Flexible valence coding by dopaminergic neurons in *Drosophila melanogaster*

Master Thesis

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08.03.2026

Abstract

Although multiple studies address functional circuits and mechanisms of valuation-signaling, how the quality of a stimulus ('valence') itself is represented at the neuronal level is insufficiently understood. Based on classical associative learning, multiple studies on *Drosophila* formulate a general concept of dopaminergic reward- or punishment neurons that encode valence signals; however, this idea of valence-segregation is challenged as the valence associated with neuronal activation can shift in operant paradigms. The present study investigates a valence reversal, expressed as a behavioral shift from avoidance to approach of neuronal stimulation, mediated by TH-D'-Gal4-positive neurons, and focusses on a subpopulation targeted by the more restricted driver TH C-AD;TH-D-DBD. Using immunohistochemistry and operant self-stimulation paradigms (T-Maze and JoyStick) the behavioral shift is isolated to dopaminergic neurons of the PPM2 and PPL1 clusters. Manipulation experiments highlight a central role of dopamine and imply operant learning circuits in the behavioral shift. Although the exact mechanisms remain unknown, this work adds evidence that a small population of neurons can drive behaviors of opposite valence, and therefore weakens the idea of segregated valence-representing neurons.

Contents

Introduction

To survive in a changing environment, animals must flexibly adapt their behavior according to its consequences. The association between an action and its outcome guides memory formation and depends on whether the outcome is desirable or not. This distinction is encoded in the valence of resulting stimuli, a term that describes the 'attractiveness' of the stimulus. For instance, the detection of sweet taste after probing a new food source represents a stimulus associated with a positive valence and may promote consumption and future foraging. Such stimulus–value associations are fundamental and shape general animal behavior. However, how valence is represented at the neural level is not fully understood.

Investigations of valuation signals have focused on dopamine (DA). The neurotransmitter plays a key role in motivation and reward processing in mammals and exerts its effects via different receptor classes (Wise 2004; Martel and Gatti McArthur 2020). Historically, debates have addressed whether DA signals aspects of 'liking', 'learning' or 'wanting' of rewards (Berridge 2007). The current conception favors the idea that dopaminergic neurons (DANs) mediate the motivational ('wanting') aspect of reward rather than basic hedonic properties ('liking') (Berridge and Kringelbach 2015; Kesner et al. 2022). DA prominently acts as a neuromodulator and influences the responsiveness of neural circuits as well as shaping synaptic plasticity (Reynolds and Wickens 2002; Marder 2012; Puig et al. 2014). Furthermore, Schultz (2016) describes midbrain DANs as encoders for a 'reward prediction error', where the discrepancy between expected and perceived reward is represented in the firing activity of these neurons. By bridging expectations and alterations of neural responses, DANs can signal, and attribute valence to stimuli.

Because DA is present in nervous systems across animal phyla (Kass-Simon and Pierobon 2007), numerous studies investigated the neurotransmitter in invertebrate systems and found conserved functions and signaling mechanisms (Barron et al. 2010; Yamamoto and Seto 2014; Verlinden 2018; Karam et al. 2020; Muralidhara and Hardege 2025). Many of these studies focus on *Drosophila melanogaster*. The comparatively simple neuronal architecture of the brain, combined with the immense repertoire of behavioral assays and genetic manipulations, make the fruit fly an ideal model organism to investigate neural substrates and signaling mechanisms. The adult *Drosophila*

protocerebrum contains approximately 120 DANs, which can be categorized into eight clusters (Mao and Davis 2009). Two clusters reside in the anterior part of the brain (PAM, PAL), while the other six (PPM1, PPM2, PPM3, PPL1, PPL2ab, PPL2c) can be found in the posterior portion. The clusters PAM, PPL1 and PPL2ab innervate the mushroom body (MB), a higher-order integration center, important for olfactory memory formation and action selection (Dubnau et al. 2003; Davis 2005). The MB is comprised of ~2000 Kenyon cells (KCs) whose axons form distinct compartments, the MB-lobes, where KCs synapse onto MB output neurons (MBONs), which, in turn, drive behavior (Turner et al. 2008; Li et al. 2020a). KCs, MBONs and DANs form a tripartite structure that provides the basis for olfactory associative learning (Aso et al. 2014a; Hige et al. 2015). In this model, DANs attribute value to different odors. They induce plasticity changes at the KC-MBON synapse which alter MBON activation, and therefore the behavioral response to the odor (Owald et al. 2015; Ichinose et al. 2021)

Consistent with this model, studies identified distinct populations of DANs, which, when stimulated artificially, were able to substitute for reinforcing stimuli. For example, sugar activates dopaminergic neurons in the PAM cluster and can be used to induce appetitive odor memory (Burke and Waddell 2011). This effect can be mimicked by a direct stimulation of PAM DANs, for example through optogenetic means (Aso and Rubin 2016). On the other hand, pairing odor presentation with stimulation of PPL1 neurons can induce odor avoidance (Claridge-Chang et al. 2009; Masek et al. 2015). Therefore, a common conception is that the valence of a reinforcing stimulus is represented by distinct sets of DANs, most prominently PAM and PPL1 DANs (see Supplementary Material 1). However, most evidence supporting valence segregation comes from classical conditioning paradigms in which an external experimenter controls the presentation of stimuli.

In contrast to this classical conditioning, operant learning requires animals to generate behavior, which in turn results in stimulation. The underlying neuronal mechanisms, as well as involved neuromodulatory systems engaged during operant learning can differ substantially from those involved in classical conditioning. Wolf et al. (1998) found that MB-less flies can learn the association of heat punishment and turning behavior, although the structure seems crucial for classical associative learning (Roman and Davis 2001; Akalal et al. 2006; Modi et al. 2020). Moreover, serotonin-depleted flies fail to operantly learn avoiding a heat-associated location, while DA seems dispensable for this

behavior (Sitaraman et al. 2008). It is therefore crucial to investigate how valence is represented across different modalities and paradigms that extend beyond the idea of a classical stimulus-stimulus association.

Rohrsen et al. addressed this issue and conducted a large-scale screen on DANs and the acute valence associated with DAN activity in different operant self-stimulation paradigms (Rohrsen et al. 2021). In their study, the authors found considerable differences between the effects reported for classical learning and results obtained in their operant paradigms. For example, they could not confirm that flies would approach an isolated activation of PAM neurons; in some experiments, flies even avoided optogenetic stimulation of these neurons. Furthermore, they found opposing effects across their own experimental setups. Flies that were crossed using the NP47-Gal4 driver approached stimulation in a group assay but showed avoidance of stimulation when tested in isolation. These observations highlight that dopaminergic–mediated valuation is complex and context dependent. Labelling individual neurons as representing positive or negative valence, simply from observations in classical studies, may be a premature conclusion.

In their screen, the authors also identified populations of DANs, which did not mediate opposing effects between, but within experiments. In the early stages of the experiment, flies avoided optogenetic stimulation of neurons targeted by the TH-D'-Gal4 driver but shifted their preference to approach over time. Although it has been shown that the same neurons can mediate opposing effects in a *timing*-dependent manner (Aso and Rubin 2016; König et al. 2018), a *time*-dependent valence reversal has not been reported, yet.

The fly strain TH-D'-Gal4 targets different populations of DANs (Liu et al. 2012b). Whether individual clusters mediate the valence shift in concert, or if a single cluster can indeed drive opposing valence, can be addressed by using more refined driver lines. The split-Gal4 driver TH-C-AD;THD-DBD drives expression in a subset of these neurons (Xie et al. 2018), and using this driver allows to narrow down TH-D'-driven behavioral effects. TH-C-AD;TH-D-DBD reportedly targets neurons which are part of the PPM2 cluster. There are multiple PPM (protocerebral posterior medial) clusters and unfortunately, not all studies precisely differentiate between them. PPM2 DANs are also addressed as "PPM1/2" (Liu et al. 2012b; Marquis and Wilson 2022), what disregards a separate PPM1 cluster. To achieve precision

as well as consistency, this study uses the more differentiated nomenclatures PPM1 and PPM2 for distinct clusters.

Anatomically, the PPM2 cluster contains 6-8 neurons per hemisphere (Mao and Davis 2009; Davis et al. 2021) and neurons in this cluster exhibit connections to the lateral horn, antennal lobe, MBONs but also as- and descending neurons (Marquis and Wilson 2022). Functionally, these DANs have received comparatively little characterization, but seem to be involved in locomotion, social spacing, circadian rhythmic and protein hunger (Liu et al. 2017; Xie et al. 2018; Marquis and Wilson 2022; Liang et al. 2023). So far, no study has linked them to valuation signaling.

To investigate the acute valence mediated by DANs, this study uses the light-gated cation channel CsChrimson to selectively stimulate target neurons (Klapoetke et al. 2014). Flies are tested in operant self-stimulation paradigms and their optogenetic preference is measured as a proxy for DAN-driven valence. The *norpA* gene encodes a phosphoinositide-specific phospholipase C (Kim et al. 1995). Mutations in this gene ablate the responses of photoreceptors to light, effectively blinding flies (Pearn et al. 1996). This allows to control for a possible bias from innate positive phototaxis (Ramin et al. 2014). To ensure that investigated effects are robust across contexts, flies are tested in two different experimental paradigms, optogenetic JoyStick and T-Maze. Together these paradigms encompass different social contexts and means to express preference. Immunohistochemistry is used to verify expression patterns and identify the neuronal substrates that drive the behavior. Additionally, manipulations of DAN function using 3-Iodo-L-Tyrosine (3IY) and an alternative stimulation protocol help to unravel the underlying mechanisms. Together, these approaches enable a systematic examination of how specific DAN populations are involved in valence representation and help understanding flexible dopaminergic signaling.

Materials and Methods

Fly stocks and maintenance

Stock flies were reared in glass vials on standard fly food consisting of cornmeal and molasses (Guo et al. 1996). Flies were housed in temperature and humidity controlled climate chambers (25°C, 60% r.h.) and a light dark cycle, with lights on at 8am and off at 8pm, was maintained. Virgin female flies were stored at a lower temperature (18°C) for up to 14 days before crossing.

Table 1 Fly Stocks

Fly strain	Origin	Reference
UAS-mCD8::GFP	Stephan Sigris	-
NorpA;20xUAS-Chrimson	Brembs Lab	(Rohrsen et al. 2021)
Gr28bd-Gal4;TrpA1-Gal4	Brembs Lab	(Rohrsen et al. 2021)
TH-C-AD;TH-D-DBD	Radostina Lyutova, Brembs Lab	(Xie et al. 2018)

The crossings were bred in plastic vials which contained standard fly food, fresh yeast and a small piece of filter paper to monitor food moisture. Egg laying was allowed for 24h after which flies were transferred to new vials to ensure similar larval densities across vials. Because T-Maze experiments require large quantities of animals, a new crossing was started every 10-12 days and multiple crossings for the same driver were kept in parallel. Flies from parallel crossings were mixed during sorting before treatment.

Reagents and antibodies

Table 2 Chemical reagents and antibodies

Chemical	Manufacturer / Origin	Article-/CAS-number
Ethanol, 99.8%	CSC JÄKLECHEMIE	64-17-5
All-*trans*-retinal (ATR)	Sigma-Aldrich	116-31-4
3-Iod-L-Tyrosine (3IY)	Sigma-Aldrich	70-78-0
Paraformaldehyde (PFA)	Electron Microscopy Sciences	30525-89-4
Triton-X-100	Merck	9036-19-5
Normal goat serum	PAN Biotech	P30-1002
Mouse-Anti-Bruchpilot	Schneuwly-Lab	-
Rabbit-Anti-GFP	Invitrogen	A-6455
Goat-Anti-Rabbit Alexa Fluor 488	Mol Probes (MoBiTec)	A-11034
Goat-Anti-Mouse Alexa Fluor 555	ThermoFisher	A-21127
VECTASHIELD Antifade Mounting Medium	Vector Laboratories	VEC-H-1000

Immunohistochemistry

To verify expression of the TH-C-AD;TH-D-DBD split-Gal4 driver line, 10 virgin female flies were crossed with five male UAS-mCD8::GFP flies and female progeny collected 1–3 days after eclosion. Flies were fixed in 4% paraformaldehyde for 2h at room temperature (RT). Brains were dissected in 1 × phosphate-buffered saline (PBS) and washed four times for 15 min each in PBS containing 0.1% Triton X-100 (PBST) at RT. To block nonspecific antibody binding, brains were incubated in 100% normal goat

serum (NGS) for 15 min, followed by 10% NGS in PBST for 1h, at RT. Brains were then incubated overnight at 4°C (protected from light) in primary antibody solution containing mouse anti-Bruchpilot (1:20), rabbit anti-GFP (1:500), and 3% NGS in PBST.

The following day, brains were washed in PBST at RT four times for 15 minutes before the secondary antibody solution containing goat anti-rabbit AF488 (1:200), goat anti-mouse AF555 (1:200) and 3% NGS in PBST was applied. Secondary AB incubation was overnight at 4°C with samples protected from light exposure.

