The Role of Octopamine in Attraction and Aversion Behavior in *Drosophila melanogaster*

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Abstract

All animals are exposed to environmental stimuli and influences at any time and place. They have to decide whether to respond to a stimulus and whether the reaction should be approach or aversion. In this thesis, the role of OA in *Drosophila melanogaster* as a reinforcer to these kinds of stimuli was investigated. Therefore the optogenetic site attraction assay, the olfactory two odor choice paradigm and feeding of pharmacological active substances were utilized.

So far OA was only known as a positive reinforcer, for example in appetitive olfactory learning and memory, while DA acts as a negative reinforcer in aversive learning and memory (Schwaerzel et al., 2003; Schneider et al., 2012). Here it is shown, that OA can also function as a negative reinforcer. OA is able to mediate attraction and aversion behavior. Optogenetic activation of a *Tdc2*-GAL4 targeted tyraminergic/octopaminergic/ cholinergic set of neurons elicited site attraction, independent of the used channelrhodopsin transgene. A smaller subset of *6.2-Tbh*-GAL4 driven tyraminergic/ octopaminergic/cholinergic neurons, the VUMa4 neurons, which is part of the site attraction eliciting set of neurons, was able to induce site aversion when activated. Both, site attraction and site aversion, are due to OA and not to TA, as activation of these two tyraminergic/octopaminergic/cholinergic subsets in the Tbh^{nM18} mutant background abolished site attraction and site aversion, respectively. So the behavioral outcome is dependent on the combination of activated neurons and OA can function not only as a positive but also as a negative reinforcer. Furthermore, OA is sufficient and necessary for olfactory attraction behavior. This was shown by pharmacological experiments. The loss of olfactory ethanol attraction phenotype in Tbh^{nM18} mutants (Schneider et al., 2012) could be restored to control level by feeding OA, while elimination of OA signaling with epinastine (an OA antagonist) abolished the natural attraction of w^{1118} control flies towards ethanol containing food odors. Thus the loss of olfactory ethanol attraction phenotype of the Tbh^{nM18} mutants is caused by the lack of OA and not due to the increased levels of TA. Mutants overexpressing the Tbh enzyme show a similar phenotype in ethanol attraction and locomotion like the mutants lacking Tbh. Therefore, it seems like a certain balance or interaction between these two neurotransmitters is needed for proper regulation of behavior. Furthermore, OA is required to switch a

behavioral response. The approaching or aversive response of a fly towards a stimulus is mediated by OA and it is also possible to shift an already existing attraction towards attractive ethanol containing food odor to another, less attractive stimulus. This suppression of a normally positive estimated response by activation of tyraminergic/ octopaminergic neurons indicates that OA is maybe not involved in attraction or aversion itself, but in the initiation and the switch between these two behaviors. Tbh^{nM18} mutants, which lack OA, consequently fail to show this switch in behavior.

Furthermore it was shown, that the obtained results in the optogenetic site attraction assay are channelrhodopsin transgene independent and thus real. Two different channelrhodopsins (ChR2 and ReaChR) were tested in more detail and neuronal light activation resulted in site attraction (activation of *Tdc2*-GAL4 targeted neurons) or site aversion (activation of *6.2-Tbh*-GAL4 targeted neurons), independent of the used channelrhodopsin. For activation of the different channelrhodopsins it is important to find a suitable wavelength and light intensity. Additionally, neuronal light activation of these tyraminergic/octopaminergic neurons is not frequency dependent. The observed differences are possibly due to the kinetics of the different transgenes.

Taken together, OA is sufficient and necessary for attraction and aversion behavior and therefore acts as a positive and negative reinforcer. It is probably not involved in these behaviors itself, but mediates the switch between an approach and an aversive reaction to a stimulus. Thus OA orchestrates the behavioral outcome by biasing the decision of *Drosophila melanogaster* towards different stimuli.

Zusammenfassung

Tiere sind überall und zu jeder Zeit den Einflüssen der Umwelt ausgesetzt. Dabei müssen sie sich entscheiden, ob sie auf einen Stimulus reagieren und ob diese Reaktion in Annäherung an den Stimulus oder in Rückzug endet. In dieser Doktorarbeit wurde die Rolle von OA in *Drosophila melanogaster* als ein Verstärker von dieser Art von Einflüssen untersucht. Dafür wurden der optogenetische Site Attraction Assay, der olfaktorische Two Odor Choice Assay und das Füttern von pharmakologischen Substanzen verwendet.

Bisher war OA nur als positiver Verstärker bekannt, zum Beispiel im appetitiven olfaktorischen Lernen und Gedächtnisbildung, während DA der negative Verstärker in aversivem Lernen und Gedächtnisbildung war (Schwaerzel et al., 2003; Schneider et al., 2012). Hier wird nun gezeigt, dass OA genauso als negativer Verstärker arbeiten kann. OA ist in der Lage sowohl attraktives, als auch aversives Verhalten zu vermitteln. Optogenetische Aktivierung von *Tdc2*-GAL4 getriebenen tyraminergen/ oktopaminergen/cholinergen Neuronen löste eine Seiten-Präferenz aus, unabhängig vom verwendeten Channelrhodopsin Transgen. Ein kleineres Set von *6.2-Tbh*-GAL4 getriebenen tyraminergen/oktopaminergen/cholinergen Neuronen, den VUMa4 Neuronen, welche Teil des Seiten-Präferenz auslösenden Set von Neuronen ist, war in der Lage bei Aktivierung Seiten-Aversion auszulösen. Beide Verhalten, Seiten-Präferenz und Seiten-Aversion, werden durch OA und nicht durch TA vermittelt, da eine Aktivierung dieser beiden tyraminergen/oktopaminergen/cholinergen Sets in einem Tbh^{nM18} mutanten Hintergrund zum Verlust der Seiten-Präferenz bzw. der Seiten-Aversion führte. Daher ist das schließlich gezeigte Verhalten abhängig von der Kombination and aktivierten Neuronen und OA arbeitet nicht nur als positiver Verstärker, sondern auch als negativer Verstärker. Des Weiteren ist OA ausreichend und notwendig für olfaktorische Präferenz. Dies wurde durch pharmakologische Experimente gezeigt. Der Verlust der olfaktorischen Ethanol Präferenz in Tbh^{nM18} Mutanten (Schneider et al., 2012) konnte durch Füttern von OA zurück auf Wildtyp Level gebracht werden, während eine Unterdrückung des OA Signalwegs durch Füttern von Epinastine (ein OA Antagonist) zum Verlust der natürlichen Präferenz für Alkohol enthaltende Futterdüfte der w^{1118} Kontrollfliegen führte. Deswegen ist der Phänotyp mit Verlust der olfaktorischen Ethanolpräferenz in Tbh^{nM18} Mutanten auf das Fehlen von OA

zurück zu führen und nicht auf den erhöhten TA Spiegel. Mutanten, die eine Überexpression des Tbh Enzyms aufweisen, zeigen einen ähnlichen Phänotyp in Ethanol Präferenz und Lokomotion, wie die Mutanten, denen das Tbh Enzym fehlt. Daher scheint es, dass ein Gleichgewicht und ein Zusammenspiel dieser beiden Neurotransmitter notwendig ist, um normales Verhalten zu zeigen. Darüber hinaus wird OA auch für den Wechsel zwischen zwei Verhaltensantworten benötigt. Die annähernde oder zurückweichende Reaktion einer Fliege auf einen Stimulus wird durch OA vermittelt und es ist sogar möglich, eine schon bestehende Präferenz für einen attraktiven, Alkohol enthaltenden Futterduft durch eine Präferenz für einen anderen, weniger attraktiven Futterduft zu ersetzen. Diese Unterdrückung von einer normalerweise als positiv bewertete Reaktion, hervorgerufen durch die Aktivierung von tyraminergen/ oktopaminergen Neuronen, deutet daraufhin, dass OA eventuell nicht selbst in Präferenz- oder Aversionsverhalten involviert ist, sondern eher an der Einleitung oder dem Wechsel zwischen diesen zwei Verhalten beteiligt ist. Tbh^{nM18} Mutanten, welche keine OA besitzen, versagen daher diesen Wechsel zwischen zwei Verhalten zu zeigen.

Außerdem wurde gezeigt, dass die mit dem optogenetischem Site Attraction Assay erzielten Ergebnisse nicht abhängig vom verwendeten Channelrhodopsin sind und daher echt sind. Es wurden zwei verschiedenen Channelrhodopsine (ChR2 und ReaChR) genauer untersucht und die neuronal Lichtaktivierung hatte im Fall von *Tdc2*-GAL4 getriebenen Neuronen Seiten-Präferenz und im Fall von *6.2-Tbh*-GAL4 getriebenen Neuronen Seiten-Aversion zur Folge, und das unabhängig vom Channelrhodopsin. Für die Aktivierung der verschiedenen Channelrhodopsine ist eine passende Wellenlänge und Lichtintensität notwendig. Zudem ist die neuronale Aktivierung dieser tyraminergen/oktopaminergen Neurone nicht von einer Frequenz abhängig. Die beobachteten Unterschiede sind eher auf die verschiedenen kinetischen Eigenschaften der unterschiedlichen Channelrhodopsine zurück zu führen.

Zusammengefasst bedeutet das, dass OA ausreichend und notwendig für attraktives und aversives Verhalten ist und somit sowohl als positiver, als auch als negativer Verstärker funktionieren kann. Es ist vermutlich nicht direkt in diese Verhalten involviert, sondern vermittelt den Wechsel zwischen einer annähernden oder einer zurückweichenden Reaktion auf einen Stimulus. Daher verändert OA die Verhaltensantwort durch das Beeinflussen der Entscheidung, die von *Drosophila melanogaster* als Reaktion auf einen Stimulus gefällt wird.

1. Introduction

1.1. The neurotransmitter Octopamine

Octopamine (OA) is a biogenic monoamine which is named after its place of discovery: the salivary glands of the octopus, whereas its function there is still unknown (Erspamer and Boretti, 1951). The synthesis of OA is shown in Figure 1. The first step of the OA synthesis is the decarboxylation of the amino acid tyrosine to tyramine (TA) by the tyrosine decarboxylase (TDC) (Livingstone and Tempel, 1983). In *Drosophila* there are two genes encoding for TDC: *Tdc1* and *Tdc2*. *Tdc1* is expressed in non-neuronal tissues and *Tdc2* in neuronal tissues (Cole et al., 2005). In the second step, TA is converted to OA by the tyramine β-hydroxylase (Tbh) (Livingston and Tempel, 1983; Monastirioti et al., 1996). The Tbh is encoded by the *Tbh* gene (Monastirioti et al., 1996), which encodes for at least five transcripts resulting in different isoforms of Tbh (Manuela Ruppert, 2013).

