to its ability to depolarize neurons, it has been recently introduced as an optogenetic tool (Nagel et al., 2003). Although this technique has an increasing application in research, little is known about its structure.

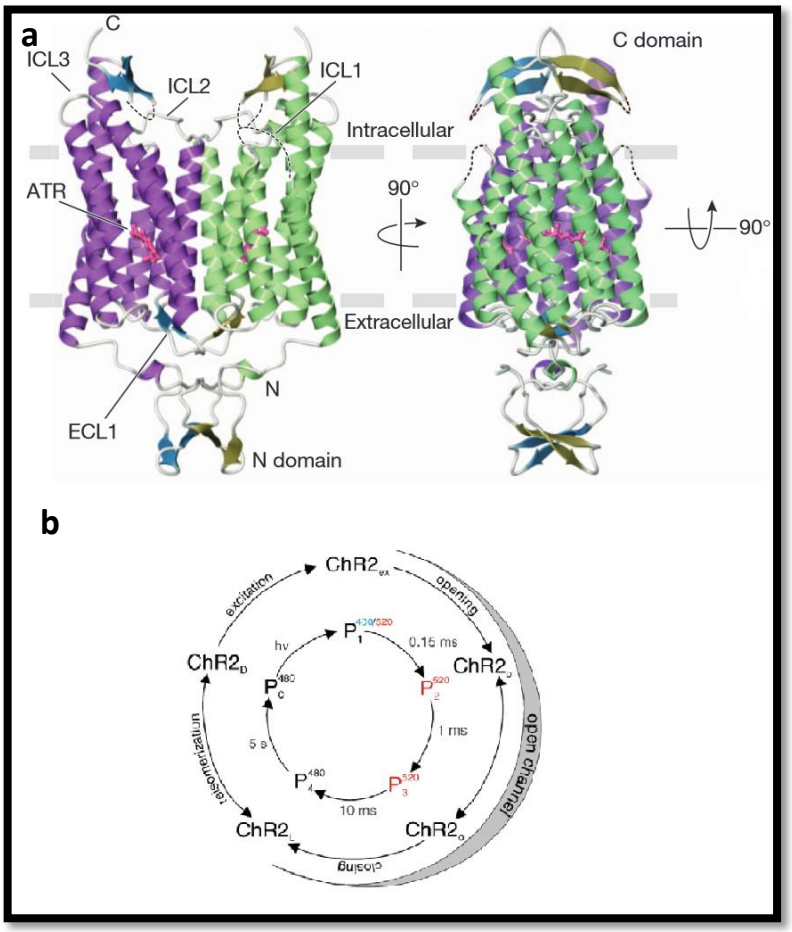

Figure 1. ChR2 structure and dynamics. a: crystal structure of the C1C2 dimmer, a chimera construct from ChR1 and ChR2. Side and frontal views of the protein are displayed with each monomer in different colours (green and violet). The ATR (red) is bound covalently in the seventh transmembrane domain. **b:** ChR2 photocycle. It consists of five different states. The opening of the channel occurs very quickly whereas the reisomerization to its basal state takes 5s (Bamann et al., 2008 and Kato et al., 2012).

It is known that ATR forms a Schiff-base with the lysine 257 sidechain in the seventh transmembrane domain of the apoprotein (Fig. 1a). When a photon targets the chromophore it leads from an all-trans to a 12-cis conformation, and this occurs in picoseconds. Thus, the cycling time is determined by the dark reactions (Bamann et al., 2010; Fig. 1b). ChR2 single channel conductance is about 60 fS (22 000 ions/s), which is 100 times smaller than that for sodium or potassium channels. Thus, a high expression is necessary for eliciting action potentials (Bamann et al., 2010).

Reward in mammals

Addressing the neurons that convey the perception of reward and aversion has never been an easy task. Around the middle of the 20th century, Olds and Milner (Olds and Milner, 1954) made a breakthrough with the introduction of an elegant technique, the intracranial self-stimulation (ICSS). With ICSS they found that rats would stimulate themselves in areas like the septal area, cingulate cortex or mammillothalamic tract.

It consequently lead to the expansion of studies assessing the neuronal populations mediating reinforcement. Many regions were found to be self-stimulated: the posterior part of medial forebrain bundle (MFB) (Olds, 1956; Olds and Olds, 1963); areas of the mesencephalic reticular system (Glickman, 1960); lateral hypothalamus; ventromedial tegmentum; caudate nucleus and neighbouring parts of the basal forebrain (Olds and Olds, 1963).

However, reliability in these studies was lacking, probably due to the nature of the technique, where an electrode activates neurons unspecifically in a diffused way, artificially and probably out of the natural ranges. With the development of optogenetics, a new way of activating neurons (and hence a new self-stimulation method) has appeared. It has the advantage of targeting neuronal populations more specifically at the same time as it provides a high temporal resolution (Boyden et al., 2005; Britt et al., 2012; Britt and Bonci, 2013; Inagaki et al., 2014).

Reward in Drosophila

In the classical theoretical framework of *Drosophila*, Octopamine and Dopamine mediate opposing roles in reinforcement: the former coding for appetitive and the latter for aversive in olfactory conditioning paradigms (Monastirioti et al., 1996; Schwaerzel et al., 2003; Heisenberg, 2003; Riemensperger et al., 2005; Schroll et al., 2006). In addition, studies in invertebrates such as crickets

and honey bees supported this view (Perry and Barron, 2013). However, studies in *Drosophila* targeted the dopaminergic population with th-GAL4, which only express in some of the dopaminergic neurons (Aso et al., 2010; Aso et al., 2012; Liu et al., 2012[a]; Waddell, 2012).

Kim found the first evidence that Dopamine was not only involved in punishment but in reward as well (Kim et al., 2007). This evidence relies on the observation that in flies lacking dDA1 receptor, the aversive and appetitive memories were robustly impaired. More recently, activation of the paired anterior median (PAM, Fig. 2a) dopaminergic cluster (which is not comprised by th-GAL4 driver) formed appetitive memory, even when Octopamine was absent (Burke et al., 2012; Liu et al., 2012[a]).

In addition, evidence points to Octopamine signaling as being upstream of that of appetitive reinforcing dopaminergic cluster, putatively the PAM cluster (Kim et al., 2007; Burke and Waddell, 2011; Burke et al., 2012; Liu et al., 2012[a]). Apparently, Dopamine signaling in this cluster mediates appetitive reinforcement by sweet taste and nutrient value whereas Octopamine only accounts for the sweet taste and not for the nutrient value (Burke and Waddell, 2011; Burke et al., 2012; Liu et al., 2012[a]).

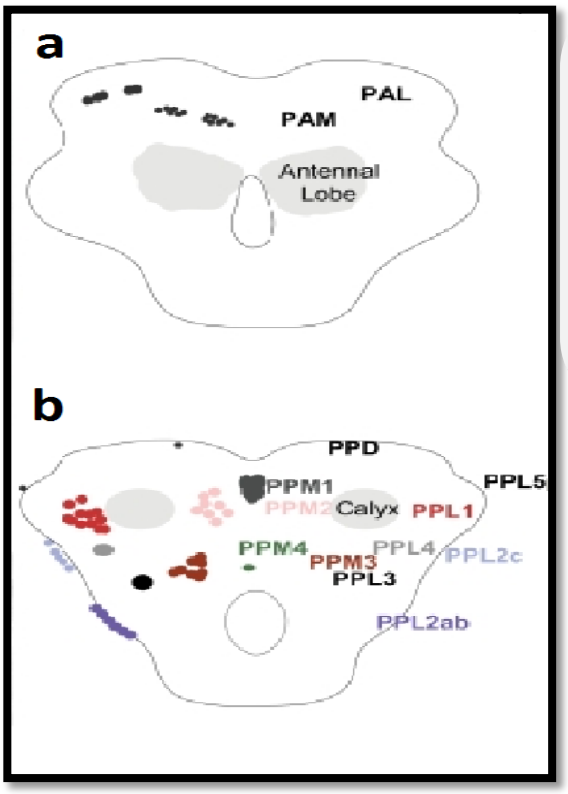

Figure 2. Schematic representation of the dopaminergic clusters in the *Drosophila* brain. Neuronal clusters are drawn in the left half of the brain whereas the right half contains the respective nomenclature **a:** two dopaminergic clusters are localized in the anterior brain: PAM (protocerebral anterior medial cluster) and PAL (protocerebral anterior lateral cluster). **b:** the posterior brain shows a high number of dopaminergic clusters: PPM1-4 (protocerebral posteromedial cluster), PPL1-5 (protocerebral posterolateral clusters) (Mao and Davis, 2009).

In the case of Dopamine, different populations of neurons seem to be required either for the acquisition or the expression of the memory (Schwaerzel et al., 2003; Krashes et al., 2009; Kaun et al., 2011; Liu et al., 2012[a]; Inagaki et al., 2012; 2009; Aso et al., 2010; Aso et al., 2012). In addition,

Dopamine plays a role in a diverse repertoire of phenotypes such as arousal (Liu et al., 2012[b]), motivation (Krashes et al., 2009; Perry and Barron, 2013; Inagaki et al., 2013), locomotion (Claridge-Chang et al., 2009; Schneider et al., 2012; Riemensperger et al., 2013) or attention-like processes (van Swinderen and Brembs, 2010), adding complexity to its characterization.

Contrary to the classical views regarding Dopamine as coding for aversion in insects, and pleasure in mammals, modern theories propose that Dopamine is coding for both aversion and pleasure independently of whether the system studied is insects or mammals. In mammals, Dopamine was attributed to reward and related processes but this view was modified with the advent of electrophysiological studies. These studies showed the diversity of dopaminergic cells within the ventral tegmental area (VTA) coding for aversion, pleasure and salience (Cohen et al., 2012; Waddell, 2013). In addition, the temporal prediction error theory (Montague et al., 1996; Schultz et al., 1997) and the actor-critic models (reviewed in Niv and Ruppin, 2002) showed that Dopamine´s processing was not as simple as coding for reward.

It was shown in recent experiments with insects, that Dopamine not only mediates aversion, but also reward (Liu et al., 2012[a]). Interestingly, many anatomical characteristics of the respective circuits in flies and rodents remain similar (Waddell, 2013). In fact, one study showed that the dopaminergic system in fruit flies might work similarly to the temporal prediction error theory proposed in mammals (Riemensperger et al., 2005).

Strikingly, in an octopaminergic cluster deep within the suboesophageal ganglion (SOG) of honey bees, the VUMmx1, has shown close similarities to the midbrain dopaminergic neurons in mammals, having the potential to shift their responses to conditioned stimuli (CS) after learning (Perry and Barron, 2013). Thus the question of how the neuronal networks that are mediated by Dopamine and Octopamine actually process reward, remains unresolved.

The finding that associative learning is strictly localized within the mushroom bodies (de Belle and Heisenberg, 1994; Connolly et al., 1996; Schwaerzel et al., 2003), directed the attention of posterior learning studies to dopaminergic neurons projecting to this neuropil (Claridge-Chang et al., 2009; Aso et al., 2010; Liu et al., 2012[a]; Aso et al., 2012).

The field of learning and memory entails extensive studies due to its well established protocols and the knowledge of sensory systems such as olfaction. Olfactory and visual systems are relatively well known in *Drosophila* as well as in other insects (Selcho et al., 2009; Yamaguchi et al., 2010; Maisak et al., 2013). Hence, the neuronal hallmarks of CS and US have been studied for some time

(Schwaerzel et al., 2003; Heisenberg, 2003; Selcho et al., 2009; Aso et al., 2012). Whereas the sensory systems account for the CS signal, dopaminergic neurons appear to be essential for the unconditioned stimuli (US) processing. Different types of dopaminergic neurons might be involved in different aspects of the US (Selcho et al., 2009).

Incentive valence, is highly dependent on reward, and refers to the motivation of an organism to pursue a given goal. In contrast to learning reinforcement, to our knowledge, there is no study addressing the neuronal populations which mediate incentive valence in flies. Therefore, bearing in mind the results from previous studies, we decided to assess which neuronal populations the fruit fly chooses to have turned on/off.

Aims of the Thesis

The aim of this thesis is twofold:

The first stage consisted in the establishment of an optogenetic model of *Drosophila*. For this we wanted to gain insights into the method by performing different experiments in order to optimize several parameters (nutritive ATR concentration, light types, light intensity and additional material).

Once this was achieved, we wanted to characterize the dopaminergic neurons coding for incentive value in *Drosophila*.

In the same way as these dopaminergic neurons determine the direction of learning, we wanted to address the preference/avoidance mediated by these neurons on their own. Because in rats distinctions were made for the coding of reinforcement (in learning) and incentive value (motivation), we considered the possibility of finding differences in the learning and preference indexes mediated by these neurons.

For that purpose we combined optogenetics, as a way to activate neurons, with a T-Maze paradigm, which is a relatively easy method to address this issue avoiding complexity and potential influential factors. Similar to rats lever pressing for a reward, the flies will just need to approach or escape from the blue illuminated area.

