The neuronal substrates of reinforcement and punishment in *Drosophila melanogaster*

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Abstract

Actions are followed by consequences, to which values are assigned. These subjective values shape our future actions in what we colloquially call “learning by doing”. But how does the assignment of values to behavioral consequences occur in the brain? In mammals as well as in three different superphyla within protostomes (Nematoda, Platyhelminthes and Mollusca) this is mediated by dopamine. However, little is known about the neural basis of value assignment to behaviors in the Arthropoda. In order to address which neurons convey punishment and reinforcement in insects, we performed four different experiments in which flies (*Drosophila melanogaster*) could control the on/off state of different subsets of dopaminergic neurons. We found that the effects of these neurons across operant (feedback) conditioning have no relation to their role observed in previously well studied pavlovian (feedforward) conditioning. These results suggest fundamentally different neuronal circuits dedicated to operant and pavlovian learning processes. The reinforcing value of most of the tested neurons is context dependent and differs among the tested operant paradigms. However, there are two exceptions: two different cell clusters projecting to different neuropils in the central complex (CX) and accessory regions, a brain area involved in multisensory integration and action selection, seem to be responsible for the reinforcement of motor commands in a context-independent way. These findings support a conserved mechanism of dopaminergic reinforcement in higher order motor centers across phyla.

1 Introduction

1.1 Neural plasticity

Homeostatic regulation in the brain allows adaptation to a changing environment, which is essential for survival. Past experiences are integrated into neural processing and decisions are taken according to the instantaneous environmental and internal context (Devan et al., 2018; Margulies et al., 2005; Rescorla & Wagner, 1972.; Solanki et al., 2015). Decisions are probabilistically influenced by evidence accumulation, which in rodents is encoded in dopaminergic neurons and dorsal striatum (Graybiel, 2016; Lak et al., 2016; Yartsev et al., 2018) whereas in flies the *αβ* lobes in the mushroom bodies (MB) integrate evidence over time for olfactory discrimination (Groschner et al., 2018). When an individual interacts within its habitat, it can learn about environmental contingencies as well as from the outcome of its actions. These learned associations are stored as probabilities which are continuously updated by new information (Fiorillo et al., 2003; Nassar et al., 2010; Zhou et al., 2018).

Formed associations are the scaffold for creating a model representation of the surrounding environment to be able to predict from past events and assess the changing conditions for its own adaptation (Cognigni et al., 2018; Devan et al., 2018; Heisenberg, 2015; Li, 2014; Margulies et al., 2005; Menzel et al., 2007; Seidler et al., 2013). Error discrimination allows an individual to know when behavior optimization is under its own control (Inoue & Kitazawa, 2018; Nassar et al., 2010; Seidler et al., 2013; Wolf & Heisenberg, 1991; Zhou et al., 2018). That means that the brain actively evaluates the confidence in the formed associations and distinguishes whether unexpected discrepancies are due to its own motor coordination or due to environmental stochasticity. Confidence is an estimation of the uncertainty of the formed associations and in mammals it is is encoded by several neurotransmitters throughout the brain: dopamine, serotonin, noradrenaline and acetylcholine (Fiorillo et al., 2003; Yu & Dayan, 2005). The ability to recall past experiences is essential for organisms to take proper decisions. The brain has evolved to dynamically update value to actions and stimuli that affect fitness (Graybiel, 2016; Paton & Louie, 2012). This valuation is the teaching signal that leads to learning by maximizing reward and minimizing punishment.

1.2 Reward and dopamine

From the ancient Greek through the Middle Ages to modern philosophy, the meaning of reward and its biological relevance has attracted a lot of interest. These reflections were

not based on brain physiology but mostly limited to observations and behavior (Marks, 2011).

There are two main paradigms for studying the physiology of hedonia: self-stimulation and conditioned place preference. The self-stimulation paradigm was developed by Olds and Milner in 1954 (Olds & Milner, 1954), and the idea behind this paradigm is that animals control the presentation of the stimulus with their own behavior. In that particular case, rodents could press a lever that was connected to a stimulation electrode placed in a certain brain region. Olds and Milner found by serendipity regions in the septal area which rodents would stimulate to the point of replacing other natural rewards like food consumption. The lateral hypothalamus, the median forebrain bundle and the dopaminergic mesolimbic system yielded the highest lever pressing rates (Paton & Louie, 2012; Wise, 2002). In conditioned place preference paradigms animals are previously conditioned with an appetitive/aversive stimulus to avoid/approach a certain place and it generally requires less training in rodents (Prus et al., 2009; Simon et al., 2009).

Although dopamine was traditionally considered to be the hedonic neurotransmitter, its function is much more varied. Self-stimulation experiments and locomotor activity measurements during differential activation of neurons expressing D1- and D2 dopaminergic receptors indicate that hedonic feelings share common dopaminergic and basal ganglia circuitry with the control of motor activity (Kravitz et al., 2012; Kravitz & Kreitzer, 2012). The basal ganglia are an essential center for motor control where the direct and indirect pathways have been traditionally seen as opposing forces for gating voluntary and involuntary movements. The direct pathway, mediated by dopaminergic receptors of type D1, increases movement and mediates positive reinforcement and reward. On the other hand, the indirect pathway, mediated by type D2 receptors, decreases movement and mediates punishment and aversion (Kravitz et al., 2012; Kravitz & Kreitzer, 2012; Paton & Louie, 2012; Saunders et al., 2018; Wise, 2002).

Dopaminergic neurons in the fruit fly are also responsible for heterogeneous functions, including reward in different classical conditioning assays (Aso et al., 2010, 2012; C. Liu et al., 2012; Vogt et al., 2016). Other tasks involve locomotion, sleep/arousal regulation, encoding of energetic state, novelty, hunger, thirst, electric shock, etc., and induce learning, memory and forgetting (Aso & Rubin, 2016; Barron et al., 2010; Berry et al., 2015; Cognigni et al., 2018; Cohn et al., 2015; Hattori et al., 2017; Hu et al., 2018; Krashes et al., 2009; Lin et al., 2014; Owald, Felsenberg, et al., 2015; Perry & Barron, 2013; Riemensperger et al., 2011; Sitaraman et al., 2015; Tschida & Bhandawat, 2015).

1.3 Reward Prediction Error (RPE)

Past experiences confer an animal the ability to predict rewards in order to anticipate its behavior. Wolfram Schultz and colleagues observed that the firing activity in dopaminergic neurons of the ventral tegmental area (VTA) and the substantia nigra pars compacta was proportional to unexpected rewards (Keiflin & Janak, 2015; Ljungberg et al., 1992; Montague et al., 1996; Schultz, 2016; Schultz et al., 1993, 1997). These neurons encode the difference between the predicted and the obtained reward, known as reward prediction error (RPE), which resembles the proposed teaching signals from older reinforcement learning models (Bush & Mosteller, 1951; Rescorla & Wagner, 1972.; Sutton & Barto, 1981; Pearce & Hall, 1980). RPE is also encoded in the anterior cingulate cortex , amygdala, globus pallidus and caudate nucleus (Belova et al., 2007; Ding & Gold, 2010; Hong & Hikosaka, 2008; Seo & Lee, 2007), whereas a region closely interconnected with the VTA, the habenula (Hb), signals punishment prediction error (Kumar et al., 2018; Tian & Uchida, 2015). Interestingly, the Hb is one of the main centers encoding relief and RPE in zebrafish, as in mammals, it works tightly with the dopaminergic system to build RPE (Li, 2014). Along with other vertebrates, RPE neurons in *Drosophila* are also dopaminergic (Cohn et al., 2015; Felsenberg et al., 2017; Galili, 2014; Keiflin & Janak, 2015; König et al., 2018; Riemensperger et al., 2005) whereas in the honey bee, the octopaminergic VUMmx1 has shown dynamic reward-predictive firing properties (Perry & Barron, 2013).

The relevance of timing is openly reflected by the RPE, and explains phenomena like punishment relief signaling, where a stimulus that occurs at the end of punishment can be appetitive (Gerber et al., 2014; König et al., 2018; Tanimoto et al., 2004). Trace and relief conditioning studies in *Drosophila* point to a widespread coding of salient stimuli (Aso & Rubin, 2016; Hattori et al., 2017; Heisenberg, 2015; Hige, Aso, Modi, et al., 2015; Hige, Aso, Rubin, et al., 2015; Konorski, 1948; Vogt et al., 2015; Zieliński, 2006). In mammals, the amygdala and the medial prefrontal cortex might use their interconnection with the VTA to encode absolute prediction errors or saliency (Mackintosh, 1975; Nasser et al., 2017; Pearce & Hall, 1980).

RPE underlies an essential neuronal correlate of learning: being able to predict indicates that associative learning has occurred before. The ubiquitous RPE correlates throughout the brain indicate that the brain is a huge associative machine and emphasizes the relevance of associative learning as a universal and essential mechanism for survival. RPE is necessary for memory reevaluation and reconsolidation by comparing previous associations with current ones (Cognigni et al., 2018; Donahoe & Burgos, 2000; Felsenberg et al., 2017).

There are two main types of learning: associative and non-associative. Associative learning is the process where relationships between events are captured and embodied in synaptic plasticity. Associative learning can be further subdivided in: classical/pavlovian- and operant/instrumental conditioning.

1.4 Pavlovian/Classical and Operant/Instrumental conditioning

Classical conditioning was firstly described by Ivan Pavlov (I. P. Pavlov, 1928; P. I. Pavlov, 2010). In his experiments he bestowed dogs with food preceded by a bell tone. After learning, the dogs started salivating upon the preceding bell tone even when no food was presented, thus anticipating the food reward. Classical conditioning describes the process where an organism captures the relationship between two external stimuli that are contingent on each other. The conditioned stimulus (CS), in this case the bell tone, whose meaning is neutral to the individual, acquires after co-presentation the value of the unconditioned stimulus (US), in this case the food reward. Hence, when an US is presented frequently enough with a CS, the subject elicits a conditioned response (CR) upon CS occurrence.

Classical conditioning consists of a feedforward process where the contingent sensory stimuli lead to a behavioral response. During operant conditioning the occurrence of behavioral and sensory events are reversed, initiating behavior bias the incoming sensory stimuli. Hence, operant learning is conceptually different in that behavioral outcomes serve as a basis to modify behavior which implies that the subject actively controls the sensory feedback, allowing to direct its cognitive resources (e.g. attention) to it (Brembs & Heisenberg, 2000; Brembs, 2008; Brembs, 2000, 2009; Heisenberg & Wolf, 1993; Wolf & Heisenberg, 1991).

Alexander Bain (1865) claimed that in our constant search for pleasure, certain spontaneous behaviors would increase their frequency. Herbert Spencer and James Mark Baldwin would develop the idea of reward-based learning until Thorndike, who showed empirical studies of trial-and-error learning in animals (1898), introduced the law of effect in 1911 (Marks, 2011). Thorndike applied the word instrumental because behavior would be instrumental to achieve rewards, whereas Skinner (1938) would deploy the term operant because behavior would operate on the environment. Contrary to many behaviorists who would try to reconcile both associative learning types in a unifying principle, Skinner made a sharp distinction between operant (emitted) behaviors and respondent (elicited) behaviors (Marks, 2011). Skinner emphasized that operant conditioning strengthened responses and not stimulus-response bonds, which was ambiguously explained by the law of effect (Marks, 2011).

Innumerable studies have behaviorally characterized operant and classical associative memories in the fruit fly. The neural substrates of classical conditioning have been extensively unraveled, pointing to the MB as the main center for classical conditioning (Aso et al., 2012; Aso, Hattori, et al., 2014; Aso, Sitaraman, et al., 2014; de Belle & Heisenberg, 1994; Claridge-Chang et al., 2009; Kirkhart & Scott, 2015; Lin et al., 2014; C. Liu et al., 2012; L. Liu et al., 1999; Mao & Davis, 2009; Margulies et al., 2005; Musso et al., 2015; Perisse et al., 2013; Pidoux et al., 2018a; Schwaerzel et al., 2003; Vogt et al., 2014; Waddell, 2013). In the operant counterpart, an extensive behavioral characterization of the different components of operant conditioning has laid the groundwork to address the neuronal mechanisms (Brembs, 2000, 2009a, 2009b; Heisenberg, 2015; Heisenberg & Wolf, 1993; Wolf & Heisenberg, 1991). The MB gate the progression from goal directed to habitual behaviors (Brembs, 2009a, 2009b) whereas in mammals this is accompanied by the activity transition from the dorsomedial to the dorsolateral striatum (Fino et al., 2018; Graybiel, 2016; Graybiel & Grafton, 2015; Thorn et al., 2010). However, the study of the neural correlates of operant behavior in *Drosophila* has been mostly limited to the mechanisms of sensorimotor transduction, recognizing the circuits eliciting behaviors (Cande et al., 2018; Giraldo et al., 2018; Lindsay et al., 2017; Namiki et al., 2018; Namiki & Kanzaki, 2018; O'Sullivan et al., 2018; von Philipsborn et al., 2011; Robie et al., 2017). The genetic components of operant learning in the fruit fly have been only partially identified, with a particular focus on dFoxP and PKC (Brembs & Plendl, 2008; Colomb & Brembs, 2016; Kottler et al., 2019; Mendoza et al., 2014). However, not that much is known about how punishment and reinforcement signaling converge to influence action selection.

Vocal learning is a well-known operant learning process in juvenile songbirds where the learn to coordinate vocal cords and muscles to imitate the adult song. Pharmacological and lesion experiments together with anatomical findings have characterized the brain regions involved in vocal learning. The cerebellum carries out a supervised error-based learning whereas the striatum involves the reinforcement system. These two regions interact with thalamocortical loops and brainstem circuits to coordinate proper motor output. This mechanism applies to other motor tasks and shows close resemblance to what is observed in other vertebrates (Jarvis, 2007; Manto et al., 2012; Pidoux et al., 2018b; Seidler et al., 2013).

In *Aplysia*, the biting behavior has been used as a model for the comparison of classical and operant conditioning mechanisms. Pairing tactile stimulation of the lips (US) with seaweed (US) leads to an increased biting frequency (CR). Concomitantly, the stimulation of the anterior branch of the esophageal nerve (En_2) after every bite induced a sustained biting increase. Interestingly, En_2 releases dopamine in the neuron B51 to induce

differential biophysical changes for both, operant and classical procedures (Bédécarrats et al., 2013; Brembs et al., 2002; Lorenzetti et al., 2006; Nargeot et al., 2009). In the zebrafish *Danio rerio* there is not much known yet but calcium imaging experiments during operant learning suggested that the lateral Hb and the pallium in the forebrain code for pain relief and prediction error (Li, 2014).

Reinforcement is essential for learning by narrowing the behavioral options to the more adaptive ones. In a changing environment this can be sometimes detrimental and the brain has therefore developed a strategy of increasing behavioral variability (Brembs, 2000; Wolf & Heisenberg, 1991). Comparing the consequences of different behaviors allows the brain to infer and discriminate the contingencies better. Thus, exploration of a wider behavioral space tends to form a more accurate internal representation and better generalization (Dhawale et al., 2017; Grunow & Neuringer, 2002; Hansson & Neuringer, 2018; Perry et al., 2010). Exploration can be reinforced and used as a reinforcer and curiosity might be a natural mechanism to drive exploration (Grunow & Neuringer, 2002; Hansson & Neuringer, 2018; Marks, 2011; Neuringer & Huntley, 1992).

1.5 Pure operant learning

Since its implementation by Olds and Milner (Olds & Milner, 1954), lever pressing experiments have been very popular for studying operant learning in rodents. However, these experiments contain a mixture of learning types, where not only the action of lever pressing is contingent on reward (“pure operant”), but also the physical event and associated cues accompanying the instrument (e.g. the lever going down, or apparatus noise, that might work as a CS). In an individual internal model, the paired bond might not necessarily be between behavior-reward but with the descending lever-reward, since a descending lever might be a better predictor of reward. Elegant experiments in fruit flies sharply dissected an operant paradigm in two different learning processes: world- and self-learning. The former referring to the bonds between sensory stimuli with the paired reward and the latter referring to the coincidence detection of ongoing motor programs and reinforcement (Brembs, 2000, 2011). In these fly experiments, during composite training which resembles lever pressing experiments, only CS-US is memorized by the fly in detriment of the behavior-reinforcement (B-R) bond. Further experiments showed that behavioral consequences are easier to retain when no other external cues are present. On the contrary, the CS-US memory is stronger when flies control the presentation with their behavior. This reveals an asymmetry where world-learning is facilitated by behavioral control of the environment whereas the presence of contingent sensory cues impair self-learning. Thus, lever pressing experiments resemble composite experiments where the CS-US association prevails over the B-R bond simulating

rather classical conditioning experiments. Disregarding this relevant facts has often led to operant paradigms that are vaguely measuring B-R associations. (Brembs, 2000, 2011; Heisenberg & Wolf, 1993; Wolf & Heisenberg, 1991).

1.6 Drosophila as a laboratory model: optogenetics

About three fourths of human disease associated genes can be found in the fruit fly, making *Drosophila* a suitable model to study (Chien et al., 2002; Fortini et al., 2000; Pandey & Nichols, 2011). The relatively simple nervous system of this insect allows us to understand in more detail how specific circuits achieve certain tasks. In addition, short life cycle and the relatively easy maintenance make this model attractive for research.

The introduction of the P-element-mediated gene transfer allowed the development of a vast set of genetic tools, permitting sophisticated experimental manipulations in *Drosophila melanogaster* (O'Kane & Gehring, 1987; Rubin & Spradling, 1983). In the last years, CRISPR/cas9 has become a major cloning strategy (Bassett & Liu, 2014).

The *GAL4-UAS* system was cloned from the yeast into *Drosophila melanogaster*, allowing the spatial control of the transcription of desired genes. In this method *GAL4*, a transcriptional activator, can be be expressed under different promoters and enhancers to yield a specific expression pattern. The Gal4-*protein* binds specifically to the Upstream Activation Sequence (*UAS*), which regulates the expression of an effector protein (Brand & Perrimon, 1993). Further variants of this system like split-G4s, MARCM and flip out techniques provided an even more accurate expression profiles (Luan et al., 2006; Pfeiffer et al., 2010; Xie et al., 2018). Many effectors have been developed to manipulate neuronal activity through temperature (Hamada et al., 2008; McKemy et al., 2002; Peier et al., 2002), drug administration (Sternson & Roth, 2014) or light (Boyden et al., 2005; Nagel et al., 2002, 2003). The latter technique, known as optogenetics, is the method chosen for this study due to its temporal resolution and decreased side effects.

Channelrhodopsins are light gated channels that were discovered in green algae in Regensburg in 2002 (Nagel et al., 2002, 2003), which were consequently further developed as a neuronal activation tool (Boyden et al., 2005). Since their discovery, the biophysical properties of these channels have been optimized according to the purposes of the scientific community. Channelrhodopsins consist of a protein channel core with its C-terminal covalently bonded with all-trans-retinal (ATR), a chromophore (Kato et al., 2012). When light hits on the chromophore, it leads to 13-cis-retinal, that induces a conformational change of the channel, making it permeable to cations, specially calcium.