On the final day, brains were washed in PBST (4x15 minutes, RT) before mounting onto standard microscope slides using VECTASHIELD Antifade Mounting Medium. Immunohistochemistry samples were imaged using a Leica SP8 confocal laser scanning microscope (RRID: SCR_018169) with a 20x objective and immersion oil. Images were finalized in FiJi (V. 1.54p) and Inkscape (V.1.4).

Pharmacological treatment

Flies for behavioral experiments were collected one day after eclosion. On the following day, flies were immobilized on a cooling platform, sorted by sex, and groups of 30-40 males transferred to small glass vials, which were prepared differently, depending on the experiment:

For initial JoyStick and T-Maze (Fig. 3), as well as exposure experiments (Fig. 4) these vials contained all-trans-retinal (ATR) supplemented standard fly food. 15µL of 200mM ATR were pipetted onto 10mL of food, and vials immediately wrapped with aluminum foil. ATR is a vitamin A derivate needed for activation of the Chrimson channel (Ullrich et al. 2013) and not produced in flies. A set of control flies received standard fly food only substituted with ethanol, serving as a negative control. Flies were kept in the foil wrapped vials for 48h before testing.

For dopamine depletion experiments (Fig. 5, Fig. 6), laboratory tissue paper (Kimtech Science, Type 7558) was cut to 10cmx10cm and compressed in a glass vial before adding 2mL of 10mg/mL 3IY dissolved in 5% sucrose solution. To prepare the 3IY solution, 2mL of sucrose solution were added to 20mg aliquots of 3IY powder. For optogenetic experiments, also 20µL of 200mM ATR were added to the 3IY solution. After vortexing for ~30 seconds, undissolved clumps of 3IY were broken down with a micro spatula and vortexed again. The mixture was pipetted onto the tissue paper using a disposable

transfer pipette with a large opening, ensuring to also transfer undissolved 3IY residues. Similar to the original ATR preparation, vials containing both ATR and 3IY were wrapped with aluminum foil and flies incubated for 48h before proceeding.

Open field monitoring of locomotion

WTB flies were used to investigate locomotion changes in response to 3IY. Flies' wings were cut to a third of their length under CO_2 anesthesia (https://dx.doi.org/10.17504/protocols.io.c7vzn5) and animals transferred to glass vials prepared as described above. After 48 hours, flies were transferred to empty vials and kept for 4h before testing.

Walking speed was measured using a locomotion assay described in (Colomb et al. 2012). In an open arena, a central platform is surrounded by water, which prevents flies from escaping. The arena is illuminated by white light and devoid of landmarks or other optical stimuli. To start the experiment, flies are placed into the middle of the platform. The tracking software is calibrated for the individual fly and tracking started. Over the time course of 15 minutes, the program records the flies' position, which is then used to calculate the median speed. Data was evaluated using BuriTrack and CeTrAn (RRID:SCR_006331)

BuriTrack data acquisition & evaluation software: https://sourceforge.net/projects/buridan/files/

Optogenetic paradigms

For optogenetic experiments, 20 female virgin NorpA;20xUAS-Chrimson flies were crossed to ten male flies of Gr28bd-Gal4;TrpA1-Gal4 (control) or TH-C-AD;TH-D-DBD. Male progeny of these flies was used for experiments described below.

JoyStick

A 0.6mm diameter monofilament line (WFT, Article #1D-C 972-060) was glued to center of the flies' dorsal thorax. The filament was aligned perpendicular to the animal's frontal plane and used to fixate the animal in the JoyStick apparatus. The JoyStick itself consists of a movable platform, a light guide that is fixated directly above the flies head and a photoelectric detector that measures the platform position. Micromanipulators were used to position the flies in the center of the platform, at a height

that allows them to manipulate position of the platform with their legs. Flies can control the stimulating light (yellow light, 595nm) by moving the platform to either the left or right, relative to their longitudinal body axis, following the principle of a light-switch (closed-loop stimulation). Stimulation parameters were adapted from previously described methods (Rohrsen et al. 2021). Light intensity was set to 800 lux, measured at the tip of the light guide using a calibrated lux meter (VOLTCRAFT, LX-1108). Light pulses were delivered at 20 Hz with a pulse width of 50ms without inter-cycle delay. Platform position signals were digitized and recorded using custom data acquisition software at a sampling rate of 20 Hz.

Acquisition software by Christian Rohrsen: https://github.com/chiser/Joystick-acquisition-software

The experimental protocol consists of two testing periods, separated by eight training periods. All periods were of similar length, which varied between 60 and 75 seconds. This variation was controlled by internal computations in the software and often varied between, but not within testing days. During training periods, one side activated the stimulating light while the other deactivated it. The light-activating side was consistent throughout training periods and changed randomly between individual experiments. Test periods measured flies' preference in the absence of optogenetic stimulation before and after training.

T-Maze

The T-Maze is a group choice assay originally designed to measure phototaxis (Gorostiza et al. 2016). The apparatus consists of a core, different cylindrical tubes and an 'elevator' (Fig. 1, https://dx.doi.org/10.17504/protocols.io.c8azsd). The elevator (dashed lines) can be aligned with the loading tube (1), choice tubes (3) or remain in a position where none of the tubes are accessible (2). The loading tube (length ≈ 10.5 cm; diameter ≈ 2.0 cm) is used to transfer flies from their incubation vial into the maze. Flies are introduced into the tube with a plastic funnel, and the tube is secured to the loading dock of the maze. Flies are left to acclimatize for 10 minutes, before being moved into the elevator, by gently tapping the maze onto a rubber foam pad. The elevator is immediately pushed down to the height where it is not connected to any of the tubes (position 2).

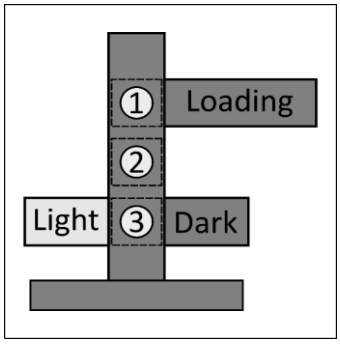

Figure 1 T-Maze apparatus

Flies have 30 seconds to recover from the tapping, during which the loading tube is emptied of potential remaining flies. After this recovery time, the elevator is pushed down all the way to the 'choice zone' (position 3) where flies can roam freely between elevator, light and dark tube. The light tube (length ≈ 6 cm; diameter ≈ 2.0 cm, same dimensions for dark) is transparent and illuminated by two opposing sets of red (660nm) or yellow (595nm) LEDs at intensities of 1600 lux and 1000 lux, respectively. Stimulation is controlled by a Grass Square Stimulator using the following parameters: Stimulation rate 20Hz, pulse width 10ms, cycle duration 9.9ms with 1ms inter-cycle delay. The dark tube is opaque and devoid of stimulation. Choice time was either one or ten minutes, after which the elevator is pulled up again and secured from moving down to the choice zone. Flies in the loading, light and dark tube are counted under CO_2.

Previous exposure - stimulation protocol

A stimulation period of five minutes was chosen to mimic exposure during ten-minute testing. For this, vials were unwrapped and placed between the yellow LEDs for the set time. Subsequent testing followed the same protocol as described above, although instead of the usual ten, flies had only five minutes to settle before testing, and flies were only tested in one-minute choice.

Data analysis

Preference indices for JoyStick experiments were calculated based on the number of data points during lights on (x) and off (y)

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

R script for JoyStick analysis: by Christian Rohrsen (adapted by Björn Brembs):
https://github.com/chiser/screen-analysis-for-yellow-Tmaze-and-Joystick/blob/master/Joystick_screen_analysis.R

Choice indices for the T-Maze were calculated by subtracting the flies in the dark tube (Dark) from flies in the light tube (Light) and dividing by the combine number of both.

$$CI = \frac{(Light - Dark)}{(Light + Dark)}$$

Statistical analysis

Our research groups applies the conservative alpha-value of 0.005 described in (Benjamin et al. 2018). However, as 3IY treatment was based on previously established protocols, the verification of the 3IY effect on locomotion used the standard α-value of 0.05.

Sample sizes for initial JoyStick and T-Maze (Fig. 3) were based on power calculations of Rohrsen et al. (2021) and a target of 30 was set for the experimental groups. For T-Maze experiments involving manipulations, power calculations were based on the effect observed in yellow light T-Maze (Fig. 3D), resulting in a target sample size of 30 to achieve 80% statistical power.

All statistical analysis was performed in RStudio (V. 2024.04.2). Normality and homogeneity of variances were tested using Shapiro-Wilk test and Levene's test. T-Maze choice indices were compared across time points or exposure conditions using either independent two-sample t-tests or Wilcoxon rank-sum tests, depending on results of normality tests. To assess whether optogenetic preference differed significantly from zero one-sample t-tests or Wilcoxon signed rank tests were applied. If not state otherwise, data is presented as mean±SD.

Results

TH-C-AD;TH-D-DBD target neurons in PPM1/2 and PPL1 cluster.

The driver line TH-C-AD;TH-D-DBD supposedly targets subpopulations of TH-D'-positive cells. I investigated the exact expression pattern with immunohistochemistry stainings of TH-C-AD;TH-D-DBD>UAS-mCD8::GFP female brains, staining for GFP and BRP to reveal target cells and neuropil. All of the analyzed brains showed strong GFP expression in at least eight cells (four per hemisphere) of the PPM1/2, and between two and three cells per hemisphere in the region of PPL1 cluster (Fig. 2A). Neurites of PPM1/2 cells project caudally and PPL1 cells have neurites extending to the midline (Court et al. 2023). These projections patterns fit the ones I observed for the stained cells. In addition, TH-C-AD;TH-D-DBD>UAS-mCD8::GFP flies showed strong expression in Kenyon cells and their neurites in the MB lobes (Fig. 2 A,B arrows), as well as multiple cells residing in the optic lobes

(Fig. 2B, ellipse). In each hemisphere, also an isolated cell in the suboesophageal zone was visible (Fig. 2B, arrowheads). Full image data is available on https://zenodo.org/records/18313591.

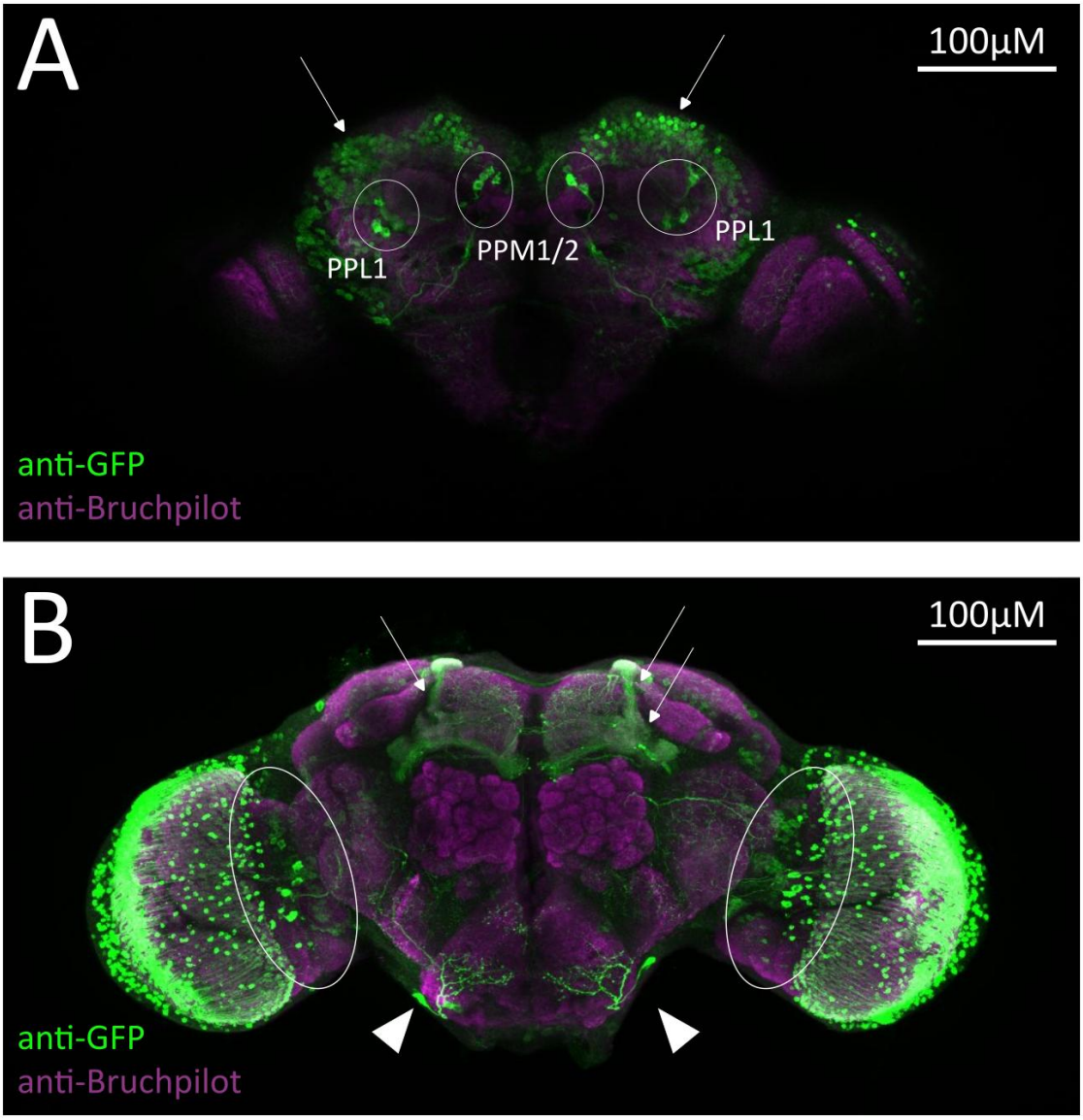

Figure 2 Immunohistochemistry staining of TH-C-AD;TH-D-DBD>mcD8::GFP

Images show expression pattern of TH-C-AD;TH-D-DBD. ***A:*** *Expression in PPL1 (lateral) and PPM2 (medial) clusters are shown by circles and cluster names. White arrows show expression in Kenyon cells.* ***B:*** *White arrows: MB-lobes comprised of KC axons. Arrowheads: Expression of one neuron per hemisphere in the suboesophageal zone. Circles: Multiple cells stained in the optic lobes. Green: anti-GFP, magenta: anti-Bruchpilot; Scalebar: 100µM*

TH-C-AD;TH-D-DBD flies consistently shift optogenetic preference across different experiments

Rohrsen et al. (2021) reported that TH-D'-Gal4 flies shift their preference in the JoyStick paradigm, from initial avoidance to later on approach. I verified the behavior using the more confined driver line TH-C-AD;TH-D-DBD.