MATERIALS AND METHODS

Fly strains for behavioral tests

Strains	Description	Reference
Tdc2-GAL4 (II)	VM, AL1-2, VL, ASM, PB1-2,PSM	Busch et al., 2009
NP5272-GAL4 (II),	PPM3, KC, neurons that project to LP	Aso et al., 2010
NP1528-GAL4 (II)/CyO	PAM	Aso et al., 2010
NP2758-GAL (X)	PAM; PPL1	Aso et al., 2010
NP6510-GAL4 (III)	PAM; PPL1	Aso et al., 2010
NP0047-GAL4 (III)	PAM; PPL1 (with MB-G80)	Aso et al., 2012
5HTR1B-GAL4 (II)	PAM, PPL1	Aso et al., 2012
NP7187-GAL4 (X)	PPL1	Aso et al., 2012
TH-GAL4 (III)	PAM; PPL1; PPL2	Aso et al., 2012
NP7323-GAL4 (II)	PAM	Aso et al., 2012
NP5272-GAL4 (II)	PAM	Aso et al., 2012
MZ840-GAL4 (III)	PPL1	Aso et al., 2012
c061-GAL4 (X)	PAM, PPL (with MB-G80)	Aso et al., 2012
DDC-GAL4 (III)	PAM, PPL2, PPL1	Aso et al., 2012
HL9-GAL4 (III)	PAM, PPL1	Aso et al., 2012
c259-GAL4 (III)	PAM; PPL1 (with MB-G80)	Aso et al., 2012
MZ19-GAL4 (II)	PAM (with $Cha^{3.3Kb}$-G80)	Aso et al., 2012
R58E02 (III)	PAM and optic lobes´s glial cells	Liu et al., 2012[a]
TH-D´ (II);	PPL1, PPM3, PPM1/2	Liu et al. 2012[b]
TH-D4 (III);	PPL1, PPM3, PPM1/2	Liu et al. 2012[b]
TH-F1 (III);	PPM3, PPM1/2, PPL2, PPL1	Liu et al. 2012[b]
TH-F2 (III);	PPM3, PPM1/2, PPL2, PPL1	Liu et al. 2012[b]
TH-F3 (III).	PPM3, PPM1/2, PPL2, PPL1	Liu et al. 2012[b]
NorpA; UAS-ChR2; UAS-ChR2	Used for optogenetic tests	Schneider et al., 2012

Table 1. Fly lines used for the behavioral experiments. Strains were obtained from their respective reference with the corresponding expression pattern. For a more detailed description of their expression pattern see figure 3. **Abreviations:** VM: ventromedial protocerebral cluster. ASM: protocerebral anterior superior medial cluster. AL: antenal lobe; PB: protocerebral bridge; PSM: protocerebral posterior superior medial cluster; VL: ventrolateral protocerebral cluster. PPM3: protocerebral posteromedial cluster; KC: Kenyon cells; LP: Lobula Plate; PAM: protocerebral anterior medial cluster; PPL1: protocerebral posterolateral cluster 1; PPL2: protocerebral posterolateral cluster 2. N.B.: although some of the lines characterized express GAL80, this study was accomplished with the GAL4 alone because the required crossings took longer than the period of the thesis.

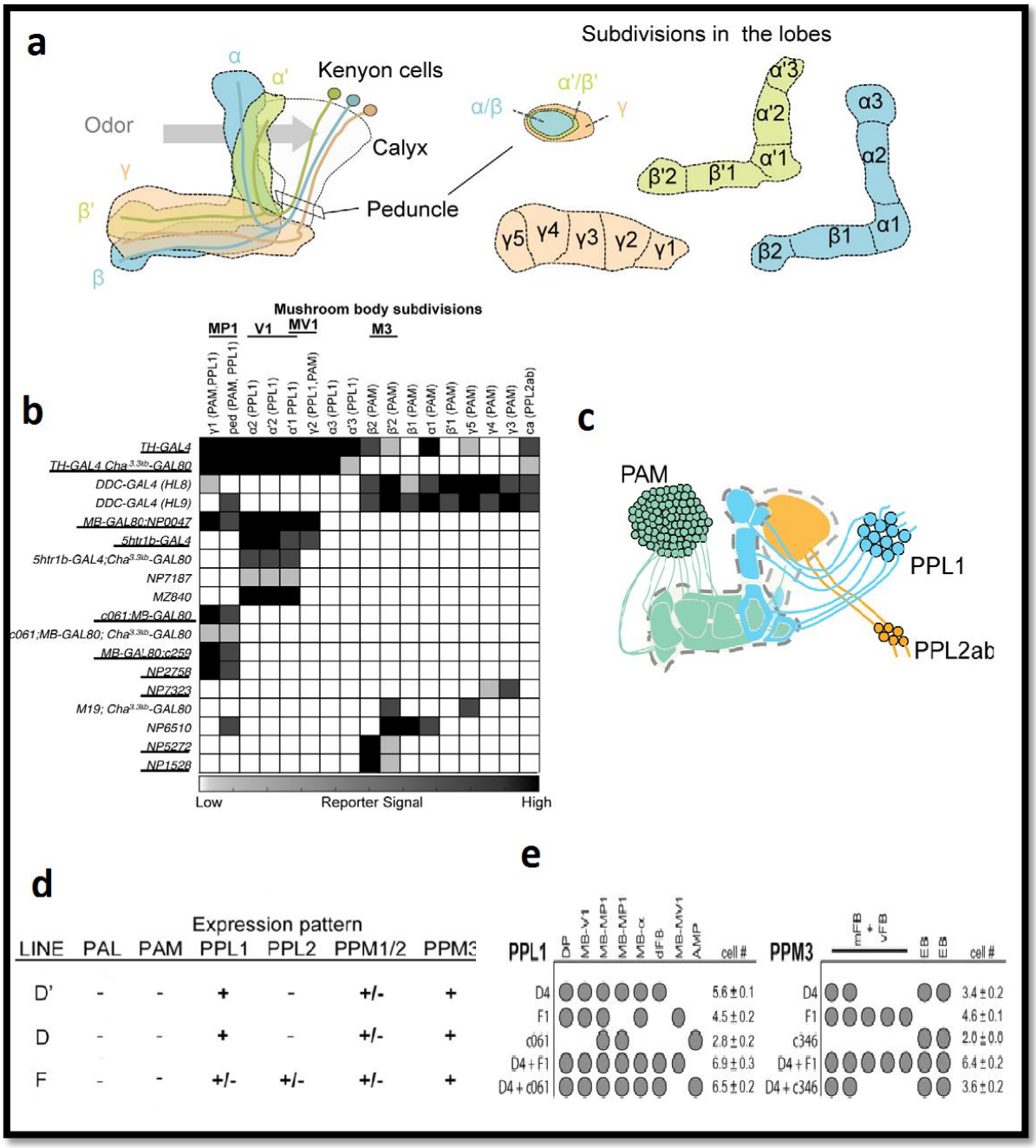

Figure 3. Schematic diagrams of the dopaminergic projections to the mushroom bodies (MB) and expression pattern of distinct dopaminergic driver lines. **a:** subdivisions of the MB. **b:** expression pattern of different dopaminergic drivers studied in aversive learning. **c:** schematic representation of the main dopaminergic projections to the MB. **d** and **e:** expression pattern of dopamiergic drivers involved in sleep/wake (modified from Aso et al., 2012 and Liu[b] et al., 2012).

Immunohistochemistry

Adult heads were fixed with 4% formaldehyde in 100 mM phosphate buffer pH 7.5 for 40 min at room temperature (RT). Brains were then extracted and rinsed three times in phosphate buffer saline with 0.1% Triton X-100 (PT) for 15 min. Samples were blocked in 7% normal goat serum (NGS) for 1 h

in PT and incubated with primary antibody at 4°C overnight. The primary antibody was a polyclonal mouse anti-GFP (1:500; Invitrogen).

Samples were washed four times for 15 min each time in PT and incubated with secondary antibody at 1:250 for 2 h at RT, and the secondary antibody was washed four times for 15 min each time in PT and mounted in 80% glycerol in PT. As a secondary antibody we used an Alexa Fluor 488 conjugated goat anti-mouse IgG (Molecular Probes, MoBiTec). Images were taken in a Zeiss LSM 510 Meta confocal microscope. After acquisition, images were selected with Fiji, an ImageJ-based image-processing environment (downloaded from http://fiji.sc/Fiji). Picture´s brightness, contrast, color and binding were accomplished with Adobe Photoshop CS6 (San Jose, CA).

Behavioral assays

Pre-test conditions

Flies were raised on a standard cornmeal/molasses/yeast/agar medium on a 12/12 h light and dark cycle at 25°C with 65% humidity. Contrary to mammals, fruit flies do not have ATR present in brain tissue. ATR is required for all rhodopsins channels to enter the photocycle (Ullrich et al., 2013). Thus, following previous studies (Schroll et al., 2006; Schneider et al., 2012), ATR was supplemented in the fly´s food.

For experiments, 30 adult male flies from 2-8 days old were collected with cold/CO_2 anesthesia and then placed for 2 days on small vials with fly media containing ethanol or all-trans retinal (ATR) diluted in ethanol. The modified fly media consisted of 30ml of fly media mixed with 150 µl of either ethanol alone, or 150 mM ATR (Sigma, Germany) dissolved in ethanol. For convenience, aliquots of ATR and ethanol were stored at -80°C. Because ATR is readily oxidized by light, these vials were wrapped with aluminum foil and thus, during the two days prior to the experiments, flies remained in darkness.

It has already been seen that flies have a preference for light in the spectrum of the blue and UV (Suh et al., 2007; Yamaguchi et al., 2010; Karuppudurai et al., 2014). Because ChR2 is opened by blue light, in order to avoid the effect of color preference, the flies contained a NorpA mutation in their genetic background. NorpA encodes for a phospholipase C in the photoreceptors of the fly and its mutation in homozygosis renders the flies blind (Schnewly et al., 1991). This mutation is localized in the X-

chromosome, for this reason, we used males from the F_1 of the cross shown in figure 4, to avoid the visual preferences effects.

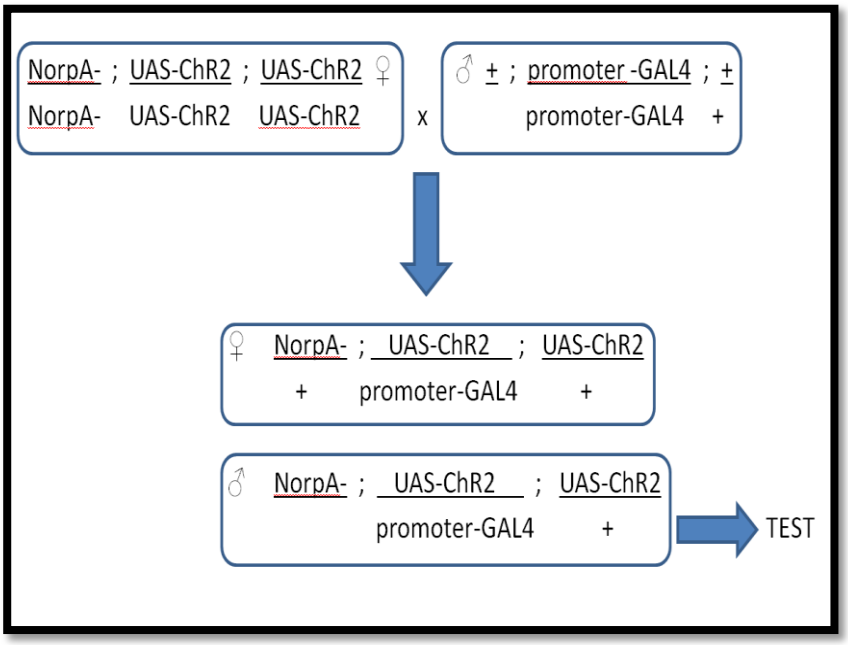

Figure 4. Example of crosses performed for the experimental lines expressing ChR2. Behavioral experiments with lines containing ChR2 used F_1 progenies of crosses between females from NorpA;ChR2;ChR2 and males from the driver lines. As a general rule, only males were tested because they were the only ones homozygous for NorpA- and thus, blind.

Only in figures 8, 9 and 14 males and females were reared and tested together. In the rest of the experiments males/females were sorted and reared in ATR containing vials and tested separately. In addition, we did not sort the males and females in the experiment with larvae (Fig. 7).

For the larvae experiment, eggs were collected and placed in a Petri dish with 1.5% agarose gel containing on the surface yeast mixed with high amount of 150µM ATR dissolved in ethanol. They were left for 2 or 3 days in darkness at 25°C and 65% humidity, until they reached second or third instar.

Experimental setup

-T-maze:

Each arm of the T-maze was illuminated by two different light sources (Fig. 5a). For the illumination we used LED lights (465–485 nm and 580-600nm spectrum; Osram Oslon SSL, Germany). During the experiments, the arms of the T-maze were illuminated under constant light in a gradient of light intensity decreasing from the end of the arm to the beginning of the arm. The lower choice arms were approximately 10cm long. From now and on, we will refer to light intensities as #/+ (being # the intensity at the outer part of the tube, and + the intensity at the inner part of the tube proximal to

the elevator, both measured in W/m²). The light intensity was measured with a luxmeter (Gossen Mavolux, Germany).

Protocol:

1. Open the vials and tap them gently and continuously in a way that the flies cannot fly away in order to put them in the resting tube with the help of a funnel.

2. Once the flies are within the resting tube, ensuring the flies do not escape, place it into the corresponding upper hole in the elevator. Then wait for 10 minutes to let them acclimatize.

3. After 10 minutes, tap the T-maze structure in a way that all the flies fall into the elevator and quickly push the elevator half way down to make sure that all the flies are within the elevator and not in the tubes. To check this, we open the resting tube and throw out the flies, in case there remain any flies in that tube. Then wait for two minutes to let the flies in the elevator acclimatize.

4. During these two minutes, adjust the desired light intensity with the luxmeter in the two arm tubes (one with yellow and the other with blue light), and place them into the two bottom holes of the T-maze. Being sure that lights are on, push the elevator all the way down to the bottom and lift it again up after 30 seconds.