Neuronal activity recording and imaging adds supplemental versatility for fly research, as well as anatomical tracing techniques like electronic microscope reconstruction and synaptically targeted GFP reconstitution across synaptic partners (GRASP) (Feinberg et al., 2008; Macpherson et al., 2015; Owald, Lin, et al., 2015). Concomitantly, recent technological advancements and a more openly shared distribution of resources have allowed higher quality research with more sophisticated analysis algorithms and high throughput experiments.

1.7 Aim of the study

The scope of this study was to find neuronal substrates for operant reinforcement (fig. 1). Subpopulations of dopaminergic neurons were screened for their reinforcing properties in four different operant paradigms. All experiments resembled self-stimulation paradigms where naive flies were in control of the neuronal activation by light.

We focus on dopaminergic neurons for several reasons:

- Evolutionary: in mammals the dopaminergic mesencephalic nuclei projecting to the basal ganglia and medial prefrontal cortex encode the teaching signals (Montague et al., 1996; Olds & Milner, 1954; Schultz et al., 1997). In birds, as in mammals, the basal ganglia encode the reinforcing signals, indicating that dopamine might be involved (Pidoux et al., 2018b). In *Aplysia*, dopamine is the learning signal involved in classical and operant conditioning (Brembs et al., 2002; Lorenzetti et al., 2006). In nematoda, platyhelminthes and crustaceans dopamine is involved in conditioning (Barron et al., 2010; Datta et al., 2018). In *Drosophila* dopamine substitutes the US and contains projections to important premotor areas like the central complex (CX) and the lateral accessory lobe (LAL) (Aso et al., 2010, 2012; C. Liu et al., 2012; White et al., 2010).
- Technical: the availability of a vast number of dopaminergic promoters for driving the expression in specific neuronal subsets, as well as the volume of literature on the effects of dopaminergic manipulation gives an excellent and extensive background framework to this study.

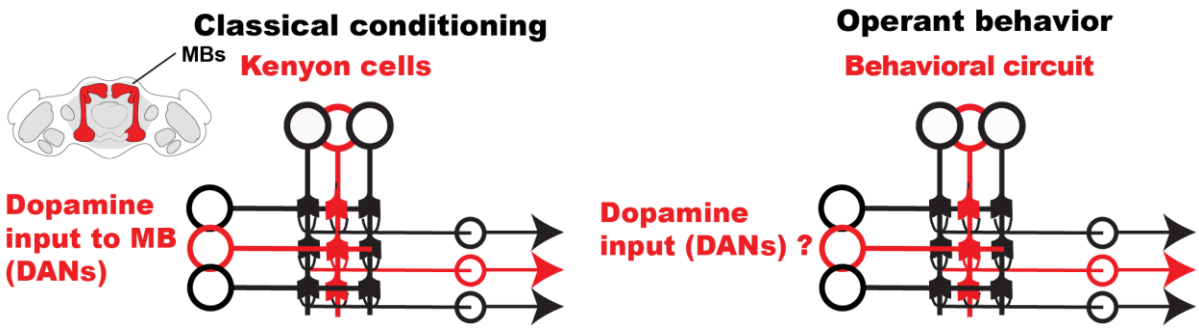


Figure 1. Circuit schematics from classical conditioning vs operant behavior. A: In olfactory classical conditioning the kenyon cells (KC) receive and convey olfactory input whereas dopaminergic neurons (DANs) provide contextual information and serve as an US. DANs consequently modify KC-MB output neurons synaptic strengths to trigger the behavioral output. **B**: Hypothetical learning mechanism for behavior: DANs projecting to a region where motor programs are selected, change synaptic strengths to increase/decrease a certain behavior (active neurons are shown in red).

2 Methods

2.1 Fly genetics

In our experiments we avoided additional contingencies other than that of the reinforcement with the fly behavior: to avoid visual cues from the stimulating light that would interfere with our reinforcement scores, we genetically blinded flies with a mutated *no receptor potential A gene* (*norpA*^{P24}). *norpA* encodes for phosphatidylinositol-specific phospholipase C that is involved in several sensory pathways, which mutation can impair vision completely (Hardie et al., 2003; McKay et al., 1995; Pearn et al., 1996; Shen et al., 2011). In addition, *norpA*^{P24} decrease olfactory discrimination (Riesgo-Escovar et al., 1995) and impair temperature discrimination at temperatures between 18°C and 26°C (Collins et al., 2004; Glaser & Stanewsky, 2005; Shen et al., 2011). This overall reduced sensory sensitivity is optimal to avoid the world-learning effects and to strictly measure self-learning, the “pure” operant learning component.

CsChrimson was the effector chosen for neuronal activation (Klapoetke et al., 2014), whereas GtACR1 and GtACR2 were deployed for inhibition experiments (Mohammad et al., 2017). Phasic stimulation activates neurons more effectively than tonic stimulation (Inagaki et al., 2014), therefore the light stimulation was pulsed.

2.2 Fly care and reagents

Males containing a dopaminergic *GAL4* (table S2) driver line were crossed to *norpA*^{P24}*;20xUAS-CsChrimson* virgin flies and kept them in standard cornmeal and molasses food media in darkness at 25°C and 70% humidity for egg laying. One to six days after hatching, groups of approx. 30 male offsprings were put in small glass vials with all-trans-retinal (ATR) supplementation for two days before testing. In mammals, retinal precursors are naturally produced in the brain whereas this is not the case in insects. Therefore, fly food is supplemented with ATR for the deployment of optogenetics. The ATR supplementation was accomplished by applying 15µl of 200mM ATR dissolved in ethanol to the food surface for intake. All the flies were fed with ATR to yield a functional light-gated channel (unless otherwise specified). In mammals, retinal precursors are naturally produced in the brain whereas this is not the case in insects. Therefore, fly food is supplemented with ATR for the deployment of optogenetics.

For each setup a previous test with a negative and a positive control was performed to confirm that the setup was working properly. Concomitantly, a positive control was always tested during the screen to check that the setup was working reliably across periods of days.

Light parameters were calibrated until the positive control showed a robust effect size relative to the negative control.

norpA^{P24};20xUAS-CsChrimson and *norpA^{P24};Gr28bd+TrpA1>Chrimson* were the negative and positive controls, respectively. Only in the red lit T-maze a different positive control was deployed, *norpA^{P24};Gr66a>Chrimson*, which expresses CsChrimson in bitter taste neurons and its activation has been shown to be aversive (Aso, Sitaraman, et al., 2014). The combination of *Gr28bd* with *TrpA1* drivers showed stronger phenotypes than *Gr66a* in our experiments and therefore was used for further screens. Whereas *Gr28bd* expresses in the hot cells in the arista of the antennae, *TrpA1* expresses in the heat sensitive neurons in the inner brain AC neurons (Galili et al., 2014; Geffeney, 2017).

Light was measured with a lux meter (table S1) which was calibrated and transformed from lux into µW/mm² with the following scripts: https://github.com/chiser/light_conversion/blob/master/lux2watts.R (fig. S1). To confirm that the light spectrum specified in the light source data sheet matched the real light spectrum, the stimulation light was with the spectrometer (table S1). This was necessary because materials such as light guides or T-maze tubes might modify the light spectrum when the light travels across them. A list of the lines used in this study is available in table S2.

2.3 T-maze

The T-maze was composed of a core and three insertable tubes. The core contained an elevator for transfering the flies from the entrance tube to the choice tubes, where they had two options: approach the dark arm or the arm with the optogenetically stimulating light (fig. 2). LEDs were adjusted for the light stimulation with a frequency generator and a power supply (table S1). LEDs were glued to a cooling plate to avoid overheating.

Whereas CsChrimson is sensitive to yellow and red light, GtACR1 and GtACR2 are both sensitive to blue and green light. Two activation screens, one with red- and another with yellow light, were performed (fig. 2). One additional experiment was performed with blue light to inhibit neurons that showed robust reinforcement across all paradigms. The light intensities were 1600 lux, 1000 lux and 2500 lux for the red- (660nm), yellow- (590nm) and blue (480nm) lit T-maze experiments respectively. The rest of the parameters were the same for the three T-maze experiments: 10ms pulse width, 20Hz stimulation frequency and 9.9ms cycle duration with 1ms delay.

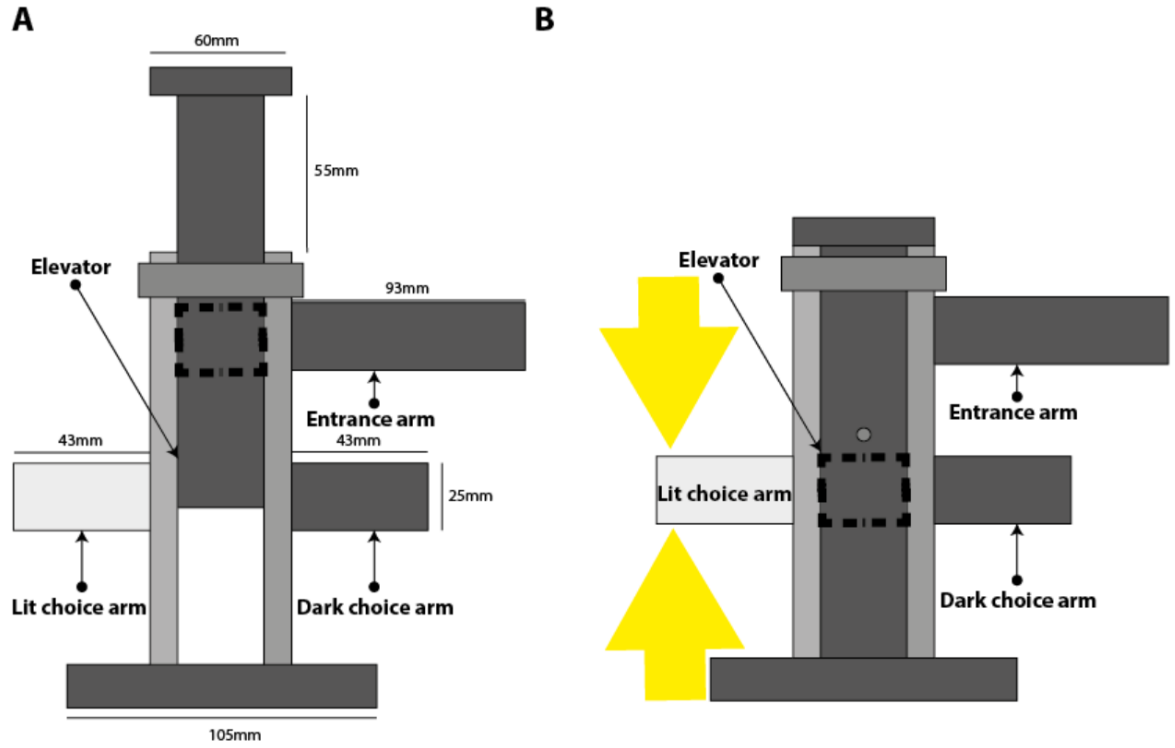


Figure 2. T-maze schematics. A: Thirty or more flies were introduced in the entrance arm for the experiment. By tapping the T-maze, flies were introduced into the elevator, which then was shifted to a middle point for 30 seconds for the adaptation of the flies. **B**: The elevator was then pushed all the way down, letting the flies move freely within the choice arms. After one minute, flies in each arm were tallied under CO^2.

The same groups of flies were tested in two consecutive days for the screen with red light as well as in the inhibition experiments with GtACRs. Since the counting was done under CO^2, and CO^2 exposure is detrimental for the flies, a day for recovery was left before the second experiment (Barron, 2000; MacAlpine et al., 2011; Perron et al., 1972). Experiments with the yellow light were conducted without repetition and blindly by two experimenters in parallel, to obtain an estimation of the handling variability.

2.4 Y-mazes

The setup consisted of a behavioral box with a rig (30x35cm) with 120 Y-mazes. The rig was backlit with an IR LEDs panel (table S1) and a diffuser to scatter light homogeneously (as in Buchanan et al., 2015; Werkhoven et al., 2019). In each Y-maze, a single fly could freely explore the three arms, one of which was illuminated with the optogenetically stimulating light (fig. 3).

The positive control showed the strongest phenotypes at 80% and 100% of the maximum projector light intensity, therefore the light intensity was set to its maximum. Light was set at [1 0 0] (RGB code) and its spectrum ranged from 570nm to 720nm with a peak at 595nm. Light stimulation frequency was set at that of the projector (75Hz), and sampling

frequency was set to 37.5 Hz, half of the refresh rate of the projector (table S1), to avoid light display inaccuracies. The duty cycle was 50%.

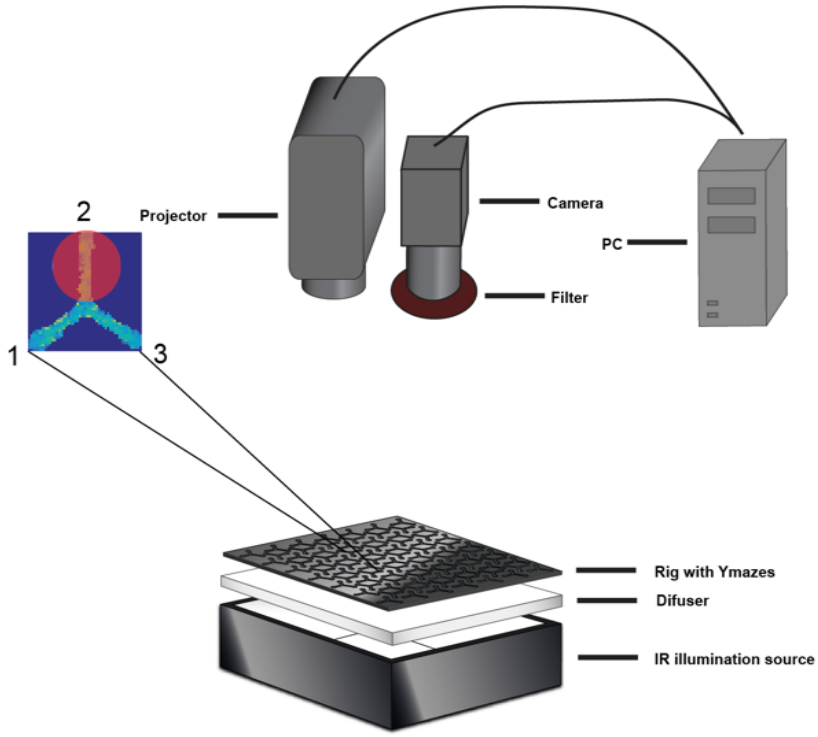


Figure 3. Y-mazes setup schematic. The behavioral box below with a zoomed-in view of a single Y-maze. The enlarged Y-maze contains the arm number as well as a red dot to simulate the stimulation light. A PC processed the camera recording online and commanded the projector for closed-loop feedback. The experiment protocol consisted of a total of 60 minutes test where each of the arms was reinforced for 20 minutes by displaying light on the arms whenever the fly entered the given arm.

Fly behavior was recorded with a digital camera and further processed with background subtraction in Matlab (Mathworks) to obtain simultaneous tracking of the 120 flies. A 850nm long pass filter (table S1) was placed in front of the camera to avoid interference of the stimulating light in the tracking.

All the Matlab scripts were run under the Matlab 2015a version in Windows 7. The projector stimulation patterns were designed with Psychtoolbox-3 toolbox (http://psychtoolbox.org/) with its third-party dependencies and an Nvidia graphic card.

Closed loop stimulation with the projector demand enough spatial resolution to display light in specific Y-maze arms. This required the pixels of the projector to match those on the camera, for which the projector displayed a black and white pattern on a white surface that was consequently captured by the camera. By knowing the coordinates of the projected patterns and recording these patterns with the camera, the corresponding camera-projector pixels were registered. For every calibration procedure, at least an R^2=0.9998 in the projector-camera pixels correlation was accomplished (approx. pixel precision).

Acquisition software scripts & parts list: https://github.com/chiser/autoTrackerV2-old-version-

Registration scripts: https://github.com/chiser/Registration-Camera-Projector.

Y-mazes video example: https://www.youtube.com/watch?v=S8uVpEOMojU.

2.5 Joystick

Tethered flies are positioned on a flexible platform that measures their leg postural lateral force (fig. 4). For the tethering, a piece of 0.7mm diameter fishing line (table S1) is glued on the dorsal side of the thorax, which is used to fix the fly to a clamp. A light guide attached to the clamp collects and directs the stimulating light to the fly head. The fly is carefully positioned with micromanipulators on the platform and inspected for proper motor activity. A photoelectric barrier detects the platform position and sends an analog signal (-5 to +5V) to the Analog-Digital converter.

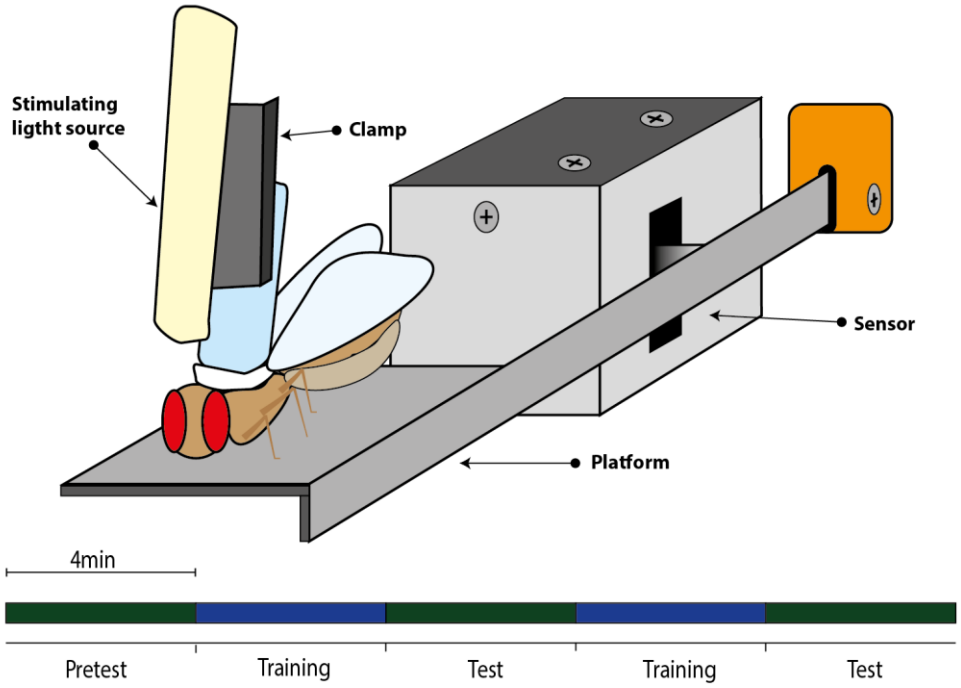


Figure 4. Joystick schematics. In a spaced training protocol, we alternate open- (green) and closed loop (blue) four-minute periods. In the closed loop/reinforcement period flies are trained by turning on the stimulating light when the fly pushes the platform to a specified side. The experiment protocol lasts for 20 min with alternating 4 min segments of Pretest, Training, Test, Training, Test as shown in the figure. The reinforced side (right/left) was alternated at each consecutive experiment.