I aimed to reproduce the behavioral shift for TH-C-AD;TH-D-DBD flies in the JoyStick paradigm with yellow-light stimulation. Across all training and test periods flies' behavior was characterized by high variability and no statistically significant preference; however, the medians for training periods 3-8 qualitatively followed the pattern of the previously observed preference shift from avoidance to approach (Fig. 3B).

In T-Maze experiments with yellow light stimulation, TH-C-AD;TH-D-DBD flies showed significant avoidance after one minute (Wilcoxon signed rank test, p = 0.0039, Fig. 3C); extending choice time to ten minutes resulted in a mild, not-significant approach of stimulation (One sample t-test, p = 0.0849). The mean choice index shifted significantly from -0.356 ± 0.54 to 0.128 ± 0.39 (Wilcoxon rank-sum test, $p = 4.33 \times 10^{-04}$). The use of red light failed to induce strong preferences (Fig. 3D). At both time points, choice indices were not significantly different from zero (1 min: Wilcoxon signed rank test, p = 0.102; 10 min: One sample t-test, p = 0.2815), and also the increase in mean choice indices from -0.201 ± 0.53 to 0.068 ± 0.34 was not significant (Wilcoxon rank-sum test, p = 0.029).

In all experiments, Gr28bd-Gal4;TrpA1-Gal4 served as a positive control. The sensation of heat, as induced by optogenetic activation of these neurons, is a stimulus generally avoided by flies (Simões et al. 2021). Flies showed robust avoidance in all experiments (Fig 3. A,C,D). In the JoyStick paradigm, the level of avoidance was consistent across training periods. Flies showed the lowest behavioral variability in training period 3, which then increased in later periods (Fig. 3A). The avoidance of flies in the T-Maze paradigm was strongest in red light condition (Fig. 3D) with mean choice indices of -0.911 ± 0.1 for one minute and -0.935 ± 0.11 in ten minute testing. Both effects were supported by statistical significance (1min: Wilcoxon signed rank test, $p = 4.73 \times 10^{-06}$; 10min: Wilcoxon signed rank test, $p = 1{,}43 \times 10^{-04}$). In yellow light, avoidance was weaker (1min = -0.552 ± 0.32; 10min = -0.676 ± 0.34) but remained significant (1min: Wilcoxon signed rank test, $p = 2.34 \times 10^{-05}$; 10min: Wilcoxon, signed rank

test, p = 4.62 x10^{-04}). Without ATR supplementation, control flies showed no preference, verifying the observed effects in positive controls and TH-C-AD;TH-D-DBD indeed do arise from stimulation of CsChrimson (Fig 3. C,D).

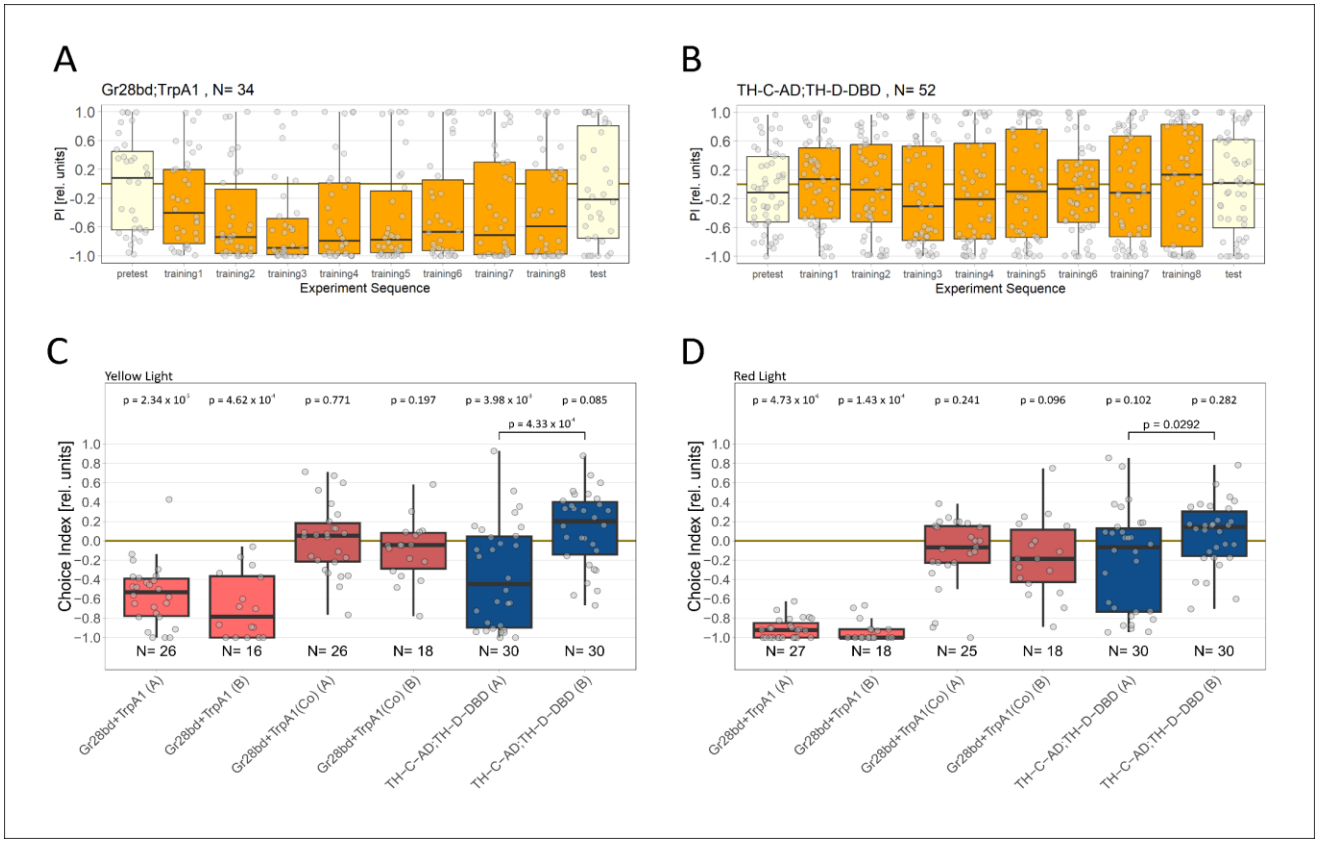

Figure 3 Behavioral shift in TH-C-AD;TH-D-DBD flies in T-Maze and JoyStick

A,B: *Preference indices (PI, y-axis) across different periods (x-axis).* ***A:*** *Control flies avoid stimulation during training periods with optogenetic stimulation (orange) but show no persisting avoidance in the test period ('test', yellow).* ***B:*** *TH-C-AD;TH-D-DBD flies show maximum avoidance in training period 3 and increasing PIs in subsequent trainings (orange). Sample sizes and group labels are shown above plots.*

C,D: *Choice indices for one or ten minutes in red and yellow light. Control flies (Gr28bd;TrpA1) show robust avoidance with, but not without ATR (suffix (Co)).* ***C:*** *Yellow light: TH-C-AD;TH-D-DBD flies avoid optogenetic stimulation in one-minute testing, and show weak approach at ten minutes.* ***D:*** *Red light: TH-C-AD;TH-D-DBD flies show weak avoidance at 1 and weak approach at 10 minutes. Suffixes (A) and (B) behind group labels indicate testing for 1 or 10 minutes, respectively. Color code: Pink: Gr28bd;TrpA1+ATR - Dark red: Gr28bd;TrpA1+EtOH – Blue: TH-C-AD;TH-D-DBD. p-values above groups indicate comparisons to zero; brackets and associated p-values indicate group comparisons. Sample sizes are shown below boxplots.*

Optogenetic stimulation before testing does not induce a behavioral shift

Extending choice time from one to ten minutes could, in principle, allow for more self-stimulation. This accumulation of neural signaling could cause the observed valence shift. To mimic increased neuronal signaling, I exposed flies to the stimulating light and subsequently tested for choice.

Gr28bd;TrpA1-Gal4 flies verify stimulation-induced avoidance, also after light exposure. Consistent with previous results, naïve control flies strongly avoided stimulation (mean CI = -0.846 ± 0.21, Wilcoxon signed rank test, p > 0.0001, Fig. 4A). A mean choice index of -0.440 ± 0.51 shows a weakened, but still significant avoidance after exposure (One-sample t-test, p = 0.0027).

Five minutes of consistent exposure to yellow light did not alter choice of TH-C-AD;TH-D-DBD flies in one-minute testing. With a mean choice index of -0.238 ± 0.52 for naïve and -0.289 ± 0.51 for exposed flies, the level of avoidance was almost identical between groups (Fig. 4, Wilcoxon rank-sum test, p = 0.657).

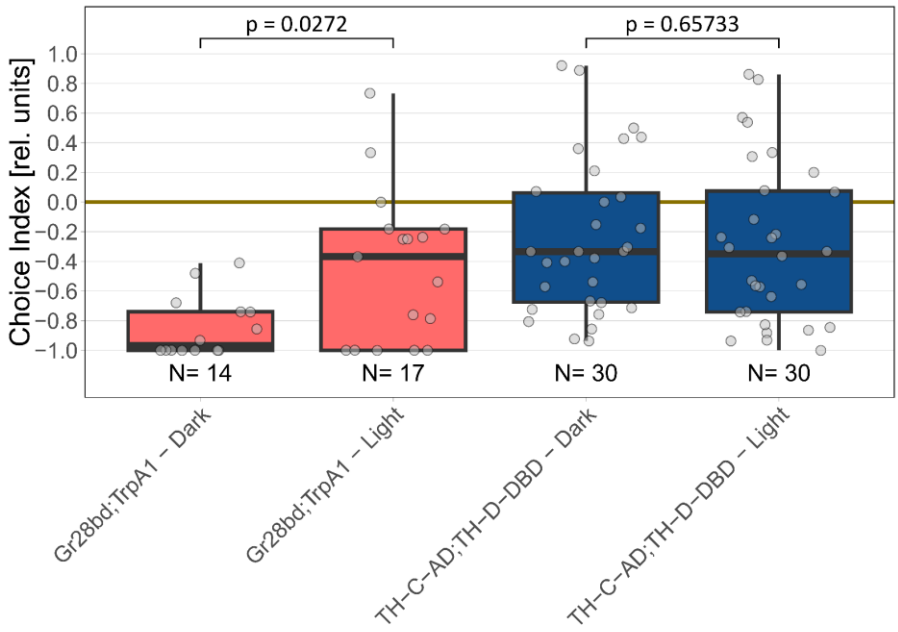

Figure 4 Exposure before testing does not induce a behavioral shift in TH-C-AD;TH-D-DBD flies

Under baseline conditions Gr28bd;TrpA1 avoid the illuminated arm of the T-Maze (suffix 'Dark'). Five minutes of exposure to the stimulating light reduces avoidance effect (suffix 'Light'). TH-C-AD;TH-D-DBD flies' behavior was independent of exposure condition. y-axis: Choice indices representing flies optogenetic preference in one-minute testing; x-axis: Phenotype – Exposure condition: 'Dark' = No exposure, 'Light' = 5-minute exposure before testing. Brackets and p-values indicate comparisons of groups. Sample sizes are displayed

Valence shift depends on dopamine

TH-D'-Gal4 and TH-C-AD;TH-D-DBD both target clusters of dopaminergic neurons. However, other neurotransmitters could, in principle, contribute to the observed valence shift. To test the specific role of dopamine, I repeated T-Maze experiments after impairing dopamine production by administrating 3-Iodo-L-Tyrosine (3IY), an inhibitor of Tyrosine hydroxylase (TH, Fig. 5A).