5. Then count the amount of flies in each tube under CO^2 anesthesia. Experiments with less than 15 flies are discarded.

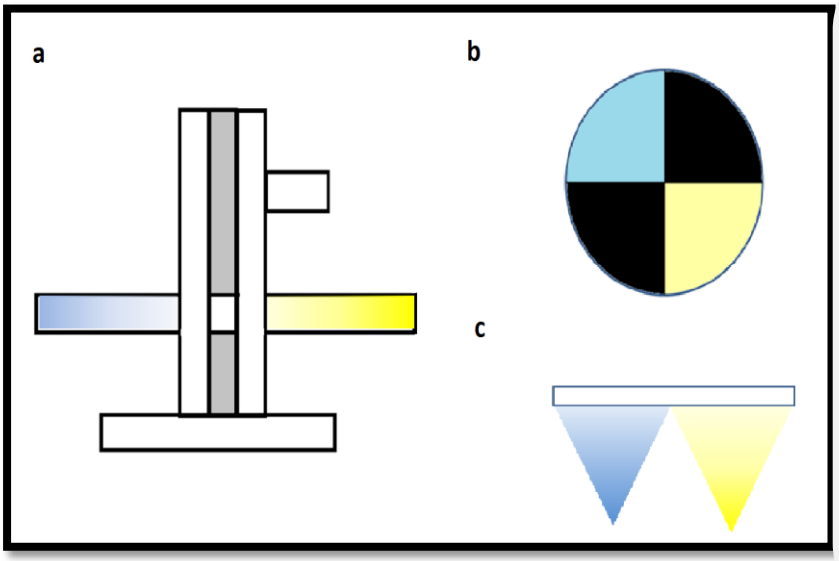

Figure 5. Instruments used for the experiments in this study. **a**: T-maze showing the three tubes connected to the elevator. The two bottom tubes where supplied with LEDs at the exterior end pointing to the elevator. The amplitude of the angle of illumination of the LED was relatively high in order to reach a steep light gradient along the tube. **b**: a schematic of the setup for the larvae experiments when viewing from above. **c:** side view schematic of the setup where the unfilled rectangle represents the Petri dish and the colored triangles represent the light illumination.

-Light-Dark T-maze:

For assessing blindness in flies carrying NorpA mutation, we adapted the T-maze to similar conditions for the study of phototaxis in *Drosophila*. It basically consisted in a T-maze similar to that used for the preference assay, but changing the two lower choice tubes. The 20cm long tubes were opaque (dark) on one side and transparent (light) on the other side. During the 30s choice time, a fluorescent light bulb 20cm above was turned on in order to illuminate the transparent tube without doing it in the opaque tube.

-Larvae preference experiment:

The larvae collected from the rearing media were gently rinsed with tap water and placed in the center of a 100x15mm Polystyrene Petri Dish filled with 1.5% agarose gel. The Petri Dish was divided into four quadrants, two were dark opaque and the other two were left transparent (Fig 5b-c). Each of the transparent quadrants were illuminated from below either with yellow or with blue light in a way that at the surface of the agarose the light intensity was maintained in between 10-15 W/m^2. Once the 10-20 larvae were placed in the middle, they were left for ten minutes and then the number of larvae in each quadrant was quantified.

Preference index:

For the results in the T-maze as well as in the larvae experiment, we calculated a preference index by the formula:

$$PI = (b-y)/(b+y)$$

b= individuals in the blue illuminated area **y**= individuals in the yellow illuminated area

The possible PI values are in the range between 1 and -1, meaning that all the flies approached or avoided the blue light, respectively. A value of zero means that the flies took each choice to a similar extent. When the results yielded in all tested groups comprised a small range of PI, the Y axis was adjusted to show more clear differences.

Flies that stayed in the elevator were not taken into account (as depicted by the formula) in order to avoid counting unhealthy/damaged flies. In addition, these flies were left out of the formula because light did indeed reach the elevator, and we do not know whether this intensity was over or below the

threshold for neuronal activation. Thus, classifying them in either side of the formula could show misleading results.

In the larvae preference test, we left out of the formula any larvae that went to the dark quadrants (the majority). As in adults, the larvae female's visual ability remains intact whereas males are blind (Fig. 4). Taking into account the negative phototaxis shown in larvae, we then expect that the females would be able to detect light and avoid it, ending in dark quadrants. Because we did not distinguish among male and female larvae during counting, we suppose that the females might be the ones comprising the majority in the dark quadrants.

Nevertheless, future experiments should only test blind males and divide the Petri dish into two halves with yellow and blue illumination in order to avoid experimental individuals not contributing to the data.

Statistical analysis

Statistical analyses were performed with the Infostat software package (InfoStat Group, FCA, National University of Córdoba, Argentina). Normality was tested using Shapiro-Wilks Test, and Levene Test was performed to assess homogeneity of variance. In the cases where the low number of experiments did not allow us to prove these assumptions (Fig. 7 and 11), no further analysis was applied.

For comparisons of two groups of normally distributed data, two sample t-tests (two-tailed, for independent samples) were performed (Fig. 8, 9 and 13). For multiple comparisons, a one way ANOVA was performed; however there were no differences in figures 10 and 12, thus no post-hoc analysis was applied. In addition, in figure 10 a one sample t-test was done in order to address its deviation from a theoretical PI=0. The reason we did this was because the blind flies´ population average should show no preference for any one arm of the T-Maze (hence PI=0). Thus, the sample mean of the experimental flies should not be significantly different from 0.

For Figure 12, due to the abnormal controls, and the fact that it is a screening, we did not consider it worthwhile analyzing statistically. The significance level of statistical tests was set to 0.05.

Bar charts (Fig. 7, 8, 9, 13 and 14) showed mean values and the standard error. The rest of the graphs were displayed in a box plot with the mean indicated as a point, the median as a horizontal bar within the box, the box containing the range within the 1st and 3rd interquartiles and the whiskers comprising the full range of the data points.

RESULTS

Octopaminergic neurons immunohistochemistry

Tdc2-GAL4 drives GAL4 under the control of a promoter region of the tyrosine decarboxylase gene (tdc). Because tdc2 encodes an enzyme involved in the synthesis of Tyramine and Octopamine, neurons expressing it can be considered octopaminergic (Busch et al., 2009).

Contrary to the dopaminergic lines, we did not have a detailed characterization from our own tdc2-GAL4 stock. A previous study (Busch et al., 2009) has already described the expression pattern of this driver. However, because we did not know if they came from the same original construct, or if they were differently handled, contaminated, or for any other reasons, we wanted to make sure that we could rely on this study for the characterization of our tdc2-GAL4 stock. For this, we undertook an immunostaining of our own tdc2-GAL4 stock and we compared it with that of Busch et al., 2009 (Fig. 6).

We did not find big differences between the clusters targeted by our tdc2-GAL4 line (Fig. 6b,c,e,f,h and i) and that of Busch et al., 2009 (Fig. 6a,d and g). Although the neuronal populations stained in each study did not match exactly, we think that this might be due to a different methodology for the staining and the material that was used for it. In addition, we assume that small differences might not make a difference when so many other neurons are targeted.

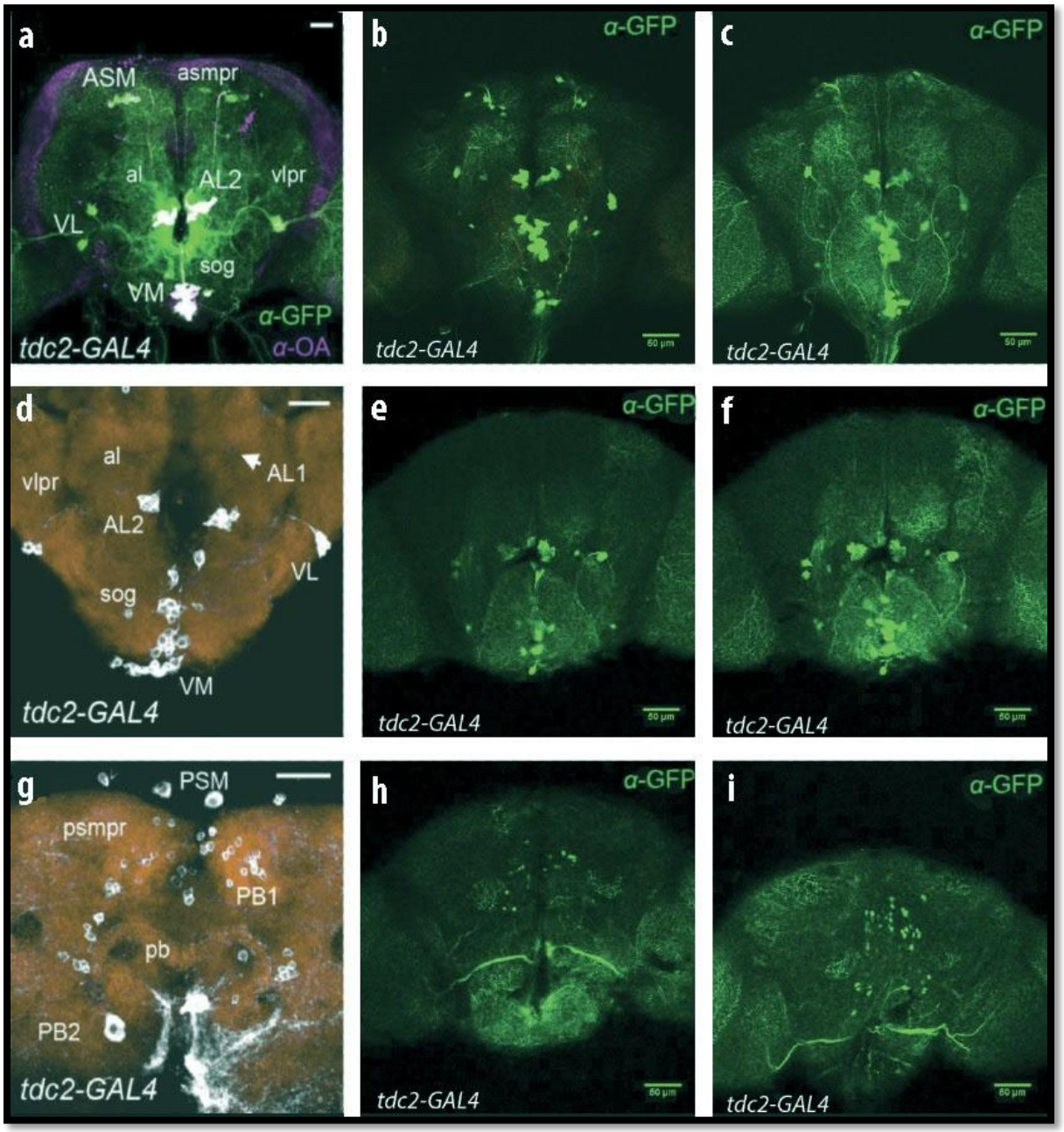

Figure 6. GAL4 expression pattern of tdc2-GAL4 in the adult brain have a similar expression to that described in Busch et al., 2009. Pictures on the left column (**a, d** and **g**) were obtained from Busch et al., 2009. In **a**, neurons were visualized with UAS-mCD8::GFP (green) and anti-OA antibody (magenta). In **d** and **g**, the neuropils and somata were visualized by Synapsin (orange) and UAS-mCD8::GFP (white), respectively. Brain regions and cell clusters are labeled with lower case and capital letters, respectively. The two remaining columns (b, c, e, f, h and i) consisted of pictures from our own immunostainings which were labeled with anti-GFP antibody (green). For facilitating the comparison, pictures in the same row match the localization in the antero-posterior axis. **a, b** and **c**: frontal view of the anterior brain with the AL2 cluster located ventromedial to the antennal lobe (al), and VL between the antennal lobe and the ventrolateral protocerebrum (vlpr). Paired somata at the anterior superior medial protocerebrum (asmpr) constitute cluster ASM. **d, e** and **f**: frontal view of the ventral supraesopagueal ganglion (SPG) and subesophagueal ganglion (SOG) , where ASM is no longer present but caudal neurons from VM, AL1-2 and VL are still present. Cluster AL1 consists of one paired soma at the anterior margin of the antennal lobe. **g, h** and **i**: posterior brain showing the octopaminergic neurons around the protocerebral bridge (PB). Scale bars of the first column pictures are 25µm long whereas the rest are 50µm.

Testing the method in larvae: Octopamine mediates preference and Dopamine aversion

A previous study (Schroll et al., 2006) showed that activating neurons under the th-GAL4 and tdc2-GAL4 drivers produced aversive and appetitive learning, respectively. This activation was in fact accomplished with optogenetics, which is a relatively simple way to measure the effects of activation of ChR2-expressing neurons by taking advantage of the transparent larvae.

For this reason we decided to perform a similar test in order to compare the reproducibility of our methods with that of others. However, we decided to measure preference or avoidance to the activation of these neurons, whereas in the aforementioned study, the larvae were tested in learning.

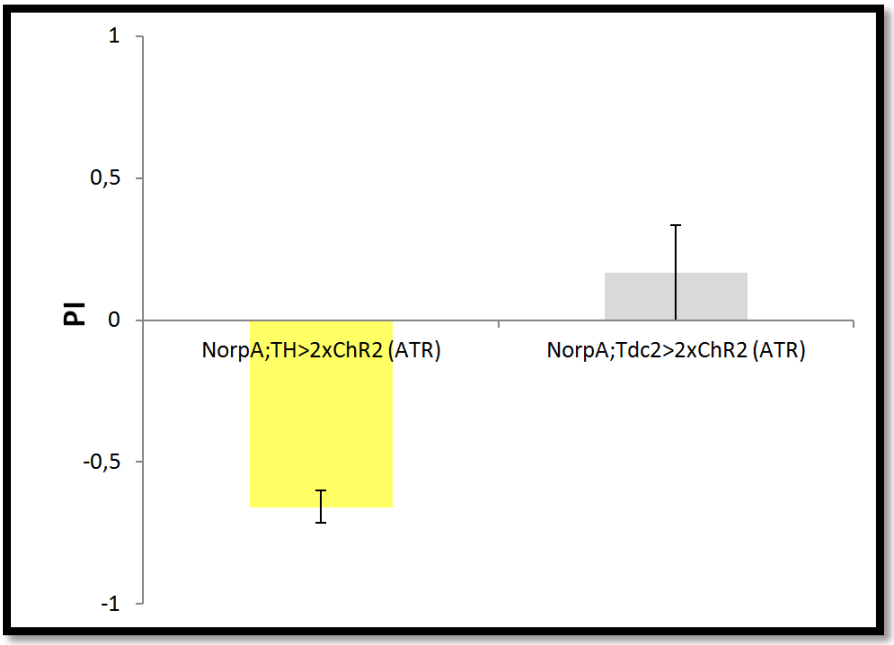

Figure 7. Light-induced activation of different neurons conveys preference in larvae. Negative PI values in the larvae expressing ChR2 in dopaminergic neurons (yellow) show that they prefer not to have these neurons activated and therefore might be mediating aversion. On the other hand, the larvae expressing ChR2 in octopaminergic neurons (grey) show a slight positive PI, meaning a tendency to approach the area where these neurons are activated. Light intensity at the agarose surface: 10-15W/m² (p=0,0428; N=2). N.B.: No test were performed for control groups.