The closed loop stimulation is gated by a microcontroller (Arduino Uno). Light guides are glued to the LEDs on one end and straight fixed next to the clamp directed to the fly head. The stimulating light intensity was 400 lux, 20 Hz, 50 ms pulse width and no cycle delay. The data acquisition software is custom written in Visual Basics (Microsoft) with a 20

Hz sampling rate. As in the T-maze with yellow light, the fly line identity was blinded to experimenters to avoid *ad hoc* bias. The experiment was conducted by two experimenters in parallel.

Joystick example video: https://www.youtube.com/watch?v=z2uOIVYrC0o.

The instructions sheet with the software scripts by Johann Schmid (modified from Mariath, 1985; Wolf et al., 1992): https://github.com/chiser/Joystick-acquisition-software.

2.6 Data analysis

Since the raw data format was different for each experiment, data was differently analyzed to obtain a score that allows comparison across setups. Hence, every score ranges from -1 to +1, where negative scores indicate light avoidance, positive scores approach, and close to zero scores indicate no preference for the light. For the T-maze we calculated a preference index:

$$PI = \frac{x - y}{x + y} \tag{1}$$

where x are the number of flies in the light and y the number of flies in the dark. The flies that did not make any choice and stayed in the middle were not considered in the formula for two main reasons: locomotor deficits would impair the flies approaching any of the arms and thus not show their preference and It is questionable whether the light intensity in the middle is over the activation threshold for CsChrimson. From the single experiment PIs we calculated an arithmetic mean and a weighted mean:

$$\overline{PI} = \frac{\sum_{i=1}^{n} PI_i}{n} \tag{2}$$

$$weighted\overline{PI} = \frac{\sum_{i=1}^{n} \beta_i PI_i}{n} \tag{3}$$

Where n is the total number of experiments and β denotes a weight proportional to the number of flies that took part in the experiment (normalized to yield a mean of 1). For the Y-mazes we had different time-stamped measures: speed, arm location and arm entry for which ratios were calculated. Only flies with at least 14 turns/arm changes were used for the analysis and speed was downsampled through window average to 3.75Hz to reduce noise.

$$Speed\ ratio = \frac{x - y}{x + y} \tag{4}$$

$$Occupancy\ ratio = \frac{x - y}{x + y} \tag{5}$$

$$Entries\ ratio = \frac{x - y}{x + y} \quad (6)$$

where x refers to mean speed, time spent, and number of entries in the lit arm for the three equations respectively whereas y refers to the mean of the mentioned parameters in the dark arms, respectively. For the Joystick we calculated the PI for each of the experiment periods by:

$$PI = \frac{x - y}{x + y} \quad (7)$$

where x is the number of data points in the lit side and y the number of data points in the dark side. We averaged all of the Reinforced periods PIs and subtracted pretest PI, to normalize for the intrinsic bias. In addition, we measured the platform wiggle as a proxy for locomotor activity:

$$wiggle = \sum_{n=1}^{N} \left| \frac{dx}{d(t - 20)} \right| \quad (8)$$

where x is the time-stamped platform position and t the time stamps. The time series was differentiated with a lag of 20 to capture the wiggle at a slower time scale that corresponded more closely to the fly behavior. We subtracted the wiggle scores during lights off to when lights were on, obtaining a ratio that is positive when flies move more during neuronal activation, and vice versa.

Power analysis was only performed for the T-maze with yellow light and the Joystick screens to predetermine the sample size. We performed a one-tailed t-test for the positive against the negative control, where the power was set to 80% and the significance to 0.05.

The estimated number of experiments for the T-maze was eight, however, since we expected smaller effect sizes for our experimental lines, we decided to do 12 experiments per line. For the Joystick the power analysis resulted in 15 experiments, and due to the time limit constraints, we kept it at 15 experiments for each line.

Most of the analysis and plotting was done in R version 3.4.2 (https://www.R-project.org) except for the analysis for the Y-mazes that was done in Matlab 2015a (Mathworks).

Analysis scripts URLs:

- Power analysis: https://github.com/chiser/power-analysis

- Y-mazes: https://github.com/chiser/matlab-analysis-on-operant-reinforcement

- Joystick: https://github.com/chiser/screen-analysis-for-yellow-Tmaze-and-Joystick.

- T-maze: https://github.com/chiser/T-maze-drosophila/tree/master/Tmaze

Tmazeplottingrepetitions.r was used for the screen with red light.

Tmazeplotting.r was used for the screen with yellow light.

All data acquisition software and analysis scripts are available at https://github.com/chiser.

All raw data can be found at https://doi.org/10.7910/DVN/RETZPG.

2.7 Neuronal clusters valence estimation

Linear models were performed to solve a system of equations with one equation per tested line. Neuronal clusters valence were the unknown variables which were weighted by the corresponding expression level from the given line, whereas the behavioral score is the response variable. Different model combinations were tested: bayesian versus frequentist, linear versus nonlinear, with- versus without interactions. Whereas in linear models the effect is proportional to the expression level, in nonlinear models we fitted nonlinear basis functions to the relation expression-effect.

Complex models tend in general to overfit the data, hence goodness of fit that takes model complexity into account, like Akaike Information Criteria (AIC), Deviance Information Criteria (DIC) and Bayesian Information Criteria (BIC) were chosen. Bayesian methods were prioritized due to their robustness to overfitting.

Analysis scripts can be found in:https://github.com/chiser/estimating-dopaminergic-clusters-valences/blob/master/modelling_scores.Rmd.

2.8 Anatomical characterization

GAL4 lines were crossed with *w⁻;6xUAS-20xGFP* flies. Fly brains were fixed in 4% paraformaldehyde for 2hs at 4°C, placed on microscope slides and mounted with antifade mounting medium (Vectashield□). Image acquisition was performed with a Leica SP8 confocal microscope. Images were scanned at a frame size of 1024x1024 pixels with a 40x oil immersion objective. Confocal stacks were viewed and analyzed using the ImageJ software. Only general adjustments to contrast and brightness were made. Anatomical identification was accomplished with the help of the Virtual Fly Brain website (www.virtualflybrain.org).

3 Results

3.1 Driver lines expression analysis

Since the *GAL4*s were obtained from different studies, some focusing on classical learning (Aso et al., 2010, 2012; Aso, Hattori, et al., 2014; Aso, Sitaraman, et al., 2014; C. Liu et al., 2012) and some on sleep/arousal (Galili et al., 2014; Q. Liu et al., 2012; Pathak et al., 2015), two different expression tables were generated. The former contains driver lines with their soma at the paired anterior medial cluster (PAM) and paired posterior lateral 1 & 2ab clusters (PPL1 and PPL2ab) projecting to the MB compartments (fig. 5A), whereas the latter contains dopaminergic lines with broader projection sites (fig. 5B).

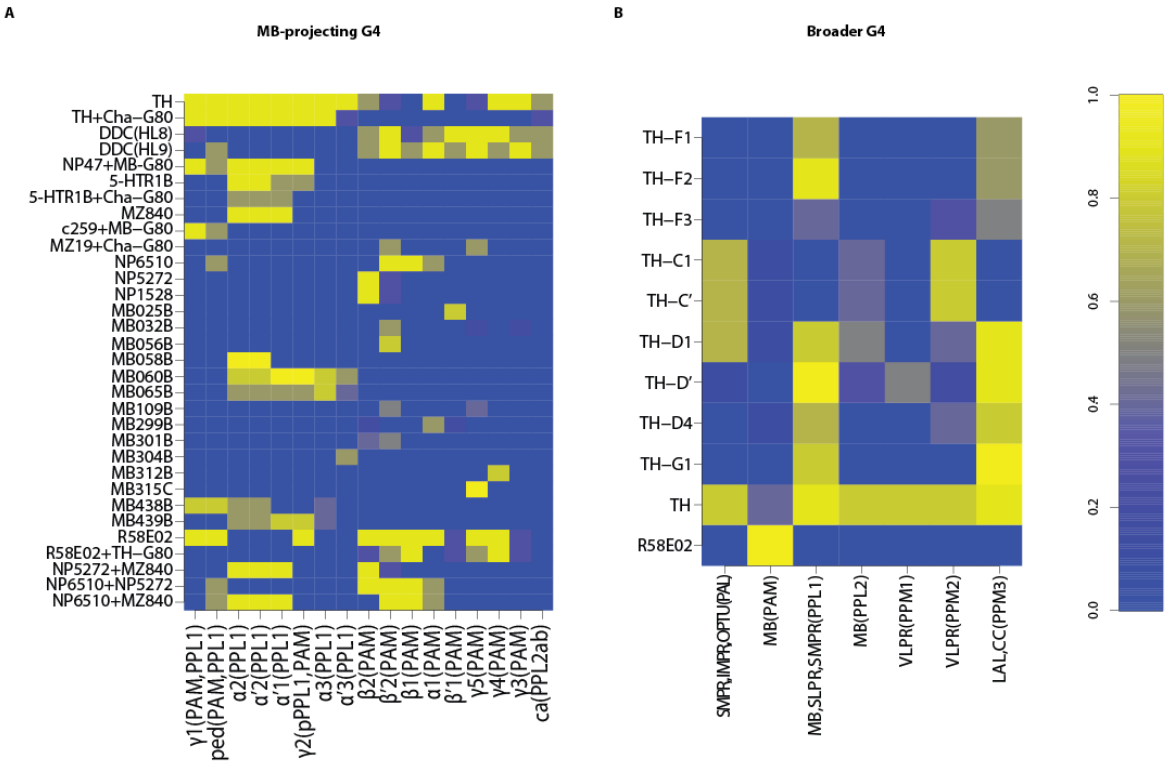


Figure 5. Dopaminergic driver lines expression pattern. On the Y-axis are the driver line names and on the X-axis each of the targeted neuronal clusters can be found. DANs are depicted by their projection sites and their cell body location (within brackets). Colorbar at the right-hand side shows normalized expression intensity from zero to one. **A**: Driver lines obtained from Aso et al. 2010, Aso et al. 2012, Aso et al. 2014 with their corresponding expression pattern. **B**: Driver lines from Liu et al. 2012. The expression pattern was estimated from the following studies: Liu 2012, Galili 2014 thesis, Pathak 2015.

Bearing in mind that protocol variations for immunohistochemistry yield different expression patterns, a summary of several studies was produced to find a consensus for the expression values for the broader expressing driver lines (fig. 5B). For a more detailed description of the generation of these driver lines see Supplemental Information in Liu 2012.

3.2 T-maze screen with red light

As explained in the methods section, a pilot experiment was performed where the positive control, *norpA^{P24};Gr66a>Chrimson*, was tested with and without ATR supplementation. As expected, the negative PI scores indicate light avoidance of the positive control and therefore indicate that the setup is functioning as expected (fig. 6).

Although we reared between 30 and 40 flies in each experimental glass, due to the handling some of the flies would not survive or would escape the test. Hence, there was a fluctuating number of flies taking part in each experiment. To test whether different numbers of flies affect the PI, a weighted PI, where the contribution to the final score was directly proportional to the number of flies that took part of the experiment, was calculated. A sharp correlation between weighted and unweighted PI analysis (r=0.99; adj. R^2=0.95; p=2.43^{-15}) indicated that weighting the PI made no difference to the result. The standard PI was chosen for further analysis, favoring simplicity since this is not detrimental.

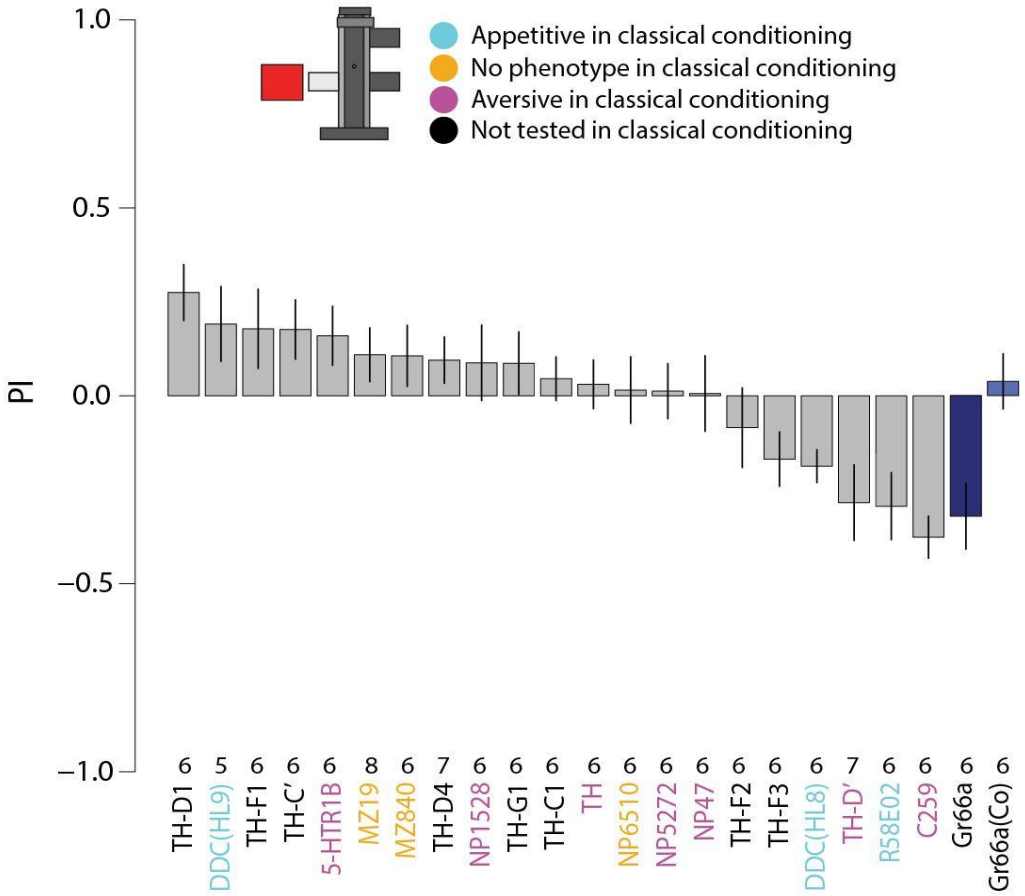


Figure 6. T-maze screen. Barplots depict each driver line means for each behavioral score in descending order with error bars depicting the standard error of the mean (SEM). Positive controls fed with and without ATR are coloured in dark- and light blue respectively. Number of experiments for each line is shown above each driver line label in the X-axis. Driver line fonts are color coded according to classical learning phenotypes as shown in the legend above. All lines contained a *norpA^{P24}* mutation which is omitted in the X-axis for simplicity.

The T-maze experiment with red light was performed twice for each group of flies to observe choice consistency between both tests (fig. 6). If flies chose differently every time they are exposed to the T-maze, one would not expect to find any correlation between first and second experiment. However, if flies developed a fixed preference for a certain neuronal activation, the first and second set of experiments should yield similar results. Two different analyses were performed on the correlations between the two tests: one across the means for each driver line, and another correlation retaining the identity of each single experiment. For the former we found a correlation ($r=0.55$; adj. $R^2 =0.55$; $p= 3.1^{-5}$), which indicates that the effects are consistent at the population level. However, for the latter there was no correlation (adj. $R^2 = 0.02$), suggesting that the effects are rather probabilistic and therefore difficult to observe in single experiments due to high decision variability.

To have an estimation of the effect of genetic background in our paradigm, the negative controls were bred in different stocks for several generations during approximately one year. Very soon after separating stocks, the fly genetic background tends to accumulate modifications that lead to divergence (Colomb & Brembs, 2014). A pilot experiment showed no effect of the genetic background in this assay. More details from these experiments are found in http://lab.brembs.net/2015/11/lab-report-optogenetics-a-screening-with-the-channelrhodopsin-chrimson/.

3.3 T-maze screen with yellow light

Since the wavelengths corresponding to yellow light activate CsChrimson more effectively, an additional T-maze screen was performed with yellow light (Klapoetke et al., 2014). The effect of octopaminergic neurons in approach/avoidance assays is dependent on stimulation parameters like frequency and intensity (Gerbera, 2018). Hence, differences between this screen and the red-lit T-maze experiments shown in the previous section might indicate if the same neurons can encode different information under different activation characteristics (fig. 7, table S3). As in the T-maze with red light, the positive control, in this case *norpA^{P24};Gr28bd+TrpA1>Chrimson*, showed a strong light avoidance, as expected, in the pilot experiments (results in http://lab.brembs.net/2018/06/tmaze-experiments-initial-results/).

Are the PIs of different lines influenced by their basal locomotor differences? This is relevant because different genetic backgrounds affecting locomotion might also influence the performance in a spatial task like this one. Movement is a necessary requirement for the flies to show their preference in the T-maze and moving less might for instance lead to more/less extreme scores. Since the starting point is the elevator, the amount of flies in the elevator at the end of the experiment was considered as a proxy for locomotor deficits. No correlation

was found between the measured PIs and the number of flies in the elevator at the end of the experiment (adj. R^2= -0.038), suggesting that basal locomotion does not influence T-maze scores.