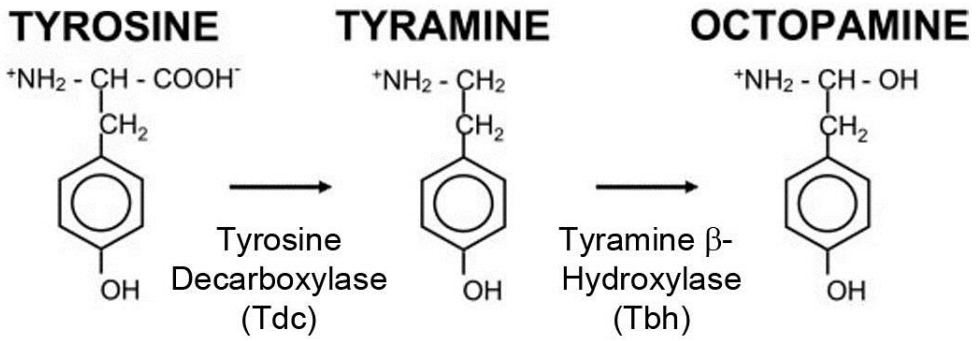


Figure 1: Synthesis of OA.

The amino acid tyrosine is decarboxylated to TA by the TDC enzyme. Then TA is hydroxylated to OA by the Tbh enzyme (modified after Cole *et al*., 2005).

OA and its precursor TA are present in invertebrates and are structurally related to the mammalian adrenaline and noradrenaline (NA) (Roeder, 1999). Both, OA and TA are independent neurotransmitters (Roeder, 2005). OA is thought to function as a

homologue of NA, since NA, an important neurotransmitter in vertebrates, has no physiological role in invertebrates and OA, an important neurotransmitter in invertebrates, has no physiological function in vertebrates (Roeder, 1999). Tbh and Dbh (dopamine β-hydroxylase, the enzyme that catalyzes the final step in noradrenaline synthesis) are highly likely to be functionally homologous and therefore evolutionary related (Wallace, 1976), which supports the homologues function of the octopaminergic and adrenergic systems in invertebrates and vertebrates (Monastirioti et al., 1996). This suggests an early evolutionary origin of the adrenergic/tyraminergic/octopaminergic system. Furthermore, they share functions in behavior, like fight or flight response, learning and memory, motivation and aggression (Roeder, 1999 and 2005). OA and TA are the only neuroactive non-peptide transmitters whose physiological role is restricted to invertebrates (Roeder, 1999). Nevertheless OA exists also in vertebrates (David and Coulon, 1985). OA is not only a neurotransmitter, but also functions as a neurohormone and a neuromodulator and is present in non-neuronal and neuronal tissue in relatively high concentrations in almost all invertebrates (Roeder, 1999). In the brain of *Drosophila melanogaster* OA is present in high concentrations, while its precursor TA is less abundant by the factor 30 (Monastirioti et al, 1996). The normal concentration of OA in nervous tissue is >10mg/g tissue, while in the insects' flight muscles or oviducts concentrations of 1mg/g were detected (Roeder, 1999).

1.1.1. Immunoreactivity of OA and Tbh

The distribution of octopaminergic neurons has been investigated in insects and it was observed that only a small number of OA containing neurons exist, but these few neurons supply almost every neuropil in the insect brain (Bräuning, 1991; Kreissl et al., 1994). The mushroom bodies (MB) and the optic lobes possess the densest innervation with octopaminergic neurons (Homberg, 1994). The most prominent and important octopaminergic neurons are the DUM (dorsal unpaired median) and VUM (ventral unpaired median) neurons (Konings et al., 1988), which are present at the dorsal and ventral midline in the suboesophageal, thoracic and abdominal ganglia and contain and release OA (Hoyle, 1975; Hoyle and Barker, 1975; Evans and O'Shea, 1977; 1978).

In the central nervous system (CNS) of adult *Drosophila* OA is synthesized and released in only a small number of 100 neurons organized in 15 clusters (Sinakevitch and Strausfeld, 2006), and these few neurons have an enormous field of innervation and cover all neuropil areas in the fly brain (Monastirioti et al., 1995; Monastirioti, 1999; Cole et al., 2005; Busch et al., 2009). Octopaminergic cells have been detected in six neuronal clusters at different levels along the dorsoventral axis. There are 12-14 OA positive cells in the suboesophageal ganglion (SOG), 4-5 cells in the antennal lobe (AL) cluster, 4-5 cells in the dorsal anterior cluster (DAC), four cells in the dorsal medial cluster (DMC), 12-16 cells in the dorsal posterior cluster (DPC) and two cells in the lateral protocerebrum (LP) (Monastirioti et al., 1995). In the thoracico-abdominal ganglion 17-19 cells were observed in a single cell and four clusters (Monastirioti et al., 1995) (see Figure 2).

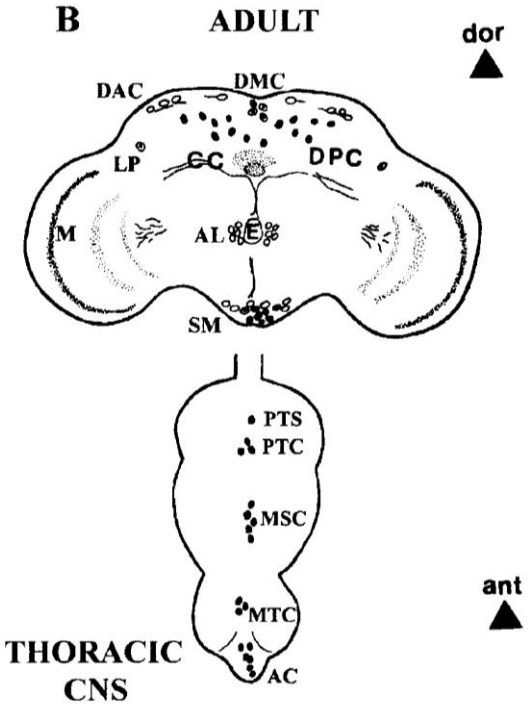


Figure 2: Schematic representation of octopaminergic neurons in the CNS of adult *Drosophila*

Octopaminergic cells are represented by dark circles in the larval CNS. In the adult brain, the filled circles indicate posterior position, circles with dots are medial and open non-filled circles are anterior cells. The stippled areas represent neuroactive neuropil (Monastirioti, 1999).

Busch and colleagues (2009) investigated the expression pattern of the *Tdc2-GAL4* driver line, a GAL4-line which contains a part of the *Tdc2* promoter and therefore targets neurons which should be tyraminergic/octopaminergic. 137 cells are targeted by the *Tdc2*-GAL4 line, but not all of them are also octopaminergic. According to Schneider and colleagues (2012), the *Tdc2-GAL4* driver line targets about 78 Tbh expressing neurons. Therefore, 59 targeted neurons are not Tbh positive. An overlap of GFP and OA was found in 21-27 cells in the ventromedial (VM) cluster, seven in the AL2 cluster, two cells in the ventrolateral (VL) cluster and 2-8 cells in the anterior superior medial (ASM) cluster. In the supraoesophageal ganglion (SPG) and SOG 27 types of octopaminergic neurons were observed. Next to the 11 types of VUM neurons, also five VPM (ventral paired median) neurons in the VM cluster were described (Busch et al., 2009).

1.1.2. The role of OA in Drosophila melanogaster

To investigate the role of OA in invertebrates, a certain *Drosophila melanogaster* mutant was generated by Monastirioti et al. (1996) – the Tbh^{nM18} mutant. These mutants have a deletion in the *Tbh* gene and therefore a disrupted OA synthesis (Figure 3). TA cannot be converted to OA, thus there is a lack of OA and six to eightfold increased TA level (Monastirioti et al., 1996). This mutant is supposed to be a null mutant as no Tbh immunoreactivity in the CNS of *Drosophila* larvae and no Tbh protein were detectable anymore (Monastirioti et al., 1996). But in a semi quantitative RT-PCR it was shown that there is no significant difference between the w^{1118} control and the Tbh^{nM18} mutants and with a quantitative RT-PCR there are 1.5% of the amount of Tbh left in Tbh^{nM18} mutants compared to the w^{1118} control (Manuela Ruppert, 2010). Thus, the Tbh^{nM18} mutant is not a null mutant. It is suggested that there are several *Tbh* isoforms and the deletion in the Tbh^{nM18} mutant does only affect some and not all of the isoforms. The phenotype of the Tbh^{nM18} mutants did not differ from wild type flies in some aspects: they survived until adulthood and had a normal appearance. But they showed a reduced viability under unfavorable, crowded conditions and female flies were sterile while male flies were fertile (Monastirioti et al., 1996; Roeder, 2005). The females retain their fertilized eggs, but this defect could be rescued by feeding OA, so the female sterility is a direct consequence of the missing OA caused by the deletion in the *Tbh* locus (Monastirioti et al., 1996). Additional existing *Tbh* mutants are the Tbh^{Del3} and the *d01344* mutant. The

Tbh^{Del3} mutant carries a larger mutation and is missing the complete first and second exon of the *Tbh* gene, but still has detectable transcripts of Tbh (Manuela Ruppert, 2013). Both *Tbh* mutants are not impaired in ethanol sensitivity but display a reduced ethanol tolerance (Scholz, 2005; Manuela Ruppert, 2013). The *d01344* mutant has a 160% upregulation of Tbh and displays increased sensitivity and normal tolerance towards ethanol (Manuela Ruppert, 2013).

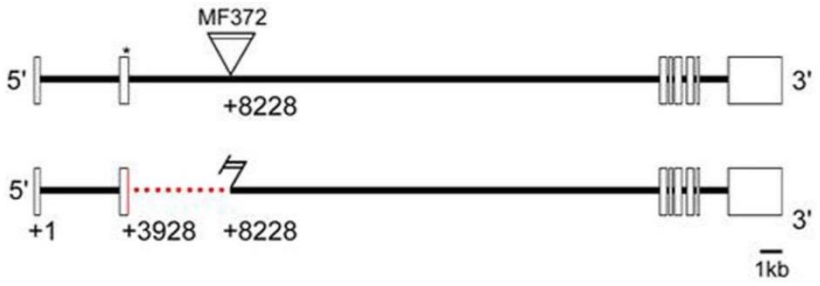


Figure 3: Deletion mapping of the *Tbh*^{nM18} mutant

The *Tbh* gene consists of eight exons. The *Tbh*^{nM18} mutant was generated through P-element mutagenesis and lacks 32 base pairs of the coding sequence of the second exons (Manuela Ruppert, 2010)

The expression pattern of the wild type Tbh was described by Monastirioti et al. (1996) and is very similar to the OA expression pattern (Monastirioti et al., 1995). Schneider and colleagues (2012) used another antibody against Tbh (Zhou et al., 2008) to identify Tbh positive neurons, which also might be OA positive. They found 112 Tbh positive neurons in the adult brain and 39 Tbh expressing cells in the ventral nerve cord (VNC). There was a match in the G0b, G3a/AL2 and VMI-III clusters. The clusters G2b and G4a showed even more Tbh positive cells, but the clusters G3b and G0 had less Tbh positive cells. Furthermore, a new neuron has been identified, which was named G0 posterior. Hence, the used antibody labels Tbh positive cells similar to the previously described octopaminergic pattern, but on average there are more Tbh positive neurons in the labeled clusters (Schneider et al., 2012).