Since our idea was to disentangle the effects of certain neurons in reward itself and in learning reinforcement, this test in larvae seemed to be a good way to start. If these results did not match that of Schroll et al., 2006, it would not mean that the method is not working rather that these neurons have different effects in reinforcement and reward. In the case that no differences among tested groups were found, one of the possible causes would be that the technique was not working properly.

By comparing Schroll et al., 2006 experiments with ours, it appears that the effect of tyrosine hidroxylase positive neurons and octopaminergic neurons correlate in incentive valence and reinforcement (Fig. 7). Thus, the reinforcement that confers the triggered neuronal activation might be independent if it is applied while the fly is making the choice or if it was previously paired to a neutral stimulus, which then acquires this value. Nevertheless, cautious conclusions must be taken from this result due to the low number of experiments carried out and the absence of a control.

Visual preference obscures the information carried by the neuronal activation

Unfortunately, a trade-off of ChR2 is that it is activated by blue light. It is a trade-off because it is known that flies have a preference for blue light over other colors (Suh et al., 2007; Yamaguchi et al., 2010; Karuppudurai et al., 2014). Therefore we considered it interesting to see to what extent this blue light is attracting the flies with normal visual function in our testing conditions.

We observed that when the visual ability remains intact, wild type flies and also the ones expressing ChR2 show a preference for blue light (Fig.8 and 9b). Indeed, this visual preference obscures the effects produced by the neuronal activation itself (Fig. 9b) showing a similar PI at around 0.5 independently of the expression of ChR2. Thus, in the following experiments, only blind individuals were tested.

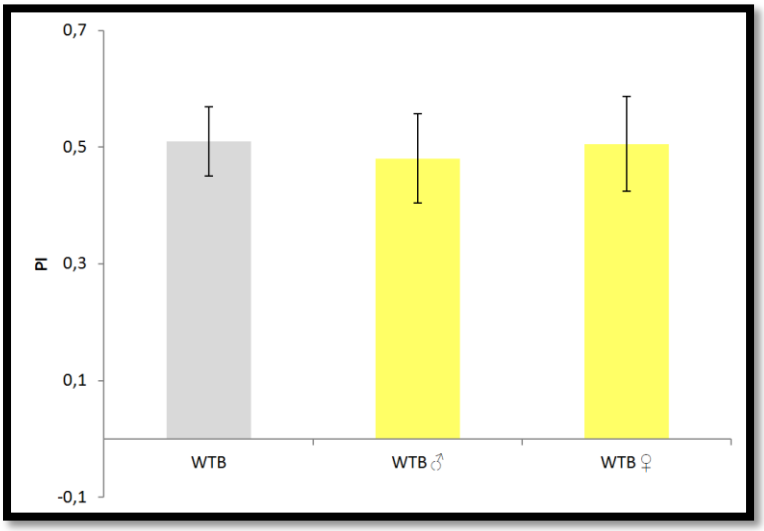

Figure 8. Wild type visual preference in the T-maze for the blue light. Males and females (yellow) were reared and tested together and then sorted during counting. This preference is similar in males and females (p=0,8249). Males and females pooled together (grey) have a similar PI to both of the other groups. The light intensity was 250/25 W/m². N=9. WTB: wild type berlin.

Octopaminergic neurons convey positive valence

Once the method was working (Fig. 7) and we addressed the visual preference in wild type flies in the T-maze conditions (Fig. 8), we decided to start the experiments with flies expressing ChR2 under the

tdc2-GAL4 driver. We thought that it was convenient to adjust the parameters with these experiments and to get some feedback before starting the screening for the different GAL4 dopaminergic drivers. We decided to start with the tdc2-GAL4 line because according to the literature it has consistent positive reinforcing effects (Schwaerzel et al., 2003; Schneider et al., 2012).

Flies showed a positive preference for the activation of tdc2 positive neurons (Fig.9a). Interestingly, the flies reared without ATR seem to have also a tendency towards the blue light (Fig. 9a). Because in other studies flies lacking ATR did not show much response to the stimulating light, we did not expect to observe this PI value in these flies. This suggests two possible explanations: either the flies are able to detect the blue light despite of the NorpA mutation, or the channel can be opened to some extent by blue light without the ATR supplement.

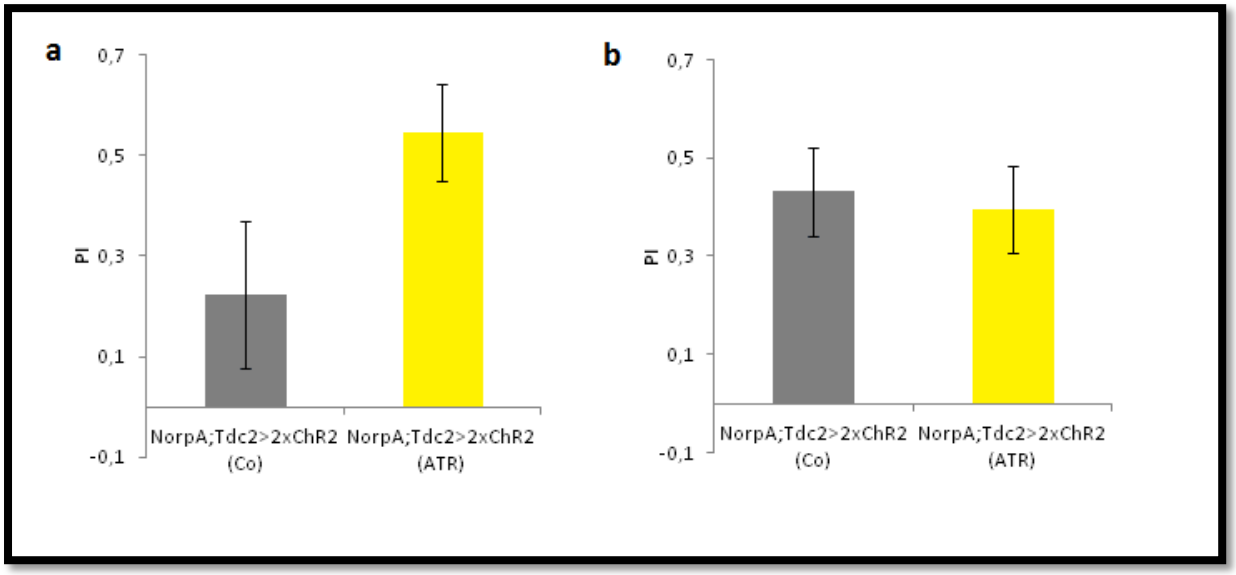

Figure 9. T-maze experiments with flies expressing ChR2 in octopaminergic neurons. The flies were reared either in fly media with ATR (yellow) or without ATR (grey). Blind males show different PI depending on the food supplement (p=0,0848; N=9). **b** Seeing females show similar PI, showing the effect of visual capability and not of the neuronal activation (p=0,7730; N=8).

To investigate this, we took advantage of the already known positive phototaxis of flies (Benzer, 1967; Markow and Merriam, 1977), and performed a Light-Dark T-maze. If the experimental flies were completely blind, they would not show any preference between light and dark tubes.

We decided to test male flies expressing ChR2 under the tdc2-GAL4 driver because we wanted to see if they were blind. In addition, we included males and females carrying NorpA mutation and UAS-ChR2 element (Fig. 4). The latter were tested because if it occurred that the tdc2>ChR2 flies were

able to see, we could get some idea by comparing the PI of tdc2>ChR2 with the PI of other lines carrying a different number of copies of the NorpA mutation. In addition we would be able to see if the different genetic backgrounds affect the PI. And because flies expressing ChR2 under the th-GAL4 driver were also used for previous experiments and planned for further experiments, we decided to include them in this test.

None of the four lines tested showed any choice preference (Fig. 10). In addition, this result was supported by experiments with the Buridan´s paradigm (data not shown). Buridan´s paradigm consists of a homogeneously illuminated circular platform with two dark vertical stripes on the walls opposed to each other. Because the platform is surrounded by water, the flies can never reach the walls were the stripes are, and keep walking from one stripe to the other (see Colomb et al., 2012 for a detailed explanation). In these experiments, flies carrying a mutated NorpA gene did not follow the stripes and their movement was slower and paused.

Considering these results, we hypothesize that this channel also works without ATR, although not so efficient as when ATR is present. Feeding the flies with ATR might activate the neurons in a more efficient way, achieving a proper stimulation of a given neuronal population.

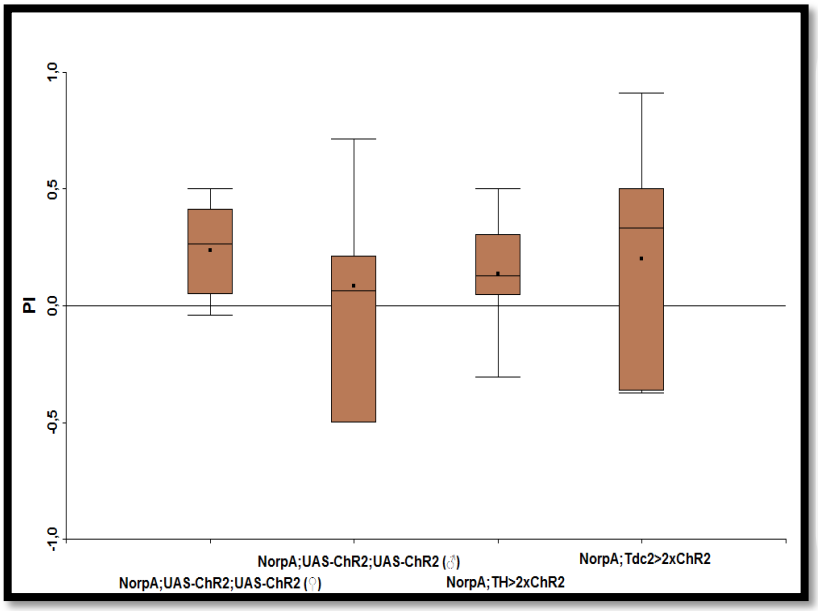

Figure 10. Light-Dark T-maze test performed in flies carrying NorpA in homozygosis (and hence blind) in different genotypes to proove the visual ability. No differences were found among them (p=0,9484). NorpA;UAS-ChR2;UAS-ChR2 males and females showed no statistical difference from a PI=0 (p=0,76 and 0,08 respectively) nor did flies expressing ChR2 in dopaminergic and octopaminergic neurons (p= 0,47 and 0,37 respectively). N=5 (for males from NorpA;UAS-ChR2;UAS-ChR2 N=4). N.B.: for simplicity, we did not show results from wild type flies with normal visual function. Their PI was around 0.6.

Different dopaminergic populations are mediating different degrees of positive valence

As an attempt to measure the reinforcement effect of the activation of different subsets of dopaminergic neurons in the fly, we expressed the ChR2 under different dopaminergic driver lines (Fig. 11 and 12).

Unfortunately, due to the fact that some of the GAL4 driver lines were located in the X chromosome, flies bred from the crosses carrying the GAL4 driver were not blind. Because the required manipulations for making these flies blind would take too long for the thesis period, we decided to see whether, even flies with normal visual function would show differences in the preference.

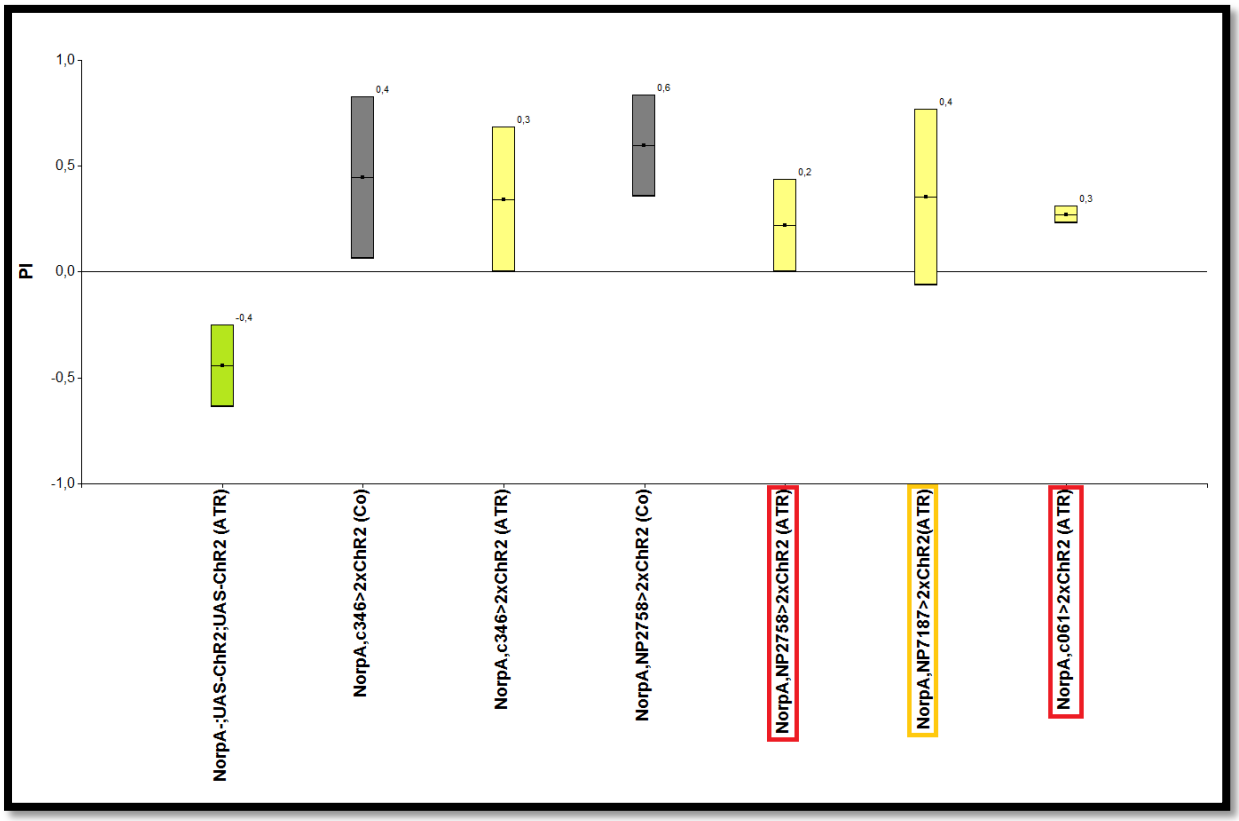

Figure 11. Screening for differences in the preference index in female flies with normal visual function expressing ChR2 in different dopaminergic populations. As a control we used a fly carrying the genetic background but without any driven expression of ChR2 (green). We used as well controls for the ATR supplementation. To save time, we just chose two fly lines randomly to rear without ATR (grey). Experimental fly lines (yellow) showed similar PI values at around 0.5. Fly lines squared in red were shown to mediate aversive learning and that squared in orange did not show any effect in learning, the rest were not tested in learning. Only females were tested and the light intensity was 1400/50 W/m². No differences were seen between the different groups (p=0,3571). N=2. *N.B.: the genetic control (green) is blind, meaning that it is not the best control, however because it was done together with the others we left it there without any further relevance.*

A pilot study was done in order to see if they remained strictly around their visual preference values or if the activation of distinct neurons might have an observable additive effect on the flies' decisions

(Fig. 11). Unfortunately, as observed previously in this study, fly´s vision made it hard to disentangle the effects of the visual preference for the blue and the intrinsic effects of the activation of the neurons. For this reason, we did not continue with these experiments. Nevertheless, the PI means of the experimental lines varied more than in figure 9, probably meaning that the increased light intensity used in this experiment, increased the neuronal activation, leading to more marked effects.