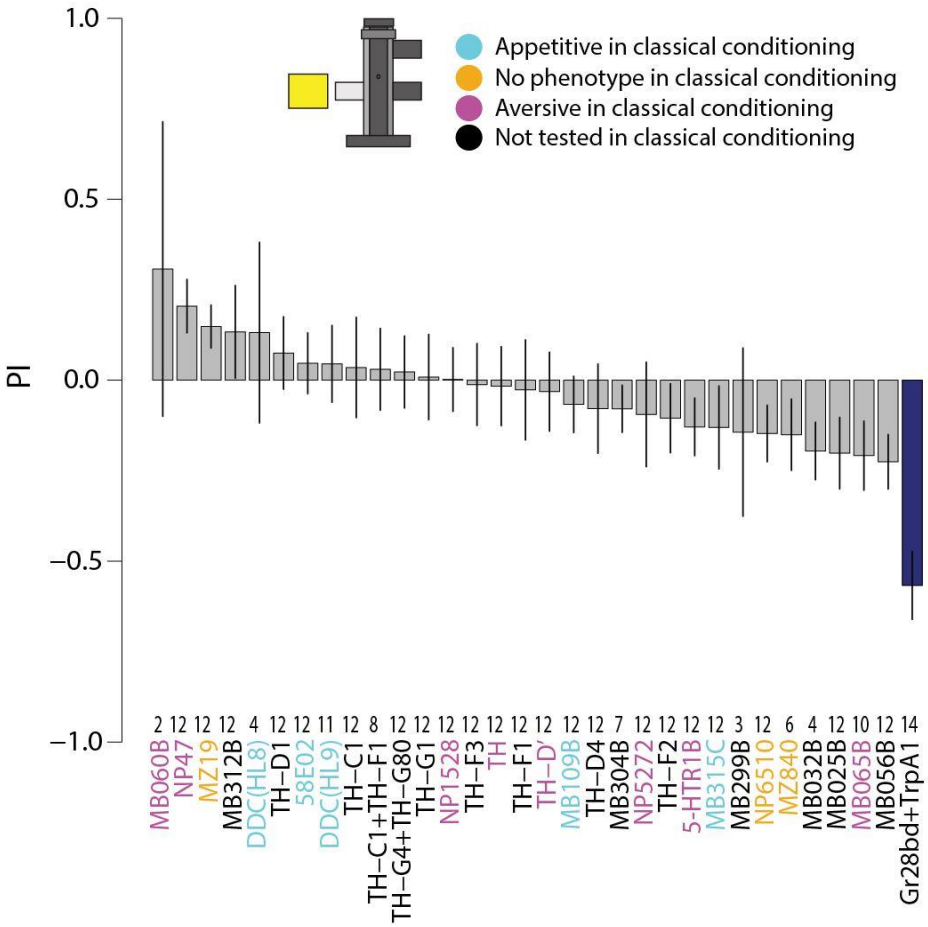


Figure 7. T-maze screen with red light. Barplots depict each driver line means for each behavioral score in descending order with error bars depicting the standard error of the mean (SEM). Positive control is coloured in blue. Number of experiments for each line is shown above each driver line label in the X-axis. Driver line fonts are color coded according to classical learning phenotypes as shown in the legend above. All lines contained a *norpA*^{P24} mutation which is omitted in the X-axis for simplicity.

Since the yellow-lit T-maze screen was performed in parallel by two different experimenters, it was interesting to see if there is consistent effect across experimenters. A correlation analysis showed no significance (adj. R^2 = 0.0086; p= 0.292 for linear correlation, and p=0.546 for Spearman´s rank correlation), indicating that overall, the yellow light creates no consistent effect on neuronal activation. To address whether the lack of consistency is due to the absence of effect size or due to a context-dependent effect further analysis was performed in sections 3.3.1 and 3.6.

3.3.1 Negative-effects screen simulation

T-maze experiments with negative controls were pooled (blind flies, either with only effector or only with any *GAL4* driver), summing up to a total of 49 experiments. The overall PI was not different from zero (fig. S2A). By sampling 12 times with repetitions from the experimental pool we can compare the obtained PI distribution to that of our T-maze experiments. The sampling with repetitions seems to be reasonable because of its unlikelihood in a real population of changes in the probability of an event after the occurrence. Fig. S2B shows the result of 32 bags of 12 samples, simulating the yellow light T-maze screen. Sampling from this null-effect fly line yield scores closer to zero, compared to the yellow lit T-maze screen (fig. 6B; fig. S2B). The presence of more extreme PIs in fig. 7 out of the range of the null effect lines suggests that neuronal manipulations in the yellow-lit T-maze certainly modify the choice biases.

3.4 Y-mazes Screen

This high throughput setup allowed to measure many single flies and to test more lines than in other setups. As explained in methods, it consists of a rig with Y-shaped mazes where single flies can choose to be in any of the three arms, one of which is illuminated with red light for optogenetic activation. As for the previous screen, the positive controls showed strong avoidance of the light (Werkhoven et al., 2019). The occupancy rates from this screen are shown in fig. 11.

Arm entries might be a favorable measure for valence because it is locomotion-independent and shows an active decision to self-stimulate. An initial experiment with non-blinded flies revealed occupancy-entries positive correlation whereas the addition of *norpA*^{P24} particularly obscured the entries effects, indicating that flies rely on their vision to navigate through the maze (Werkhoven et al., 2019). Furthermore, arm entries rate did not show any correlation to occupancy rate in the performed screen (adj. $R^2 = 0.07$; $p=0.046$), nor to speed (adj. $R^2 = 0.052$; $p= 0.082$) neither at the population level nor at the single lines (fig. S3). Although the presence of *norpA*^{P24} was a major hindrance for the flies to navigate, the positive correlation of entries and occupancy in seeing flies indicates that occupancy rates are a good proxy for valence. Moreover, occupancy rate is more similar to T-maze PI, where its locomotory effects cannot be separated from its valence.

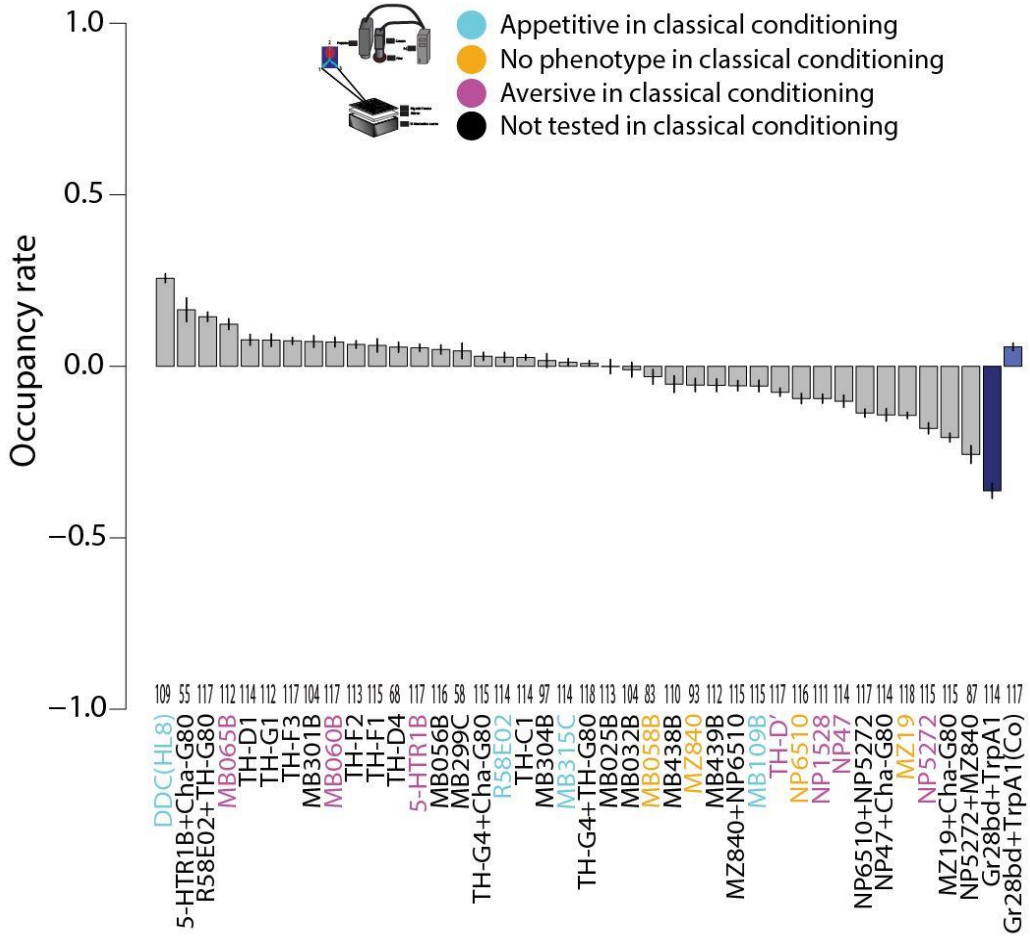


Figure 8. Results from the operant behavior screens. Barplots depict each driver line means for each behavioral scores in descending order with error bars depicting the standard error of the mean (SEM). Positive controls fed with and without ATR are coloured in dark- and light blue respectively. Number of experiments for each line is shown above each driver line label in the X-axis. Driver line fonts are color coded according to classical learning phenotypes as shown in the legend above. All lines contained a *norpA*^{P24} mutation which is omitted in the X-axis for simplicity.

A tight inverse correlation between occupancy and speed rates (r=-1.57, adj. R^2 = 0.94, p= < 2.2^{-16}) indicate that flies that avoided the lit arm also tend to run faster in this arm and vice versa. To investigate whether neuronal activation directly induced effects in walking speed, yoked experiments were carried out. Yoked experiments consist of a paired experiment where one individual controls its own feedback as well as that for the paired individual. This allows the former individual to learn from behavioral outcomes whereas this is not possible for the latter individual. Thus, half of the flies were tested with one of the arms lit, whereas the other half of the flies received light stimulation according to the ubication of the paired fly rather than based on their own ubication. These results unambiguously reveal that neuronal activation changes walking speed (fig. S4). In summary, neuronal activation induces changes in occupancy and speed. Running more in a specific Y-maze arm might lead to leaving that arm sooner, thus affecting occupancy scores. However, experiments in

non-blinded positive controls reveal that neuronal activation has a valence effect that is independent of locomotion.

3.5 Joystick screen

In contrast to the previous paradigms, in the Joystick flies are tethered, which might facilitate the measure of a locomotion-independent valence (fig. 9). To avoid handling biases we corrected the training PI by subtracting the pretest PI from it (as explained in Methods). Reinforcement PIs with and without pretest normalization were only slightly correlated (adj. R^2=0.10; p=0.059), indicating that pretest biases have a strong effect in reinforcement scores.

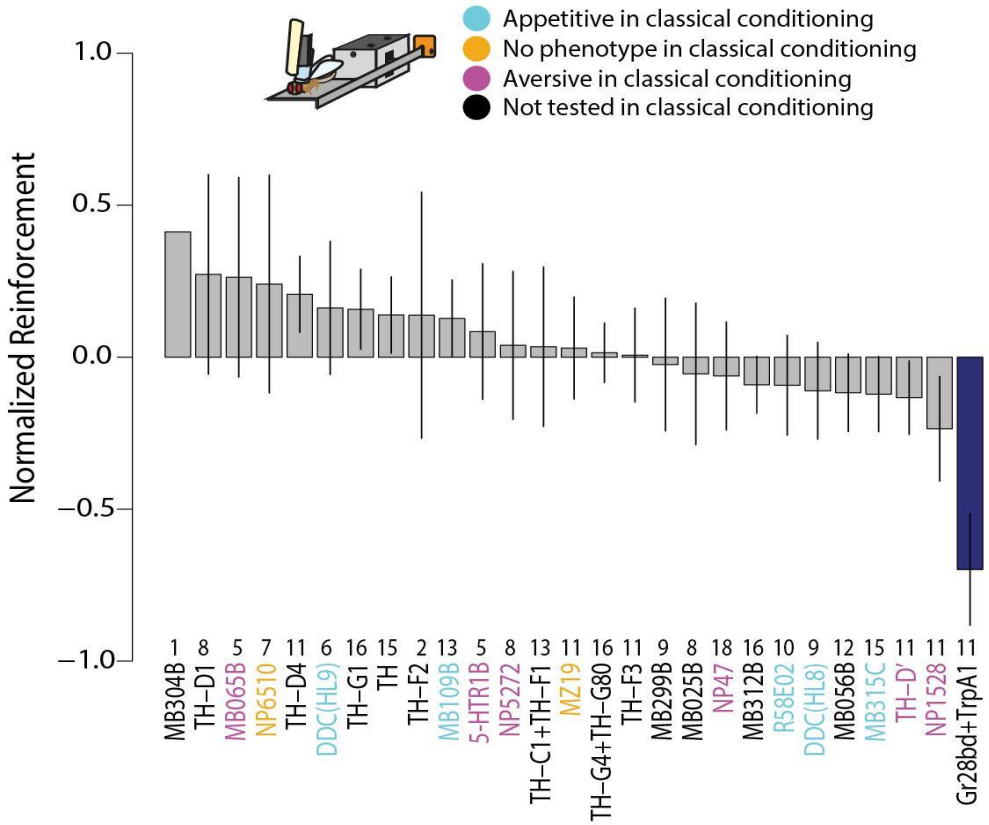


Figure 9. Results from the Joystick screen. Barplots depict each driver line means for each behavioral score in descending order with error bars depicting the standard error of the mean (SEM). Positive control is coloured in blue. Number of experiments for each line is shown above each driver line label in the X-axis. Driver line fonts are color coded according to classical learning phenotypes as shown in the legend above. All lines contained a *norpA*^{P24} mutation which is omitted in the X-axis for simplicity.

For assessing the correlation between locomotion and valence, as it was done for previous setups, a correlation analysis between wiggle and reinforcement scores was carried out. Contrary to the Y-mazes, there was no correlation locomotion-valence and hence it is a paradigm suitable for segregating these two features (wiggle versus normalized reinforcement adj. R^2=0.01; p=0.27).

3.6 Context-dependency: Effect on the mean and variance

All the results from the performed screens were uncorrelated (fig. 10; table S3 for statistics). The reason behind this could be: either these neurons do not encode for reinforcement, or the reinforcement is context-dependent and thus, effects vary across setups. To address this, we analyzed how much these phenotypes deviate from zero for each line and compared them to the negative controls.

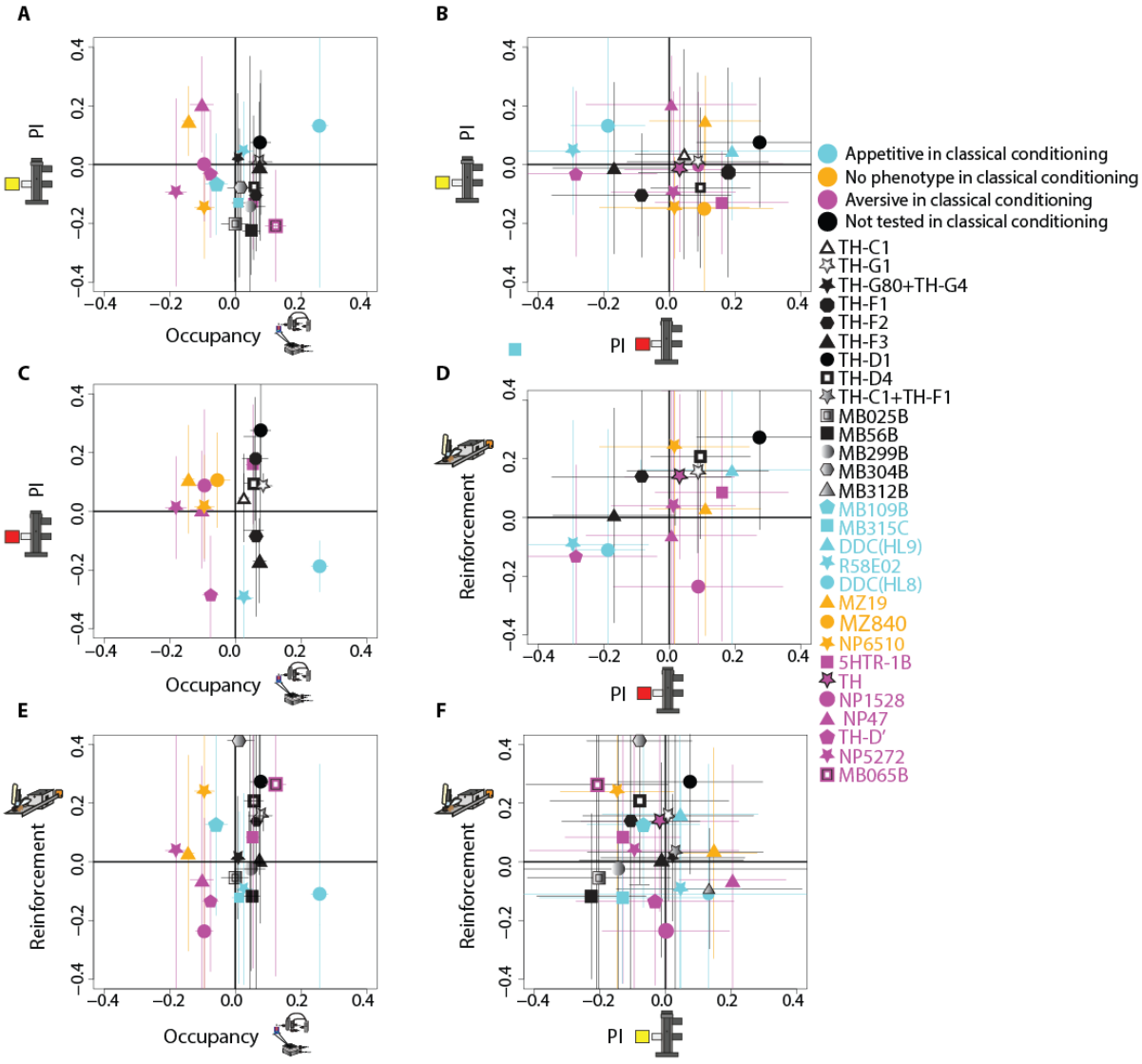


Figure 10. Double comparison between operant behavior paradigm scores. A-F: Eight combinations of biplots projecting two behavioral axes. Axes ranges were truncated to -0.4 to +0.4 for visualization purposes. Legend on the right, with colours indicating classical learning phenotypes and a corresponding symbol for each driver line. Only lines that were tested in both paradigms are shown in the graphs.

If the neuronal manipulation has no effect, one would expect close to zero variance across experiments for all the lines tested. This variance will only be dependent on the noise, which should in principle affect all lines to the same degree. However, if the manipulation has an effect, we would expect an increment of this value proportional to its

effect size. Interestingly, our negative control, *TH-G4+TH-G80*, never showed extreme phenotypes in any of the four screens performed, contrary to the two positive controls, Gr28bd+TrpA1 and Gr66a (fig. 11).

Since context-dependent behavior shows variable phenotype, activation of these neuronal populations might not shift overall means but variance across paradigms. Hence, we expect the lines with higher scores in fig. 11 to be the more influenced by the situation.