The function of OA has already been investigated in different species and in *Drosophila melanogaster* the *Tbh*^{nM18} mutants are a good opportunity to investigate the role of OA. It is involved in the regulation of many different behaviors like sleeping (Crocker and

Sehgal, 2008), egg laying (Monastirioti et al., 1996; Monastirioti, 2003; Middleton et al., 2006), stress response (Möbius and Penzlin, 1993), motivation (Roeder, 1999; 2005), associative appetitive and aversive learning and memory (Dudai, 1988; Schwaerzel et al., 2003; Honjo and Furukubo-Tokunaga, 2009; Sitaraman et al. 2010; Iliadi et al., 2017), fight or flight response (Brembs et al., 2007), aggression (Baier et al., 2002; Hoyer et al., 2008; Zhou et al., 2008), decision making between aggression and courtship behavior (Certel et al., 2007; 2010), response to olfactory and gustatory stimuli (Scheiner et al., 2014), locomotion (Saraswati et al., 2003), starvation induced hyperactivity (Yang et al., 2015), development of tolerance towards alcohol (Scholz et al., 2000; Scholz, 2005) and innate attraction to odors such as ethanol (Schneider et al., 2012). Thus, OA modulates almost every physiological process in peripheral or sense organs in invertebrates which are studied until now (Roeder, 1999; 2005). Furthermore, OA is involved in the modulation of sensory input and the outcome of the olfactory pathway in the insect brain, which is processed in the olfactory lobes (for olfaction) and MB (for learning and memory) (Farooqui et al., 2003; Schwaerzel et al., 2003). Sombati and Hoyle (1984) postulated the "Orchestration Hypothesis" which describes the function of OA and which says that for every set of behavior there is a neural network and those can be selectively activated or inhibited by the release of OA, which thus allows the suppression of opposing behaviors. Stress factors like heat, starvation, mechanical or chemical influences lead to an increase in OA concentration in many organisms (Davenport and Evans, 1984; Hirashima and Eto, 1993).

In the *Drosophila* larvae, Saraswati and colleagues (2003) investigated the locomotion phenotype of Tbh^{nM18} mutants and compared it to their genetic control. Tbh^{nM18} mutants showed a reduced speed and track length and more pausing and directional changes. This severe phenotype could be partially rescued by feeding OA. Simultaneously feeding of TA nullified the rescue, while feeding only yohimbine (TA antagonist) also partially rescued the locomotion phenotype. When the larvae were fed with OA and yohimbine at the same time, the rescue was further improved. From these experiments, they concluded that OA and TA both influence locomotion in *Drosophila* larvae and that they possibly have antagonistic effects. Thus, for a normal locomotion behavior a balance between these two neurotransmitters is needed (Saraswati et al., 2003). An interaction of both neurotransmitters is also required in the flight initiation and maintenance of *Drosophila*, as Tbh^{nM18} mutants are impaired in this behavior (Brembs et al., 2007). This

phenotype could be restored to control levels by substituting OA with heat shock inducible Tbh or blocking TA receptors. Simultaneously feeding yohimbine and giving a heat shock even improved the performance over control levels. Feeding of OA did not rescue the mutant phenotype. Elimination of all tyraminergic and octopaminergic neurons by using a *Tdc2* mutant resulted in the same phenotype like in Tbh^{nM18} mutants (Brembs et al., 2007).

The right amount of TA and OA also is important in the courtship behavior of male flies. OA is necessary to respond appropriately to presented information. Male flies lacking OA showed courtship behavior towards both, female and male flies, instead of showing aggression towards other males (Certel et al., 2007; 2010). A small subset of three octopaminergic VUM neurons in the SOG, which also expresses Fru^{M} (the male form of neural sex determination factor) is involved in this behavior (Certel et al., 2007). The SOG is the primary taste-processing center. The sensory information which is sent to this neuropil includes the female pheromone recognition cues, which are necessary for males to identify females and show courtship behavior (Bray and Amrein, 2003). Hence, OA is needed in this neuronal subset to accurately transmit contact gustatory pheromone information, as without OA the same information can lead to two different behaviors – aggression and courtship (Certel et al., 2007). Too low and too high levels of OA have the same effect, which is why the level of OA signaling has to be in a certain range. An increase in OA levels or activation of octopaminergic neurons also led to elevated courtship behavior of male flies towards other males (Certel et al., 2010). But the subset of VUM neurons described by Certel et al. (2007; 2010) is not the same subset of octopaminergic neurons in the SOG as mentioned by Zhou et al. (2008) in their aggression studies with *Drosophila*. Here, a reduction of OA signaling led to a decrease in aggression behavior in both males and females, while locomotion, olfaction, sexual discrimination and courtship behavior were unaffected. Tbh^{nM18} mutants did not initiate fighting and did not fight other males even when provoked. An increase in OA levels (feeding OA agonist, overexpression of *Tbh* or activation of OA neurons) resulted in elevated aggressive behavior in grouped flies, but not in socially isolated flies. By combining the *Tdc2-GAL4* driver line with the *Cha-GAL80* driver, Zhou et al. (2008) narrowed the number of involved neurons down to five neurons in the SOG and indicated that neural OA is needed for aggression. This small subset of octopaminergic

neurons seems to mediate aggression in a direct way, as other behaviors were not affected (Zhou et al., 2008).

OA is also involved in appetitive and aversive learning, where *Tbh*^{nM18} mutants displayed severe defects (Schwaerzel et al., 2003; Iliadi et al., 2017). Habituation (a simple form of learning) of *Drosophila melanogaster* is indirectly affected by OA (Scheiner et al., 2014). *Tbh*^{nM18} mutants are less responsive to sucrose and thus showed a faster proboscis extension response (PER) habituation than their controls. This phenotype could be rescued by feeding OA or inducing *Tbh* expression in suboesophageal neurons in the VM cluster. So the mechanism of habituation is intact in *Tbh*^{nM18} mutants, only the gustatory responsiveness and therefore the evaluation of the sweet component of sucrose reward in associative appetitive learning is impaired (Scheiner et al., 2014).

The role of OA was also shown for mediating ethanol related behavior, like tolerance development (Scholz et al., 2000; Scholz, 2005) or innate olfactory ethanol attraction (Ogueta et al., 2010; Schneider et al., 2012). *Tbh*^{nM18} mutants are impaired in tolerance development towards alcohol (50-60% compared to genetic control), but the ethanol sensitivity is normal (Scholz et al., 2000). Furthermore, the *Tbh*^{nM18} mutants had a repression in the initial startle response and a prolonged and increased hyperactivity phase when exposed to ethanol (Scholz, 2005). Thus, Tbh is necessary for proper regulation of locomotor activating and repressing effects of alcohol, OA is required for tolerance development and TA is involved in the regulation of ethanol-activating effect (as *Tbh*^{nM18} mutants already are more active without being exposed to alcohol) but not in ethanol sensitivity or startle response (Scholz et al., 2000; Scholz, 2005). Regarding the attraction of *Drosophila* towards ethanol, wild type flies show an attraction towards natural alcohol concentration, when offered a choice between 5% ethanol containing food odor and alcohol free food odor (Ogueta et al., 2010; Schneider et al., 2012). This concentration of up to 5% of ethanol can be found in the natural environment of the fly, for example in rotten fruits (Dudley, 2002). Ethanol in food odor mixtures is a key odorant, which regulates food attraction (Giang et al., 2017). But *Tbh*^{nM18} mutants failed to show attraction towards alcohol, but preferred food odor over water and ethanol over water, so the odor perception is not impaired and they just might be less sensitive to ethanol (Schneider et al., 2012). The mutant phenotype could be rescued by expressing *Tbh* in a *Tdc2-GAL4* dependent manner, which targets 78 Tbh positive neurons. So the

function of Tbh in these 78 neurons is required for olfactory alcohol attraction. Reducing the number of targeted neurons by using the *Cha-GAL80* driver line did not restore the mutant phenotype. But this subset of neurons is involved in aggression (Zhou et al., 2008), so it is known that olfactory ethanol attraction and aggression are mediated through different kinds of subsets. By testing further GAL4-driver lines, Schneider and colleagues (2012) could narrow down the number of ethanol attraction mediating neurons to 26 *Tbh* expressing neurons in the G3a/AL2 cluster and in the VMI-III, whereas the Tbh positive neurons in the VNC are not involved in mediating ethanol attraction. From this it can be concluded, that the lack of attraction towards ethanol is not due to deficits in execution of motor tasks, as the VNC is the main region involved in locomotor output and is not involved in attraction behavior. Additionally, they assumed that Tbh^{nM18} mutants are able to sense environmental changes and to perform motor related tasks, but they are unable to respond to these stimuli in an appropriate way, so the function of Tbh is probably at the interface between sensory information and response selection and acts as a reward center. So far it is still unknown, how exactly the reinforcer works (Schneider et al., 2012).

1.2. The reinforcing properties of OA

Reinforcement systems drive the synaptic plasticity within neuronal circuits that form memories (Waddell, 2013) and more similar in flies and mammals than thought (Burke et al., 2012). Earlier studies showed that OA acts as a positive reinforcer for reward/appetitive learning and DA acts as the negative reinforcer in aversive learning (Schwaerzel et al., 2003). But more recent studies revealed that DA is also involved in appetitive learning and memory (Burke et al., 2012) and that OA can also mediate aversive learning (Iliadi et al., 2017).