Pulsing the stimulation has been shown more effective in the activation of neurons (Milner, 1991; Inagaki et al., 2014). Therefore, in future studies pulsing the light might yield relative differences enough, even in flies with normal visiual function. Another option is to use a non-visible light to stimulate ChR, avoiding the effects of vision, as done in a recent study (Inagaki et al., 2014). However, the slight differences among genotypes cannot be taken literally due to the low number of experiments.

Because of our previous findings, we focused on the screening with blind flies (Fig. 12). To our considerable surprise, we observed that the controls without any driven expression of ChR2 had unexpected values considering that the flies were blind and they did not have any ChR2 expression (Fig. 12). It had been expected that they would remain in PI values around zero (for a detailed reasoning of this observations see the pertinent chapter in the Discussion).

At higher light intensities, the effects of feeding the flies with ATR disappears (Fig. 12). Taking into account that the light intensity used for these experiments was several fold higher than in that if figure 9a, we presume that at this high intensity it causes the maximum open state possible, making the effect of ATR superfluous. So that even with ATR, there cannot be more activation than that already produced by blue light alone.

Surprisingly, all the experimental fly lines in the screening showed either a preference for the activation of the neurons or no apparent preference but none of them avoided the activation of these neurons. This shows different results to that observed in olfactory classical conditioning.

Interestingly the GAL4 fly lines that were involved in appetitive learning: 58E02, DDC and HL9 (Claridge-Chang et al., 2009; Aso et al., 2012; Liu et al., 2012[a]), are congruent with the degree of approach to the blue light that this screening shows (Fig.12). Because DDC and HL9 label many cell types, the roles of individual dopaminergic neurons might be antagonized by each other and this could be the reason that they do not show strong phenotypes in appetitive learning and positive valence (Claridge-Chang et al., 2009; Aso et al., 2012; Liu et al., 2012[a]). These two drivers label serotoninergic neurons as well, but these serotoninergic neurons do not appear to play any role either in learning (Claridge-Chang et al., 2009; Liu et al., 2012[a]) or in arousal (Liu et al., 2012[b]). Hence, it could be that they are not involved in the approach measured in our study either.

R58E02 strongly labels glial cells in the optic lobes and almost all PAM neurons targeted by DDC and HL9, except the MB-M3 which entails neurons involved in aversive learning (Claridge-Chang et al., 2009; Aso et al., 2012; Liu et al., 2012[a]).

On the other hand, dopaminergic clusters involved in aversive learning do not show any correlation to the degree of avoidance of the blue illuminated arm in this study (Aso et al., 2010; Aso et al., 2012). The most logical conclusion is that classical conditioning is correlated with the value of the US. Thus, if a US produces appetitive learning, US alone should be then preferred. The same might hold true for aversive learning and negative valence of the US.

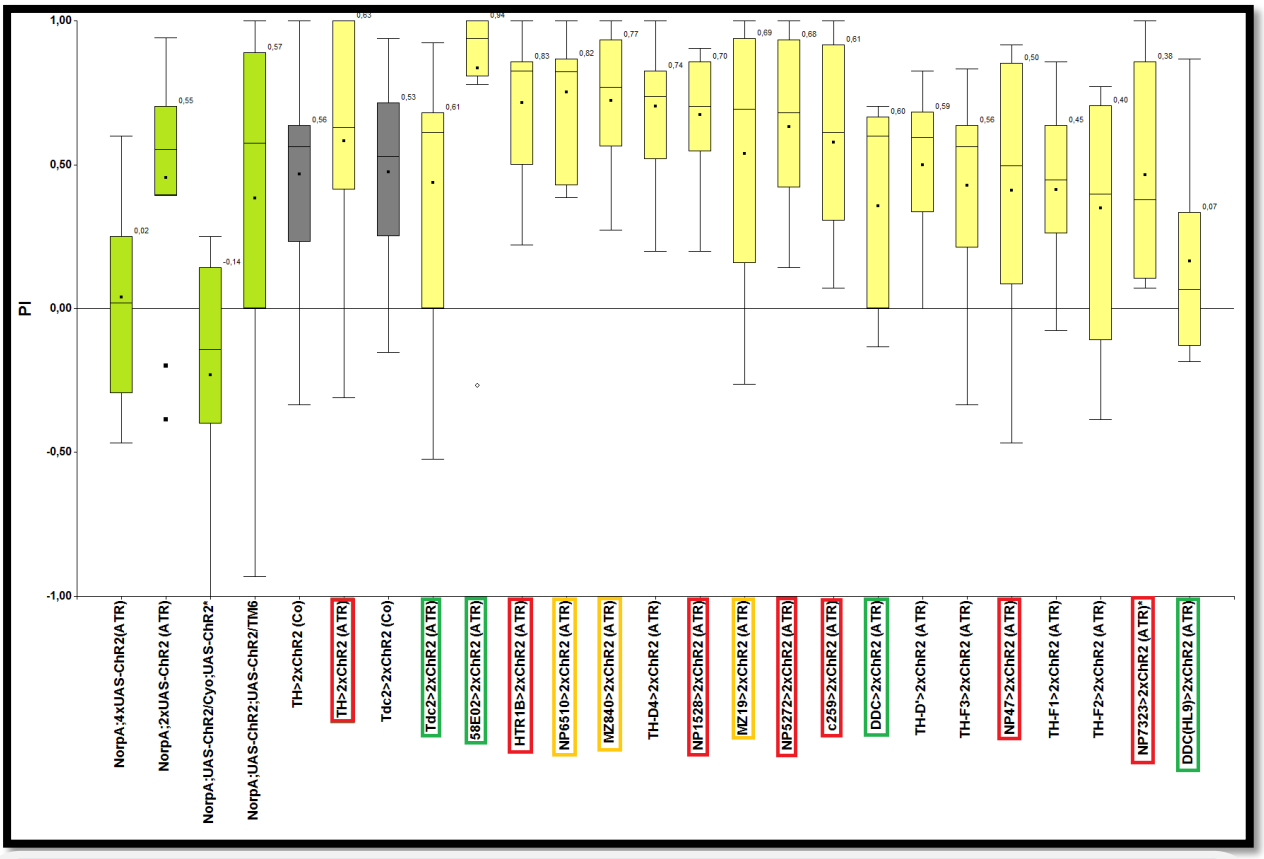

Figure 12. Light-induced activation of different subset of dopaminergic neurons shows different reinforcement degree. Genetic controls without driven expression of ChR2 (green) on the left, control for the ATR supplement in flies expressing ChR2 (grey) and experimental lines fly (yellow). The light intensity was 1400/50 W/m². Lines squared in green, red and yellow were lines previously shown to be involved in appetitive, aversive and no learning, respectively. The ones without squares were not tested in learning. Due to the non-parametric display of the graph, the experimental groups were ordered by a decreasing median. N=8-12.

But the question is, can pleasant stimuli be avoided and can unpleasant stimuli be chosen? Is it possible that the information processed by the dopaminergic neurons for a US during classical conditioning acquires a different meaning in other circumstances? For instance in the case of this experiment, is activation of dopaminergic neurons immediately reinforcing the movements (operant

learning) the animal is choosing to make? In short, could dopaminergic neurons carry different meaning depending on the type of learning (classical or operant)?

In classical conditioning, learning occurs by the association of an event with its significance, independent of the animal´s actions. This significance, whether rewarding or punishing, is mediated by dopaminergic neurons in *Drosophila* (Schwaerzel et al., 2003; Riemensperger et al., 2005; Schroll et al., 2006; Claridge-Chang et al., 2009; Krashes et al., 2009; Aso et al., 2010; Aso et al., 2012). In the case of operant learning, elicited actions receive a feedback from the environment, which is then processed in the brain modifying future actions (Brembs, 2011). Feedback from the environment can be positively, negatively or not reinforcing at all, and dopaminergic neurons are involved in mediating this reinforcement provided by external stimuli (at least in classical conditioning). Then, activating these neurons artificially, without the trigger of any external stimuli, might generate the consequent reinforcement in classical conditioning, and perhaps in operant behavior as well.

As this study is measuring the approach of the animal depending on the reinforcement that a cluster of neurons is mediating, it appears that every dopaminergic neuron positively reinforces the operant behavior. If this holds true, the Dopamine´s role in reinforcement should be considered depending on the learning approach.

It is important to distinguish what we mean by classical learning, since in the previous learning studies, the memory was tested either 2 min, 2 hours or one day after the training period (Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012; Liu[a] et al., 2012). In our paradigm the fly´s choice takes thirty seconds, a very brief time period for the learning processes previously studied. Since calling it learning can be confused with the longer term memories tested in previous studies, we decided to call it preference, where the actions are instantly reinforced.

Nevertheless, we cannot forget that even in such a short period as in our test, learning and memory is present. If not, the fly would not be able to approach a certain goal because it would forget constantly its intended action. Indeed, visual working memory in flies lasts only a few seconds, and without it, flies would lose their visual orientation while approaching a landmark (Thran et al., 2013).

In the case that Dopamine is conveying the same reinforcement, independent of its operant or classical nature, these results would contradict studies in classical conditioning. However, it could be that the US mediating aversive learning is indeed producing an aversive effect on the fly, but that the fly has a preference to be punished rather than having no stimulus at all. This will show the preference for stimuli (either bad or good) over nil valence information. This idea could be a possible explanation, although it might not be fully satisfactory.

Neurons under the expression of *th*-GAL4 show an activation-dependent meaning

In the previous screening (Fig. 12), we found activation of *th*-positive neurons as preferred, which is in contrary to all of the experiments to our knowledge so far (Schwaerzel et al., 2003; Heisenberg, 2003; Schroll et al., 2006; Claridge-Chang et al., 2009; Krashes et al., 2009; Aso et al., 2010; Aso et al., 2012; Schneider et al., 2012). Therefore, we tried to stimulate this line with another light intensity (Fig. 13 and 14).

What we found is that the PI observed for these flies carrying the ChR2 in tyrosine hydroxylase positive neurons was different to the values seen in the screening. Hence, if might well be that the information carried by these neurons depends on the level of activity, which to our knowledge has not been tested so far.

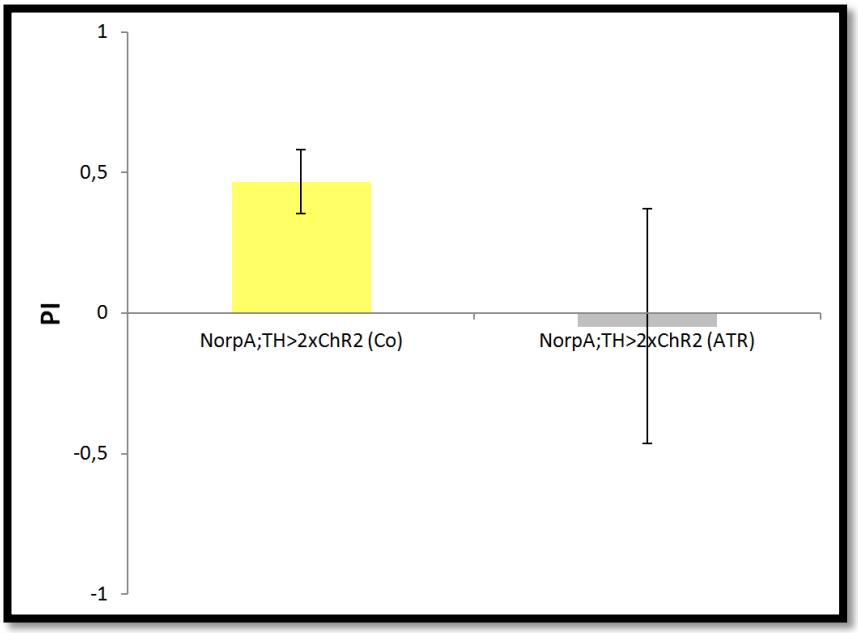

Figure 13. Dopaminergic neurons show different meaning under different light intensity: 500-28W/m². When not reared with ATR (yellow) flies show a preferred activation of the neurons whereas when reared in ATR (grey) they show neither positive nor negative valence of these neurons. This shows nevertheless a big difference to what is seen in Figure 12. The flies in this figure were reared and tested together with females. N=4.(p=0,2802).

In fact, we see that even reared without ATR (Fig. 14) the flies can yield negative PI values. This could be because at lesser light intensities (because without ATR, the channel opening might be less effective) the *th*-positive neurons might mediate aversion. Hence, studies that used TrpA1 as a tool for activating these neurons might activate them less than with this technique.