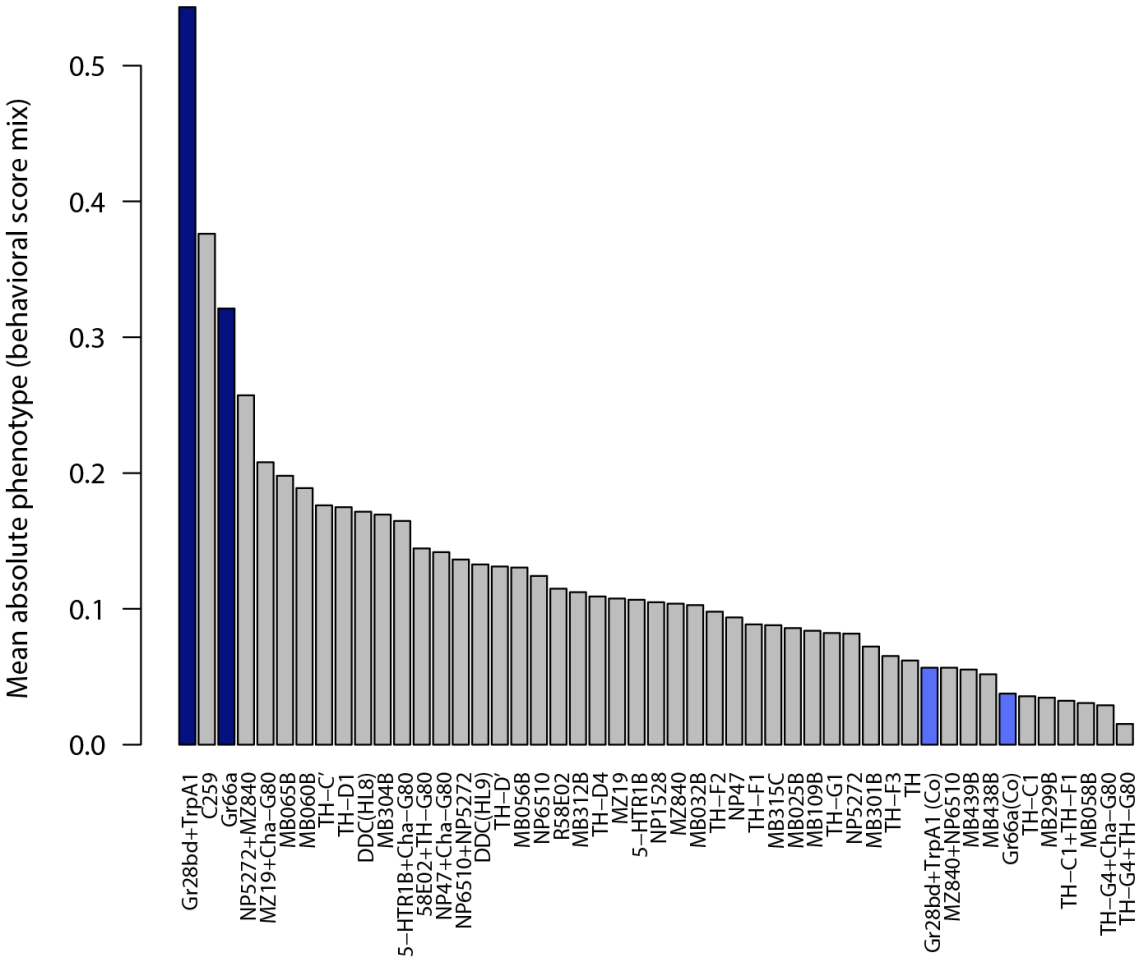


Figure 11. Mean absolute phenotype score across the four operant paradigms. Lines are ordered from higher to lower absolute phenotype. These scores consist of an average of the unsigned behavioral scores for each fly line. Dark blue are positive controls and light blue positive controls without ATR supplement.

3.7 Context-independent reinforcers

Previous analysis indicated that reinforcement is predominantly influenced by the context. However, our interest lies in the neuronal manipulations that produce consistent effects, that is, the general reinforcers. We focused on lines with big phenotypic effects and balanced contribution from each experiment. TH-D', TH-D1 and TH-G1 were the only lines with consistent avoidance/attraction across the four tests (fig. 12). Unfortunately, not all lines were tested in all setups due to different technical reasons. Considering that only lines with

four consistent results were chosen, some interesting lines might have been missed, which is a major setback of this study. Nevertheless, this data is still valuable to select promising candidate lines in more detail in the future.

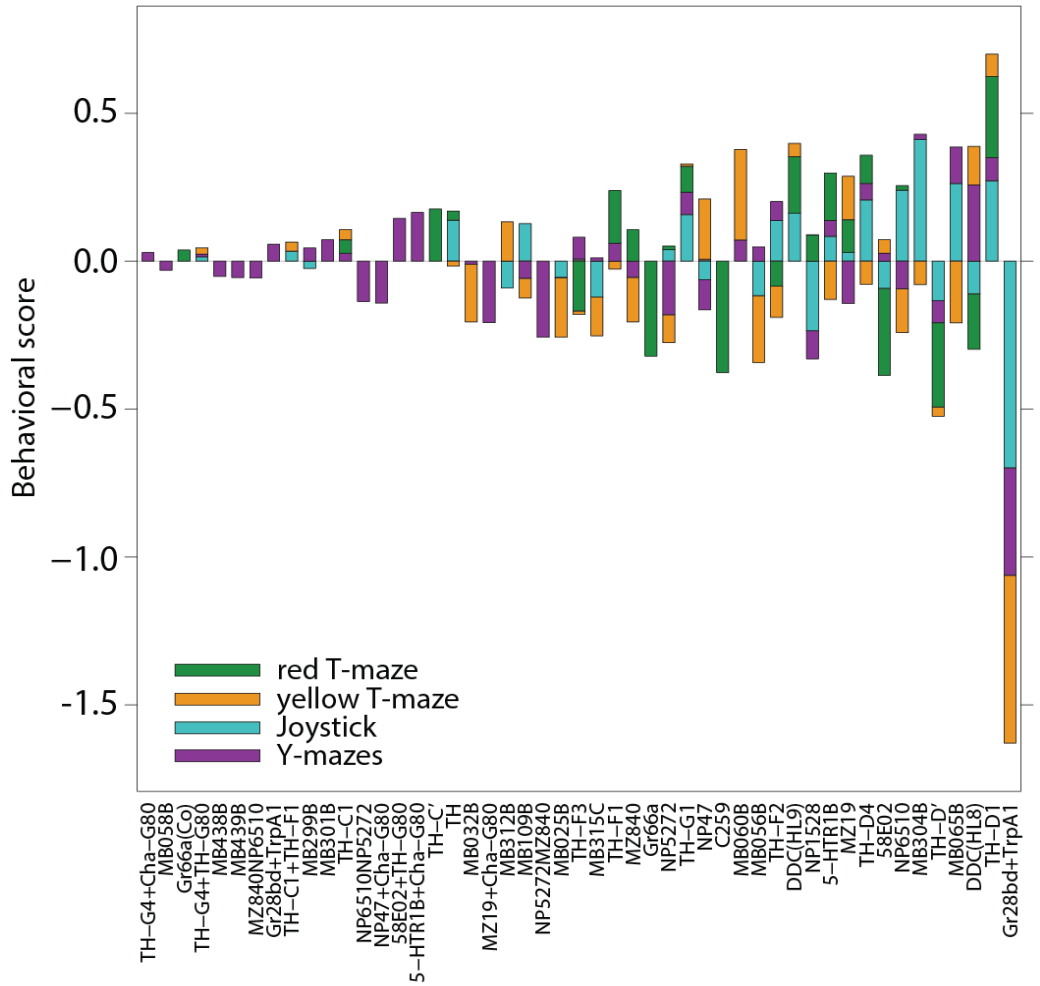


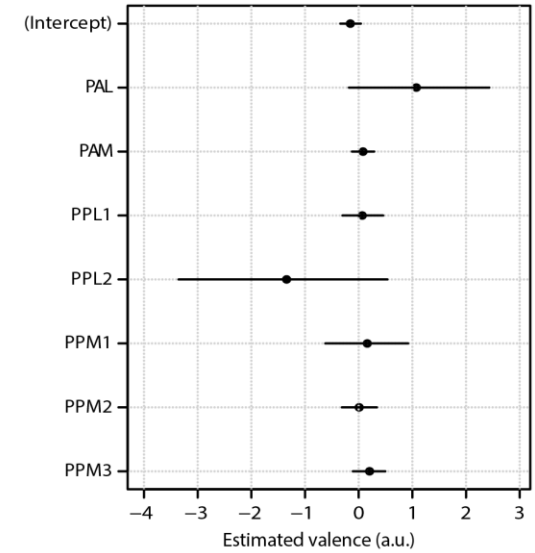
Figure 12. Superimposed barplot with the driver line scores for each experiment. On the Y-axis the corresponding score unit for each paradigm in arbitrary units. On the X-axis the driver lines tested across all screens. Note that not all lines were tested in all paradigms for different reasons. Legend below left show colours attributed to each paradigm score.

3.8 Estimation of the valence of dopaminergic clusters

We created a model with the lines broadly targeting dopaminergic clusters (fig. 5B) to have a numerical estimate of the valence of these populations. The overall behavioral score from the previous section was chosen as the response variable, since it combines data from the four screens, emphasizing the effect of consistent lines. AIC, DIC and BIC are relative values for goodness of fit, therefore one cannot consider them alone but in comparison with other models. Models without interactions yielded lower AIC/DIC/BIC scores, supporting the idea that the effect of these neurons is exclusively additive (fig. 13B).

Since neurons often work in a nonlinear fashion (Benda et al., 2010; Birman, 2005; Zhang et al., 2013), we modelled them with nonlinear basis functions. Using a general additive model (GAM; *gam* function in R) and linear models (*lm* function in r) yield the same goodness of fits, probably because GAM failed to fit nonlinear basis functions to the linear model variables (fig. 13B). We therefore assume that the effect of these neurons is approx. proportional to the expression level. Allowing ourselves to compare AIC and DIC equally, bayesian models fitted slightly better than frequentist models (fig. 13B). Considering adj. R^2 is an absolute fit score where approx. one would be a perfect score, an adj. R^2 of 0.3 seem to be a low score. This means that these models are probably not capturing properly the behavior of these neurons or, the measured effect of these neurons is very noisy.

A



B

R function	Interactions	AIC	BIC	LogLik	Adj.R^2	DIC
bayesglm	-	-51.801	-37.063	34.9004 (df=9)		
	+	-12.112	37.016	36.0559 (df=30)		
gam	-	-53.642	-38.904	35.8209 (df=9)		
	+	-51.310	-33.297	36.6551 (df=11)		
lm	-	-53.642	-38.904	35.8209 (df=9)	0.3091	
	+	-51.310	-33.297	36.6551 (df=11)	0.2916	
MCMCglmm	-					-52.309
	+					-50.018

Figure 13. Valence estimation of major dopaminergic neuronal clusters. A: On the Y-axis the major dopaminergic clusters and on the X-axis the valence scores in arbitrary units (a.u.). The dots represent posterior means with 95% credible intervals bars. **B**: Different goodness of fit values for the different models tested. The lower the AIC/BIC/DIC value, the better the trade-off information-overfitting. Note that some of the fields are empty because the R function would not calculate these. bayesglm: bayesian general linear model; gam: general additive model; lm: linear model; MCMCglmm: Markov Chain Monte Carlo general linear mixture model.

All valence estimates credible intervals were overlapping with the zero line. Thus, we could not be be certain of any neuronal effect. The low adj. R^2 suggests that the effects might not have been captured by our model. Hence, only focusing on the interesting lines might give a better characterization for reinforcement neuronal correlates, which we will deal with in the next section.

3.9 Anatomical characterization of the context-independent reinforcers

Interestingly, the three lines of interest highlighted the same regions although it was not possible for us to assure if they labeled the same neurons (fig. 14). Morphological features at the light microscope-level resolution are often insufficient to distinguish cell types and additional information about synaptic connectivity and genetic expression profiles are necessary to fully define cell identity (Wolff & Rubin, 2018). The cell bodies labelled belonged mostly to dopaminergic clusters PPL1 and PPM3. TH-D1 and TH-D' labelled additionally PPM1 cluster neurons that project to the ventrolateral protocerebrum (White et al., 2010). Unfortunately, different microscope settings during image acquisition did not allow comparison for absolute intensity, and we therefore compared intensities relative to other brain areas.

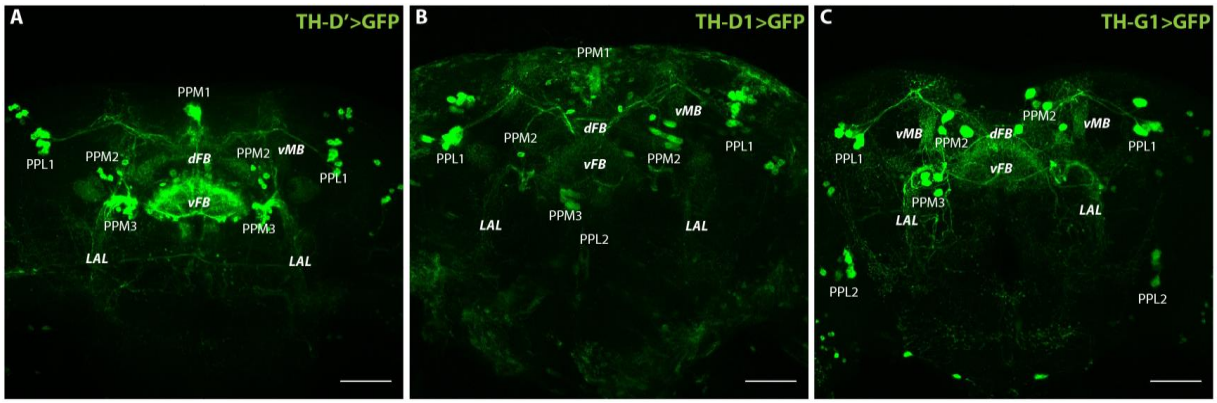


Figure 14. Staining of the brain regions for reinforcement. GFP staining for TH-D' (**A**), TH-D1 (**B**) and TH-G1 (**C**). The cell clusters and neuropils (bold italics) are in white. PPL: paired posterior lateral; PPM: paired posterior medial; dFB: dorsal fan-shaped body; vFB: ventral fan-shaped body; LAL: lateral accessory lobe; vMB: vertical lobes of the mushroom bodies. Scale bar = 50µm.

At the neuropil level, the three lines labeled specific layers in the FB. The PPM3-vFB (2-4 layers) and noduli was particularly intense for TH-D' and TH-D1. Unfortunately, the penetration of our anti-nc82 antibody, used to mark synaptic regions, was not enough to distinguish subdomains in central brain regions. We believe that TH-G1 stains a more medial layer, maybe fifth or sixth. An additional FB layer from the PPL1-dFB projection, was stained by all the drivers. The signal in the ellipsoid body (EB) is stronger in TH-G1 and TH-D1 whereas the LAL is targeted in all lines (fig. 14). TH-G1 stained conspicuously what we believe is the prow, located within the subesophageal zone. The MB vertical lobes and pedunculus projection from the PPL1 was clearly distinguishable in the three lines. TH-D1 had more unspecific targets whose effects are not known.

3.10 Optogenetic inhibition of dopamine neurons

To gain more insights into the physiology of the reinforcement candidate neurons we performed an additional experiment with two of the candidate lines: TH-D' and TH-D1. Instead of letting the flies choose whether they want to depolarize neurons with CsChrimson, this time they had to choose whether they want to hyperpolarize the neurons with gtACR (fig. 15).

Flies expressing inhibiting channelrhodopsins, gtACR, in TH-D' neurons, chose to approach the light. Thus, whereas TH-D' activation is avoided (fig. 6-10; fig. 12) its inhibition is appetitive (fig. 15), depicting a preference for reduced activity for these neurons. Interestingly, flies with *TH-D1>gtACR*, which approached light in activation experiments, also did so in inhibition experiments. In this case, the neuronal inhibition as well as the activation was chosen by the flies.

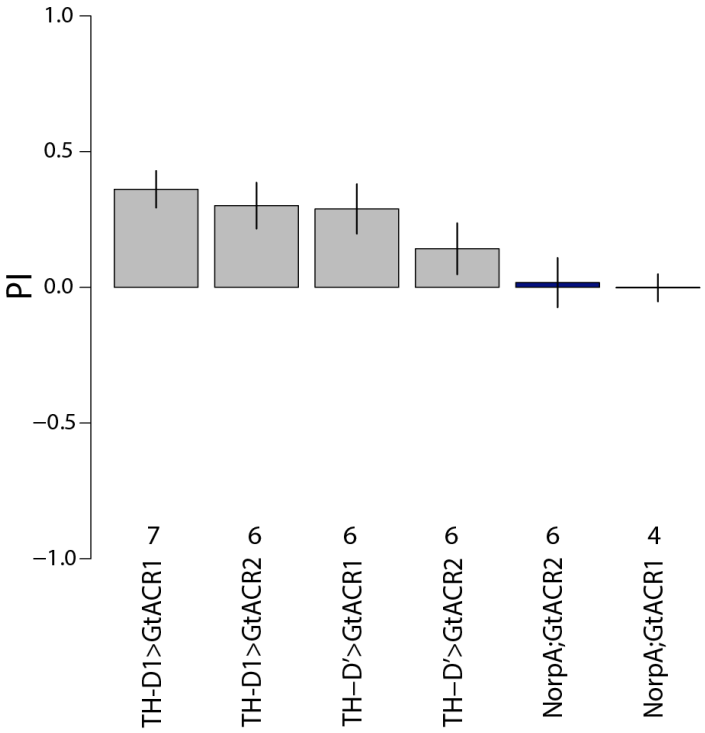


Figure 15. Preference for inhibition of context-independent reinforcers and punishers. Barplot indicating the mean score with error bars the standard error of the mean (SEM). In blue the negative controls and in grey the experimental lines. On the Y-axis, a positive PI indicates approach to light and hence, preference for inhibition. Number of experiments for each line depicted above the fly line names.

4 Discussion

4.1 The neural circuits underlying classical and operant conditioning

4.1.1 The role of the MB

The MB are the major center where gustatory, olfactory and visual classical conditioning takes place, with a strong predominance of olfaction (Aso et al., 2010; Kirkhart & Scott, 2015; Vogt et al., 2016). This is supported by the anatomy, since abundant olfactory input converges into the MB calyx. The other major input constitutes dopaminergic neurons, which project to the MB lobes. Their activity encodes internal states and values of sensory cues shaped by the instantaneous circumstances and previous experiences (Aso, Sitaraman, et al., 2014; de Belle & Heisenberg, 1994; Hige, Aso, Modi, et al., 2015; Hige, Aso, Rubin, et al., 2015; Ito et al., 1998; Mao & Davis, 2009; Schwaerzel et al., 2003; Takemura et al., 2017; Waddell, 2013; Yao Yang et al., 1995). So far, no motor-related inputs have been found for the MB which suggests that the MB might not be a structure for motor plasticity and operant learning. Hence, circuits for both associative learning types are probably segregated. In this study, all the driver lines projecting to the MB showed no robust reinforcement value across paradigms (fig. 5A; fig. 10; fig. 12), which supports this idea. The markedly sensory input into the MB implies a specific role for classical conditioning. Within the MB, the reciprocal interconnection of DANs, KCs and MB output neurons in every combination complicates the oversimplified classical view of KCs and DANs transmitting feedforward information to the MB output neurons (Takemura et al., 2017). This suggests that not only CS-US associations take place in the MB but also CR-CS and CR-US associations, whose roles are still not fully elucidated.