The reinforcing properties of OA have been investigated and shown in different experiments. For the first time it was revealed in the honey bee *Apis mellifera*, where it was shown that activation of the octopaminergic VUMmx1 neuron was able to substitute for the unconditioned stimulus (US) (Hammer, 1993). In *Drosophila* larvae, it was

discovered, that pairing an olfactory stimulus with light activation of tyraminergic/octopaminergic neurons induced appetitive memory formation, while activation of dopaminergic neurons elicited aversive learning. From these findings it was concluded, that these two modulatory systems act antagonistically and are moreover sufficient to substitute for appetitive and aversive reinforcement in an olfactory learning and memory paradigm (Schroll et al., 2006). The same observation was made in adult flies. Schneider et al. (2012) showed that activation of tyraminergic/octopaminergic neurons in a *Tdc2-GAL4* dependent manner in adult *Drosophila melanogaster* is sufficient to elicit site attraction and to increase locomotor activity. Activation of dopaminergic neurons in a *TH-GAL4* dependent manner instead led to site aversion, but also to an increase in locomotor activity in a similar way as activation of the tyraminergic/octopaminergic neurons. With this result, they confirmed that the observed site attraction which resulted from the activated tyraminergic/octopaminergic neurons is not due to hyperactivity of the flies. Furthermore, they concluded that tyraminergic/octopaminergic neurons act as a positive reinforcer, while dopaminergic neurons have the opposing effect and act as a negative reinforcer. In experiments with the OA deficient Tbh^{nM18} mutant (Monastirioti et al., 1996), the effect of missing OA on sugar reward learning was investigated in adult flies. Tbh^{nM18} mutants displayed normal aversive learning scores, but were severely impaired in sugar learning (Schwaerzel et al., 2003). By blocking dopaminergic neurons using *UAS-shi*^{ts}, electric shock learning was severely impaired (Schwaerzel et al., 2003). So they concluded that positive association of an external odor stimulus depends on OA signaling, while negative association is mediated by DA. This is conform to the results of Schroll et al., (2006) and Schneider et al., (2012). But new findings revealed that OA is also involved in aversive learning, as Tbh^{nM18} mutants showed a reduced performance index in electric shock test. This phenotype was not rescuable with feeding OA, but by expressing *Tbh* in a *Tdc2-GAL4* dependent manner and is not due to sensorimotor defects, as Tbh^{nM18} mutants showed normal sensorimotor abilities and taste perception (Iliadi et al., 2017).

In addition to the fact, that OA functions as a positive and negative reinforcer, it was discovered, that DA also is involved in aversive and reward learning. Next to three different dopaminergic pathways of forming aversive memories with different temporal stabilities (Aso et al., 2012), there has to be a way to form appetitive memories including dopaminergic neurons. It was shown that OA signaling in aversive learning requires

signaling via the OAMB receptor located on dopaminergic neurons in the MB (Burke et al., 2012). Activation of dopaminergic neurons can also substitute for sugar in an appetitive learning process and form a robust memory, even if the flies are lacking OA and dopaminergic neurons are also involved in the short-term reinforcing effect of OA (Burke et al., 2012). The fact that OA receptors on dopaminergic neurons are involved in memory formation shows that DA signaling is downstream of OA in the appetitive learning process (Burke et al., 2012). Regarding the DA receptors, the receptor dDA1 is the key receptor for aversive and appetitive learning in *Drosophila* and is located in the MB (Kim et al., 2007). The MB is the main integrative center for learning and memory (Menzel et al., 1988; 1990; Roeder, 1999) and both, appetitive and aversive olfactory memories are localized to the same neuropil in the MB (Schwaerzel et al., 2003). Hence, both neurotransmitters are involved in positive and negative reinforcement and interact which each other to form appetitive and aversive memories.

1.3. OA receptors in adult *Drosophila*

Mediation of this broad range of behavior in invertebrates by OA signaling requires receptors specific for OA to pass on the signal from one neuron to the next cell – the OA receptors. *Drosophila* has four known OA receptors: OAMB and three OctβRs (Han et al., 1998; Balfanz et al., 2005; Maqueira et al., 2005) and all of them are G protein-coupled receptors (GPCRs) (Roeder, 1999 and 2005; Evans and Maqueira, 2005; Maqueira et al., 2005). These receptors are similar to mammalian adrenergic receptors (Dudai and Zvi, 1982; Han et al., 1998) and are classified into the OA1 and OA2 receptor family (Evans and Robb, 1993, Balfanz et al., 2005, Maqueira et al., 2005). The OA1 receptor family contains α-adrenergic-like receptors (OctαRs) (e.g. OAMB) and is known to increase intracellular Ca^{2+} levels, while the OA2 receptor family contains β-adrenergic-like receptors (OctβRs) and is able to stimulate the adenylyl cyclase which results in an increase of intracellular cAMP (Evans and Robb, 1993, Balfanz et al., 2005, Maqueira et al., 2005). The three OctβRs (Octβ1Rs, Octβ2Rs and Octβ3Rs) and the OAMB receptor show a strong preference for OA over TA (Blenau and Baumann, 2001; Maqueira et al., 2005; Balfanz et al., 2005; 2014). The group of OctβRs mediates a major amount of

octopaminergic functions during the development of *Drosophila* (Ohhara et al., 2012). There are also receptors which are sensitive to OA and TA or which are even more sensitive to TA than OA. These receptors are called octopamine/tyramine (Oct-Tyr) receptors or tyraminergic receptors (TyrR, TyrRII and TyrRIII) (Monastitiroti, 1999; El-Kholy et al., 2015; Evans and Maqueira, 2005; Roeder, 2005; Bayliss et al., 2013). Just like the OA receptors, the Oct-Tyr and TA receptors are also G protein-coupled, but both neurotransmitters mediate their effect through different GPCRs (Saudou et al., 1990; Blenau and Baumann, 2001). These receptors belong to the α2-adrenergic receptors and can be divided in two classes: the Type 1 TA receptor (Oct-Tyr receptor) and the Type 2 TA receptor (TA receptor) (Blenau et al., 2017). The Type 1 TA receptor is better activated by TA than OA and has the opposite effect of the OA receptors, as it inhibits the adenylyl cyclase and therefore decreases the intracellular cAMP level, while the Type 2 TA receptor can be almost only activated by TA and is able to mediate Ca^{2+} and cAMP levels (Blenau et al., 2000; 2017). On the other hand, TA is also able to bind to and activate an OA receptor at high concentrations (Han et al., 1998; Blenau et al., 2000; Blenau and Baumann, 2001; Balfanz et al., 2005; Maqueira et al., 2005).

All OA receptors are expressed throughout the whole development in the CNS of the fly, especially in the MB (Ohhara et al., 2012; El-Kholy et al., 2015). Additional, expression in other organs varies depending on the OA receptor type.

In adult flies the Octβ1R was found additionally in the ovaries and testis, the muscles, the intestine, the trachea, the pars intercerebralis, the AL and the thoracic-abdominal ganglion, while the optic lobes were innervated by Octβ1R neurons (Ohhara et al., 2012; El-Kholy et al., 2015). But compared to the other OctβRs it shows only low levels in the female reproductive organ (Li et al., 2015). It was shown, that Octβ1R is required for acute changes in synaptic structure in response to OA and for the increase in locomotion velocity due to starvation (Koon and Budnik, 2012).

The Octβ2R could be found in adult flies in the male and female reproductive system in the skeletal muscles, the trachea, the intestine, the fat body, the salivary glands, the malpighian tubes, the maxillary muscular system, the third antennal segment, the pars intercerebralis, the AL, the optic lobes and with only a few cells in the thoracic-abdominal ganglion (Ohhara et al., 2012; El-Kholy et al., 2015; Li et al., 2015). The Octβ2R plays a pivotal role in the fertilization and ovulation of female flies (Lim et al.,

2014; Li et al., 2015). The homozygous Octβ2R mutant females display normal pre- and post-mating behavior, but they are unable to lay eggs (Lim et al., 2014). They have a delay in copulation rate and enlarged ovaries compared to w^{1118} and Tbh deficient flies and the few laid eggs do not develop as they are not fertilized, which indicates a sperm delivery problem (Li et al., 2015). This phenotype could be (partially) rescued by expression of Octβ2R or ectopic expression of the other three OA receptors (Lim et al., 2014). It was suggested, that there might be an interaction of Octβ2R and OAMB in the female reproductive system (Lim et al., 2014; Li et al., 2015).

In the adult fly, the Octβ3R was detected in the ovaries and testis and in the AL and the mechanosensory center in the CNS. The trachea, the malpighian tubes, the muscles and the pars intercerebralis showed only a weak expression and the optic lobes and the thoracic-abdominal ganglion almost none (Ohhara et al., 2012; El-Kholy et al., 2015). The expression levels in the female reproductive organs were slightly decreased compared to Octβ2R or OAMB (Li et al., 2015). It was shown that Octβ3R is involved in the regulation of ecdysone synthesis in the prothoracic gland, a hormone which is essential for the metamorphosis in *Drosophila*. A knock down of Octβ3R in the prothoracic gland leads to an arrested metamorphosis in the stage between larva and prepupa. As a knock down of TA synthesis resulted in a similar effect, it is likely that not only OA but also TA is able to activate Octβ3R (Ohhara et al., 2014).

The OAMB belongs to the OctαR family and is a α1-like receptor (Kim et al., 2013; Lim et al., 2014). In the adult fly, it was detected in in the pars intercerebralis, the ellipsoid body of the central complex, in some skeletal muscles in the legs, in the reproductive organs (epithelium of the oviduct), in the trachea, in the intestine and in the thoracic-abdominal ganglion. The outer and inner medulla and lobula of the optic lobes were innervated by OAMB neurons (Strauss and Heisenberg, 1993; Han et al., 1998; Lee et al., 2003; El-Kholy et al., 2015; Li et al., 2015). The OAMB seems to be involved in several functions in *Drosophila*. It is involved in synaptic modulation underlying behavioral plasticity (Han et al., 1998), in associative learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994; Davis, 1996; Kim et al., 2013), in motor activities (Strauss and Heisenberg, 1993) and ovulation in female flies (Lee et al., 2003; Lim et al., 2014; Li et al., 2015). Comparable to Octβ2R mutants, OAMB mutant females exhibit normal courtship and copulation behavior, but have a defect in ovulation and thus retain the

eggs in their abdomen (Lee et al., 2003; Lim et al., 2014; Li et al., 2015). But it was shown, that only expression of OAMB in the body and not the high expression levels in the brain are required for normal ovulation (Lee et al., 2003). Furthermore, the OAMB is also involved in appetitive (but not aversive) olfactory learning in *Drosophila*, as OAMB null mutants have a severely impaired learning phenotype (Kim et al., 2013). They showed that the αβ-lobe and the γ-lobe are the functional sites of OA signaling in appetitive olfactory learning and that the OAMB is the key molecule.

1.4. Channelrhodopsins – a tool for light induced neuronal activation

To investigate the role of tyraminergic/octopaminergic neurons in attraction and aversion behavior in *Drosophila melanogaster*, the genetic tool of neuronal light activation via three different channelrhodopsins was used.