Nevertheless, in the experiments represented in figures 13 and 14 the PIs are not congruent with each other. The only difference between these two studies is the presence of females during rearing and testing conditions. Because we do not think that the presence of females is causing the differences observed among these two figures, we assume that the number of experiments

performed was too low to be seriously studied. Thus, the only conclusion that we can extract from these two experiments is that at different levels of activation, *th*-positive neurons convey a different incentive value. Preferably, a second way of measuring this behavior should reaffirm or contradict these findings.

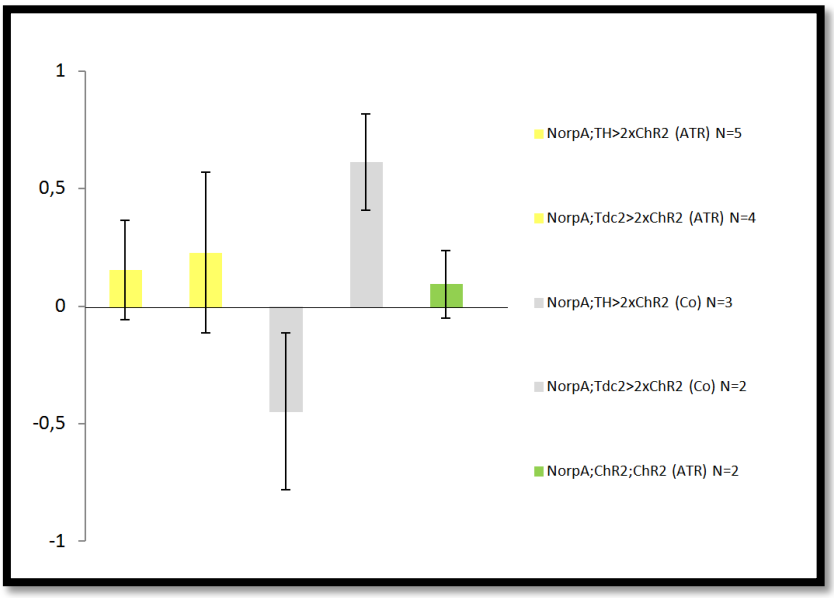

Figure 14. Dopaminergic and octopaminergic neurons show modulation depending on the neuronal activation. Octopaminergic neurons always show a positive preference either with (dark green) or without ATR (blue). However, activation of dopaminergic neurons show positive valence with ATR (dark green) and negative without ATR (blue). The light intensity was 500-28W/m² but in contrast to figure 13, tested male flies were reared and tested separated from females. (p=0,3889) N=2-5.

DISCUSSION

Effects of ATR on ChR2

All rhodopsins require all-trans-retinal in the dark state covalently linked so that when a photon targets it, the channel can enter its photocycle. The presence of ATR is required for the appropriate conformation of ChR2, which is needed for two reasons: to avoid degradation and to be fully functional. Light-gated currents in ChR2 are ten-fold higher than in Chop2 in cell culture (Ullrich et al., 2013).

Our study showed evidence that an ATR supplement increases the channel opening probability. In a mammalian brain an ATR supplement is not necessary, but in C. elegans and Drosophila, ATR is necessary for activating the light-gated currents of this channel (Boyden et al., 2005; Schroll et al., 2006; Ullrich et al., 2013). As in this study, these other studies discussing the relevance of ATR for the channel function were based on behavioral observations. Because no isolation of the molecule from brain tissue has been done, we cannot state that ATR is completely absent in the brain of *Drosophila* and *C.elegans*. Contrary to Schroll et al., 2006, we were able to show that even in the absence of supplemented ATR the channel is still functional. The differences could be explained by the high intensities of light used in this study as well as that instead of adults, Schroll et al., 2006 worked with larvae.

ATR is not only necessary for channel function but also for the channel's resistance to degradation. If the newly formed Chop2 does not find an ATR molecule in the cellular environment to bind with, the apoprotein will be degraded, probably recognized because of its abnormal conformation (Ullrich et al., 2013). For this reason, rearing flies in ATR must be for a long enough time to cover the DNA expression period. Indeed flies lacking chromophore synthesis enzymes show a decrease in the eye rhodopsins that is rescued by feeding them with ATR. And the possibility that ATR deficiency could affect the gene expression has been discarded (von Lintig et al., 2001). Interestingly, a supplement of only 0.2 mM ATR was enough to rescue their eye rhodopsin levels (Wang et al., 2007).

Dopamine is not just coding for reward

The classical view of Dopamine mediating reward has changed as a result of numerous studies in rodents and primates which have shown dopaminergic neurons coding aversive, noxious stimuli,

salient stimuli and stress as well (Cohen et al., 2012; Waddell, 2013). In addition, the temporal prediction error theory postulates that Dopamine is not correlated with the reward itself rather with the error from the predicted outcome, and therefore it seems to be essential for learning by trial and error (Montague et al., 1996; Schultz, 1997).

Pleasure networks are activated by US through the senses. When this US is associated with a neutral stimulus, this neutral stimulus then activates the pleasure networks gaining its own incentive valence.

The firing of dopaminergic neurons just after but not during consummation of the reward brought the idea that Dopamine was mediating the wanting, as a motivational aspect, and not the liking. The degree of liking and wanting interact but are distinct elements of the reward system and can be independently assessed (Laviolette and van der Kooy, 2004; Perry and Barron, 2013).

In theory, the hedonic value (liking) determines the extent of wanting. The degree of wanting and the hedonic value determine the acquisition and retrieval of memories. Internal drive states of the animal at a precise moment as well as “provoking” factors such as conditioned cues modulate the liking and the wanting. Thus, depending on the context, the extent to which something is liked and wanted varies, and this determines to a big extent the learning rate (Toates, 1986; Perry and Barron, 2013).

In flies, a dopaminergic cluster has been found that indeed modulates the behavior depending on the feeding state, in a way that motivation is a result of satiation/starvation degree, which then determines the performance in a certain task (Krashes et al., 2009; Inagaki et al., 2012). It is also interesting that Dopamine seems to mediate the acquisition of a memory but also the retrieval. Hence, it is not only important for learning but also for the response output to a learned event (Krashes et al., 2009; Aso et al., 2010; Kaun et al., 2011; Aso et al., 2012; Liu et al., 2012[a]; Inagaki et al., 2014). In *Drosophila*, the contribution of different dopaminergic neurons in learning does not appear to be additive, and sometimes their individual roles become redundant (Aso et al., 2012).

The discussion about Dopamine has attributed it to certain roles which paradoxically are not the same. The reward of “wanting” (motivation), the reward of “liking” (hedonic aspect of reward) and the reward of learning (reinforcement) in rodents have different neural substrates (Smith et al., 2011; Perry and Barron, 2014). This could explain why the results obtained in this study are differing to the results observed in classical conditioning (Claridge-Chang et al., 2009; Aso et al., 1010; Aso et al., 2012). The difference lies in the fact that these mentioned studies measured learning (thus, reinforcement), whereas in this study the fly´s choices were without previous training.

Unfortunately, studies in flies discriminating liking, wanting and learning are lacking as well as difficult to address (Perry and Barron, 2014). Therefore, one of the neuronal populations mediating aversive learning might be dependent of the motivation of the flies towards distinct stimuli that is controlled by other circuits. And these interactions might occur reciprocally in all of liking, wanting and learning.

Although the segregation of these concepts is understandable at the level of circuits, at the conceptual level this distinction is not that trivial. If we allow ourselves to anthropomorphize the fly's situation, if we like to approach one arm of the T-Maze because it causes certain neurons to be activated, it follows that also if we have a neutral cue that is associated with the activation we will also like it. It also makes sense that if we do not like something, we will escape from it rather than going for it.

An interesting phenomenon has been observed in addiction studies made with invertebrates and vertebrates. These studies give new insights into the complexity of the reward system. It was found that after drug consumption there is a brief period of avoidance from the cue conditioned to the drug administration, however, after a period of time, the cue acquires a positive valence (Jorenby et al., 1990; Shoaib and Stolerman, 1995; Cunningham et al., 2000; Laviolette and van der Kooy, 2004; Pautassi et al., 2008; Kaun et al., 2011).

And the interesting thing in flies is that the dopaminergic neurons targeted by DDC-GAL4 and the th-GAL4 are necessary for the conditioned preference that appears in the long-term after drug administration. On the other hand, when the neurons of these two clusters were inhibited, it did not affect the early conditioned aversion which is surprising given the vast amount of literature which demonstrates the opposite (Schwaerzel et al., 2003; Heisenberg et al., 2003; Schroll et al., 2006; Aso et al., 2010; Aso et al., 2012; Schneider et al., 2012).

This could explain some of our findings (Fig. 12) where *th*-positive neuron activation is preferred, because this neuronal population mediates expression of the preference but not the learning of it (Kaun et al., 2011). From our study we can deduce as well that the same population can mediate two opposite meanings and how different populations mediate the same meaning with distinct features.

Effects of arousal in the preference index

Interestingly, the TH-D positive neurons stimulation has demonstrated arousal (Liu et al., 2012[b]) and positive preference (Fig. 12). In the case of the TH-F neurons, their stimulation did not affect arousal (Liu et al., 2012[b]) but showed a positive preference, although lower than that of TH-D (Fig. 12).

Apparently, the neurons promoting arousal are dopaminergic PPL1 neurons projecting to the FB (Liu et al., 2012[b]), in the PPL1 cluster there are also neurons involved in aversive learning projecting to the MB (Aso et al., 2012). Although the localization of neurons promoting arousal and memory appear to be segregated, it might occur that overlapping neurons comprised by both drivers are mixing the effects in both phenotypes.

In addition, just the trigger of one phenotype could influence the performance in the other phenotype. For instance, by exciting neurons that are arousing might improve or impair the memory performance, as memory is known to be influenced by the arousal level (Jhean-Larose et al., 2014). Thus, it is important to undertake a more accurate characterization of these neurons in order to avoid mixing influencing aspects.

This could explain some of the interactions found in learning studies (Aso et al., 2010; Aso et al., 2012). Indeed, DopR has been shown to be essential for both, arousal (Liu et al., 2012[b]) and learning (Kim et al., 2007; Claridge-Chang et al., 2009; Qin et al., 2012), but for arousal its expression is important in the fan shaped body whereas in learning it is in the MB. It appears that Dopamine effects are segregated in different structures, but nevertheless as we already mentioned, just the effect of being aroused can modulate the reward system or cognitive-like tasks.

Effects of motivation in the preference index

The two MB-MP neurons from the PPL1 (targeted by c061-GAL4;MB-GAL80) that project to the α/β lobes of the MB are modulated by the feeding state (Krashes et al., 2009). Under starvation conditions there is a retrieval of the appetitive memory conveyed by sugar reward whereas with food satiation this memory is blocked (Claridge-Chang et al., 2009). What this shows to us, is that the wanting, modulated by internal states, is selecting at each moment the memories that may be important to retrieve.

It is known that the PAM neurons (targeted by DDC, HL-8 and 58E02) are involved in the acquisition of appetitive learning (Claridge-Chang et al., 2009; Aso et al., 2012). And it appears that the memory block with food satiation could be explained by a greatly decreased activation of PAM neurons in response to food (Liu et al., 2012[a]).

MP neurons stimulation suppresses appetitive memory, probably because it resembles satiation (Krashes et al., 2009). In our study, the c061 driver is in the X-chromosome and therefore we could not address its effects properly. If anything, it shows an aversion to blue light compared to other lines (Fig. 11). However, if MP neurons are mediating satiation, its activation might be rather rewarding. In addition, the robust aversive learning conveyed by these neurons is in agreement to what seen in figure 11 (Aso et al., 2012). Because neurons mediating aversion might not be mediating satiation, further experiments need to elucidate this question.

The effects of motor activity in the preference index

Already long ago (Milner, 1991), the correlation of reward and motor activity was evident, by facts like the potentiation of reward by psychomotor stimulants such as amphetamine. Apparently, low dopaminergic stimulation increased reward without affecting motor activity. The striatum is a link between the cortex and the extrapyramidal motor system, so it would be the prime candidate for associating non-rewarding stimuli with approach response, a feature of incentive motivation (Milner, 1991; Nieh et al., 2012).

In rodents, striatal medium spiny neurons are classified by the ones expressing D1 dopaminergic receptor (dMSN, involved in the direct pathway) and the ones with D2 receptors (iMSN, involved in the indirect pathway). Whereas the former is rewarding and facilitating movement, the latter is aversive and inhibiting movement. Dopamine may promote reinforcement through activating dMSNs, and increased motor activity by activating the direct pathway along with the inhibition of iMSNs implicated in the indirect pathway (Nieh et al., 2012; Kravitz and Kreitzer, 2012).

In flies, inhibition of th-GAL4 and HL9-GAL4 decreases locomotor activity and their activation increases it (Claridge-Chang et al., 2009; Schneider et al., 2012). The former mainly targets the PPL1 cluster whereas the latter the PAM cluster.

A recent study, however, pointed to the PAM cluster as the one increasing locomotion with the other dopaminergic clusters not playing a significant role (Riemensperger et al., 2013). This recent study supports the Dopamine´s role being conserved in rats and flies, where the circuits conveying reward are also increasing locomotion whereas the ones mediating punishment are decreasing it.

The fact that th-GAL4 mediates a negative reinforcement in flies at the same time as it increases locomotion is different with what was found in rats (Kravitz and Kreitzer, 2012). Surprisingly, in our study, th-GAL4 activation produced a positive valence, that together with an increased locomotor effect would correlate to what occurs in rats. Due to inconsistencies in the role of certain

dopaminergic neurons in locomotion (Claridge-Chang et al., 2009; Schneider et al., 2012; Riemensperger et al., 2013) and rewarding properties (Aso et al., 2010; Aso et al., 2012; and our study), further experiments should clarify the interaction between the two phenotypes.