4.1.2 Reinforcer versus Unconditioned stimulus

The MB seem to gate the transition from goal-directed to habitual behavior. In section 1.5 a few representative interactions of classical and operant conditioning were demonstrated. These intricate interaction between classical and operant learning components foreshadow bidirectional projections, direct or indirectly, between the MB and regions involved in operant conditioning. Hence, despite its conceptual distinction, operant and classical conditioning frequently interact in natural environments. Flies can recognize in a complex environment the effect of different predictors in order to obtain reward. Interestingly, flies can learn that B-R contingencies only hold in the presence of a specific environmental cue (Brembs & Plendl, 2008). Behavior and sensory cues are potential predictors of the reward and searching the temporal contingencies in sensory signals in the wrong moments could reduce the efficiency of behavior-outcome associations. Adding

contextual cues can yield better learning to the detriment of poor generalization (Brembs, 2000, 2011, 2009a; González et al., 2003; Rescorla, 1994; Wolf & Heisenberg, 1991). The dilemma of whether a common formalism can be applied to operant and classical conditioning has been a discussed issue over many years and only recently started to clarify (Brembs, 2000, 2011; Gormezano & Tait, 1976; Hebb, 1956; Heisenberg & Wolf, 1993; Rescorla, 1994; Rescorla & Solomon, 1967; Skinner, 1935, 1937; Wolf & Heisenberg, 1991).

In our paradigms we observed that neurons that have shown robust effects in classical learning did not necessarily reinforce spontaneous behavior in the same way, that is, reinforcement evoked by dopaminergic neurons has no relation to their role as an US in classical conditioning (fig. 6-10; fig. 12). These findings emphasize that, in addition to the segregation of behavior and stimuli as predictors, reinforcement and US should be equivalently teased apart. This implies the need to deploy different terminology for these two learning types in order to rigorously describe segregated mechanisms. Here we accentuate that the combinations of positive/negative punishment/reinforcement should be strictly deployed in operant situations whereas appetitive/aversive US should refer to classical conditioning. Conceptually this means that behavior, reinforcement, CS and US should be referred as different entities that might interrelate according to the situation. In fact, stimuli like heat have shown to punish and act as an aversive US at the same time (Galili et al., 2014; Wolf & Heisenberg, 1991). A different aversive US, electric shock, is signalized in two different pathways, the MB and the FB, which might indicate that different structures convey different learning types (Galili et al., 2014; Hu et al., 2018; Perry & Barron, 2013).

Contrary to what is seen in the fruit fly, *Aplysia´*s feeding behavior can be classically as well as operantly conditioned by the same dopaminergic neuron En_2, which hints at a shared operant-classical associative circuit. The esophageal nerve En_2 targets B51, a decision-making neuron for eliciting feeding. Operant and classical conditioning induce differential changes in the biophysical properties of B51 which indicates that the signal segregation occurs at the cellular level (Brembs et al., 2002; Lechner et al., 2000; Lorenzetti et al., 2006).

Therefore, the brain might come up with different mechanisms to segregate operant from classical learning. Dedicating different signaling pathways within the same cell might explain how the same circuits could accomplish different functions polivalently and in parallel. In general, organisms with less number of neurons compensate by deploying their neurons more polivalently, which might explain why in *Aplysia* both conditioning types occur in the same circuit. Interestingly, in the adult fruit fly, Protein Kinase A (PKA) pathway is necessary for classical learning whereas PKC signaling pathway is essential for operant learning, however it is not clear if these pathways overlap in the same neurons (Brembs &

Plendl, 2008; Colomb & Brembs, 2016). Based on our findings that different neuropils seem to be required for operant reinforcement and classical learning, both pathways might take place in different neurons.

4.2 Reinforcement substrates in *Drosophila melanogaster*

Different motor programs might share overlapping reinforcement substrates. However, the absence of relationship across the performance of fly lines in the different operant paradigms do not support a shared reinforcement circuitry for the performed tasks (fig. 10, table S3). This is striking if we consider the case of both T-maze screens, where flies were exposed to the same conditions except for the light stimulation. The differences between both T-maze experiments suggest that these neurons might be sensitive to the stimulation patterns. Gerbera 2018 showed that attraction/aversion effects in specific octopaminergic neuronal clusters were dependent on intensity and/or frequency. It is also possible that the real correlations of shared sparse circuits might be obscured by the many driver lines that did not show any effect and thus, added noise to the correlation.

Our approach/avoidance paradigms also permitted us to find other reinforcers that are independent of the context. The ubication of these neurons in central brain regions suggest a more general reinforcement system (fig. 5, fig. 14), yet further tests should elucidate what kind of reinforcement these neurons encode. We identified three fly lines encoding a generalized reinforcement: TH-D' for punishment and TH-D1 and TH-G1 for positive reinforcement (fig. 12). The expression pattern of TH-D' can be compared to those of the other two driver lines in two different ways: 1) the set difference, that is the regions that distinguish them, and 2) the intersection, that is the regions they share in common. Interestingly, the model in section 3.8 exploits the regions that are differentially expressed to explain the behavioral scores and the low adj. R^2 obtained, is indicative of a very modest fit (fig. 13B), which suggests that the set difference might not explain the reinforcement scores. An alternative strategy would be to inspect the intersection, that is the common regions expressed. In this case, the lines expressing in the common regions, are expected to have the more extreme reinforcement/punishment effect sizes. We will therefore focus on common regions expressed by the three candidate lines.

Fig. 5B and fig. 14 depicts PPL1 and PPM3 populations as the common regions targeted by the context-independent reinforcement lines. Since other lines with PPL1-MB projections (fig. 5A) did not show consistent reinforcement, only PPL1-dFB projections could explain the reinforcement effect from lines expressing in the PPL1 cluster. The PPL1-dFB projection, stained by the three drivers, has a main role in sleep regulation (Jeffrey M. Donlea et al., 2014; Q. Liu et al., 2012; Nguyen, 2017; Qian et al., 2017). If we compare the

staining patterns of all driver lines tested (fig. 5), these three share a very similar pattern, which suggest these common regions might mediate the observed positive reinforcement and punishment.

The projection PPM3-vFB (2-4 layers) and noduli were particularly intense for TH-D' and TH-D1. vFB, which consist of layers 1 to 5, is highly responsive to electric shock and nociceptive heat and mediate innate avoidance, which explains the avoidance behavior in TH-D'. Interestingly, electric shock signal is also relayed to the MB by dopaminergic neurons and their manipulation does not affect acute avoidance (Aso, Sitaraman, et al., 2014; Cohn et al., 2015; Galili et al., 2014; Hu et al., 2018; Schroll et al., 2006; Schwaerzel et al., 2003; Waddell, 2013). TH-G1 might stain a layer from mFB (fifth or sixth), but our nc82 counterstaining did not penetrate the tissue enough to confirm this. In addition to avoidance, the FB is reported to be important for other functions, including locomotion control (Strauss, 2002), visual feature recognition (Liu et al., 2006) and processing (Weir & Dickinson, 2015), courtship maintenance (Sakai & Kitamoto, 2006), quiescence regulation (Berry et al., 2015; Donlea et al., 2011; Ueno et al., 2012) sleep homeostasis (Qian et al., 2017).

The EB and LAL are, together with the FB, the most outstanding regions labelled by these three driver lines (fig. 14). The EB encodes heading orientation and feature detection with the corresponding associated memories (Seelig & Jayaraman, 2015; Strauss, 2002; Wolff et al., 2015). Dop1R1 signaling in the EB ring neurons affect the temporal organization of motor actions, exploration and turning behavior (Kottler et al., 2019). Hence, the dopaminergic projections might reinforce different behaviors by biasing the action selection process in the ring neurons of the EB. Manipulating specific dopaminergic inputs into the ring neurons might show differential effects. The EB and the FB, as the other CX structures (PB and No), show a strikingly compartmentalized layout with restricted connections, which suggest a high degree of functional specialization (Wolff et al., 2015; Wolff & Rubin, 2018). Hence, dopaminergic projections to the CX and LAL might be topographically organized to differentially drive avoidance and approach (Hu et al., 2018; Wolff et al., 2015). The structure and function of the CX and LAL is quite conserved across insect's species. They process spatial aspects of complex multisensory information and integrate it with information about the insect's internal state and past experiences, to drive proper motor outputs (Buchanan et al., 2015; Hu et al., 2018; Namiki et al., 2018; Namiki & Kanzaki, 2016, 2018; Seelig & Jayaraman, 2015; Strauss, 2002; Wolff et al., 2015; Wolff & Rubin, 2018).

The LAL is the major output site of the CX and its bilateral coordinated activity mediates signals to the thoracic motor centers. The LAL is closely interconnected with other CX structures but it also receives input from AOT, SMP, Lobula Plate, PS and thoracic motor centers. In addition, the LAL receives ascending feedback about proprioceptive information

to coordinate downstream motor commands in locomotion, flight, phonotaxis and pheromone orientation in several insects. The characteristic flip-flop signals observed in the LAL and downstream DNs are correlated with turning maneuvers and its role seems to be locomotion-biased, which points to this region as a major candidate where reinforcement might converge (Namiki et al., 2018; Namiki & Kanzaki, 2016, 2018; Wolff et al., 2015; Wolff & Rubin, 2018).

In the search for candidate lines for reinforcement we sought lines that showed a consistent behavior across the four paradigms (fig. 12). Interestingly, none of the lines projecting to the MB stood out. Emphasizing the anatomical commonalities and differences from our candidate lines (TH-D', TH-D1 and TH-G1), we outlined putative regions encoding reinforcement (fig. 16). We hypothesize that topographically organized dopaminergic projections (PPL1 and PPM3) into the CX and LAL allow the convergence of motor programs and reinforcement.

If the neuronal targeting by the *GAL4-UAS* system can only be coarser than the topographical organization of the reinforcement system, only a model exploiting commonalities might yield effective results. It is suggested that the implementation of a similar model to fig. 13 for a test with more specific GAL4s, labelling only PPM3 and PPL1-dFB subpopulations, could show the topography of reinforcement within these clusters.

Concomitantly, optogenetic inhibition experiments revealed that flies prefer to inhibit neurons targeted by the TH-D' driver when they have the opportunity to do so (fig. 15), which agrees with the previous result that neuronal activation is avoided (fig. 12). On the other hand, flies have shown a preference for the activation of TH-D1 positive neurons, as well as a preference of the neuronal inhibition (fig.15). An explanation might be that the baseline state of TH-D1 neurons is punishing and any disturbance that bias activity away from this baseline might lead to a preference state. In fact, it has been shown that only at optimum levels of the dopamine receptor 1 (DopR1) in the EB leads to appropriate action selection (Kottler et al., 2019).

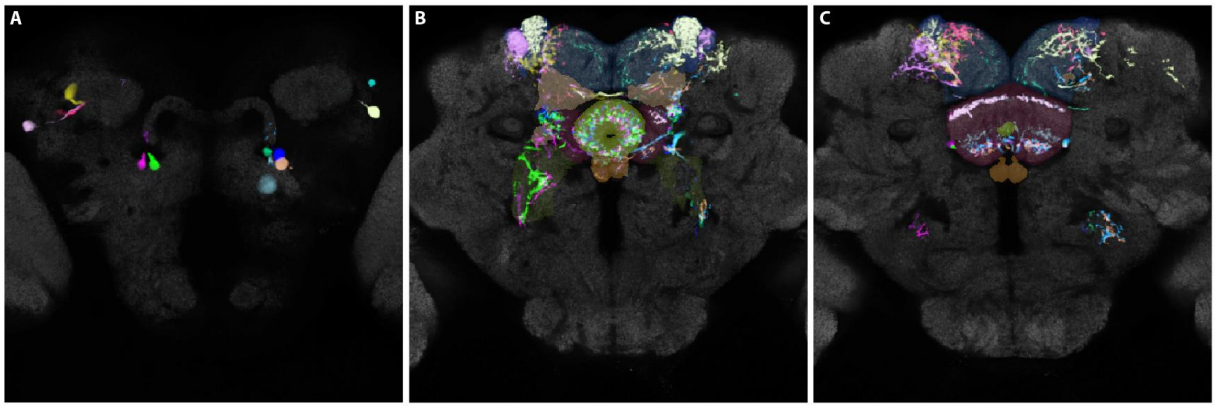


Figure 16. Identification of the brain regions for reinforcement. A: Template of a posterior brain section with a few representatives of PPL1 and PPM3 cell bodies colored. **B**: Brain section template highlighting a few representatives of PPM3 and PPL1 projections into the LAL, EB, No, vertical lobes of the MB, and SMP. **C**: Brain section template depicting a few epitomes of PPL1 and PPM3 projections into the FB, SMP and ventrolateral protocerebrum (vlpr). blue: superior medial protocerebrum (SMP), light brown: crepine, green: lateral accessory lobe (LAL), purple: fan-shaped body (FB), orange: Noduli (No).

4.3 Centralized versus distributed behavioral control

In the previous sections we glided over the anatomy of reinforcement and observed that several of the tested lines contribute to reinforcement. Thus, reinforcement might be broadly encoded by the dopaminergic system. RPE signal, a neural correlate of associative learning, can be ubiquitously found in the brain. In fact, previous models already postulated a distributed associative memory storage without implying a locationalistic theory of memory (Allport, 1985; Sutton & Barto, 1981).

Allport (Allport, 1985) advocated the distributed nature of brain physiology, supported by the observations from lesion studies, where they often resulted in partial impairments and not total impairments of specific brain functions. Allport claimed that, alluding hebbian plasticity, neurons that fire together frequently, potentiate their connections yielding in an auto-associative network, what he calls an engram (Allport, 1985).

Karl S. Lashley's unsuccessful efforts to find an engram led him to conclude that memory is not located in specific regions but rather distributed throughout the brain (Bruce, 2001). However, Thompson discovered that the lateral interpositus nucleus (LIP) activity in the cerebellum is necessary for the eyelid CR in rabbits and thus, it might be the region where the association occurs (Devan et al., 2018). In recent "capture" studies, fear-conditioned freezing response was recovered by activating a neuronal ensemble from the hippocampus and amygdala. Other brain areas such as the striatum and the cerebral cortex are also involved in memory engram formation and retrieval, in what seems to be a more distributed associative circuit than previously thought (Bruce, 2001; Devan et al., 2018; X. Liu et al., 2012, 2014; Ramirez et al., 2015; Redondo et al., 2014; Tonegawa et al., 2015).

Hunting these engrams might depend on the nature and complexity of the memory. A general consensus is that certain memory types may be contained in specific regions in the brain whereas more complex and multifaceted memories are likely to be distributed (Gerrig & Zimbardo, 2005). The octopus seems to share this idiosyncrasy in motor circuits, where the more brain regions which are simultaneously stimulated, the more behavioral complexity is observed (Zullo et al., 2009). Whereas studies mostly focused on engrams for episodic memories, less is known for other memory types. Our interest lies in behavioral engrams, if they exist as such, since knowing about their location might facilitate the understanding of regions and mechanisms of reinforcement.

Descending control mechanisms in invertebrates bear similarities to that of vertebrates. *Drosophila* has a large behavioral repertoire but only around 0.5% of all neurons control the full range of movements (Orlovsky et al., 1999). Recent efforts combining artificial activation of neurons with high throughput ethomics allowed screening the effects of the triggering behaviors of many brain regions: some with transient and others with steady behavioral effects (Cande et al., 2018; Robie et al., 2017).

Artificial activation of neurons in the fruit fly does not deterministically trigger a certain behavior but rather changes the transition probabilities according to the current state, once again showing the relevance of the instantaneous context (Cande et al., 2018). Often, descending neurons trigger very specific commands constrained to small behavioral spaces and might therefore be a likely target for reinforcement. Combinatorial activity of descending neurons might lead to high variety of motor commands, similar to what is seen in the octopus, where the more regions stimulated, the more complex behaviors elicited. Interestingly, motor regions in the octopus do not have a central topographical organization but are distributed over wide regions (Berman et al., 2014, 2016; Bidaye et al., 2014; von Philipsborn et al., 2011; Zullo et al., 2009).

A few projections from the brain relayed via descending interneurons are important for both the initiation and modulation of central pattern generators (CPG) activity for goal-directed behavior. With the help of sensory/proprioceptive signal, and its modulation by descending pathways, CPGs coordinate motor sequences with temporal precision (Bidaye et al., 2018). During fast movements, central mechanisms can control the CPGs effectively without the need of feedback, yet increasing behavioral variability (Bidaye et al., 2018). This modular and hierarchical architecture is supported by the dynamic analysis of high resolution ethograms in the fruit fly (Berman et al., 2014, 2016; Namiki et al., 2018; Namiki & Kanzaki, 2018; Wolff et al., 2015).

Even reflexes, the most fixed behaviors, can be conditioned in mammals (Wolpaw, 1997, 2018) as well as in insects (Gorostiza et al., 2016; Martin et al., 2015). The current model claims that the brain and the spinal cord negotiate a balance between flexibility and stability for the tuning of motor commands (Wolpaw, 2018).

The behavior-anatomy correlation maps in the fruit fly indicate that different behavioral circuits are located in different brain regions (Robie et al., 2017). How can reinforcement adjust circuits coordinated throughout the brain? One possibility is that specific reinforcement signals are dedicated to each behavioral circuit, however, this solution might be costly. The other option is to have a central reinforcement system that controls the major motor centers, delegating more intricate adjustments to downstream areas like CPGs.

The dopaminergic neurons deployed in this study are located in higher cognitive brain areas (Aso et al., 2010, 2012; Aso, Hattori, et al., 2014; Q. Liu et al., 2012; White et al., 2010) (fig. 5; fig. 14) and descending signals from the CX in the insect brain can modify and trigger walking patterns. Hence, the projection from the PPL1 and PPM3 to major motor areas like the CX and the LAL supports the idea of a centralized reinforcement of motor commands, affecting the action selection process (Bidaye et al., 2018; Namiki et al., 2018; Namiki & Kanzaki, 2018; Robie et al., 2017; Wolff et al., 2015). Operant conditioning experiments in flies suggest that flies try to achieve a desired state through behavioral action selection. This point of view suggest that reinforcement should occur at this higher level to guide goal-directed behavior. The difference from the instantaneous to the desired states might serve to inform the reinforcement system to assign values to motor actions (Brembs, 2000; Wolf & Heisenberg, 1991; Wolf et al., 1992).

In vertebrates, reinforcement-based learning occurs in the striatum whereas the cerebellum is dedicated to a reinforcement-free motor skill tuning (Fino et al., 2018; Graybiel, 2016; Graybiel & Grafton, 2015; Pidoux et al., 2018b; Thorn et al., 2010). If we imagine that the reinforcement system in *Drosophila* is an archetype for the vertebrate counterpart, the striatal reinforcement-based learning could be accomplished by the dopaminergic projections to the CX and LAL in insects. In fact, Strausfeld and Hirth (Strausfeld & Hirth, 2013) alleged several genetic, anatomical and physiological parallelisms to endorse the homology of nigrostriatal pathways of mammals with the PPL1/PPM3-CX/LAL pathways in insects. Moreover, the descending neurons would connect this central system to the CPGs, a region involved in tuning weights for more skilled maneuvers, analogous to the cerebellum. Dopamine also modulates the control of pattern-generating interneurons associated with movement in mollusks and crustaceans (Hills, 2006). Additional motor tuning could occur directly in downstream regions (e.g. CPG, LAL or posterior slope), for single muscular commands, but whether this involves reinforcement-based or reinforcement-free learning is

not known. For fine motor skills, intricate coordination of different muscles requires the recruitment of many neurons, where reinforcement could act *in situ*.