Open-field monitoring of WTB flies, treated with 3IY, revealed a reduced walking speed in flies. The Wilcoxon rank-sum test showed a significant difference in median speed between treated and untreated WTB flies ($p = 0.0067$). Movement speed in the treated group was typically lower than in controls group with an estimated difference of 3.7mm/s (Fig 6B). Because T-Maze experiments required supplementation with both ATR and 3IY, it is important to account for potential interactions between the two compounds. Combined administration of both compounds induced a visible color change from yellow to orange (Fig. 5C). To examine whether this change in color reflect a loss of function, I treated WTB flies with both compounds, and found no difference in walking speed between the group treated with both compounds and the group treated only with the dopamine inhibitor (Two-sample t-test, $p = 0.438$). Likewise, 3IY does not interfere with ATR, as positive control groups avoided optogenetic stimulation with and without 3IY supplementation (Fig. 6).

Impairing dopamine production in TH-C-AD;TH-D-DBD flies abolished the effects of optogenetic stimulation at both time points (Fig. 5E). With a mean choice index of -0.162 ± 0.54 flies treated with 3IY showed no preference in one minute testing (Wilcoxon rank sum test, $p = 0.2501$). For the extended choice time of ten minutes, the mean choice was -0.123 ± 0.33, which was also not significantly different from zero (One Sample T-Test, $p = 0.1113$). Choice indices were not different between testing times (Wilcoxon rank sum test, $p = 0.9587$). Consistent with previous experiments, mean choice indices of untreated flies shifted from -0.262 ± 0.48 to 0.0734 ± 0.29 (Two Sample t-test, $p = 0.0022$).

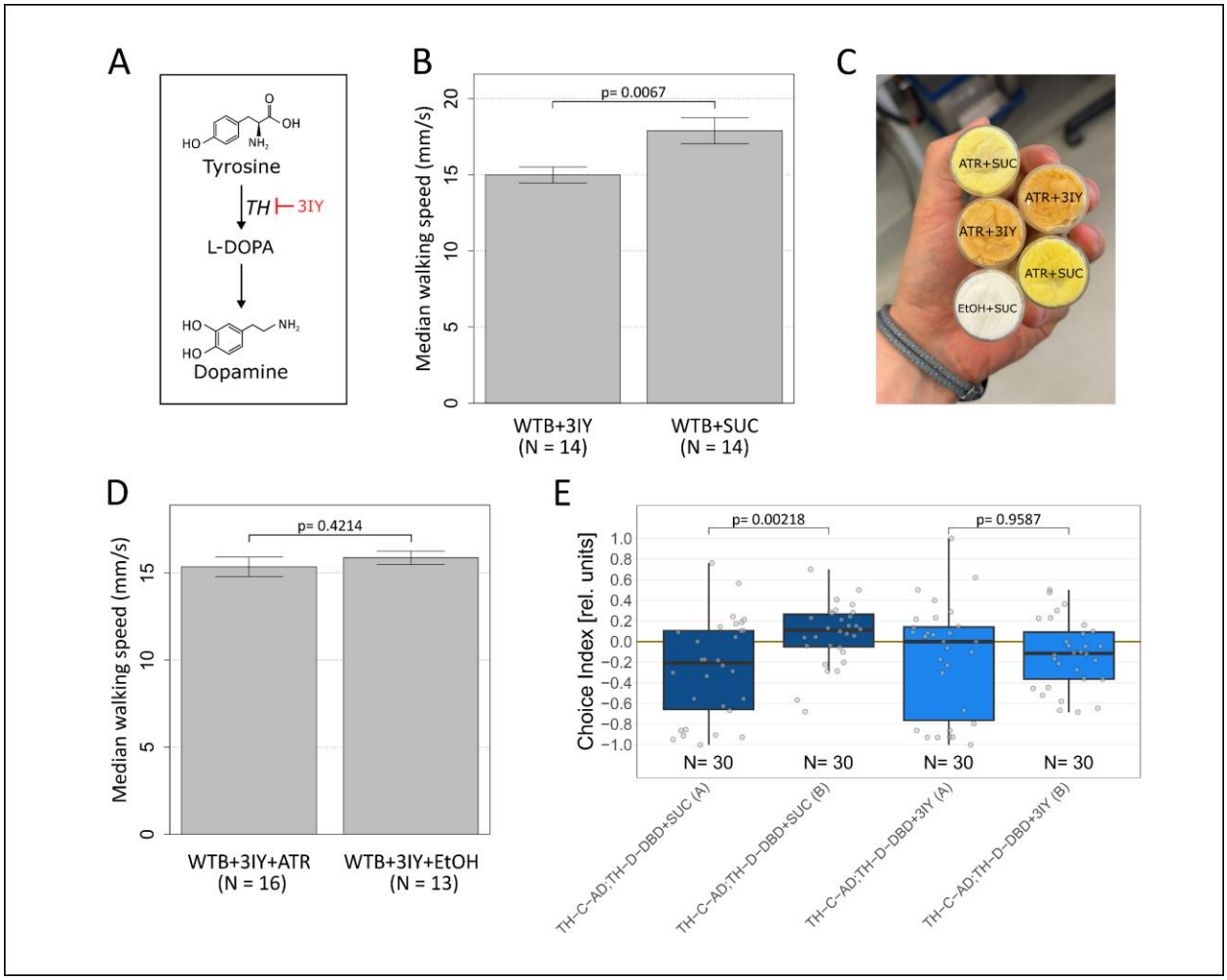

Figure 5 Dopamine depletion experiments

***A:** Schematic of dopamine synthesis via L-DOPA and inhibition of TH via 3-Iodo-L-Tyrosine (3IY). **B-D:** Open-flied locomotion assay with WTB flies. **B:** 3IY treatment reduces median walking speed in WTB flies compared to controls. **C:** Representative image of substrate preparations. Combined application of 3IY and ATR induces color change from yellow to orange. **D:** Combined application of 3IY and ATR does not significantly alter median walking speed compared to flies treated with 3IY and EtOH. The y-axis In **B** and **D** shows median speed (mm/s) and x-axis shows treatment of WTB flies. Brackets and p-values denote statistical comparisons. Sample sizes are indicated blow group labels. **E:** Dopamine depletion alters choice behavior in TH-C-AD;TH-D-DBD flies. In untreated flies (+SUC) behavior shifts from one (A) to ten minutes (B). Impairing dopaminergic signaling with 3IY abolishes the preference shift. Suffixes (A) and (B) denote 1-min and 10-min testing, respectively. Brackets and p-values indicate group comparisons. Sample sizes are shown below boxplots.*

Heat-“punishment” signaling is independent on dopamine

To verify ATR action in the presence of 3IY and simultaneously test whether heat avoidance is affected by dopamine depletion, I treated Gr28bd-Gal4;TrpA1-Gal4 flies with both compounds and tested their optogenetic preference in all three assays.

Despite dopamine inhibition, flies exhibited robust avoidance in the JoyStick paradigm. I observed constantly negative preference indices across training trials and a negative median PI during the test phase (Fig. 6A). As also observed in flies with unimpaired dopamine synthesis, choice exhibited the lowest variability in training 3 and became more variable in later training periods.

In red light T-Maze, 3IY treated flies showed avoidance in both 1 min (Wilcoxon signed-rank test, p = 0.0036) and 10 minute testing (Wilcoxon signed-rank test, p = 0.0014, Fig. 6B). Mean choice indices of -0.973 ± 0.06 at one minute and -0.946 ± 0.08 at ten minutes indicate strong avoidance. In the yellow-light condition, avoidance levels were slightly lower with a mean choice index of 0.840 ± 0.17 for one minute and -0.794 ± 0.29 for ten minute testing (Fig. 6C). The low sample size achieved in the yellow light condition most likely accounts for the increased variability and lack of significance (Wilcoxon signed-rank tests: $p_{1min} = 0.125$, $p_{10min} = 0.0355$).

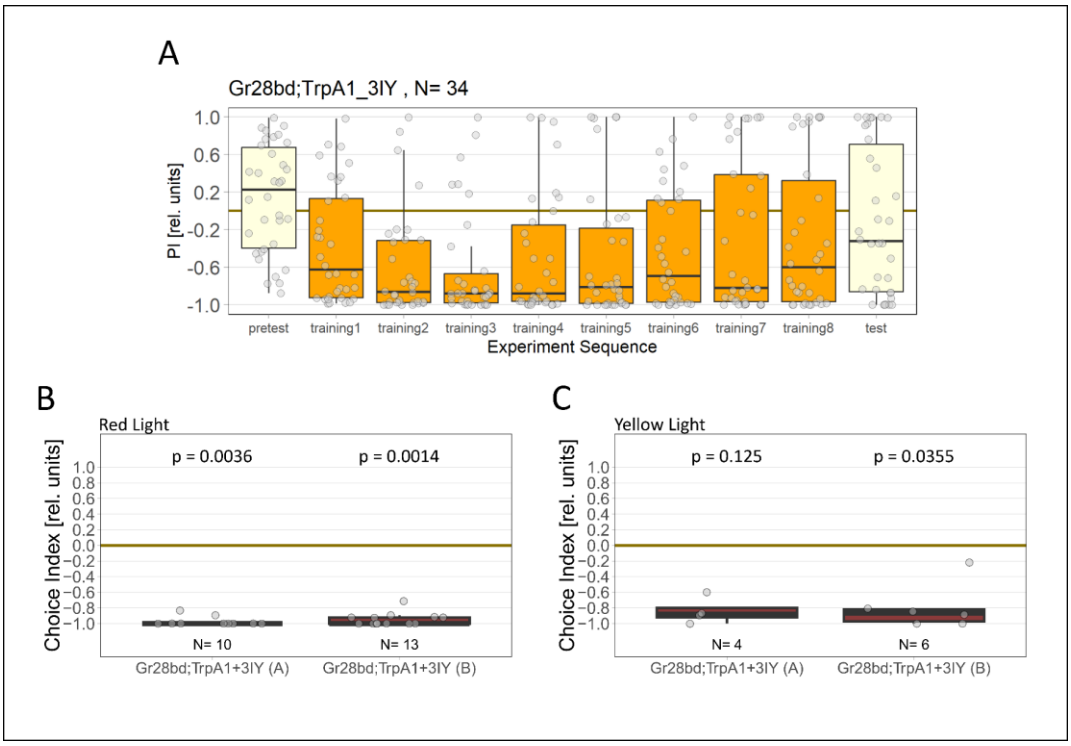

Figure 6 Heat avoidance of Gr28bd-Gal4;TrpA1-Gal4 flies is independent of dopamine

***A:** Preference Indices (PIs, y-axis) of dopamine depleted Gr28bd,TrpA1 flies across training and test periods. In periods with optogenetic stimulation (orange), flies avoid stimulation. In the test without optogenetic stimulation (yellow) flies exhibit decreased avoidance but negative median PIs. Experiment sequence is shown on the x-axis; Sample size is shown above plot **B, C:** Dopamine depleted control flies avoid the illuminated arm of the T-Maze in both conditions. Suffixes (A) and (B) of group labels denote 1-min and 10-min testing, respectively. Respective LED color is named above plot. p-values indicate comparisons to zero. Sample sizes are shown below boxplots.*

Discussion

TH-C-AD;TH-D-DBD phenocopies TH-D' effects

Rohrsen et al. (2021) identified TH-D'-Gal4 as one of the few lines to produce robust behavior across different self-stimulation paradigms. In these experiments, the valence associated with activation of TH-D'-Gal4-positive neurons shifted over time, reflected in a behavioral transition from stimulation avoidance to approach. The present study shows that targeting a subpopulation of TH-D'-Gal4 neurons using the split-GAL4 line TH-C-AD;TH-D-DBD can reproduce aspects of this behavioral shift.

A previous JoyStick study in our lab (Guyton 2023) showed that in the JoyStick paradigm, TH-C-AD;TH-D-DBD flies behave qualitatively similar to TH-D'-Gal4 flies, although with a reduced effect size. While I was unable to fully reproduce these findings, elements of the behavioral trajectory were still apparent (Fig. 3B). Compared to the results reported by Guyton (2023), my data was characterized by higher variability, which may have compromised a reliable detection of the shift in preference. Deviations, for example the lack of initial avoidance, likely can be attributed to technical mistakes and suboptimal fly placement on the platform. For this type of experiment, it is crucial to position the fly low enough to maintain good control over the platform, but not so low that it becomes constrained. I used the reliable avoidance of Gr28bd-Gal4;TrpA1-Gal4 flies to confirm that the positioning was sufficient to allow flies to manipulate the platform. However, as also indicated by T-Maze results (Fig. 3, Fig. 4, Fig. 7) activation of these heat-sensing neurons has a strong effect on optogenetic preference. This strong drive to avoid heat-sensation may allow flies to overcome suboptimal positioning. Because the observed effects in TH-C-AD;TH-D-DBD are comparatively small, this choice of reference was not ideal. Furthermore, the observation that in control flies with and without 3IY supplementation, behavioral variability increased in the late stages of the experiments (Fig. 3A, Fig. 6A) supports this

interpretation. After several minutes of avoiding the heat stimulus, flies may become too exhausted to compensate suboptimal positioning. However, because the general behavioral patterns of TH-C-AD;TH-D-DBD flies align with those reported by both Guyton and Rohrsen et al., suboptimal placement likely hindered but did not eliminate the behavioral expression of preference.