Microbial-type rhodopsins are found in archaea, prokaryotes and eukaryotes and have some structural similarities to the rhodopsin in animals, but no sequence homology (Nagel et al., 2003). They are also found in fungi and algae (Bieszke et al., 1999; Hegemann et al., 2001). The prototype of these microbial-type rhodopsins is the light-driven proton pump bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971). Other ion channels are Channelrhodopsin-1 (ChR1) and the related Channelrhodopsin-2 (ChR2). ChR1 mediates the high-intensity response, whereas ChR2 is responsible for low-intensity photocurrents (Sineshchekov et al., 2002). Both were isolated from the green algae *Chlamydomonas reinhardtii* (Nagel et al., 2003) and are involved in generating photocurrents (Sineshchekov et al., 2002). The ChR2 is a blue light gated cation channel with seven transmembrane domains (α helices) and has a covalently bound all-*trans* retinal (ATR), which is necessary for opening and closure of the ChR2 (Nagel et al., 2003). After absorption of a photon with a wavelength of 460-480nm, the retinal changes from all-*trans* to *cis*-conformation, which leads to the opening of the channel and cations can get inside the cell (Figure 4). This leads to a depolarization and thus activation of the cell. But ChR2 has only a low conductance and therefore a higher expression of ChR2 is required for stronger depolarization (Nagel et al., 2003; Pulver et

al., 2009). Furthermore it is said, that blue light does not penetrate the cuticle as well as red or amber light (Eichler et al., 1977; Inagaki et al., 2014). Inagaki and colleagues (2014) measured the penetration of different wavelength through the cuticle of a fruit fly and showed that blue light penetrance is much weaker (about 2%) than green (5%), amber (8%) or red (7%) light. To avoid the high absorption and scattering problem of blue light activatable ChR2, modified channelrhodopsins were created and tested (Lin et al., 2013; Dawydow et al., 2014; Inagaki et al., 2014; Klapoetke et al., 2014). One of these new channelrhodopsins is Chrimson from the algae *Chlamydomonas noctigama*, which has a spectral peak at 590 nm (Klapoetke et al., 2014). A new variant of the channelrhodopsin from *Chlamydomonas reinhardtii* was engineered by Lin and colleagues (2013) – the red-activatable channelrhodopsin (ReaChR), which has an optimum excitation wavelength of 590-630 nm, which is orange to red light (Lin et al., 2013) and does not interfere with normal visual function (Inagaki et al., 2014). But additionally, it is also activatable with green light (Inagaki et al., 2014; Krause et al., 2017). ReaChR has faster kinetics, higher photocurrents and better membrane trafficking compared to other red-shifted channelrhodopsins. A slow channel closure rate after the ending of stimulation is the only limitation of this new red light activatable channelrhodopsin (Lin et al., 2013).

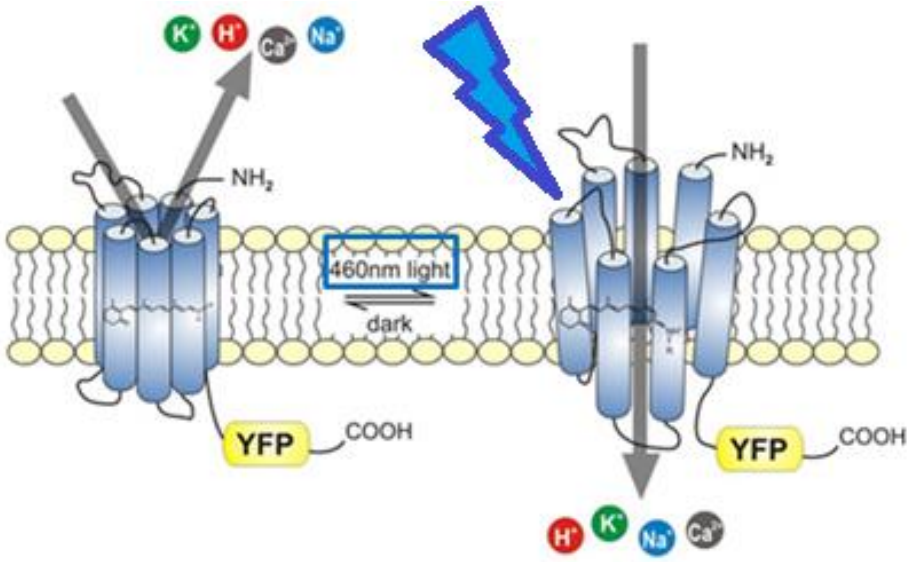


Figure 4: Schematic drawing of Channelrhodopsin-2.

Closed (left) and open (right) state of the blue light gated ChR2 as an exemplary presentation of all channelrhodopsins. Upon light exposure, the ATR changes its conformation and leads to an opening of the channel which allows cations to enter the cell and which thus is activated. (Modified after http://www.biophys.mpg.de/en/bamberg. html).

Using light stimulation to activate neurons has advantages and disadvantages. Due to their characteristics, channelrhodopsins are a non-invasive, rapid and reliable tool with a good spatial and temporal resolution (Nagel et al., 2003, 2005; Boyden et al., 2005; Wang et al., 2007). Compared to temperature sensitive channels like dTRPA1 (*Drosophila* transient receptor potential A1) (Hamada et al., 2008; Viswanath et al., 2003), it is possible to switch the light activation on and off in the range of milliseconds and therefore create a faster and more precise stimulation of the neurons. Experiments with different channelrhodopsins have been done a lot in *Drosophila melanogaster*, regarding different developmental stages and behaviors (Schroll et al., 2006; Zhang et al., 2007; Crisp et al., 2008; Pulver et al., 2009; Schneider et al., 2012; Dawydow et al., 2014; Inagaki et al., 2014; Klapoetke et al., 2014; Hsiao et al., 2015; Xu et al., 2016; Saras et al., 2017).

Light activation of neurons via a channelrhodopsin can be done with constant or pulsed light. In *Drosophila* larvae, constant light led to a higher spike frequency adaptation than light pulses, thus pulsed light might be more effective for long time stimulation (Pulver et al., 2009). Pulsed light was also used by Schneider et al. (2012) and Xu et al. (2016). They used an activation pattern of 2s 40Hz, 16s 8Hz, 2s 0Hz, which derives from a study performed by Hammer (1993) with the honey bee *Apis mellifera*. In this experiment, the conditioning of the PER occurs after a single pairing of an odor (CS – conditioned stimulus) with a food odor US (in this case sugar). Through electrophysiological recordings, a single neuron attracted attention because of its specific response and unique morphology – the octopaminergic VUMmx1 neuron (ventral unpaired median cell of maxillary neuromere 1) – which responded to the US with a long burst of action potentials and fired in the pattern mentioned above. To test its qualities of substituting sugar as the US, a supra-threshold depolarization of the VUMmx1 was elicited instead of offering sugar. There were no differences between the results of eliciting the PER, no matter if using either sugar or depolarization of the VUMmx1, so stimulation of the VUMmx1 neuron is equally effective as an US as sugar. So Hammer (1993) showed that a single neuron is sufficient to mediate reinforcement in associative learning and that OA might be the neuronal representative of the US. Since stimulation of this single neuron could substitute for the US, it is possible that the used frequency is a reward firing pattern. Furthermore, the VUMmx1 neurons has similarities to the VUMa2 neuron in the *Drosophila* brain and both innervate olfaction related structures like the AL, the lateral

horn (LH) and the MB (Hammer, 1993; Busch et al., 2009), therefore it was decided to use this frequency pattern for our experiments.

In addition to the blue light activatable ChR2 used for example by Schneider and colleagues (2012), experiments with Chrimson and ReaChR, two red light activatable channelrhodopsins, have been done. These red activatable channelrhodopsins represent an additional possibility of neuronal light activation and might be a more convenient one. Klapoetke et al. (2014) investigated the function of Chrimson. They used pulsed light for neuronal activation and showed that not only orange (617 nm) or red light (720 nm) stimulation could activate Chrimson, but also blue light (470 nm). Blue and orange light were able to elicit the investigated behavioral response in larvae and adults even at short light pulse durations and low intensities, while red light stimulation needed longer pulses and a higher intensity (Klapoetke et al., 2014). The ReaChR was tested by Inagaki et al. (2014) with pulsed red (627nm), green (530nm) and blue (470nm) light. Green and red light are both sufficient to activate ReaChR and achieved higher scores in all experiments at all times compared to blue light activation of the blue light activatable ChR2. Green light even has the strongest capacity to activate ReaChR when the intensity was not normalized (Inagaki et al., 2014), although it is a red light activatable channelrhodopsin. Consistent with the results obtained by Pulver and colleagues (2009) with the blue light activatable ChR2, Inagaki and colleagues (2014) also observed a dependence of light intensity and pulse frequency in the performance of the flies with the red light activatable ReaChR. Therefore, the broad dynamic range of frequencies and intensities which can be used for ReaChR stimulation leads to a more quantitative and temporally controlled approach to investigate neuronal controlled behavior (Inagaki et al., 2014).

1.5. Aim

The aim of this thesis was to investigate the role of OA in attraction and aversion behavior of *Drosophila melanogaster.* So far it is known that OA is involved in mediating reward learning and memory (Schwaerzel et al., 2003) and in innate odor attraction (Ogueta et al., 2010; Schneider et al., 2012). But it is not known, which kind of set of tyraminergic/octopaminergic neurons is involved in mediating this behavior and if this set is sufficient and necessary to elicit attraction. Furthermore, there is only little information about OA being involved in aversive behavior (Iliadi et al., 2017). These questions are investigated in this thesis.

To answer the first question, which subset of neurons mediates site attraction, the optogenetic site attraction assay was used. Using neuronal light activation, different tyraminergic/octopaminergic sets of neurons were activated via two different channelrhodopsins – a blue (ChR2) and a red (ReaChR) light activatable one. Activation of 78 Tbh positive neurons targeted by the *Tdc2-GAL4* driver line elicited site aversion (Schneider et al., 2012). To narrow down the number of neurons, additional GAL4 driver lines were tested (*Feb15-GAL4, fru-GAL4* and *6.2-Tbh-GAL4*). The achieved results should give further insight into which set of neurons is responsible for attraction behavior and if there probably might be also a Tbh positive subset of neurons which is able to elicit aversive behavior. This would answer the next question, whether OA is also capable of mediating site aversion. To further verify the role of OA, the experiments were repeated in a Tbh^{nM18} mutant background with lacking OA levels (Monastirioti et al., 1996). By combining the TH-GAL80 or Cha-GAL80 driver line to the Tbh positive neurons targeting driver lines, putative effects of dopaminergic or cholinergic neurons were ruled out. Furthermore it was tested, if OA is able to switch an already existent decision by shifting the reinforcer to another option. For all these experiments, an activation pattern of 2s 40Hz 16s 8Hz 2s 0Hz (successfully used by Schneider et al. (2012) and Xu et al., (2017)) was used to stimulate the flies and thus to elicit the desired behavior. Regarding this activation pattern, it was also tested if modifications or only parts of it were able to elicit the same behavior as the original light stimulation pattern. Additionally to the potential frequency dependence, the influence of different light intensities was investigated. From these results, it could be told whether the observed behaviors are really due to neuronal activation or if they are just artefacts caused by the channelrhodopsin transgenes. The

results also give the opportunity to compare the functionality of the two different channelrhodopsin with each other.