Reinforcement might be able to reinforce at different levels of the hierarchy, in fact, neuroanatomy in the fruit fly larva shows that there is a complex multilevel multimodal convergence architecture enabling selective tuning to combinations of cues (Ohyama et al., 2015). Experiments with decapitated cockroaches and locusts show that there is a high degree of local control of leg movements by segmental ganglia and that not all proprioceptive control of the leg needs to ascend the brain (Horridge, 1962). Concomitantly, operant conditioning of the vertebrate H-reflex shows that even the simplest learning is accompanied by plasticity at multiple sites allowing compensatory changes to incorporate new behaviors without affecting older behaviors (Wolpaw, 1997). Although reinforcement was found to occur centrally through dopamine, further research at other levels in the hierarchy might show a multilevel reinforcement system. Alone the dopaminergic projections to several neuropils from the CX and LAL might reinforce different aspects of motor programs.

4.4 Operant activity versus conditioning

Wolf 1991 proposed a conceptual framework of operant behavior: firstly, it requires a goal which deviates from the actual state, which prompts the initiation of motor programs whose outcomes inform about the deviation from the goal. During the search for predictors, attempting certain behaviors over others in order to reach a preferred state in different situations suggests that initiating activity is essential to achieve a desired state. Motor programs are selected to modify the sensory input in the direction towards the goal. Consistent control of biological important stimuli to achieve the goal, that is operant activity, may drive to a lasting behavioral change, namely, conditioning. This implies that conditioned preferences are preferred states rather than modified motor patterns. Whereas operant activity occurs as soon as the operant contingencies can be captured by the animal, long lasting modulation of the behavior does not necessarily need to occur (Brembs, 2000; Wolf & Heisenberg, 1991; Wolf et al., 1992). In the Joystick paradigm, positive controls showed operant activity and conditioning, however our interesting lines did not show discernible results. Future experiments with more effective protocols should elucidate this.

Interestingly, in previous versions of this setup with heat beams used to reinforce left/right platform positions, operant conditioning was not detectable although operant activity was. Mariath (Mariath, 1985) claimed that although it is not behaviorally exhibited the association might be stored. Evidence of memory in operant conditioning was proven through the effect of a training session on the performance of further training, that is, a

previous exposure to the training contributed to a faster learning (Mariath, 1985; Wolf et al., 1992). Natural and complex learning tasks are easier to solve than the more artificial, single-association tasks, and they need less familiarization training. The joystick might not be a natural behavior and it provides only a single contingency, thus, it might be more difficult to store in the long term. In the flight simulator flies can avoid a heat beam with the yaw torque which proves the relevance of the task for manifestation of the operant lasting effects. However, the yaw torque operating an attractive odor does not show the conditioning effect. In this case, although the task is the same (yaw torque), the reinforcement is different. In summary, all these experiments suggest that the combination of both, behavioral task and reinforcement feedback type, determine whether the conditioning would show up after operant activity (Brembs, 2000; Wolf & Heisenberg, 1991; Wolf et al., 1992).

Moreover, we could not observe the behavioral after-effect in the Y-mazes with the positive control (data not shown), yet operant activity was evident. However, in a previous study, freely walking single flies were conditioned to avoid one side of a small test chamber with heat (Wustmann et al., 1996). There might be two reasons why in the Y-mazes flies did not exhibit conditioning: blind flies might not able to orient themselves or the control of artificial stimulation by entering one of the Y-maze arms might be too difficult to learn.

Successful operant conditioning needs in general less training than classical conditioning but the extinction of the instrumental response in the absence of stimulus is rapid. This does not imply an absence of memory, but an absence of display of the experience (Brembs, 2000; Mariath, 1985; Wolf & Heisenberg, 1991; Wolf et al., 1992).

4.5 Evolutionary effects of dopamine in valence and locomotion/arousal

Pleasantness or unpleasantness, according to Wundt (1896), is only one aspect of affect. According to Wundt's tridimensional theory, all feelings, including those associated with sensory experiences, may be characterized by quantitative values on each of three bipolar dimensions: pleasantness-unpleasantness, strain-relaxation, and excitement-calm (Marks, 2011).

Whereas we usually perceive valence and locomotion as two segregated bipolar dimensions, manipulation of dopaminergic neurons in fruit flies (Fuenzalida-Uribe & Campusano, 2018; Gomez-Marin et al., 2016; Kong et al., 2010; Lebestky et al., 2009; C. Liu et al., 2012; Q. Liu et al., 2012; Riemensperger et al., 2011, 2013; Sun et al., 2018; Van Swinderen & Andretic, 2011) and in mammals (Draper et al., 2007; Hikida et al., 2016; Kravitz et al., 2012; Kravitz & Kreitzer, 2012; Tran et al., 2002) reveal that these two

dimensions are to some extent correlated. Y-mazes experiments reveal locomotor effects of most of the dopaminergic populations (fig. S5), in contrast to other studies that only attribute locomotor effects to particular clusters (Berry et al., 2015; Fuenzalida-Uribe & Campusano, 2018; Sun et al., 2018). We found a correlation that flies under unpleasant situations, increase locomotion and the opposite for pleasant ones, similar to what is observed in rodents (fig. S3)(Kravitz et al., 2012; Kravitz & Kreitzer, 2012).

Dopamine projection to the EB regulates different locomotor traits. DopR1 effects seem to account for the increased arousal and depletion of dopamine decreases both locomotor activity and reactivity and increases sleep (Kong et al., 2010; Q. Liu et al., 2012; Riemensperger et al., 2011). A small population of R2/R4 ring neurons in the EB elicit an increase in walking probability whereas locomotor reactivity is influenced by several dopaminergic clusters but not the EB (Robie et al., 2017; Sun et al., 2018). In our study, the optogenetical activation of dopaminergic neurons targeting the EB showed changes in walking speed (fig. S5). Interestingly, DopR1 levels in the EB ring neurons affect centrophobism and exploration, altering place preference. Dopamine effects are difficult to reconcile with only one single behavioral dimension and thus, a shared action selection is the most parsimonious explanation for the effects of dopamine (Kottler et al., 2019).

The ancestral role of dopamine is the reaction to salient stimuli followed by the corresponding modulation of motor circuits. In the nematode *Caenorhabditis elegans* dopaminergic mechanosensory neurons are sensitive to food abundance and modulate the crawling speed and turning behavior, as well as affecting memory systems involved in food-seeking behavior. Dopamine seems to be involved in reinforcement in the flatworm *Dugesia japonica* and in the cnidarian Hydra japonica, dopamine affects the extent of mouth opening in response to food stimuli (Barron et al., 2010).

In animals with simpler brains, dopamine seem to signal more concrete rewards, often food resources. During foraging, the lack of information about the location of food resources might lead to the exploration of the environment. Reward or novelty, to represent information about resources, can be exploited by coincident networks to form association with other predicting stimuli or behavior and tune motor circuits to adopt optimal food search strategies. This *modus operandi* can be generalized with any other goal-directed behavior (Hills, 2006).

All but the Joystick are spatial experiments where flies move freely, simulating foraging where instead of encountering food sources, the flies receive dopaminergic stimulation. Similar spatial experiments tracking fruit fly larvae for temperature and odor preference have found some general hallmarks of larval locomotion to find the preferred

places. When appetitive odor gradients decrease, larvae increase turning to avoid moving away from highly concentrated regions. When gradients increase, they keep a straight line to approach the higher concentrations. Similarly for temperatures, larvae regulate their turning rate and straight runs to occupy the prefered temperatures. For both *C. elegans* and *D. melanogaster* dopamine modulates turning behavior in response to a perceived reward, either in the form of food or drugs (Gomez-Marin et al., 2016; Hills, 2006; Shen et al., 2011). Whereas larvae seem to tweak its turning behavior for place preference, the adult fly might modify its speed accordingly. In this way, a relation of pleasure and aversion with decreased- and increased locomotion respectively, might be a valid standard approach to escape harming and dangerous regions and stay in sheltered and advantaged areas. Interestingly, a computational model was recently developed to simulate how the EB would exploit sensory inputs to orchestrate spatial navigation (Fiore et al., 2017).

It is remarkable how dopamine´s ancestral role early in the history of metazoans has derived from concrete events to more processed representations of reward. Together with the detection of environmental stimuli to modulate motor commands, dopamine sets the appropriate arousal levels (Barron et al., 2010; Hills, 2006). Interestingly, TH-D1 and TH-D' drivers (as well as TH-D4) promote wakefulness and this effect is mediated by an increase in arousal through PPL1-dFB (Q. Liu et al., 2012). In the Y-mazes we observed decreased speed when stimulating TH-D1 and TH-D4 whereas TH-D' increased the speed (fig. S5). TH-G1 on the contrary did not exhibit any effect in sleep (Q. Liu et al., 2012)Liu 2012). The regulation of arousal states allows quick responses when these are needed and modulate attention according to the demands. Adjusting arousal states to affective states is necessary for proper response selection (Birman, 2005; Lebestky et al., 2009; Van Swinderen & Andretic, 2011).

Although dopamine´s role is similar across phyla, it is unlikely to be a true homology. Molecular evidences suggest that higher order brain regions have evolved independently. Consequently, the mechanisms of reward in these regions should have evolved independently as well. In both cases the evolutionary process may have made use of a common molecular toolkit to lead to a convergent evolution of this general behavioral mechanism (Barron et al., 2010; Hills, 2006).

4.6 Technical constraints and outlook

4.6.1 Optogenetics

By activating neurons artificially we overlook the complexity and relevance of neuronal dynamics. Under experimental light settings, depolarization via optogenetics might not be within the physiologically meaningful ranges. A tailor-made adjustment of stimulation

parameters for a given neuronal type to resemble its physiological state is time consuming and thus, barely done. Therefore, as in other studies our proof of concept was performed empirically by testing positive controls. However, while our positive controls targeted mostly neurons in the periphery, dopaminergic neurons lie within the brain, where the cuticle and brain tissue diffuse and scatter the light, reducing the light intensity. We believe that all these obstacles are inconspicuous since there is a broad range of stimulation protocols, with different effectors and stimulation patterns in the same neurons, that trigger the same result (Aso et al., 2010, 2012; Aso, Sitaraman, et al., 2014; Berry et al., 2015; Cande et al., 2018; Inagaki et al., 2014; Klapoetke et al., 2014; C. Liu et al., 2012; X. Liu et al., 2014; Ramirez et al., 2015; Redondo et al., 2014; Robie et al., 2017).

From a theoretical perspective, activating neurons out of context of other neuronal activity might have no physiological meaning. On the other hand, activating a neuron that belongs to an auto-associative network might be followed by the activation of the rest due to the strong interconnections among them, thus, the stimulation would not be out of context (Allport, 1985). According to the hebbian rule, artificial activation of one single neuron repeatedly without any context, might decrease all its synaptic weights to a dysfunctional state. As an example, activation of the neurons encoding the US alone lead to memory extinction, an idea already proposed by Randich (Aso & Rubin, 2016; Baggett et al., 2018; Berry et al., 2015; Randich & Haggard, 1983; Schleyer et al., 2018; Vogt et al., 2015).

Recent work on dopaminergic neurons focusing on the activity dynamics have produced interesting insights in how the preceding context influences action selection in the fruit fly (Cohn et al., 2015; Hige, Aso, Modi, et al., 2015; Hige, Aso, Rubin, et al., 2015). Imaging allows observation of dynamic brain activity while an individual is learning. Using the advantages of the transparent fish *Danio rerio*, Li (Li, 2014) recorded the whole brain activity during operant learning, allowing the anatomical distinction of sensory, relief prediction, RPE, action selection and motor neurons.

4.6.2 Experimental design and analysis

Looking retrospectively, there are a few changes that would have helped to improve this study. We would have tested the same positive control and a complete screen of all lines in the four paradigms. This would allow a better comparison across setups with more statistical power. We would have designed the experiments in blind with at least two experimenters in parallel for every screen, to avoid biases and have an approximate estimation of noise. We would have tested behavioral consistency over days to assess the origins of reinforcement variability: reinforcement might be specific for each individual or have probabilistic effects that are only observable at the population level. In addition, there have been some recent developments for more accurate neuronal targeting techniques

(Aso, Hattori, et al., 2014; Xie et al., 2018). Follow up experiments targeting subpopulations of our candidate lines might show a topographical organization of reinforcement.

In the red lit T-maze we observed that flies choice was consistent at the population level, but not at the level of individual experiments. This could be due to a high decision variability in individual flies. Hence, activation of these neurons might change decisions probabilistically and thus only be observable at the population level in a similar way as it has been seen in descending neurons (Cande et al., 2018).

A featureless Y-maze hinders a fly's self-orientation unless it cumulatively tracks its movements. Flies have been shown to keep their orientation in working memory for around 90 seconds (Seelig & Jayaraman, 2015; Wolff et al., 2015). When flies are genetically blind, the lack of spatial reference impairs the appearance of entries preference. If they could associate neuronal activation with arm location, this could be a valid reference, unfortunately, there is no evidence that flies can achieve this. Since our setup is spatial and is dependent on fly movement, this correlation could indicate that the occupancy rates might be due to effects in locomotion rather than in valence. Yoked experiments incontestably demonstrated that dopaminergic activation produces a locomotion phenotype. This emphasizes the relevance of the Joystick experiment where locomotion and valence can be disentangled. It might be interesting to test the candidate lines with and without $norpA^{P24}$ to see if phenotype differences might be due to the presence of a CS due to the visual perception of the optogenetically stimulating light.

In the Joystick, we often encountered unwanted biases due to inappropriate positioning of the flies on the platform. Hence, we subtracted the pretest PI values from the training PIs. Unfortunately, in the absence of any closed-loop stimuli, flies would spend long periods of time exploring with the platform on one side. This led to random strong pretest biases that were not averaged out even after testing several flies. For future experiments, to avoid big fly generated pretest biases one could perform experiments with longer pretest times.

For the overall scores (fig. 12) we considered whitening (centering and normalizing the variance) all paradigms scores because in this way all paradigms would contribute equally in the overall phenotype. However, whitening involves centering the data, which would assume that our selected *GAL4*s contribute as much to approach as to avoidance, a strong assumption that is not necessarily true. In addition, since score means and variances were not drastically different across experiments, we opted for no data whitening. Another option was to normalize to the positive control, but since the positive controls seem to have similar effect size, we did not apply any normalization.

All in all, we took care in reproducing results; digitizing the data collection when possible to avoid bias that could occur in certain steps of the experiment. Moreover, we applied as much high throughput as we could to allow powerful statistics. Our analysis was very extensive taking in many points of view, biased to an explorative analysis but being conservative about the findings. The high amount of data generated during this thesis should allow to reanalyze data for new perspectives and follow up experiments.

4.6.3 Outlook

- Screen with sparse GAL4s from PPL1-dFB and PPM3.
- Classical learning experiments with the same driver lines.
- Counterstaining and GRASP stainings of the candidate lines to identify their reciprocal connections.
- Joystick experiment to test memory and habit formation in the interesting lines.
- Calcium imaging of interesting regions while the fly is being reinforced for a certain behavior.
- Do long term individual experiments to observe its own individual variability.

5 Conclusion

The study revealed that dopamine functions as a reinforcer of behaviors in the fruit fly, supporting the idea that dopamine plays a conserved role.

It was demonstrated that reinforcement and US signals are spatially segregated in different populations of dopaminergic neurons and therefore accurate terminology should be adopted by the scientific community.

Although most of the dopaminergic neurons do not have a reinforcement effect, or if they do, it is context dependent, projections from two dopaminergic clusters (PPL1 and PPM3) to the central complex and lateral accessory lobe accounted for robust reinforcement across several testing conditions. The central complex and lateral accessory lobe are neuropils involved in sensory integration and action selection.

Dopamine shows additional effects in other behavioral dimensions, in this case walking speed, which seem to be evolutionarily conserved. Therefore, we propose a model in which operant conditioning occurs in the central complex and lateral accessory lobe with the reinforcement of dopamine.

This work has laid down valuable foundations for future investigations about the circuit level mechanisms of different operant learning processes.

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7 Attachments

7.1 Figures

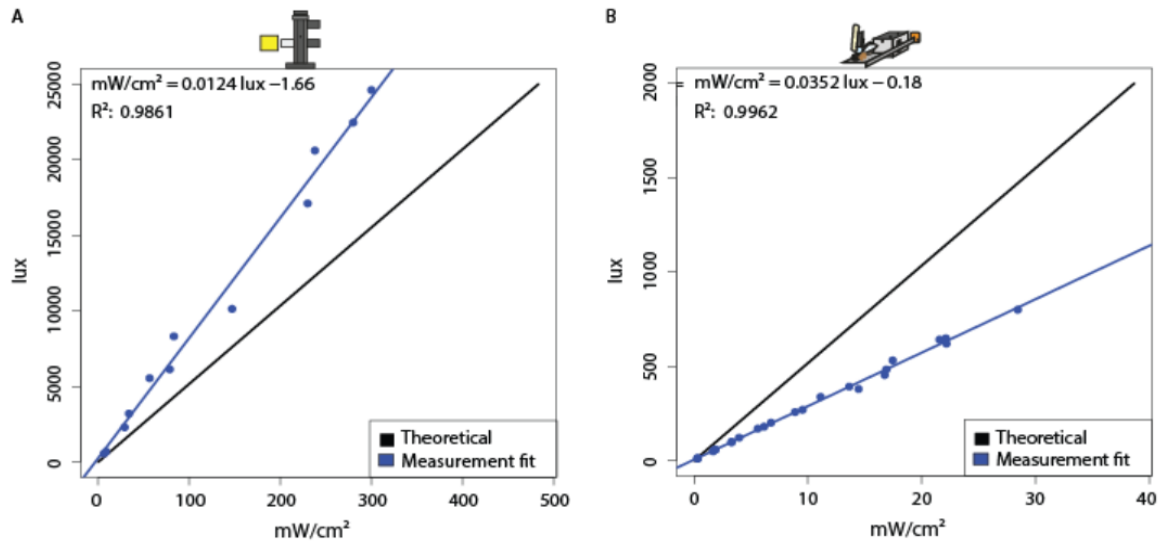

Figure S1. Conversion from Lux to mW/cm² in the Joystick (A) and T-maze with yellow light (B). The black line is the theoretical conversion as explained in the corresponding R script in the Methods section, and the blue line is the empirical calibration with the dots being each single measurement.