Dopaminergic systems are known to respond to social interactions in vertebrates (Torquet et al. 2018; Antunes et al. 2022; Padilla-Coreano and Martínez-Rivera 2025) and invertebrates (Wada-Katsumata et al. 2011; Fernandez et al. 2017). Therefore, it was crucial to examine behavior in varied social and sensory contexts. Using the optogenetic T-Maze with different stimulation wavelengths revealed that the behavioral response of flies was influenced by the wavelength of the stimulating light, but qualitatively followed the previously described shift (Fig. 3, Fig. 5E, Fig. S2). The early-on avoidance effect was notably weaker in red light, which could reflect different levels of CsChrimson activation. Examination of the activation spectrum of CsChrimson shows that yellow light should induce 4-fold stronger activation of the channel than red light (Klapoetke et al. 2014). Although red light intensity was set to 1600 Lux and therefore approximately 1.5x as high as yellow light (1000 Lux), a different activation strength could still account for these initial differences. However, in the baseline experiments Gr28bd-Gal4;TrpA1-Gal4 flies showed stronger avoidance for red than yellow light (Fig. 3). Because these observations contradict each other, they suggest a more complex interaction of the wavelength of the stimulating light and the resulting neuronal activation. Longer wavelengths, such as red light, generally penetrate tissue more effectively than shorter wavelengths (Mustafa and Jaafar 2013). Studies in *Drosophila* support this, showing that cuticle penetration increases with wavelength (Lin et al. 2015). Consequentially, the cellular localization of target neurons might affect the activation evoked by the stimulating light. Unfortunately, unresolved issues with Gr28bdGal4;TrpA1-Gal4 flies (discussed later) make comparisons of control flies between the initial T-Maze screen and 3IY experiments, and therefore clear conclusions concerning wavelength effects impossible. Although determining whether neuronal location systematically affects optogenetic efficacy was not the focus of this project, these observations highlight potential complications in the design of optogenetic stimulation paradigms, as activation of neuronal targets could differ between control and

experimental groups. However, despite potential wavelength-depend differences in activation strength, TH-C-AD;TH-D-DBD flies show a similar behavioral shift in JoyStick and T-Maze paradigms.

In both T-Maze conditions, flies' behavior in one minute testing was more variable than at ten minutes. To initiate the experiment, flies are moved into the choice zone by pushing down the elevator, which can lead to startle-induced arousal (Lebestky et al. 2009) and might bias initial decision making (Wemm and Wulfert 2017). In addition, the choice period begins the moment flies encounter the choice zone for the first time. At this stage, the primary motivation may be to escape the crowded elevator rather than to make a choice based on the experienced stimulation. Therefore, during the first minute of testing, flies that end up in the illuminated arm do not necessarily have approached activation. However, even with these limitations, flies express initial aversive behavior across different experiments, demonstrating the robust initial negative valence signaled by TH-C-AD;TH-D-DBD-positive neurons.

Although T-Maze and JoyStick nominally measure the same behavior, the way in which choices are quantified differs substantially. In the JoyStick results, each individual data point represents the choice of one fly, while data points in the T-Maze represent the mean choice of a group of flies. Therefore, positive PIs in the JoyStick represent flies that chose to activate the light *relatively* longer than keeping it off. In contrast, a positive choice index in the T-Maze reflects that, at this moment, overall *more flies* made the decision to approach stimulation than avoided it. One possible interpretation is that the population consists of two subgroups: one that consists of flies that learned to approach the light and would do so constantly, while the other represents flies that did not learn and kept avoiding. If this were the case, JoyStick data would be expected to show a bimodal distribution. Although the present data shows some tendency towards such clustering (Fig. 3D), this pattern was not observed in (Guyton 2023) or (Rohrsen et al. 2021), therefore the aforementioned inaccuracies in the experimental preparation likely skewed individual flies' preference to extreme values. Instead of having two differently behaving subgroups, the data indicates that in the JoyStick flies move the platform to both sides but gradually prefer the illuminated one. Accordingly, positive T-Maze choice indices do not arise from two distinct groups, but from flies moving between compartments and staying longer in the illuminated one. Taken together these observations suggest that the valence represented by TH-C-

AD;TH-D-DBD-positive neurons does not shift in absolute terms, such that it is initially completely aversive and becomes fully attractive over time, but instead reflect a probabilistic bias. Flies push the platform *relatively* longer to the light-activating side and spend *relatively* more time in the light compartment after prolonged testing. Nevertheless, this highlights that the overall valence associated with TH-C-AD;TH-D-DBD activation does shift from negative to positive.

From a statistical perspective, red light T-Maze experiments were not supported by significant p-values and the effect of approach did not reach a significant level in any of the experiments. Because there are differences in the exact protocols for experiments (wavelength, food substrate), the data from these experiments cannot be formally pooled. However, these experiments are nominally repetitions and pooling would most likely lead to robust and statistically significant results. Additionally, it should be noted that our laboratory applies a more restrictive significance threshold ($\alpha = 0.005$), whereas other studies using the more common threshold of $\alpha = 0.05$ might interpret effects as statistically significant.

This overcoming of not only technical limitations, but also higher statistical threshold, highlights that, even though the observed effects are relatively small, flexible valence coding by TH-C-AD;TH-D-DBD-positive neurons is a robust phenomenon.

There are two main hypotheses that could possibly explain the observed valence shift:

1) Individual components of approach and avoidance are mediated by distinct subsets of neurons, which are all targeted by our driver. Consequentially, the balance between these subsets dictates the animals' behavior, a principle proposed for *Drosophila* action selection (Yamazaki et al. 2018). Initially, it favors avoidance and later approach. This balance-model prompts three interactions for our observed behavior: (a) Downregulation of avoidance-promoting signals while approach remains at baseline; (b) strong upregulation of approach while avoidance stays at baseline, ultimately approach overcomes avoidance; or (c) simultaneous down-regulation of avoidance and up-regulation of approach.

2) If there are no distinct, separate subsets that mediate approach and avoidance behavior, respectively, this suggests that the very same neuronal populations can drive behaviors of opposite

valence. This scenario would require complex reweighting of downstream circuits or parallel output pathways.

Manipulations experiments are a promising approach to unravel which of these hypotheses is the most likely to underlie the observed behavior. Because of greater effect sizes in T-Maze, I focused on this paradigm for the manipulation experiments.

Behavioral shift reflects learning, not changes in synaptic activity due to increased neuronal stimulation

As a prolonged testing time is likely associated with more exposure to light and thereby activation of target neurons, it is possible that the shift reflects a behavioral change in response to accumulating signaling. This mechanism would be a straightforward way to explain the idea that with distinct neuronal populations mediating positive and negative valence, thus approach and avoidance behavior separately, one of them is affected by prolonged signaling while the other remains at baseline. Fading avoidance could arise from a simple depression of the signaling mechanism that drives it, e.g. through depletion of dopamine pools (Hypothesis 1a). An increase in approach prompts a threshold model in which approach promoting neurons need to be sufficiently activated (Hypothesis 1b); Prolonged activation could also induce these individual alterations in both neuronal populations in parallel (Hypothesis 1c). Because all these hypotheses are based on the idea of excess neuronal activation, I decided to expose flies to the stimulating light before testing.

There are multiple ways to mimic the stimulation flies experience during experiments. The simplest two are to stimulate either for four or ten minutes. Stimulation for ten minutes would align with the length of the baseline experiments, and is a justifiable approach. Four minutes, on the other hand, is more sophisticated and represents the sum of stimulation better. Over the course of the JoyStick experiments, mean preference indices average out to approximately zero, meaning LEDs are activated for roughly half of the training duration (Fig. 3) (Rohrsen et al. 2021; Guyton 2023) resulting in a total on-time of four minutes. As a third option, exposure could mimic the *pattern of stimulation* flies actually experience. This method should elicit a more ‘organic’ stimulation, but requires calculation of the mean activation pattern of JoyStick experiments and playing this back to flies before testing, a protocol I was unable to establish within the time limit of this project. Also, evidence for inter-fly

variability (Rihani and Sachse 2022; Okuno et al. 2025) questions whether this pattern would be the same for each individual fly. The behavioral shift could, in theory, also reflect a delayed change in signaling that is triggered by a single instance of activation, as a single burst of activation can induce long lasting changes in downstream targets (Remy and Spruston 2007). Regardless of how I mimic the increased stimulation, flies will have a short recovery period after they are introduced into the Maze. The combined length of a five-minute exposure and recovery time does represent the temporal endpoint of the experiments and should be sufficient to also capture a delayed effect. As this is also close to mean stimulation time of four minutes, I chose five minutes to mimic overall exposure and capture potential effects of a delayed signaling cascade.

Strikingly, exposure before testing did not affect flies' choice during one-minute choice (Fig. 4), ruling out that the valence shift is merely due to prolonged activation of the target neurons or delayed signaling. Specifically, if a depletion of DA pools would underlie the behavioral shift, the strong activation of these neurons should have affected choice.

The fact that TH-C-AD;TH-D-DBD flies behavior does not change after uncontrollable exposure implies that a correlation between the flies' behavior and the stimulus is necessary for a change in valence. Only when the stimulation is elicited by the flies' own actions and can be avoided, the aversive aspect weakens and the approach behavior emerges. This distinction of, whether own actions affect the outcome or not, is what separates operant from classical learning. Because both learning forms can employ different neuronal pathways (Wolf et al. 1998), it is possible that the shift between the opposing behaviors may also depend on a distinct operant circuit. Moreover, activity of DANs depends on context and internal state (Siju et al. 2020) and endogenous neural activity can affect stimulus perception as well as neural tuning of specific circuits (Li et al. 2020b). The ability to control the stimulus, rather than being simply exposed to it, may therefore affect dopaminergic signaling in a circuit-specific manner. Finally, if the opposite behaviors require control and a behavior-stimulus association, it is clear why classical conditioning studies have not reported similar observations.

Chrimson-driven ion transport relies on the isomerization of ATR in response to light (Oda et al. 2018) and exposure did weaken the avoidance in control flies. Given that stimulation of these neurons is strongly aversive for flies (Choice Indices close to -1 in unexposed flies), this weakened avoidance is

likely caused by a behavioral change rather than a mechanistic failure of the channel. Animals can exhibit 'learned helplessness', a protective mechanism where they stop evading an aversive stimulus, if they cannot control it (Silveira and Joca 2023) and this has also has been shown in flies (Yang et al. 2013). After five minutes of uncontrollable heat sensation, flies could be either too exhausted to properly express preference or have learned that there is no escaping the stimulation. Moreover, light-exposed TH-C-AD;TH-D-DBD flies showed similar preference behavior as the non-exposed group, therefore altered optogenetic efficacy due to exposure is unlikely.

Roles of neurotransmitter signaling in the valence shift

TH-C-AD;TH-D-DBD and TH-D'-Gal4 are both driver lines that are derived from the TH promotor. Consequentially, they drive expression in different sets of dopaminergic neurons (Liu et al. 2012b; Xie et al. 2018). Importantly, the behavioral consequences of activating neurons classified as dopaminergic are not necessarily mediated exclusively by DA. A study using both operant self-stimulation and classical learning showed that the reinforcing effect of PAM DAN activation during olfactory conditioning only partially depends on DA, with octopamine and glutamate contributing substantially to the behavioral outcome (Mohammad et al. 2024). These findings demonstrate that the valence resulting from DAN activation can arise from multiple transmitter systems and those DA-independent components may mask or shape the observed behavior.

Given this complexity, a critical step in dissecting the observed behavior in TH-C-AD;TH-D-DBD flies was to determine whether—and to what extent—it depends on dopaminergic signaling. For example, if DA only signals negative valence but does not instruct the subsequent shift, depleted flies should exhibit an (increasingly) positive preference, already from minute one. This would suggest that in untreated flies valence is represented in the signaling of multiple neurotransmitters, which ultimately outcompete DA-driven negative valence. On the other hand, if impairing DA signaling generally abolishes preference, it would strengthen the idea of DA being the main signal for valuation processes. This would further support the notion that the valence mediated by DA is not static but flexible within the same neurons. Establishing DA dependence (or independence) therefore provides necessary insight to untangle the mechanisms of valence signaling.

Dopaminergic signaling can be impaired by substituting fly food with 3IY (Neckameyer 1996). Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis and catalyzes the conversion of tyrosine to L-DOPA, the immediate precursor of dopamine. 3IY competitively blocks TH and therefore impairs dopamine production (Richelson 1976). As locomotion is strongly affected by dopaminergic signaling, the reduced locomotion speed in WTB flies verified the action of the compound (Fig. 5B) (Wicker-Thomas and Hamann 2008).