The results of the optogenetic site attraction assay revealed that OA is sufficient to elicit site attraction. To answer the question about the necessity, the role of OA in innate olfactory attraction was investigated. The *Tbh*^{nm18} mutants and their genetic control *w*^{1118} were tested in the two odor choice paradigm (Ogueta et al., 2010). *Tbh*^{nm18} mutants fail to show attraction towards ethanol containing food odors in natural concentrations (Ogueta et al., 2010; Schneider et al., 2012). The flies were pre-fed with different pharmacological substances to either activate or block the OA or TA receptors. Next to OA and TA, two OA agonists (clonidine and naphazoline), an OA antagonist (epinastine) and a TA antagonist (yohimbine) were administered to the flies. Furthermore, different *Tbh* mutants (*Tbh*^{nm18} and *Tbh*^{Del3} with decreased OA levels and *d01344* with increased levels of OA) and a *Tdc2* mutant (which lacks both OA and TA) were compared to each other and the effect of an overexpression of Tbh was investigated. This should also provide evidence about the relation of OA and TA in attraction behavior. In a last step, the four different OA receptors were eliminated in the olfactory sensory neurons (*Orco-GAL4*) by using RNAi. In the two odor choice paradigm it was investigated which OA receptor is involved in mediating olfactory attraction towards ethanol containing food odors.

2. Material and Methods

2.1. Material

2.1.1. Fly strains

Name of fly stock (named in this thesis)	Origin	Chromo-some	Stock list
w^{1118}	Lindsley & Zimm	X	#4
w^{1118}; $Tdc2^{RO54}$/CyO (*$Tdc2^{RO54}$*)	Cole et al., 2005	II	#535
w^{1118}, Tbh^{nM18}/FM7(*Tbh^{nM18}*)	Monastirioti et al., 1996	X	#1
w^{1118}, $Tbh^{R3\text{-}XPdel}$/FM7 (*Tbh^{Del3}*)	Manuela Ruppert, 2013	X	#536
w^{1118}, XP^{d01344} (*d01344*)	Exelixis Collection at HMS	X	#16
w^{1118};; UAS-Tbh/TM2	Henrike Scholz	III	#22
norpA^{1}; UAS-ChR2; UAS-ChR2 (*UAS-ChR2*)	Nuwal, 2010	X + II + III	#318
w^{1118}, Tbh^{nM18}; UAS-ChR2; UAS-ChR2	Gerbera Claßen	X + II + III	#40
w^{1118}; UAS-ReaChR; UAS-ReaChR (*UAS-ReaChR*) (original Stocks: *w; UAS-ReaChR* and *w;; UAS-ReaChR*)	Gerbera Claßen (original stocks: Inagaki & Anderson)	II + III	#536
w^{1118}, UAS-CS Chrimson (*UAS-Chrimson*)	Vivek Jayaraman	X	#506
w^{1118}; dTdc2-GAL4 (*Tdc2-GAL4*)	Cole et al., 2005	II	#35
w^{1118};; 6.2_2-Tbh-GAL4	Hampel, 2007	III	#278
w^{1118}; Feb15-GAL4	Siegmund & Korge, 2001	II	#217

w^{1118};; NP0021/TM6 (*fru-GAL4*)	Kimura et al., 2005	III	#392
w^{1118}; dTdc2-GAL4/CyO; $Cha^{3,3kb}$-GAL80/TM6,Tb1 (*Tdc2-GAL4; Cha-GAL80*)	Andrea Schneider	II + III	#30
w^{1118}; TH-GAL80; 6.2-Tbh-GAL4	Gerbera Claßen	II + III	
norpA^{1}; UAS-ChR2; fru-GAL4	Gerbera Claßen	X + II + III	
norpA^{1}; UAS-ChR2; 6.2-Tbh-GAL4	Gerbera Claßen	X + II + III	
norpA^{1}; UAS-ChR2; Cha-GAL80	Gerbera Claßen	X + II + III	
w^{1118};; Orco-GAL4 11.17 (*Orco-GAL4*)	Vosshall, 2008	II	#89
BDSC = Bloomington *Drosophila* Stock Collection			
w[1118]; P{y[+t7.7] w[+mC]=GMR29H06-GAL4} attP2 (GMR29H06-*GAL4*)	#49506, Pfeiffer et al., 2008	III	#394
w[1118]; P{y[+t7.7] w[+mC]=GMR47A10-GAL4} attP2 (*GMR47A10-GAL4*)	#50289, Pfeiffer et al., 2008	III	#395
w[1118]; P{y[+t7.7] w[+mC]=GMR51D07-GAL4} attP2/TM3, Sb[1] (*GMR51D07-GAL4*)	#48186, Pfeiffer et al., 2008	III	#393
y[1] v[1]; P{y[+t7.7] v[+t1.8]= TRiP.JF01673}attP2 (*UAS-OAMB-RNAi)*	#31171, Ni et al., 2008	III	#457
y[1] v[1]; P{y[+t7.7] v[+t1.8]= TRiP.JF01571}attP2 (*UAS-Octβ1R-RNAi*)	#31106, Ni et al., 2008	III	#455
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]= TRiP.HMS0115 1}attP2 (*UAS-Octβ2R-RNAi*)	#34673, Ni et al., 2011	III	#459
y[1]v[1];P{y[+t7.7] v[+t1.8]=TRiP.JF01573} attP2/TM3,Sb[1] (*UAS-Octβ3R-RNAi*)	#31108, Ni et al., 2008	III	#456

2.1.2. Fly husbandry

The flies were raised on standard fly food prepared after the following recipe for 30l (filled up with water):

agar-agar (fiber)	240g
brewer's yeast	449g
polenta	1800g
sugar beet syrup	2400ml
propionic acid (E280) (bactericide)	87ml
30% sodium methylparaben (E219) in H2O dest. (fungicide)	240ml

The environmental conditions were 25°C and 60% humidity at a 12 hours light/dark cycle. For amplifying and crossing big food vials (Ø 5cm) were used. Exceptions are the crossings for the locomotion experiments, which were set up in medium vials. For collecting virgins or males medium food vials (Ø 3,5cm) were used.

Crosses were set up in big food vials with 35-40 virgins and 15 male flies (15 virgins and 7-8 males for crosses in medium food vials), which were collected for one week. The crosses were held on 25°C and flipped thrice every two days. After 10-12 days, the flies started hatching and the males were collected in medium vials (50 males for the olfactory two odor choice paradigm and for the optogenetic site attraction assay, 10-15 males for the locomotion assay). After a recovery of minimum 24h the flies could be tested.

2.1.3. Chemicals

All chemicals, if not mentioned otherwise, were ordered from Sigma-Aldrich. The all-*trans* retinal was ordered from Bettersyn, Shanghai.

2.2. Methods

2.2.1. Olfactory two odor choice paradigm

In the two odor choice paradigm (Figure 5) the flies were offered two food odor sources in two medium vials which were in a glass beaker (Ø 10,5cm). The first food odor source was apple mango juice (1,5ml) and the second food odor source was apple mango juice with 5% ethanol (in one experiment 23% ethanol) (1,5ml). The apple mango juice consisted of 75% apple juice and 25% mango juice (Alnatura, Germany GTIN: 4104420071841) and stored at -20°C. The vials were closed with a lid with a hole in the middle, were a blue pipette tip was inserted. The opening of the cut pipette tip was 1,8mm, through which the flies could enter the food odor trap. Once a fly made a decision to enter one vial, it was trapped and the choice was not reversible as they were not able to leave the vial again. The experiment was executed at 25°C and 60% humidity overnight (at least 19h) on a light source. On the next day, the number of flies in each vial and of undecided flies in the beaker was counted and the attraction index (AI) was calculated (Figure 6). If more than 10% of the flies did not decide during the night, this experimental run was not used.

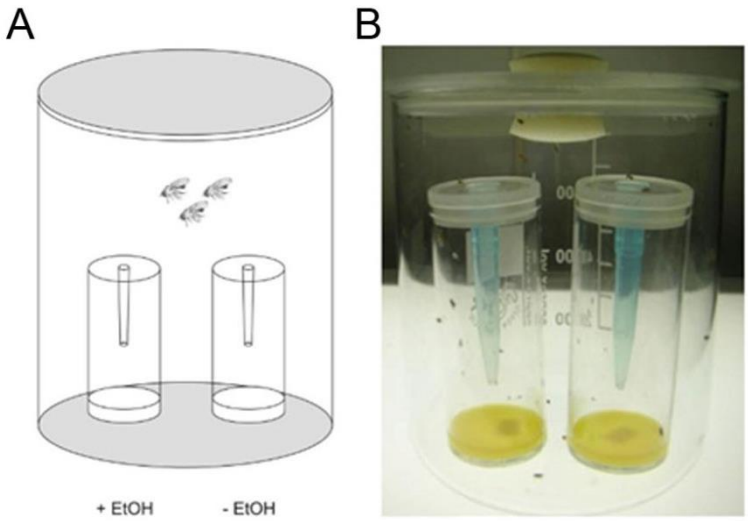


Figure 5: Two odor choice paradigm.

(A) Schematic drawing (Schneider et al., 2012) and (B) picture (Ogueta et al., 2010) of the two odor choice paradigm for testing olfactory ethanol attraction. The flies had the choice between a food odor containing trap and a trap with a food odor supplemented with ethanol. Once they decided, they could not undo their decision and the AI could be calculated.

$$AI = \frac{\text{number of flies in trap A - number of flies in trap B}}{\text{number of flies in trap A + number of flies in trap B}}$$

Figure 6: Calculation of the attraction index (AI)

2.2.2. Feeding of pharmacological substances

For some experiments, pharmacologically active substances were fed to the flies prior to the olfactory two odor choice assay. If not mentioned otherwise, the control group was fed on 5% sucrose and 5% red food color in water added on a filter paper. The experimental group was fed on the same stock solution but with the added drug on filter paper. The drugs were fed as hydrochlorides. The amount of pipetted solution on the filter paper was 300µl. If not mentioned otherwise, the flies were previously starved for 3h without any access to water.