The fact that activation of these neurons modulates locomotion, arousal, reward, etc. might mask effects in our tests. For instance, if flies expressing ChR2 in motor-activating neurons approach the blue illuminated arm, the flies will be more mobile due to the activation of these neurons. Meanwhile, these same flies moving away from the blue illuminated arm would move slower and thus, because no motor-activating neurons are turned on, would remain a longer period of time far from the blue illumination.

Neuronal dynamics should not be overlooked

We can see often in neuroscience the attributing of distinct roles to distinct neuronal populations disregarding the potential of the different neuronal dynamics within one population. Now there is increasing evidence that Dopamine is mediating both punishment and reward. However, studies in *Drosophila* and rodents have focused on distinguishing which neurons are mediating what, overlooking the effects of the firing pattern. An explanatory case can be found in memory, where the same circuit or synapse can mediate LTD or LTP depending on the spatio-temporal firing dynamics.

In fact, within a single brain region, the VTA, nicotine can have rewarding or aversive effects as a function of nicotine concentration (Laviolette and van der Kooy, 2004). If we assume that all the neurons expressing nicotinic Ach receptors within the VTA have the same receptor subtypes, the different concentrations of nicotine will activate the same neurons but to different extent. And then we would be able to state that different degrees of activation convey different meaning. But, another explanation for these results could be that high affinity and low affinity receptors are differentially expressed within the aversive and rewarding dopaminergic neurons of the VTA,, activating differentially dopaminergic neurons depending on the nicotine concentration.

Several studies activating neurons directly have shown different effects under different stimulation patterns within the same population (Chaudhury et al., 2013; Bass et al., 2013). This highlights the importance of the firing of the neuron and that neurons cannot be classified as mediating one meaning or the other because they might have the potential to function in a bidirectional way. These observations support the idea that different levels of activation of neurons can convey opposing poles of the same scale.

Even if many neurons comprising many circuits have been very well functionally characterized, there is a step further to go, and this is to understand the meaning of the dynamics of these neurons. Even the same neuron could convey opposing values depending on their spatial and temporal firing or their contextual state. With the avenue of this fancy technique, optogenetics, we are able to control the activation of neurons to a temporal and spatial precision that we never before had.

Recent studies including the results of our own studies highlight distinct functions for the same dopaminergic neurons. For instance, MB-MP neurons are suppressing the retrieval of appetitive memory when flies are not starved (Krashes et al., 2009), they form aversive odor memory (Aso et al., 2012) and regulate the long-term memory by synchronized spontaneous activity (Plaçais et al., 2012).

Comments about the technique

A disadvantage from all the techniques that stimulate neurons is that they activate the neuronal network in an artificial way, ignoring the natural pattern of activity of the network, and stimulate neurons simultaneously, that otherwise would show different temporal patterns. Hence, we might lose sight of network functions that require asynchronicity or different firing frequency among the neurons comprising the network.

These functions might be obscured particularly when big neuronal populations are targeted, probably due to opposing effects or inhibiting effects of activated neurons. An interesting observation by Milner with experiments with ICSS in rats (Milner, 1991), is that approach dominates avoidance when targeting big neuronal populations. Nevertheless, we do not consider the neuronal clusters targeted in this study to be large. In this respect, inhibition instead of excitation of neurons can offer another more reliable approach to find functions processed by these networks.

As Milner pointed out (Milner, 1991), it was clear that a single impulse from the electrode, even if intense, was not enough to induce a response in the ICSS paradigm. Apparently, the rewarding effect arises from several pulses in a short period. For instance, the optimum of stimulation in the VTA for the release of Dopamine in the Nacc was 50Hz in a sine wave (Milner, 1991).

One feature of our experiments was that light was not pulsed, rather constant. The constant light might bring the neurons in a depolarization block state (Bianchi et al., 2012; Inagaki et al., 2014), leaving them unresponsive. Nevertheless, the steep gradient of light intensity provided in the T-maze might make the activation of neurons more dynamic depending on the movement of the fly towards or away from the blue light.

Finally, it is important to remark the difference between the olfactory learning in a T-maze used in many other studies and the preference test performed in this study. The association in the former occurs with the presentation of the CS and US and it is controlled by the experimenter and independent of the actions of the animal (similar to conditioned place preference in rats). This contrasts with our experiments that consist rather of an operant task where the animal gets the feedback from their actions, more similar to the lever pressing studies in rats.

Although, this seems to be a slight difference, behavioral studies from rodents have shown that there are important distinct aspects behind these two approaches (Milner, 1991; Prus et al., 2009).

Possible problems with the screening controls

Looking to the genetic controls of the screening we found out that some of them were not around zero as expected. We thought about possible reasons for this and came up with the genetic background as being the most logical explanation.

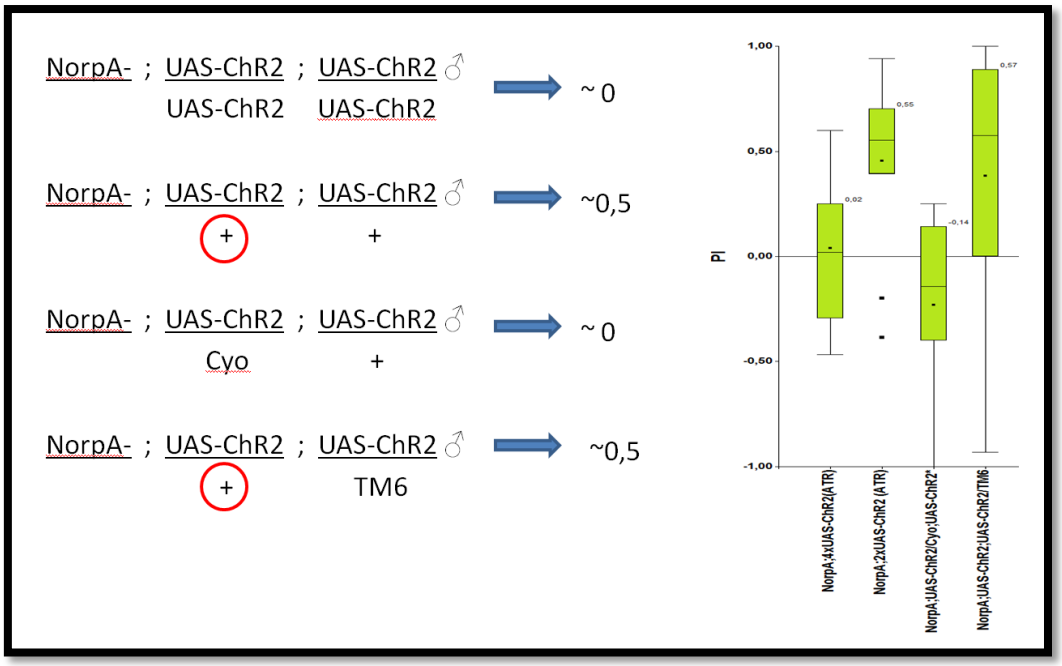

Figure 15. Diagram showing the comparison of the control genotypes to their corresponding phenotypes. On the left, the detailed genotype of the controls tested in Figure 12, shown in the right of this figure as well. By examining the commonalities found in the unexpected control lines (the ones with PI around 0.5), we can see that a wild type second chromosome can explain it.

We decided to see which chromosome could explain the abnormal phenotypes (Fig. 15). The only chromosome that could explain the unexpected approach to the blue light of the flies was a wild type second chromosome. What it is very interesting is that the approach was at around a PI of 0.5, similar to what was previously found in flies with visual function (Fig. 8, 9b and 12).

One of the most plausible explanations is that there is a contamination of the parental line containing the NorpA mutation and the P-element containing the UAS-ChR2. For instance, one hypothesis is that the NorpA mutation is lost only in some of the X chromosomes and an additional mutation in the second chromosomes carrying the UAS-ChR2 makes them blind. Then, the flies carrying a wild type second chromosome would be rescued from blindness, which would otherwise not occur when the flies are in homozygosis for the second chromosome containing ChR2.

Hence, the flies which have a double copy of UAS-ChR2-containing second chromosome are blind whereas in heterozygosis the flies´ vision is rescued by the wild type chromosome. In addition, all the manipulations done to the balancer containing the CyO might impair the rescue of vision. This will make all the experimental lines able to see and that might be the reason they are all approaching the blue light.

In addition, figure 16 shows the difference between flies staying in the elevator compared to the average number of flies that made a choice. What we can see is that the putative control blind flies without ChR2 driven expression stay more in the elevator than the rest of the tested groups. This is evidence that they are really blind because we expect that if a fly is blind, and no other information is affecting their choice they would not see any reason to move from its original situation.

In contrast, the two other control lines (NorpA;2xUAS-ChR2 and NorpA;UAS-ChR2;UAS-ChR2/TM6) might get a valence, and because there is no expression of ChR2, it might be their ability to detect the blue light. This is similar to what is seen in the experimental lines, where they have the valence of the neuronal activation, and this drives them to make the choice.

Although loss of NorpA mutations in some of the X-chromosomes and a mutation in the second chromosomes containing ChR2 that makes the flies blind could explain many of the unexpected results, the odds of occurring this are low. Other hypotheses are open, but none of them are fully convincing. Therefore, we think that we should perform further experiments in order to be able offer more accurate explanations.

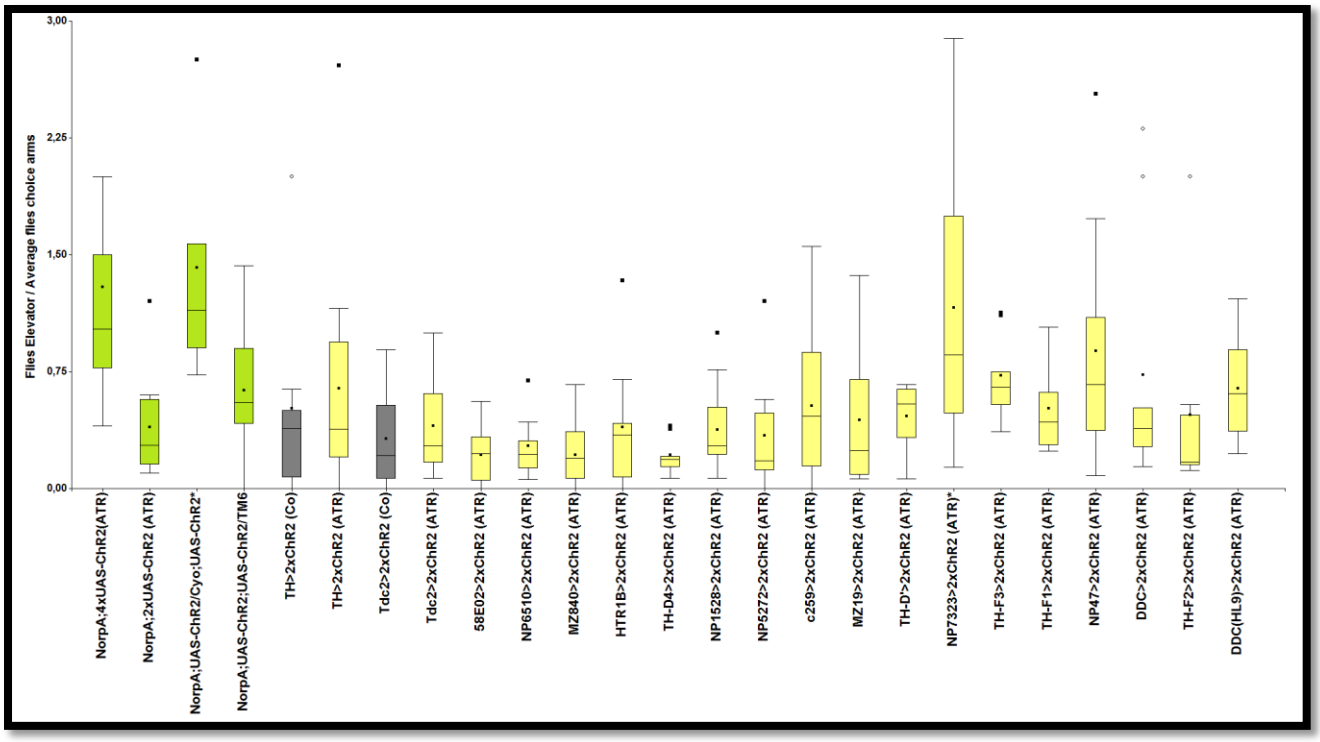

Figure 16. Number of individuals that stayed in the elevator in comparison to the ones that made a choice. It was evident already during the experiments that in two of the genetic controls (green), more flies stayed in the elevator. Thus we plotted for each line the number of flies counted in the elevator divided by the average of flies that went to either arm (elevator/((blue illuminated arm+ yellow illuminated arm/2)). The two control lines that showed PI around zero in figure 12 and 15, NorpA;4xUAS-ChR2 and NorpA; UAS-ChR2/Cyo; UAS-ChR2/+, contain the larger number of flies staying in the elevator in comparison to the total amount of flies. In the other two genetic controls with PI around 0.5, (Fig. 12 and 15) the ATR controls (grey) and in the experimental lines (yellow), this value was not as high as in the above mentioned controls.

Additional experiments with second chromosome deficiencies for instance or with other neurogenetic techniques like ReaChR, TrpA1 or Shibire might help to address this question.

ACKNOWLEDGEMENTS

I want to thank…

Prof. Dr. Brembs, for offering himself as my supervisor, his suggestions during the project and for offering the possibility to work in such a fascinating field.

Prof. Dr. Schnewly, for assuming the second evaluation and for his help during the master program issues.

Axel, for hearing my daily complaints, for encouraging me when the results frustrated me, for teaching me concepts and methods in Drosophila.

Juan, for his help and kindness during the master program, for giving me material during my master thesis uninterestedly.

José, Beibs, Lars, Olli, etc. for facilitating a daily friendly working environment.

Max Planck Institute in Munich and Henrike Scholtz, for providing me with the experimental lines.

Carey, who revised thoroughly my master thesis correcting my english and making me review my incomprehensible explanations.

My family, in particular Peter, Carey, Sonja and Anja because they deserve much more than what I offer to them and because I wish I could spend more time with them.

Natalia, for her support in every aspect of my life and for coping with my boring interests and understanding my unlimited dedication to my work.