In TH-C-AD;TH-D-DBD flies, 3IY abolished the preference shift (Fig. 5E). Although DA depleted flies showed no early avoidance, there are a few data points that drag the distribution of CIs to negative values. These may represent individual experiments where depletion was insufficient. It took several trials to establish a protocol that was able to reliably reduce walking speed and applicable for the T-maze preparation protocol. 3IY is a powdered substance and only dissolved in the sucrose solution when it was vortexed for an hour. However, it rapidly sedimented after mixing, and therefore different fractions from a stock solution would likely have drastically different concentrations. To resolve this issue, I shortly vortexed small batches of the solution, and pipetted them onto the prepared substrate, including also undissolved clumps of powder. This ensured that at least the total amount of 3IY was consistent within each prepared vial. Still, because flies might not feed on the dry powder, the magnitude by which DA levels were decreased could have differed between preparations, potentially explaining the negative choice indices. Additionally, it is important to note that 3IY does not completely abolish DA signaling, it inhibits DA production, therefore flies do not completely lack DA, but exhibit decreased levels (Neckameyer 1996). The residual DA pool could potentially mitigate the effects of DA depletion in the early stages of the experiment, adding another explanation why I observed a mild tendency towards avoidance. However, as there is strongly impaired replenishment of releasable pools, 3IY has a greater effect during prolonged testing (Xiao and Venton 2015). Consequentially, the effect of depletion is stronger for the 10-minute group. Without DA impairment, positive mean choice was robustly observed across all experiments at ten minutes (Fig. 3, Fig. 5), but after treatment with 3IY, flies showed negative mean choice indices, in both light conditions (Fig. 5, Fig. S2). Considering that depletion might been insufficient in some preparations, it implies that without DA, not only is the approach behavior abolished, flies might even avoid stimulation after

prolonged testing. Because depleted flies do not avoid the light at one-minute this suggests that the negative medians choice indices at ten minutes represent a separate, avoidance-mediating circuit that is independent of DA. This would also fit the observations of Mohammad et al. (2024) that DANs can rely on different co-transmitters, and reflect a major claim of the study that the same neurons can signal opposing valence. However, with the achieved sample size, the individual aspects of avoidance during early and approach in the late phase of the experiment, and the involvement of other signals cannot be reliably addressed. I based the target sample size on the magnitude of the time-dependent shift in choice behavior, which is greater than the individual effects, therefore a sample size of 30 was too low to detect small effects. Nevertheless, the results make it evident that DA is indeed the essential for the observed valence shift, supporting the populations of DANs targeted by both TH-C-AD;TH-D-DBD and TH D' as the primary candidates underlying this behavior.

Neuronal substrates of the valence shift

I chose the driver line TH-C-AD;TH-D-DBD based on the reported expression pattern. In their study, Xie et al. reported a restricted expression in four neurons that are part of the PPM2 cluster (Xie et al. 2018), one of the main clusters targeted by TH-D'-Gal4 (Liu et al. 2012b). As behavior of TH-D'-Gal4 and TH-C-AD;TH-D-DBD flies was comparable across different optogenetic self-stimulation screens (Fig. 3)(Guyton 2023; Rohrsen et al. 2021), these PPM2 neurons might cause the observed effects. Surprisingly, the expression pattern observed in my immunostainings of TH-C-AD;TH-D-DBD>UAS-mCD8::GFP flies' brain differed from the one originally described. There was additionally expression of GFP in the optic lobes, suboesophageal zone, populations of Kenyon cells, and importantly neurons that are potentially part of the PPL1 cluster (Fig 3). Based on these differences, I examined the data associated with the literature that originally described the driver. In contrast to what was reported, I found a similar expression pattern as in my stainings. Furthermore, in the publication, the authors label the genotype of their driver as 'TH-C-AD;TH-D-DBD', whereas in the available data it is listed as 'TH-D-AD;TH-C-DBD'. Although grave differences in the expression pattern between the two genotypes are unlikely, this discrepancy, together with the additional, unreported expression, creates uncertainty. Importantly, the authors' original images show cells that may also well correspond to PPL1 neurons. A particular neuron that resides in this cluster has been

reported to promote arousal (Liu et al. 2012b), which would fit the observation of increased activity in TH-C-AD;TH-D-DBD neurons (Fig. S3). This particular neuron projects to the fan-shaped body and is targeted by TH-D'-Gal4. Because I could not identify such projections in my immunostainings, it is unlikely that the TH-C-AD;TH-D-DBD driver targets this specific neuron. However, TH-D'-Gal4 also expresses in other PPL1 neurons. Because of this overlapping expression, the observed behavior cannot be isolated to only PPM2 neurons, as I initially assumed. Because TH-D'-Gal4 does not label Kenyon cells, optic lobe neurons, or suboesophageal neurons, and nevertheless produces the same behavioral phenotype, these populations are unlikely to contribute to the observed behavioral shift. Therefore, using TH-C-AD;TH-D-DBD reduces the candidates for the TH-D'-Gal4 mediated shift to PPM2 and PPL1 neurons.

The initial targets of this study, PPM2 neurons, have been weakly characterized in the literature. The study which originally described the TH-C-AD;TH-D-DBD driver reports effects on social spacing (Xie et al. 2018). The authors claim that activation of a subset of PPM2 neurons can reverse the effects of social isolation during development. However, because of the aforementioned issues with the driver line, this has to be interpreted with caution. Marquis and Wilson (2022) report that PPM2 DANs are activated by locomotion, and activity in these neurons encodes changes in movement speed. Aspects of locomotion and social context differ strongly between T-Maze and JoyStick. Consequentially, PPM2 neuron activation may differ as flies walk across tubes or detect conspecifics, which in turn could affect signaling. However, because there were no substantial difference in fly behavior between my experiments, these particular aspects of PPM2 signaling are unlikely to play a role in preference. Together with (Rohrsen et al. 2021) the present study and marks the first to report a role of PPM2 DANs in valuation processes.

PPL1 neurons, on the other hand, have been subject to numerous studies that investigate valuation signals. At least five different types of PPL1 DANs have been discovered in the adult fly protocerebrum, innervating the MB lobes, as well as heel and distal peduncle (Li et al. 2020a). Many of these target locations also respond to electric shock and odors (Mao and Davis 2009; McCurdy et al. 2021). In conclusion, it is not surprising that activation of these neurons can substitute for aversive teaching signals in classical conditioning (Aso et al. 2012; Villar et al. 2022). It would be logical to assume that

the avoidance component of the behavior in the present study might depend on PPL1 signaling. However, one of the main findings of (Rohrsen et al. 2021) is that effects in associative learning differ from operant settings. Also, apart from a timing-dependent valence reversal (König et al. 2018), there are no reports that the same PPL1 neurons can signal opposite valence in a time-dependent manner. Because the reflexive effect of isolated activation of PPL1 neurons has not been studied, it is impossible to attribute the avoidance effect to PPL1 neurons.

The shared expression of TH-D'-Gal4 and TH-C-AD;TH-D-DBD promotes that concurrent activation of PPL1 and PPM2 neurons could be required for the observed behavioral shift. This implies a convergence onto shared downstream circuits that can drive behavior, for example mushroom body output neurons (MBONs). Via synaptic modulation by DANs, these output neurons regulate approach and avoidance of an odor in classical olfactory conditioning (Owald et al. 2015). Aso et al. provided a comprehensive anatomical and functional characterization of MBONs and identified 21 distinct types with defined valence associations (Aso et al. 2014b). I analyzed the connectivity of TH-C-AD;TH-D-DBD-positive neurons and found MBON20 as a shared downstream target of both PPM2 and PPL1 neurons. MBON20 itself was not directly manipulated in the original optogenetic valence screen by Aso and colleagues (Aso et al. 2014b). However, a recently published study has tested multiple MBON20 driver lines in spatial preference, but could not find evidence for valence guided by activation (Rubin and Aso 2024), making it unlikely that this MBON is the main driver of our observed behavior. Still, this does not exclude other MBON derived effects in the valence reversal. Although they prominently guide behavioral output by synapsing onto descending motor pathways, many MBONs also feedback recurrent feedback loops with other DANs (Li et al. 2020a) which may alter the input balance. Consequentially, the opposite behavior may spring from complex circuit changes in DAN-MBONs signaling.

Extending the scope beyond populations

A possible mechanism how these complex changes manifest can be found in dopamine receptor subtypes and signaling. Four types of DA receptors have been characterized in *Drosophila*: Dop1R1, Dop1R2, Dop2R and DopEcR (for a review see Karam et al. 2020). Although D1-like receptors Dop1R1 and Dop1R2 are characterized based on their common ability to increase cAMP, they also exert

different functions and Dop1R2 receptors have been shown to also elevate intracellular Ca^{2+} (Feng et al. 1996). Because Ca^{2+} is central to the regulation of neurotransmitter release and plasticity events, these fluctuations may affect signaling of DANs and alter behavior (Cavazzini et al. 2005; Neher and Sakaba 2008; Südhof 2012). In addition, both types of D1-like receptors are attributed opposing roles in the formation of memory and active forgetting (Berry et al. 2012). Furthermore, Dop1R2 activation can have opposing, time-dependent effects that occur on a scale from 3-30 minutes (Han et al. 1996; Reale et al. 1997) fitting the coarse time frame of the observed valence shift. D2-like receptors Dop2R (D2R), on the other hand, decrease cAMP levels, and have an inhibitory role. They function as both auto- and postsynaptic receptors in mammals and flies (Vickrey and Venton 2011; Ford 2014; Qi and Lee 2014) and can regulate the activity of DANs via auto-inhibition (Ford 2014). This inhibitory role of Dop2R receptors could play a role in the behavioral shift. With accumulating neural signal, inhibition of the target neurons could reach a threshold that silences downstream activity and therefore alters behavior. Although the observations of the exposure experiments make such a straightforward interaction unlikely, it illustrates how the functional heterogeneity of the dopaminergic system and involved receptors can shape opposing behaviors.

DA has also been shown to signal the discrepancy of predicted and experiences rewards in mammals (Schultz 2016) and similar mechanisms are proposed for *Drosophila* (Riemensperger et al. 2005; Bennett et al. 2021). Some TH-C-AD;TH-D-DBD-positive neurons could act as such punishment-predictors. However, because the prediction is not further reinforced by an actual occurring punishment, this could lead to a ‘relief reward’ which might explain how the initial aversive stimulus can attain an attractive aspect (Leknes et al. 2011; Willems et al. 2025)

DA depletion experiments suggest that the scope has to be extended beyond dopaminergic signaling. Even if the main driver of the behavioral shift seems to be DA, the fact that other neurotransmitters could potentially drive opposite behaviors implies that they might also shape behavior in the presence of DA. There is accumulating evidence that behavioral effects of DAN activation also rely on octopamine, glutamate and GABA (Mohammad et al. 2024; Yamazaki et al. 2023). Specifically, PPL1 neurons have been found to co-transmit glutamate and GABA, and GABAergic signaling in these neurons can shape the calcium oscillations of DANs, ultimately affecting function (Pavlowsky et al.

2018; Yamazaki et al. 2023). Whether similar aspects affect PPM2 neurons, has not been yet investigated.

Although the mentioned studies do not allow the formation of a defined hypothesis how TH-C-AD;TH-D-DBD-positive neurons can induce opposing behaviors, they highlight that circuit- and cell-specific effects, different receptors types as well as different transmitter systems can affect dopaminergic signaling and potentially drive complex and flexible behavior.

Limitations & Conclusion

The present study revealed that the valence signaled by TH-C-AD;TH-D-DBD-positive cells can shift from negative to positive, as reflected by the shift from avoidance to approach; however, there are limitations that need to be addressed.

Most importantly, the behavior was overall variable and effect sizes, especially for the approach behavior, were small. As already stated, this is partially compensated by the fact that we robustly observe the same behavior across repetitions and variations of the experiments. The approach behavior might be too weak to be of evolutionary relevance; however, the aim of this study was not to detect a strong shift, it aimed to explain how the valence signaled by specific populations of neurons can shift over time.

The major limitation of the T-Maze is that it only shows flies' choice at the measured time point, without any information about how the choice of individual flies changed over time. It is well possible that some of the flies caught in the dark tube never experienced optogenetic activation and therefore did not necessarily avoid it. For flies caught in the illuminated tube this consequently means that they might not have actively approached the stimulation. Because initial avoidance is robust across repetitions, the second option is less likely. Unfortunately, with the current state of the experiment, it is impossible to track individual flies' movements during the experiment. Single-fly T-Maze experiments with repeated measures could prove a valuable tool to address this aspect. The 'FlyVac' is a single fly assay and designed to repeatedly measure phototaxis-based choice in the same fly (Kain et al. 2012). Because after each choice, the fly is transported back to the null position, it is forced to

repeatedly form a decision. Adapting this paradigm to measure not phototaxis-driven, but optogenetic choice could reveal how for individual flies the effect of optogenetic stimulation changes over time.

A more technical constraint lies in the optogenetic stimulation method itself. The simultaneous activation of large populations of neurons in response to the opening of optogenetic channels hardly mimics natural stimulation (Guo et al. 2015; Ceto and Courtine 2021). In addition, as discussed before different neuronal populations might differently respond to stimulation patterns and/or wavelengths. Unfortunately, I could not address the wavelength-dependent avoidance in control flies. Over the course of this study, flies without ATR supplementation began to occasionally show avoidance (Supplementary Figure 4). This was not consistently observed throughout experiments, and renewing fly stocks did not resolve the issue. However, this seemed to only affect control and not the experimental flies. Nevertheless, a potential interaction of wavelength and activation capacity, especially whether activation differs between neuronal populations should be further addressed in optogenetic studies.