Pharmacological Drug	Molarity/ Concentration	Feeding Time
Octopamine	53mM	1h
Epinastine	3mM	48h
Clonidine	50mM	3h
Naphazoline	200nM	3h
Yohimbine	25mM	2h
Tyramine	288mM	18h
Ethanol	10%	0.5h

Yohimbine was given without sucrose. Also the control group was pre-fed with only water and red food dye. To dissolve yohimbine in water, the water had to be heated to 30°C and the yohimbine was added bit by bit under constant stirring, as it is not very good soluble in water. In this case, the flies were starved overnight with a moist filter paper.

Epinastine was administered for 2d with daily remoistening of the filter paper. In this case, the flies were not starved beforehand.

Ethanol was fed to the flies for 30 minutes. Afterwards the flies had a recovery time of 3,5h with access to a wet filter paper containing pure water.

Tyramine was supplied without sucrose. Also the control group had no access to sucrose and both groups were not starved beforehand.

2.2.3. Optogenetic site attraction assay

Food vials used for optogenetic experiments had to be prepared beforehand. Two different crosses were set up: a control group and an experimental group. Channelrhodopsins need all-*trans* retinal (ATR) to function, which is soluble in ethanol and had to be added to the food. The food in the vials was perforated with pipette tips and 250mM of ATR was added on the food (200µl for big vials and 100µl for medium vials). To rule out influences of the absolute ethanol the ATR was dissolved in, the vials for the control group were supplemented with the same amount of 100% ethanol. As ATR is light sensitive, all vials used for the optogenetic experiments were wrapped in aluminum foil. This was also done for the control group to eliminate differences in the development due to light influences.

In the optogenetic site attraction set up the flies were offered two identical food odor sources (1,5ml apple mango juice), but the food odors were illuminated with different LEDs (Figure 7). If other food odor combinations were used, it is mentioned in the text. The experiment was executed at 25°C and 60% humidity overnight (at least 19h). The testing chambers are made of light proof dark grey plastic, so the light of the diodes was the only light sources in this experiment. One LED was supposed to activate the used channelrhodopsin and the other one served as a negative control without an effect on the transgene. Both diodes had the same activation pattern and light intensity in one experiment. Among the different experiments, the activation patterns and intensities could change. The activation pattern was controlled by a LED controller to which the diodes are connected and which itself was connected to a computer. The computer program LPTfreq (Andrea Schneider, 2011) controlled the activation pattern, which

consisted of the 2s 40Hz 16s 8Hz 2s 0Hz pattern or modifications of this original pattern and was repeated during the whole experiment. The light intensity of the diodes were adjusted at the LED controller and was checked by a light-meter (5052, PeackTech). The used light intensities ranged from 300 lux to 1700 lux.

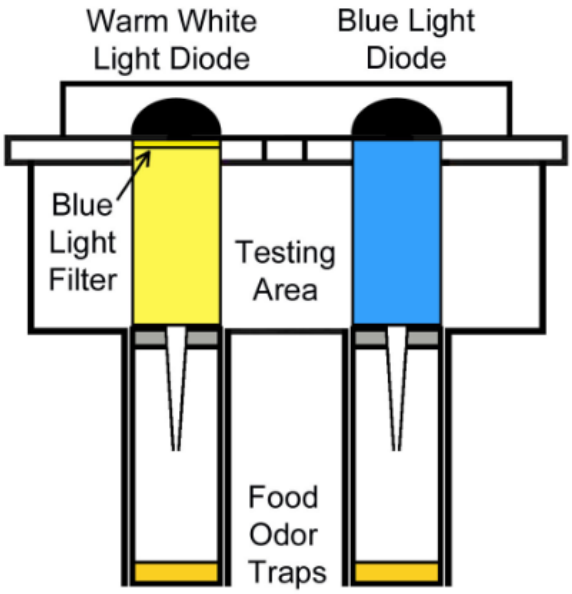


Figure 7: Optogenetic site attraction setup.

Schematic drawing of the optogenetic site attraction assay (modified after Schneider et al., 2012). The blue light diode and the warm white light diode with a blue light filter are representative for the two different diodes used in every experiment.

2.2.4. LEDs

All diodes were obtained from Cree, Munich, Germany.

Warm white light diode (2600-3700K, XREWHT-L1-0000-008E7) with a blue light filter (high pass filter, 510 nm, HEBO, Aalen, Germany) (resulted in yellow light)

Blue light diode (465-485nm, XREBLU-L1-0000-00K01)

Red light diode (620-630nm, XPEBRD-L1-R250-00801)

Green light diode (520-535nm, XREGRN-L1-0000-00P01)

Amber light diode (585-595nm, XRCAMB-L1-0000-00J01)

2.2.5. All-*trans*-Retinal

For the stock solution of 250mM all-*trans*-Retinal 1g of the Retinal was filled up to 14ml with 100% Ethanol and dissolved completely. The solution was stored it at -20°C. All these steps had to be done in the dark as Retinal is light-sensitive.

2.2.5. Cuticle penetration of blue and red light

To measure the penetrance of blue and red light through the cuticle of a fly, the head of a fly was placed on top of an optical fiber (Ø = 1mm) (Figure 8). The light source, either a blue or a red light diode, was placed in the same distance (4cm) to the head of the fly as the distance existing between the diodes and the top of the food odor traps in the optogenetic site attraction assay. A power meter (indicating instrument: SoloPE, sensor: PH100-Si, Gentec-EO) measured the energy arriving at the optical fiber. In total, a number of eight fly heads were measured for blue and red light.

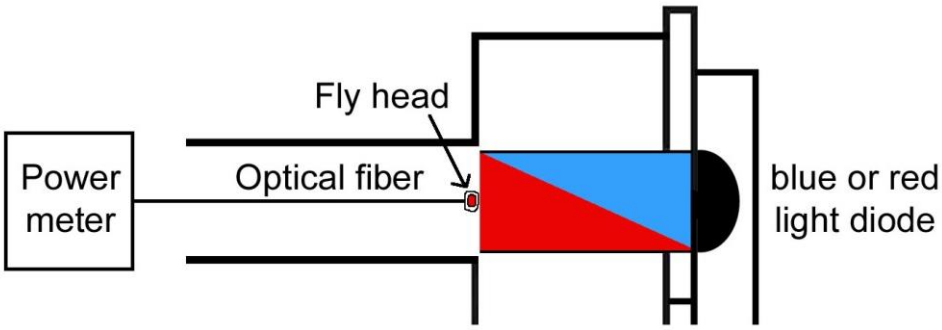


Figure 8: Penetration of red and blue light setup

Schematic drawing of the setup for measuring the penetration of red and blue light through the cuticle of the head of a fly. The blue light or red light diode were placed at a distance of 4cm from the fly head and a power meter measured the light energy still reaching the top of the optical fiber.

2.2.6. Locomotion assay

The setup for the locomotion assay consisted of a video camera (Sony Network Handycam DCR-TRV950E) which was connected to a computer, a cold white light plate as a light source from below and the test arena (glass petri dish (Ø 3,6cm) on a glass plate) containing the fly (Figure 9).

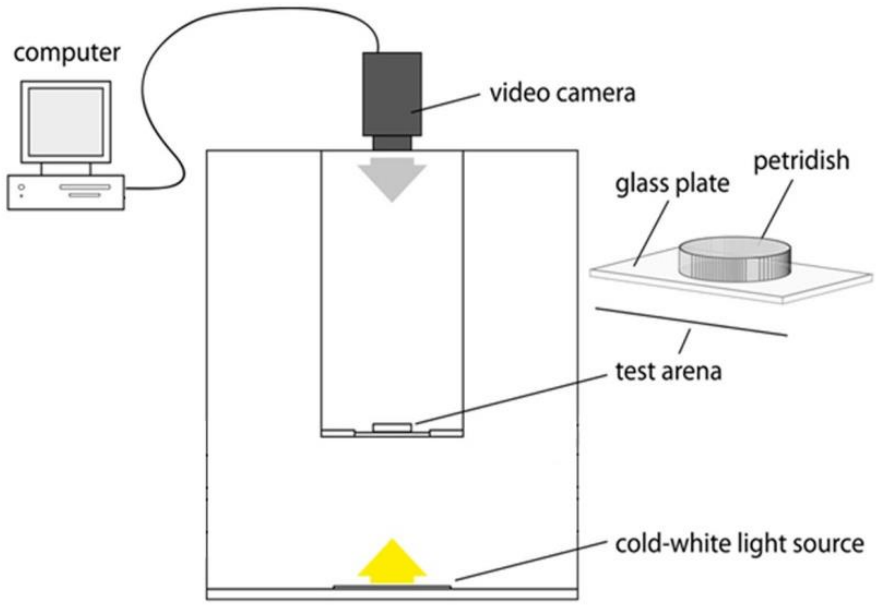


Figure 9: Locomotion Setup.

The flies could move freely in the glass Petri dish while they were illuminated by a cold white light source from the bottom and filmed from above by a camera connected to a computer (modified after Andreas Klein, 2013).

At the day of the experiment the flies which should be tested were transferred one by one into the test arenas and were allowed to adapt to the new environment for five minutes. To start an experiment one test arena containing a fly was transferred carefully into the setup between the camera and the light source. There the fly was allowed to calm down again if necessary. Then they were filmed for 1:05 minutes with a frame rate of 25 pictures per second. Only a filmed sequence of one minute was needed, but to avoid too short videos, additional five seconds were recorded.

The analysis was done with four different programs. For more detailed information see "Cell specific ChR2 light activation of freely moving flies" (Protocol by Andreas Klein, 2013). "VirtualDub 1.9.11" was used for cutting the video into a fragment of exactly one minute duration. The videos were recorded in color, but for further analysis only black/white videos were needed. For splitting the channels (further using the red channel) and clearing the background "Fiji" was used. Tracking of the flies was done with the program "Tracker". Five of the many more analyzed parameters in this program (frame, time, x-coordinate, y-coordinate and radius) had to be copied and pasted in this order into an "Excel" file. The covered distance of the fly was calculated via the x and y axis parameters between two time frames and from this the speed was calculated via the distance and the time point. The x and y axis parameters were also used to display the walking pattern of a fly (Figure 10). The p-values were determined by the student's t-test. For the results a closer look on the travelled distance and the velocity of the flies was taken.