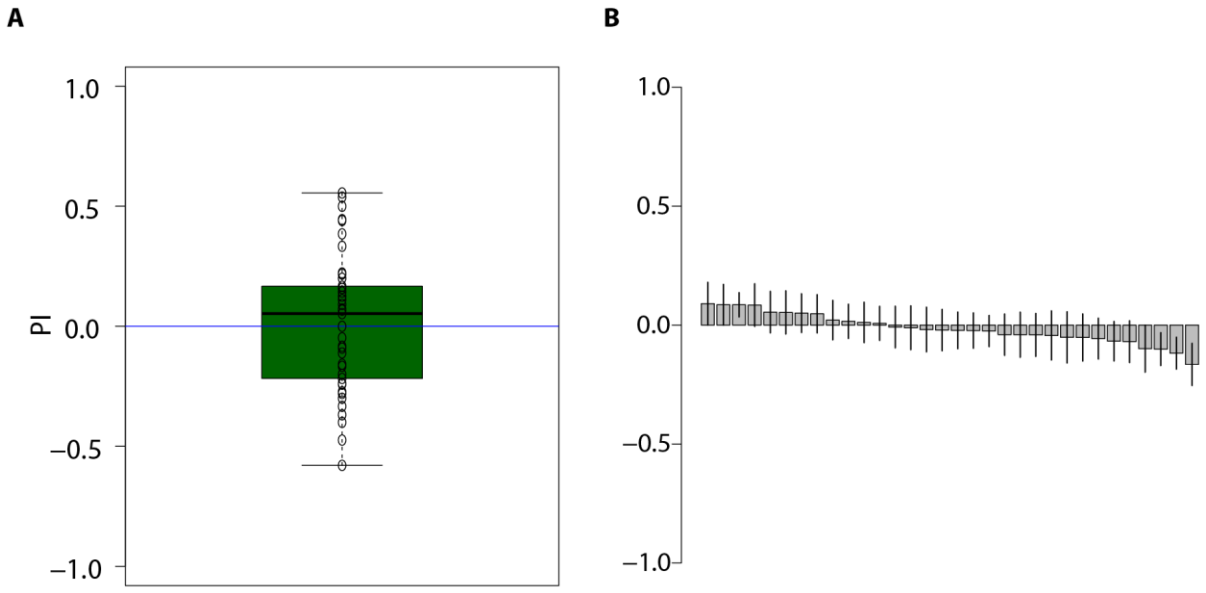

Figure S2. Barplot of fictive lines obtained from the bootstrap. A: Boxplot for the total of 49 experiments with blind negative controls. **B**: Twelve samples with repetitions for 32 experiments (as many experiments and lines that were tested in the T-maze screen with yellow light). The range and the distribution of PIs is similar, slightly lower, to that obtained for the tested screens in the T-maze in Fig 7.

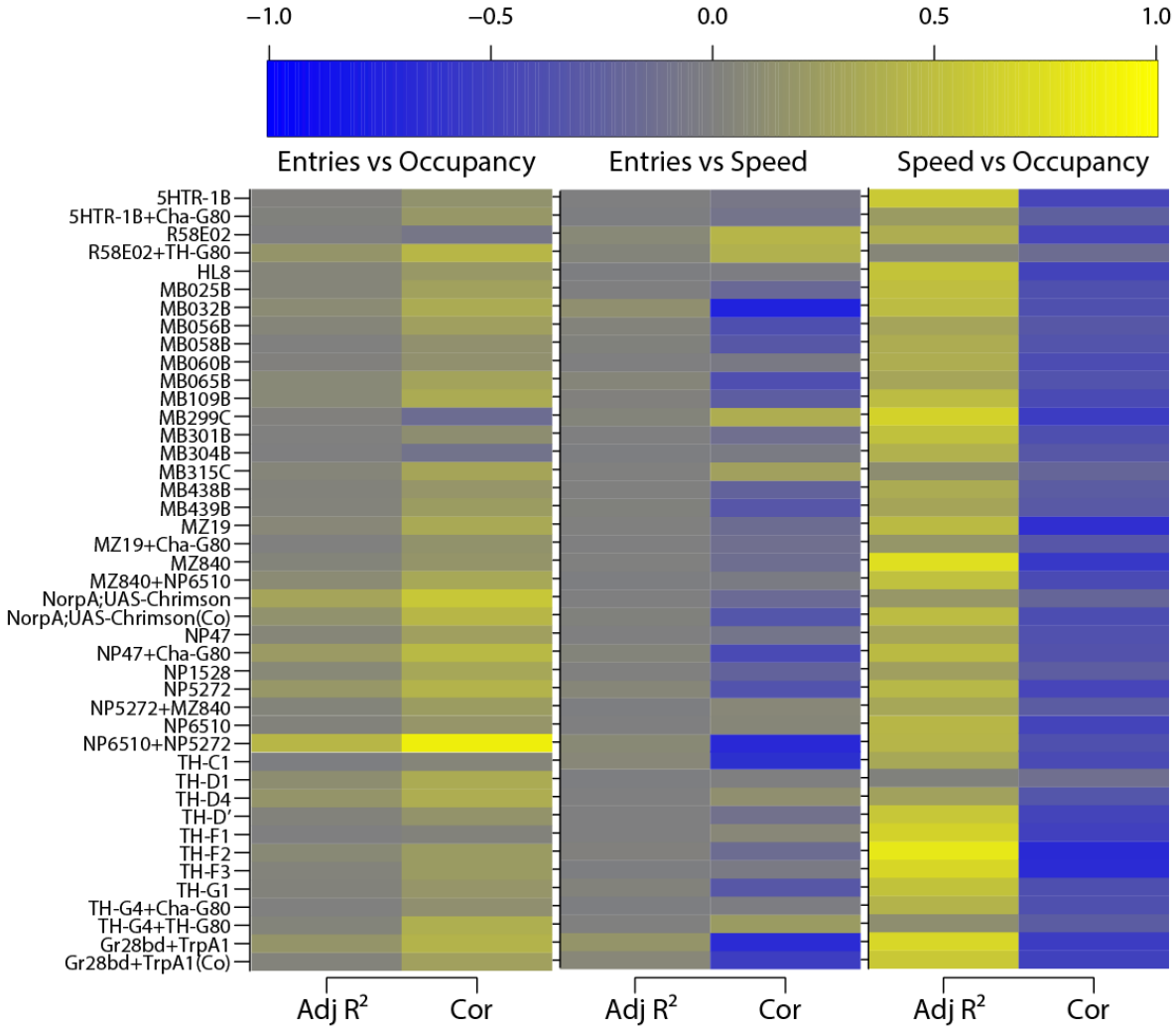

Figure S3. Combination of correlation pairs between speed, entries and occupancy rates for each screened line. Color bar at the top indicate the color code for the correlation score. For each correlation pair, the first column shows the adj. R^2 and the second column show the correlation between parameters.

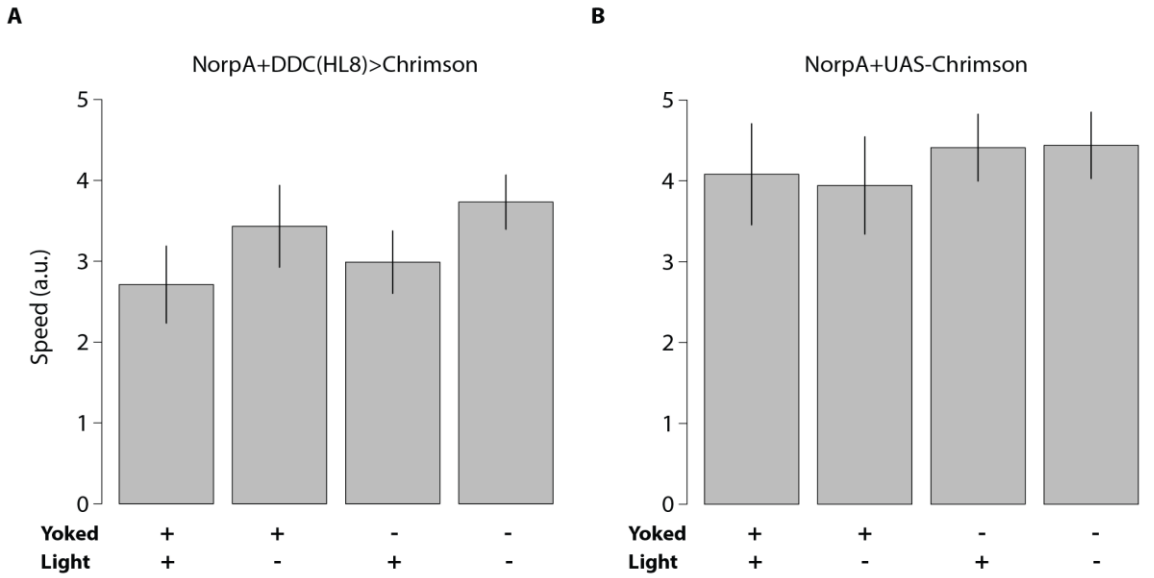

Figure S4. Yoked experiments between norpA+DDC>Chrimson and the negative control without GAL4. Barplots and error bars showing means and SEM respectively. **A**: Experiments with norpA+DDC(HL8)>Chrimson. When the light is on, flies walking speed decreases, independently of if they are yoked experiments or experiments with regular closed loop. **B**: This is not the case with the negative control NorpA;UAS-Chrimson where lights do not influence the average walking speed.

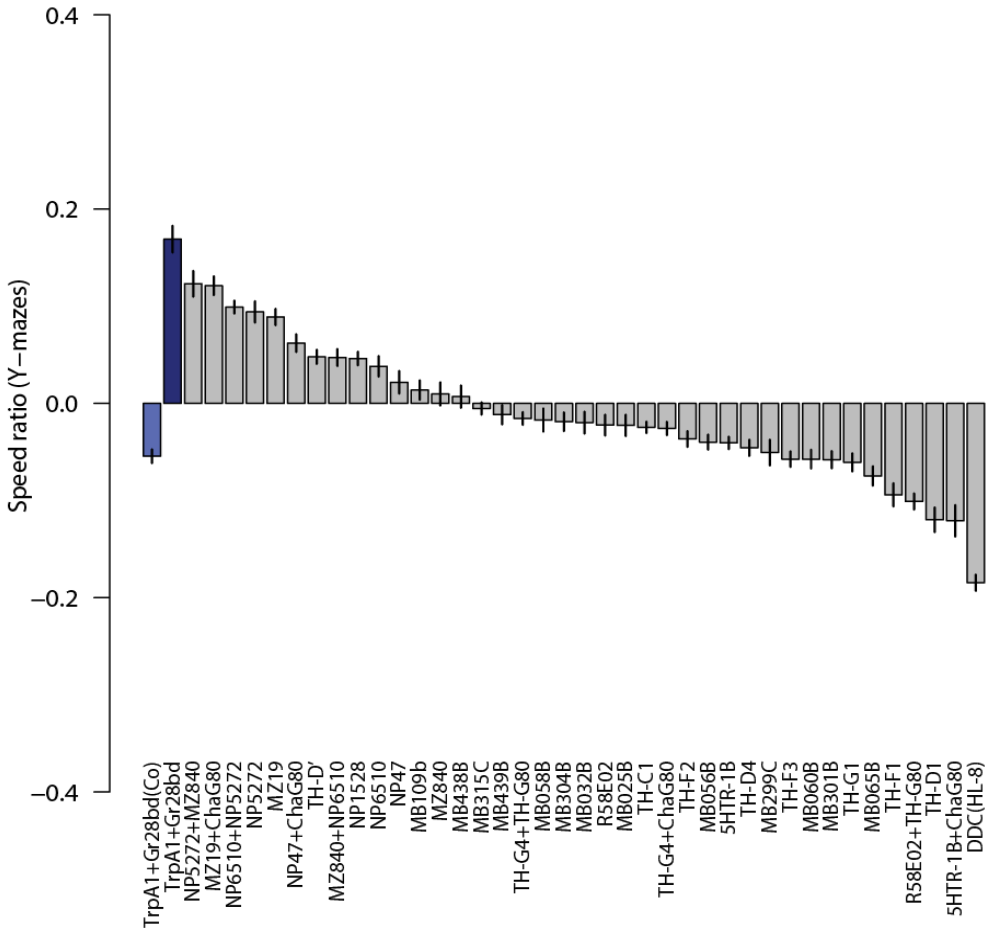

Figure S5. Speed effects in the Y-maze. Ordered barplots by decreasing means with error bars depicting the standard error of the mean (SEM). Experimental lines in grey, in dark and light blue the genetic control with and without ATR supplementation, respectively. On the Y-axis the speed ratio and on the X-axis the fly lines tested.

7.2 Tables

Product	Company	URL	article number
Cosine Corrector for SMA-Connectorized Fiber	Thorlabs	https://www.thorlabs.com/newgrouppage9.cfm?objectgroup_id=3482&pn=CCS200	CCSA1
Wide range spectrometer 200-1000nm	Thorlabs	https://www.thorlabs.com/thorproduct.cfm?partnumber=CCS200#ad-image-0	CCS200/M
Coherent FieldMaxII-TO Laser Power Meter	Edmund Optics	https://www.edmundoptics.de/lasers/laser-measurement/laser-power-meters/coherentreg-fieldmaxii-to-laser-power-meter	#88-411
all trans-Retinal powder, >=98%	Sigma	http://www.sigmaaldrich.com/catalog/product/sigma/r2500?lang=de&region=DE	R2500
Osram Oslon SSL 145lm yellow 10x10mm platine LEDs	Osram	https://www.leds.de/osram-oslon-ssl-smd-led-mit-10x10mm-platine-145lm-gelb-69643.html	69643
Osram Oslon SSL 80lm blue 10x10mm platine LEDs	Osram	https://www.leds.de/osram-oslon-ssl-smd-led-mit-10x10mm-platine-80lm-blau-69635.html	69635
Philips Lumileds red LEDs	Philips	http://de.mouser.com/ProductDetail/Philips-Lumileds/LXM3-PD01/?qs=sGAEpiMZZMu4Prknbu83y5qjVwwqxj2nEpXkn zdNQZg%3d	LXM3-PD01
Digital Luxmeter 0-400000lx	Voltcraft	https://www.conrad.de/de/luxmeter-voltcraft-lx-1108-bis-400000-lx-kalibriert-nach-werksstandard-ohne-zertifikat-121885.html?WT.mc_id=xplosion&hk=ARX&insert=RA&utm_campaign=xplosiondetailansicht&utm_medium=cpc&utm_source=xplosion&utm_term=121885	LX-1108
Camera Pointgrey Firefly MV	Pointgrey	https://www.ptgrey.com/firefly-mv-usb2-cameras	FMVU-03MTC-CS
Voltcraft DC Power Supply 1,5 to 15V	Voltcraft	http://www.voltcraft.com/de/labor-netzgerate/	
Grass Instrument S44G Square Pulse Stimulator	Grass Instrument		
Fishing Line	Cormoran Seacor Schockleader	http://www.cormoran.de/co/de/produkte/angelschn%C3%BCre/seacor_shockleader/5,1,62,63,1,1__products-model.htm?ovs_prdrows2=10&sid=btarcbwsn&stamp=1513450435	
IR LED panel	Luminous Film	https://www.luminousfilm.com/custom-led-modules.htm	

Table S1. Parts list. In the first column are the product names from the components used for this study with the corresponding manufacturer (second column), website (third) and article number (fourth).

Fly line	bloomington/flybaseID	source
w;;TH-GAL4		Berlin
w;TH-D1-G4/Cyo		Tanimoto
w;;TH-D4-G4/TM6		Tanimoto
w;TH-D'-G4/Cyo		Tanimoto
w;TH-C1-G4/Cyo		Tanimoto
w;;TH-C'-G4/TM6		Tanimoto
w;;TH-F1-G4/TM6		Tanimoto
w;;TH-F2-G4/TM6		Tanimoto
w;;TH-F3-G4/TM6		Tanimoto
w;;TH-G1-G4/TM6		Tanimoto
w;it/Cyo;DDC-G4(HL9)		Tanimoto
w;MZ840-G4		Tanimoto
w;NP1568/Cyo		Tanimoto
w;MZ19-G4		Tanimoto
w;;DDC-G4(HL8)		Tanimoto
w;sp/Cyo;NP0047		Tanimoto
w;;NP6510-G4		Tanimoto
wy;NP5272-G4;		Tanimoto
w;MB438B (split G4)		Grunwald-Kadow
w;MB032B (split G4)		Grunwald-Kadow
w;MB299B (split G4)		Grunwald-Kadow
w;MB439B (split G4)		Grunwald-Kadow
w;MB304B (split G4)		Grunwald-Kadow
w;MB60B (split G4)		Grunwald-Kadow
w;MB065B (split G4)		Grunwald-Kadow
w;5HTR1B-G4		Tanimoto
w-;R58E02-G4		Tanimoto
w;;c259-G4		Tanimoto
w;MB312B (split G4)		Grunwald-Kadow
w;MB301B (split G4)		Grunwald-Kadow
w;MB058B (split G4)		Grunwald-Kadow
w;MB109B (split G4)		Grunwald-Kadow
w;MB315C (split G4)		Grunwald-Kadow
w;MB056B (split G4)		Grunwald-Kadow
w;MB025B (split G4)		Grunwald-Kadow
w;;Gr66a-G4/TM6		bloomington
w;Gr28bd-G4/Cyo;Gr66a-G4/TM3		bloomington
w;TrpA1-G4		bloomington
w;TH-G80		Tanimoto
w;MB247-G80/Cyo		Tanimoto
w;;Cha-G80		Tanimoto
w;Cha-G80		Tanimoto

norpA^{P24}		Heisenberg
norpA^{P24};20xUAS-Chrimson		crossed stock
w;20xUAS-Chrimson		bloomington
w;TH-C1/cyo; TH-F1/tm3		crossed stock
w;TH-G80;TH-G4		crossed stock
w20xUAS-GtACR1(attP2)	2026	bloomington
w;;20xUAS-GtACR2(attP2)/TM3(Sb)		bloomington
w;;20xUAS6xGFP	52261	bloomington
w;Gr28bd-G4;TrpA1-G4		crossed stock

Table S2. Fly lines deployed in this study. The first column depicts all the fly names with the genetic manipulations for the three first chromosomes. w^-: white mutation. The second column contains the ID number for the flies that were ordered in Bloomington (https://bdsc.indiana.edu/) in the third column.

Comparison	Adj. R^2	R	P-value
red- vs. yellow lit T-maze	-0.05	-0.11	0.77
Y-mazes vs. red lit T-maze	-0.05	-0.08	0.66
Joystick vs. yellow lit T-maze	-0.003	-0.26	0.35
Y-mazes vs. Joystick	-0.03	0.08	0.54
Y-mazes vs. yellow lit T-maze	-0.04	-0.07	0.73
Joystick vs. red lit T-maze	0.29	0.65	0.02

Table S3. Correlation statistics from the operant screens. The first column indicates the two operant screens that were used to correlate the scores from the respective driver lines tested. The adj. R^2: (adjusted R^2) close to zero indicate no correlation whereas the closer to one, the higher the correlation is, in which case the slope is given in the second column by R: (correlation between both variables). The P-values where all not significant after the Bonferroni correction.

8 List of figures

9 List of tables

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11 Eidesstattliche Erklärung

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