Applying 3IY globally depletes DA levels. While this might be the justifiable approach to get an idea of general interactions, it does not reveal population-specific effects especially since different DA receptors might have different, cell-specific functions. Furthermore, 3IY experiments lacked the sample size required to fully detect DA effects in the individual components of the shift, making clear interpretations whether the individual behaviors differently depend on DA signaling difficult. Future studies may want to adjust their target sample size to the small effect of the approach behavior to ensure that all aspects are covered. Also, if feasible, a target specific inhibition of dopaminergic signaling, using Gal4-driven RNAi for TH or specific dopamine receptors, is crucial to further disentangle the valence shift.

Immunohistochemistry revealed that the driver line that should specifically target PPM2 neurons has additional targets. As there are no other driver lines that specifically label the PPM2 cluster, a possible approach to isolate the effects can be the inhibition of the additional targets. A commonly applied method to refine the expression of a driver is the use of a Gal80 construct (Fujimoto et al. 2011), allowing to intersect Gal4 expression in subsets of neurons. Introducing another genetic construct into the TH-C-AD;TH-D-DBD driver might not be feasible. However, because we can assume that the same

neurons in TH-D'-Gal4 mediate the behavioral shift, a possible approach to narrow down candidates might be the combination of TH-D'-Gal4 and $Cha^{3.3kb}$-Gal80 (Aso et al. 2010). This should, in principle, allow to target TH-D'-positive cells and simultaneously exclude PPL1 neurons. Because I could not identify the specific types of PPL1 neurons targeted by TH-C-AD;TH-D-DBD, it is not clear whether these exact neurons are targeted by $Cha^{3.3kb}$-Gal80. Nevertheless, silencing individual populations of neurons in the broader-expressing driver can help to narrow down the drivers of the observed behavior.

In the present study, I investigated TH-C-AD;TH-D-DBD flies to address whether the valence associated with an activation TH-D'-positive neurons can be isolated to certain subpopulations. As observed with the broader driver, TH-C-AD;TH-D-DBD flies initially avoid, but later on approach optogenetic activation of target neurons, reflecting a valence shift from negative to positive. Treatment with the TH inhibitor 3IY identified DA as the major driver of the behavioral shift. However, there are implications for additional signaling-pathways in the targeted DANs. Observations from stimulation experiments hint that operant learning circuits might underlie the behavioral shift. Comparing immunohistochemistry stainings to the expression pattern reported for TH-C-AD;TH-D-DBD revealed discrepancies to literature but still allowed to isolated the effect to PPL1 and PPM2 neurons. While the underlying mechanisms how the same neurons could represent opposite valence signals remain unknown, this study shows that a small set of neurons is able to drive such opposite behaviors in a time-dependent matter.

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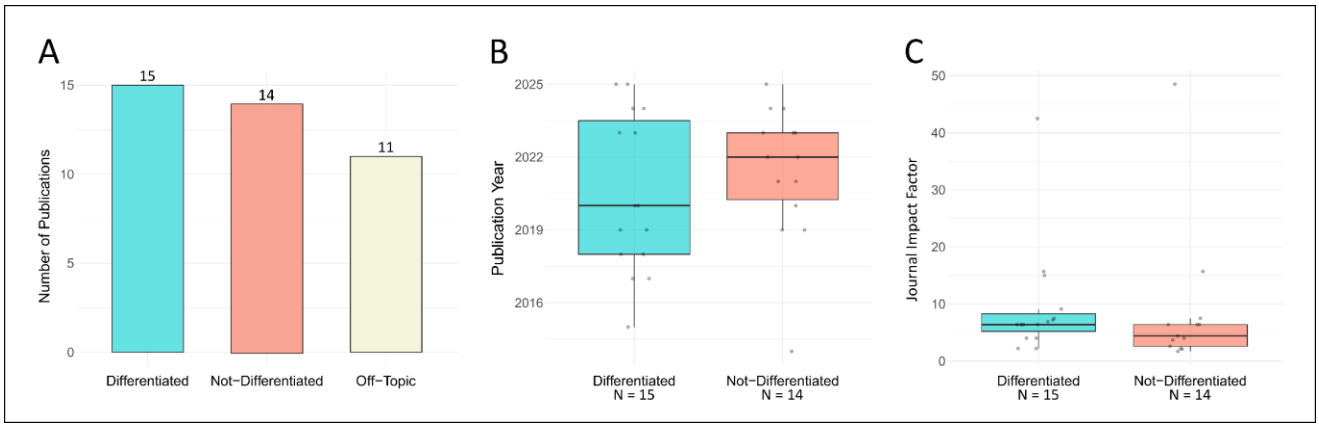
Appendix

Supplementary Material 1: Ambiguous use of terminology in dopamine targeting literature

A PubMed query "Drosophila optogenetics dopamine behavior" returned 40 publications. Out of these, I excluded 11 as they did either established a new method (e.g. Sun et al. 2018; Sun et al. 2020) or investigated neuronal populations and behaviors that did not fit the idea of the analysis. From the remaining 29 publications, 15 were categorized as "Differentiated" and 14 as "Not-Differentiated", based on their use of terminology describing dopaminergic function. A mean publication year of 2021 for both categories did not indicate a change in terminology over time. The mean journal impact factor was higher for the *Differentiated* (9.46), compared to *the Not-Differentiated* category (8.59) but too little to imply alternative publishing. Overall, the fraction of *Not-Differentiated* publications was substantial (14/29). A complete list of analyzed publications is provided in Supplementary Table 1.



Supplementary Figure 1 Analysis of literature employing optogenetics and targeting dopamine-dependent behavior in Drosophila

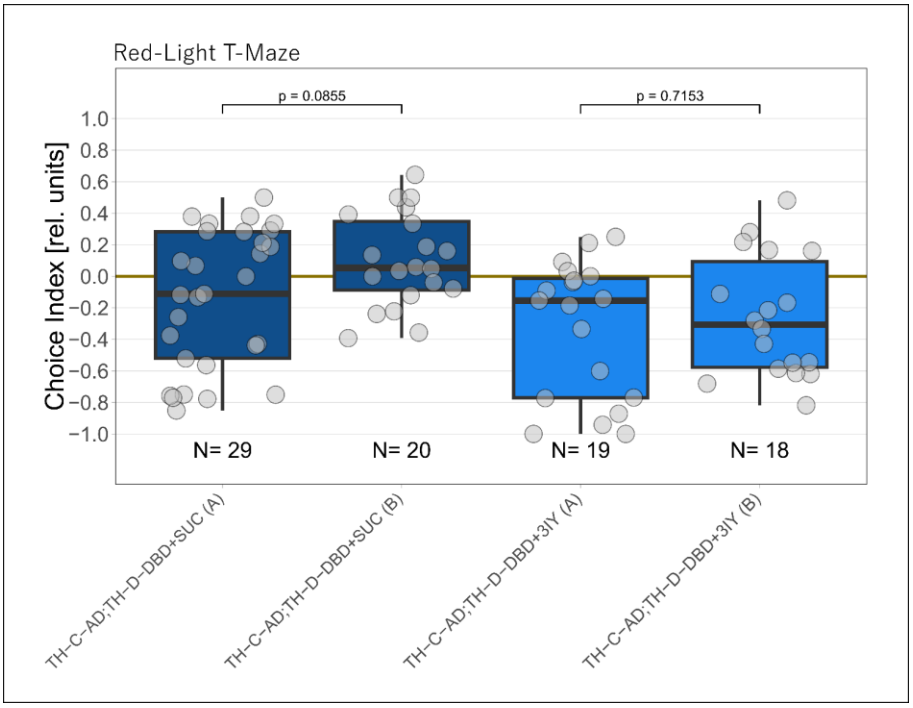
A: Categorization of 40 publications returned by the PubMed Query 'Drosophila optogenetics dopamine behavior'. Publication counts for each category are shown above plots; B, C: Comparison of publication years (B) and Journal Impact Factors (C) for categories 'Differentiated' and 'Not-Differentiated." x-axis: Category, y-axis: respective metric. Sample sizes are indicated below group labels.

Many of the analyzed studies formed general principles for dopaminergic neurons in reward- or punishment signaling. However, in most cases, the effects of those neurons was only tested in olfactory conditioning. It is known that classical and operant conditioning different underlie neuronal

populations and neurotransmitters (Sitaraman et al. 2008; Ma et al. 2020). Many of the analyzed studies attribute valuation mainly to dopaminergic neurons of the PAM and PPL1 cluster and generally label them as reward- or punishment-neurons, respectively. Whether this classification holds true beyond the investigated function remains unknown. PPM2 neurons have been originally described for mediating sugar reward, and Rohrsen et al. could not detect appetitive reflexive behavior in satiated animals (Liu et al. 2012a; Rohrsen et al. 2021). Therefore, these neurons might simply signal appetitive values of food, rather than reward in general. The present study implies that PPL1 neurons might mediate behaviors of opposite valence. These observations draw a complex picture of dopaminergic signaling, which seems to be often oversimplified in literature. Importantly, the analysis does not challenge an involvement of PAM, PPL1 or other currently unreported dopaminergic neurons in reward- or punishment-related behavior. Rather, it highlights a recurring mismatch between the specificity of the experimental paradigms employed and the generality of the terminology used to describe neuronal functions. Although I applied the criteria as consistently as possible, the categorization can be debated as it was overall based on a subjective assessment. However, this also shows that the literature on dopaminergic neurons in *Drosophila* lacks the precision required for an unambiguous classification.

Supplementary Material 2: Dopamine depletion experiments with red light

Before the decision to focus manipulation experiments on yellow light, I measured dopamine-depleted flies' choice in the red light-Maze. Untreated flies showed the similar, non-significant behavioral shift as observed in baseline conditions. 3IY treated flies showed consistently negative CIs.

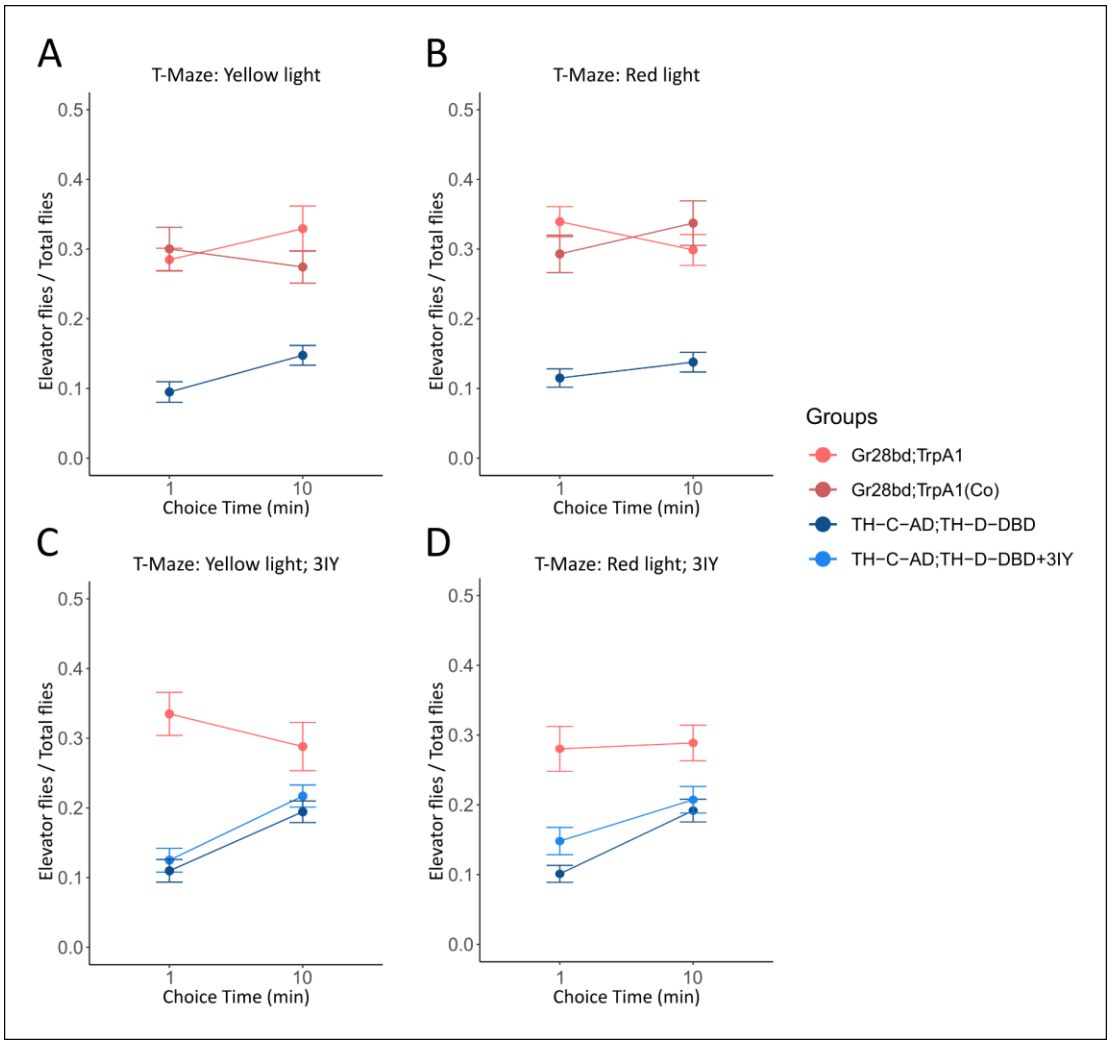


Supplementary Figure 2 Red Light T-Maze dopamine depletion experiment

Suffixes (A) and (B) denote 1-min and 10-min testing, respectively. Brackets and p-values indicate group comparisons. Sample sizes are shown below boxplots.