BIBLIOGRAPHY

Aso Y, Herb A, Ogueta M, Siwanowicz I, Templier T, Friedrich AB, Ito K, Scholz H, Tanimoto H: Three dopamine pathways induce aversive odor memories with different stability. PLoS Genet 2012, 8:e1002768.

Aso Y, Siwanowicz I, Bräcker L, Ito K, Kitamoto T, Tanimoto H. Specific dopaminergic neurons for the formation of labile aversive memory. Curr Biol. 2010 Aug 24;20(16):1445-51.

Aso Y, Siwanowicz I, Bracker L, Ito K, Kitamoto T, Tanimoto H: Specific dopaminergic neurons for the formation of labile aversive memory. Curr Biol 2010, 20:1445-1451.

Bamann C., Kirsch T., Nagel G., Bamberg E. Spectral characteristics of the photocycle of Channelrhodopsin-2 and its implication for channel function. J Mol Biol, 375 (2008), pp. 686–694

Bass CE, Grinevich VP, Gioia D, Day-Brown JD, Bonin KD, Stuber GD, Weiner JL, Budygin EA. Optogenetic stimulation of VTA dopamine neurons reveals that tonic but not phasic patterns of dopamine transmission reduce ethanol self-administration. Front Behav Neurosci. 2013 Nov 26;7:173.

Benzer S. Behavioral mutants of Drosophila isolated by countercurrent distribution. Proc Natl Acad Sci U S A. Sep 1967; 58(3): 1112–1119.

Bianchi D, Marasco A, Limongiello A, Marchetti C, Marie H, Tirozzi B, Migliore M. On the mechanisms underlying the depolarization block in the spiking dynamics of CA1 pyramidal neurons. J Comput Neurosci. 2012 Oct;33(2):207-25.

Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci. 2005 Sep;8(9):1263-8.

Brembs B. Spontaneous decisions and operant conditioning in fruit flies. Behav Processes. 2011 May;87(1):157-64.

Britt JP, McDevitt RA, Bonci A. Use of channelrhodopsin for activation of CNS neurons. Curr Protoc Neurosci. 2012;Chapter 2:Unit2.16.

Burke CJ, Huetteroth W, Owald D, Perisse E, Krashes MJ, Das G, Gohl D, Silies M, Certel S, Waddell S: Layered reward signaling through octopamine and dopamine in Drosophila. Nature 2012, 492:433-437.

Burke CJ, Waddell S: Remembering nutrient quality of sugar in Drosophila. Curr Biol 2011, 21:746-750.

Busch S, Selcho M, Ito K, Tanimoto H. A map of octopaminergic neurons in the Drosophila brain. J Comp Neurol. 2009 Apr 20;513(6):643-67.

Chaudhury D, Walsh JJ, Friedman AK, Juarez B, Ku SM, Koo JW, Ferguson D, Tsai HC, Pomeranz L, Christoffel DJ, Nectow AR, Ekstrand M, Domingos A, Mazei-Robison MS, Mouzon E, Lobo MK, Neve RL, Friedman JM, Russo SJ, Deisseroth K, Nestler EJ, Han MH. Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. Nature. 2013 Jan 24;493(7433):532-6.

Claridge-Chang A, Roorda RD, Vrontou E, Sjulson L, Li H, Hirsh J, Miesenböck G. Writing memories with light-addressable reinforcement circuitry. Cell 2009 Oct 16;139(2):405-15.

Cohen JY, Haesler S, Vong L, Lowell BB, Uchida N. Neuron-type-specific signals for reward and punishment in the ventral tegmental area. Nature 2012 Jan 18;482(7383):85-8.

Colomb J, Reiter L, Blaszkiewicz J, Wessnitzer J, Brembs B. Open source tracking and analysis of adult Drosophila locomotion in Buridan's paradigm with and without visual targets. PLoS One. 2012;7(8):e42247.

Connolly JB, Roberts IJ, Armstrong JD, Kaiser K, Forte M, Tully T, O'Kane CJ. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science 1996 Dec 20;274(5295):2104-7.

Cunningham CL, Howard MA, Gill SJ, Rubinstein M, Low MJ, Grandy DK. Ethanol-conditioned place preference is reduced in dopamine D2 receptor-deficient mice. Pharmacol Biochem Behav. 2000 Dec;67(4):693-9.

de Belle JS, Heisenberg M. Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. Science 1994 Feb 4;263(5147):692-5.

Glickman SE. Reinforcing properties of arousal. J Comp Physiol Psychol. 1960 Feb;53:68-71.

Heisenberg M: Mushroom body memoir: from maps to models. Nat Rev Neurosci 2003, 4:266-275.

Hirsh J, Riemensperger T, Coulom H, Iché M, Coupar J, Birman S. Roles of dopamine in circadian rhythmicity and extreme light sensitivity of circadian entrainment. Curr Biol. 2010 Feb 9;20(3):209-14.

Inagaki HK, Ben-Tabou de-Leon S, Wong AM, Jagadish S, Ishimoto H, Barnea G, Kitamoto T, Axel R, Anderson DJ. Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. Cell. 2012 Feb 3;148(3):583-95.

Inagaki HK, Jung Y, Hoopfer ED, Wong AM, Mishra N, Lin JY, Tsien RY, Anderson DJ. Optogenetic control of Drosophila using a red-shifted channelrhodopsin reveals experience-dependent influences on courtship. Nat Methods. 2014 Mar;11(3):325-32.

Jhean-Larose S, Leveau N, Denhière G. Influence of emotional valence and arousal on the spread of activation in memory. Cogn Process. 2014 Apr 9.

Joel D, Niv Y, Ruppin E. Actor-critic models of the basal ganglia: new anatomical and computational perspectives. Neural Netw. 2002 Jun-Jul;15(4-6):535-47.

Jorenby, D. E., Steinpreis, R. E., Sherman, J. E. & Baker, T. B. Aversion instead of preference learning indicated by nicotine place conditioning in rats. Psychopharmacology 1990; 101, 533–538.

Karuppudurai T, Lin TY, Ting CY, Pursley R, Melnattur KV, Diao F, White BH, Macpherson LJ, Gallio M, Pohida T, Lee CH. A hard-wired glutamatergic circuit pools and relays UV signals to mediate spectral preference in Drosophila. Neuron 2014 Feb 5;81(3):603-15.

Kaun KR, Azanchi R, Maung Z, Hirsh J, Heberlein U. A Drosophila model for alcohol reward. Nat Neurosci. 2011 May;14(5):612-9.

Kim YC, Lee HG, Han KA: D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. J Neurosci 2007, 27:7640-7647.

Laviolette S.R., van der Kooy D. The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. Nat. Rev. Neurosci. 2004; 5, 55–65.

Liu[a] C, Placais PY, Yamagata N, Pfeiffer BD, Aso Y, Friedrich AB, Siwanowicz I, Rubin GM, Preat T, Tanimoto H: A subset of dopamine neurons signals reward for odour memory in Drosophila. Nature 2012, 488:512-516.

Liu[b] Q, Liu S, Kodama L, Driscoll MR, Wu MN. Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in Drosophila. Curr Biol. 2012 Nov 20;22(22):2114-23.

Maisak MS, Haag J, Ammer G, Serbe E, Meier M, Leonhardt A, Schilling T, Bahl A, Rubin GM, Nern A, Dickson BJ, Reiff DF, Hopp E, Borst A. A directional tuning map of Drosophila elementary motion detectors. Nature. 2013 Aug 8;500(7461):212-6.

Mao Z, Davis RL. Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. Front Neural Circuits. 2009 Jul 1;3:5.

Markow TA, Merriam J. Phototactic and geotactic behavior of countercurrent defective mutants of Drosophila melanogaster. Behav Genet. 1977 Nov;7(6):447-55.

Mercer AR, Menzel R. The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee Apis mellifera. J. Comp. Physiol. 1982 A 145:363–68

Milner PM. Brain-stimulation reward: a review. Can J Psychol. 1991 Mar;45(1):1-36.

Monastirioti M, Linn Jr CE, White K. Characterization of Drosophila tyramine β-hydroxylase gene and isolation of mutant flies lacking octopamine. J Neurosci 199616: 3900-3911.

Montague PR, Dayan P, Sejnowski TJ. A framework for mesencephalic dopamine systems based on predictive Hebbian learning. J Neurosci. 1996 Mar 1;16(5):1936-47.

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., and Bamberg, E. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc. Natl. Acad. Sci. USA *100* , 13940– 13945.

Olds J, Milner P. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. J Comp Physiol Psychol. 1954 Dec;47(6):419-27.

Olds J. A preliminary mapping of electrical reinforcing effects in the rat brain. Journal of Comparative and Physiological Psychology, Vol 49(3), Jun 1956, 281-285

Olds, M.H., Olds, J. Approach-avoidance analysis of rat diencephalon. Journal of Comparative Neurology 1963, 120, 259-295.

Pautassi, R.M., Molina, J.C. & Spear, N. Infant rats exhibit aversive learning mediated by ethanol's orosensory effects but are positively reinforced by ethanol's post-ingestive effects. Pharmacol. Biochem. Behav. 2008; 88, 393–402.

Perry CJ, Barron AB. Neural mechanisms of reward in insects. Annu Rev Entomol. 2013;58:543-62.

Plaçais PY, Trannoy S, Isabel G, Aso Y, Siwanowicz I, Belliart-Guérin G, Vernier P, Birman S, Tanimoto H, Preat T. Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in Drosophila. Nat Neurosci. 2012 Feb 26;15(4):592-9.

Prus AJ, James JR, Rosecrans JA. Conditioned Place Preference. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience. 2nd edition. Boca Raton (FL): CRC Press; 2009. Chapter 4. Frontiers in Neuroscience.

Qin H, Cressy M, Li W, Coravos JS, Izzi SA, Dubnau J. Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in Drosophila. Curr Biol. 2012 Apr 10;22(7):608-14.

Radu I, Bamann C, Nack M, Nagel G, Bamberg E, Heberle J. Conformational changes of channelrhodopsin-2. J Am Chem Soc. 2009 Jun 3;131(21):7313-9.

Riemensperger T, Issa AR, Pech U, Coulom H, Nguyễn MV, Cassar M, Jacquet M, Fiala A, Birman S. A single dopamine pathway underlies progressive locomotor deficits in a Drosophila model of Parkinson disease. Cell Rep. 2013 Nov 27;5(4):952-60.

Riemensperger T, Völler T, Stock P, Buchner E, Fiala A. Punishment prediction by dopaminergic neurons in Drosophila. Curr Biol. 2005 Nov 8;15(21):1953-60.

Roeder T. Octopamine in invertebrates. Prog Neurobiol. 1999 Dec;59(5):533-61.

Schroll C, Riemensperger T, Bucher D, Ehmer J, Völler T, Erbguth K, Gerber B, Hendel T, Nagel G, Buchner E, Fiala A. Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae. Curr. Biol. 2006 16:1741–47

Schneuwly S, Burg MG, Lending C, Perdew MH, Pak WL. Properties of photoreceptor-specific phospholipase C encoded by the norpA gene of Drosophila melanogaster. J Biol Chem. 1991 Dec 25;266(36):24314-9.

Schultz W, Dayan P, Montague PR. A neural substrate of prediction and reward. Science. 1997 Mar 14;275(5306):1593-9.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M: Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci 2003, 23:10495-10502.

Selcho M, Pauls D, Han KA, Stocker RF, Thum AS. The role of dopamine in Drosophila larval classical olfactory conditioning. PLoS One. 2009 Jun 12;4(6):e5897.

Shoaib, M. & Stolerman, I. P. Conditioned taste aversion in rats after intracerebral administration of nicotine. Behav. Pharmacol. 1995; 6, 375–385.

Smith KS, Berridge KC, Aldridge JW. Disentangling pleasure from incentive salience and learning signals in brain reward circuitry. Proc Natl Acad Sci U S A. 2011 Jul 5;108(27):E255-64.

Suh GS, Ben-Tabou de Leon S, Tanimoto H, Fiala A, Benzer S, Anderson DJ. Light activation of an innate olfactory avoidance response in Drosophila. Curr Biol. 2007 May 15;17(10):905-8.

Thran J, Poeck B, Strauss R. Serum response factor-mediated gene regulation in a Drosophila visual working memory. Curr Biol. 2013 Sep 23;23(18):1756-63.

Tindell AJ, Berridge KC, Aldridge JW. Ventral pallidal representation of pavlovian cues and reward: population and rate codes. J Neurosci. 2004 Feb 4;24(5):1058-69.

Toates FM. Motivational Systems. Cambridge, UK: Cambridge Univ. Press. 1986 188 pp

Ullrich S, Gueta R, Nagel G. Degradation of channelopsin-2 in the absence of retinal and degradation resistance in certain mutants. Biol Chem. 2013 Feb;394(2):271-80.

van Swinderen B, Brembs B. Attention-like deficit and hyperactivity in a Drosophila memory mutant. J Neurosci. 2010 Jan 20;30(3):1003-14

von Lintig J, Dreher A, Kiefer C, Wernet MF, Vogt K. Analysis of the blind Drosophila mutant ninaB identifies the gene encoding the key enzyme for vitamin A formation invivo. Proc Natl Acad Sci U S A. 2001 Jan 30;98(3):1130-5.

Waddell S. Reinforcement signalling in Drosophila; dopamine does it all after all. Curr Opin Neurobiol. 2013 Jun;23(3):324-9.

Wang T, Jiao Y, Montell C. Dissection of the pathway required for generation of vitamin A and for Drosophila phototransduction. J Cell Biol. 2007 Apr 23;177(2):305-16.

Wright GA, Mustard JA, Simcock NK, Ross-Taylor AA, McNicholas LD, Popescu A, Marion-Poll F. Parallel reinforcement pathways for conditioned food aversions in the honeybee. Curr Biol. 2010 Dec 21;20(24):2234-40.

Yamaguchi S, Desplan C, Heisenberg M. Contribution of photoreceptor subtypes to spectral wavelength preference in Drosophila. Proc Natl Acad Sci U S A. 2010 Mar 23;107(12):5634-9.