Supplementary Material 3: Genotype and Testing Time affect distribution in T-Maze

Not all flies that participate in T-Maze experiments contribute to the calculation of the choice index. Flies trapped in the movable elevator compartment cannot be counted to either arm of the maze and were therefore excluded from index calculation. I compared elevator ratios across experiments and found that the ratio of excluded flies differed systemically (Two-way ANOVAs are summarized in Supplementary table 2).



Supplementary Figure 3 Distribution of flies in T-Maze changes with increased testing time

A-D: *Prolonged testing affects ratio of flies that are caught in the elevator compartment. Ratio of Gr28bd;TrpA1 flies with and without ATR supplementation does not in- or decrease consistently across experiments and wavelengths. Elevator ratios of TH-CAD;TH-D-DBD consistently increase in initial experiments* ***(A,B)*** *as well as manipulation experiments* ***(C,D)*** *In all panels y-axis denotes ratio of flies caught in the elevator from total flies. x-axis denotes testing times. Color-coding is summarizes in the legend. Plots show mean ± SE. Statistics are detailed in Supplementary Table 2 and 3.*

Supplementary Table 2. Summary of Two-Way ANOVA of Elevator Ratios as a function of Groups and Testing Time/Exposure

The table summarizes results of two-way ANOVAs assessing the main effects of 'Group', 'Testing Time' and their interaction on Elevator Ratio across experiments. The Experiment *column indicates the corresponding experiment (Fig. 5).*

Experiment	Variable 1: Group	Variable 2: Testing Time	Interaction
A: Yellow light	significant	n.s.	significant
B: Red light	significant	n.s.	n.s.
C: Yellow light; 3IY treatment	significant	significant	significant
D: Red light; 3IY treatment	significant	significant	n.s

Supplementary Table 3 Summary of Post hoc test

The table summarizes positive post hoc test carried out with the emmeans-package in R. Columns indicate the corresponding experiment ('Exp', see Fig. 5), which groups were compared ('Contrast') and main result of a comparison of the estimated marginal means of groups ('p-value'). The table only summarizes comparisons that revealed significant differences between groups. Because the ANOVA showed only an effect of the variables group in Experiment B comparisons between were performed collapsed across time points.

Exp	Co	ntrast	p-value
A	Gr28bd;TrpA1 - 1 min	TH-C-AD;TH-D-DBD - 1 min	< 0.0001
	Gr28bd;TrpA1(Co) - 1 min	TH-C-AD;TH-D-DBD - 1 min	< 0.0001
	Gr28bd;TrpA1 - 10 min	Gr28bd;TrpA1(Co) - 10 min	0.0032
	Gr28bd;TrpA1 - 10 min	TH-C-AD;TH-D-DBD - 10 min	< 0.0001
	Gr28bd;TrpA1(Co) - 10 min	TH-C-AD;TH-D-DBD - 10 min	0.0022
	Gr28bd;TrpA1 - 1 min	Gr28bd;TrpA1 - 10 min	0.0415
	Gr28bd;TrpA1 - 1 min(Co)	Gr28bd;TrpA1(Co) - 10 min	0.0235

B	Gr28bd;TrpA1	TH-C-AD;TH-D-DBD	< 0.0001
	Gr28bd;TrpA1(Co)	TH-C-AD;TH-D-DBD	< 0.0001
C	Gr28bd;TrpA1 – 1 min	TH-C-AD;TH-D-DBD – 1 min	< 0.0001
	Gr28bd;TrpA1 – 1 min	TH-C-AD;TH-D-DBD+3IY– 1 min	< 0.0001
	Gr28bd;TrpA1 – 10 min	TH-C-AD;TH-D-DBD – 10 min	0.0031
	Gr28bd;TrpA1 – 10 min	TH-C-AD;TH-D-DBD+3IY– 10 min	0.0208
	TH-C-AD;TH-D-DBD – 1 min	TH-C-AD;TH-D-DBD – 10 min	0.0015
	TH-C-AD;TH-D-DBD+3IY– 1 min	TH-C-AD;TH-D-DBD+3IY– 10 min	0.0006
D	Gr28bd;TrpA1 – 1 min	TH-C-AD;TH-D-DBD – 1 min	< 0.0001
	Gr28bd;TrpA1 – 1 min	TH-C-AD;TH-D-DBD+3IY– 1 min	< 0.0001
	Gr28bd;TrpA1 – 10 min	TH-C-AD;TH-D-DBD – 10 min	< 0.0001
	Gr28bd;TrpA1 – 10 min	TH-C-AD;TH-D-DBD+3IY– 10 min	0.0004
	TH-C-AD;TH-D-DBD – 1 min	TH-C-AD;TH-D-DBD – 10 min	0.0299

Throughout all experiments, TH-C-AD;TH-D-DBD flies consistently exhibited lower elevator ratios. Because I counted fewer flies the 'no-choice zone', this initially suggests more definitive decision-making. However, elevator ratios did not correlate with choice indices in this group. Moreover, control flies with and without ATR supplementation did not differ in elevator ratios. Thus, low elevator ratios and optogenetic preference appear to reflect different aspects of behavior.

The elevator is considerably shorter than the light and dark tubes. If optogenetic activation were the sole determinant of distribution, I would expect flies without preference to distribute evenly across compartments. A comparisons of dimensions reveals that this would lead to ratios that are comparable to the TH-C-AD;TH-D-DBD groups'. However, control flies without ATR supplementation - which are indifferent to optogenetic stimulation - showed substantially higher elevator ratios than expected by chance (Fig. S2A,B). This suggests a systematic bias in distribution.

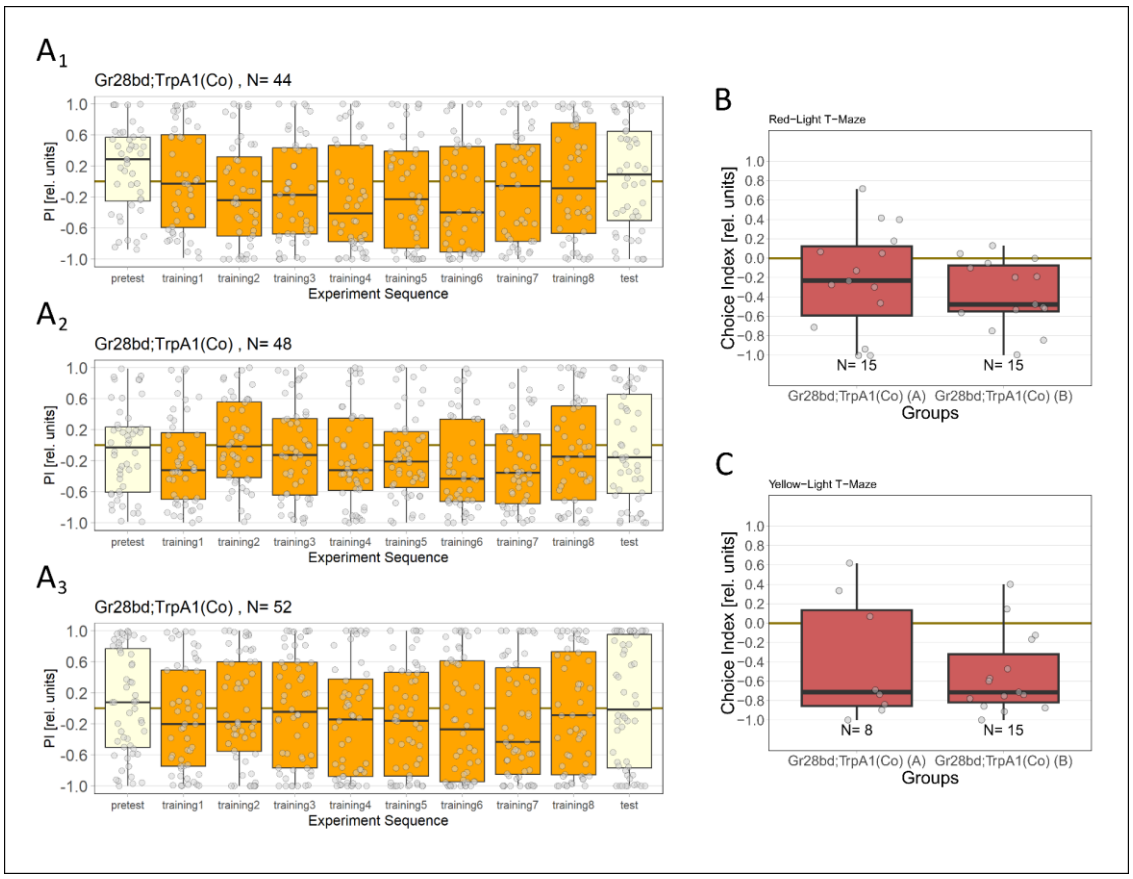
High ratios could reflect flies that never left the compartment after the experiment was initiated.

However, it is unlikely that flies would not move at all (Martin et al. 1999; Lebestky et al. 2009). A potential source of bias lies in the experimental preparation. After introduction into the loading tube, flies are left for 10 minutes to adjust before testing. During this phase, my main focus was that the elevator should descend to the choice zone as this would mean premature testing and I would have to discard this experiment. Less attention was given to whether the elevator was moved upwards during loading. This could allow some flies to enter the compartment where they could leave chemical cues that could be attractive to conspecifics (Mercier et al. 2018). Although I cleaned T-mazes occasionally, I did not perform a cleaning cycle after every testing day or after each individual experiment. Residual chemical marking could therefore have biased the distribution of flies across compartments.

If chemical cues attract flies to the elevator zone, TH-C-AD;TH-D-DBD flies seem to be indifferent towards them. Alternatively, increased activity or social spacing could overcome the drive to stay. Both aspects are partially supported by literature (Xie et al. 2018; Marquis and Wilson 2022). However, because the elevator zone is mostly devoid of illumination and interference with dopamine signaling did not affect ratios, an effect of the target DANs seems unlikely. In addition, no study that used this driver did report abnormal activity. Nevertheless, this phenotype might only develop in the specific combination of driver and effector.

Supplementary Material 4: Negative Control Issues

Over the time course of this study, issues concerning the control flies Gr28bd;TrpA1> NorpA;20xUAS-Chrimson emerged. While during initial tests, flies that were not treated with ATR showed no preference (Fig. 3), flies started to avoid optogenetic activation in JoyStick (Fig. S4 A_1,A_2; experiments in A_2 were carried out by a visiting student) initial red light manipulation experiments (Fig S4B). New stock flies for both the driver and the effector line led to a partial improvement, however in subsequent yellow light T-Maze manipulation experiments the issue persisted (Fig. S4C). Experimental flies seemed to be not affected by this phenomenon as they showed similar behavior than in the original screens (Fig. 3, 4, 5).



Supplementary Figure 4 Avoidance in control flies without ATR supplementation

A: *Preference indices (PI) of Gr28bd;TrpA1(Co) from different JoyStick screens.* ***A_1:*** *June-August 2025;* ***A_2:*** *July 2025;* ***A_3:*** *October 2025. x-axis denote experiment sequence, y-axis indicate PIs. Orange: training periods with light- stimulation; yellow: test periods without light stimulation. sample sizes are indicated above plots .* ***B,C:*** *Choice indices of Gr28bdTrpA1(Co) flies in red* ***(A)*** *and yellow* ***(B)*** *T-Maze. x-axis denote group and testing time: suffix (A): 1-minute choice, suffix (B): 10-minute choice; y-axis indicate CI. Sample sizes are shown below group labels.*

Acknowledgments

I want to thank all of the members of the Brembs laboratory for their continuous support throughout this project.

I specifically want to thank…

Prof. Björn Brembs for giving me the opportunity to work in his lab, as well as for always having an open ear (and door) for any concerns, questions, or simply reassuring words whenever I was in doubt. Thank you also for giving me the freedom to develop my scientific potential and for providing so many opportunities that extend well beyond the classical framework of a master's thesis.

Prof. Veronica Egger for kindly agreeing to serve as the second evaluator of this thesis and for enriching Thursday morning journal clubs.

Dr. Radostina Lyutowa for her unconditional support in every aspect of my time in the lab. Thank you not only for being an invaluable source of knowledge, but also for being a friend in every other way.

Lastly, I want to thank my partner **Anna-Lena**. Your patience, encouragement, and unwavering belief in me have been a constant source of strength throughout this journey.

I hereby confirm that the printed copies and the digital version of the thesis submitted are identical; that the thesis is my own work; that I have used only those sources and aids cited; and that I have not already submitted the work to another university to obtain an academic degree. I am aware of the legal consequences of this declaration being inaccurate provided for in accordance with § 26 Para. 5 of the relevant examination regulations.

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