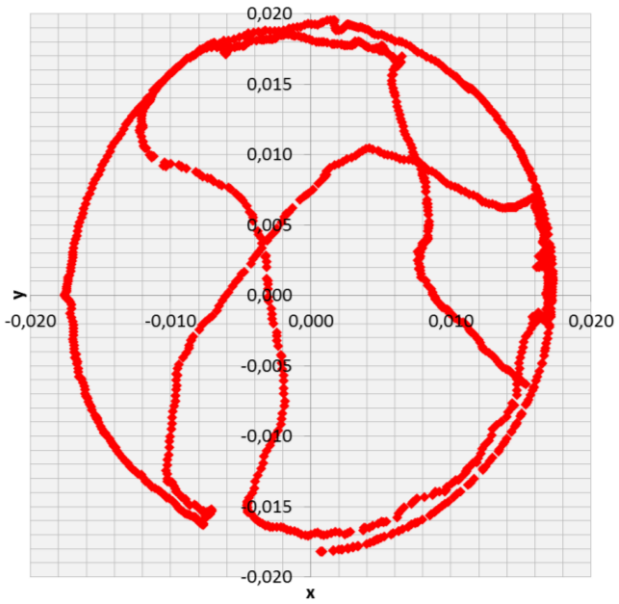


Figure 10: Walking pattern of a fly.

This exemplary picture shows the walking trace of a filmed w^{1118} fly over the time of one minute.

2.2.7. Statistics

Errors are indicated as standard error of the mean (s.e.m.). Except for the locomotion experiment, where the standard deviation (STDV) is shown. Bars labelled with an **a** are significantly different from zero according to the One-sample sign test (Statview). Due to the normal distribution of the data, the Student's *t*-test (Excel) was used for comparison of the control group and the experimental group in the optogenetic site attraction experiments and some of the olfactory attraction experiments. If more than two experimental groups were tested in the olfactory two odor choice assay, ANOVA post hoc Tukey test was used (Statistica). The statistical significances are marked by $*P = 0.05$, $**P = 0.01$ and $***P = 0.001$.

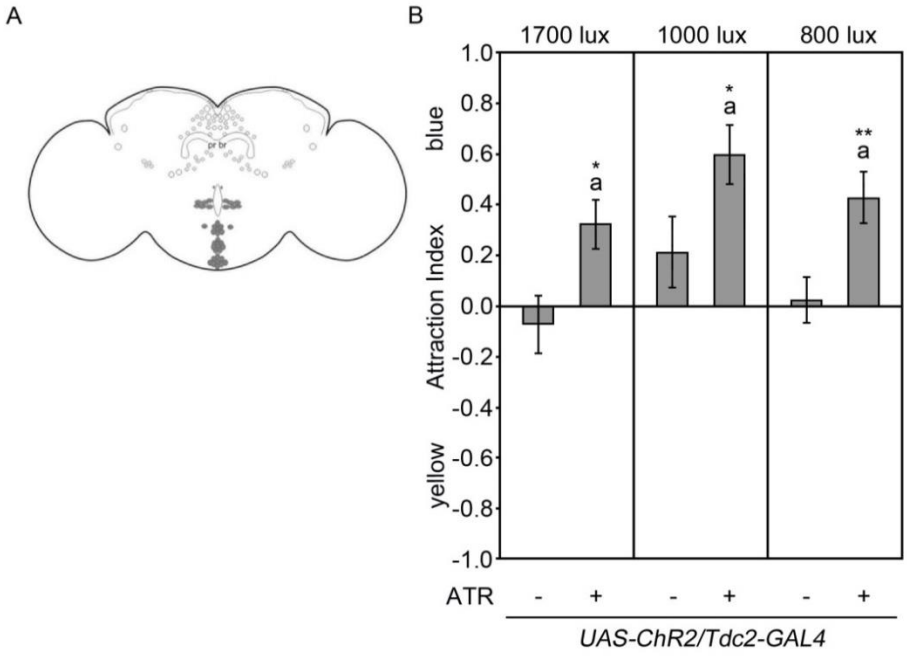
3. Results

3.1. A subset of tyraminergic/octopaminergic/cholinergic neurons mediates site attraction and aversion

3.1.1. Site attraction elicited by *Tdc2*-GAL4 targeted neurons is light intensity independent but frequency dependent when using the blue light activatable ChR2

Expression of *UAS-Tbh* in a *Tdc2*-GAL4 dependent manner in Tbh^{nM18} mutants restored the mutant phenotype of loss of attraction towards ethanol containing food odors to control level in a binary choice assay, showing that Tbh is required for olfactory attraction in adult flies (Schneider et al., 2012). The *Tdc2*-GAL4 driver line targets around 78 Tbh expressing neurons in the CNS of adult *Drosophila melanogaster*: in the G3a/AL2 cluster, the G3b cluster and the VMI-VMIII cluster (41 in the brain and 37 in the VNC) (Figure 11A). They also tested, if neuronal activation of this Tbh positive set of neurons is sufficient to induce attraction. Therefore *UAS-ChR2* was expressed under the control of the *Tdc2*-GAL4 driver line using the UAS/GAL4 system (Brand and Perrimon, 1993) in the no receptor potential A ($norpA^{1}$) mutant background. This was done to eliminate influences of optical stimuli, as this mutation leads to an inability to generate receptor potentials (Bülthoff, 1982) and thus the flies are blind. Only when the flies enter the blue light illuminated area of the setup, the neurons expressing *UAS-ChR2* are activated and should induce attraction behavior. For stimulation of these neurons Schneider and colleagues (2012) used a frequency of 2s 40Hz 16s 8Hz 2s 0Hz, which derives from experiments with the honey bee, where the VUMmx1 neuron showed a similar firing pattern and could substitute for sucrose as the US (Hammer, 1993). The used light intensity in the optogenetic site attraction assay was 1800 lux. The combination of the activation pattern and light intensity resulted in site attraction, thus activation of *Tdc2*-GAL4 targeted neurons in the SOG and AL is sufficient to induce site attraction (Schneider et al., 2012). The AL and SOG are of special interest in olfactory attraction behavior, as the AL are the primary olfactory input region and the SOG is the main region of gustatory input (Busch and Tanimoto, 2010).

As 1800 lux is a rather high light intensity, the same experiment was repeated in this study with the same activation pattern of 2s 40Hz 16s 8Hz 2s 0Hz at three lower light intensities: 1700, 1000 and 800 lux (Figure 11B). All these different light intensities were sufficient to induce site attraction for the blue illuminated food odor trap. Therefore light activation of the *Tdc2*-GAL4 driven neurons expressing the *UAS-ChR2* is not intensity dependent.



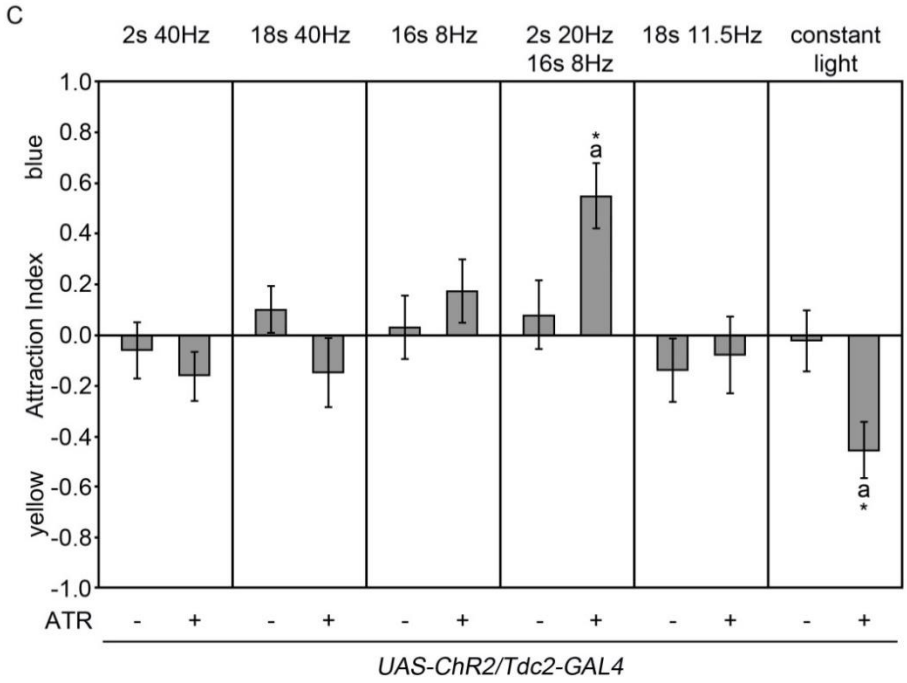


Figure 11: Site attraction elicited by activation of *Tdc2*-GAL4 dependent neurons is not light intensity dependent but frequency dependent when using the *UAS-ChR2* transgene

(A) The *Tdc2*-GAL4 line drives expression in 41 neurons in the SOG and AL in the adult fly brain. The white cells are Tbh positive cells and the grey cells indicate a co-localization of Tbh and GFP (Schneider et al., 2012). (B) Light activation of *UAS-ChR2* in a *Tdc2*-GAL4 dependent manner with a 2s 40Hz 16s 8Hz pattern with different light intensities led to site attraction towards the blue illuminated trap (AIs for the control group and the experimental group at 1700 lux: -0.07 ± 0.12 and 0.32 ± 0.1 respectively, n = 20, 20; AIs at 1000 lux: 0.21 ± 0.14 and 0.6 ± 0.12, n = 34, 31; AIs at 800 lux: 0.02 ± 0.09 and 0.43 ± 0.1, n = 39, 33). (C) Only activation of *norpA*^{1}*; UAS-ChR2/Tdc2*-GAL4*; UAS-ChR2/+* flies with a 2s 20Hz 16s 8Hz 2s 0Hz pattern led to the same site attraction behavior as observed beforehand while activation with constant light leads to site aversion (AIs for the control group and the experimental group at 2s 40Hz: -0.06 ± 0.11 and -0.16 ± 0.1 respectively, n = 20, 21; AIs at 18s 40Hz: 0.1 ± 0.09 and -0.15 ± 0.14, n = 29, 24; AIs at 16s 8Hz: 0.03 ± 0.13 and 0.17 ± 0.13, n = 23, 26; AIs at 2s 20Hz 16s 8Hz: 0.08 ± 0.14 and 0.55 ± 0.13, n = 27, 23; AIs at 18s 11,5Hz: -0.14 ± 0.13 and -0.08 ± 0.15, n = 24, 16; AIs at constant light: -0.02 ± 0.12 and -0.46 ± 0.11, n = 14, 13). The result of 2s 40Hz, 18s 40Hz, 18s 11.5Hz and constant light are taken from Thomas Giang (2014). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. **P* < 0.05 and ***P* < 0.01.

To check if the observed site attraction towards the blue illuminated site is caused by a specific activation pattern of 2s 40Hz 16s 8Hz pattern, different frequencies were used to activate the *UAS-ChR2* expressing neurons (Figure 11C). Using the high frequency pattern of 40Hz has no effect on the attraction behavior, no matter if stimulated for 2s or 18s (Thomas Giang, 2014; Figure 11C first and second panel). Activation with a lower frequency of 8Hz for 16s also had no effect (Figure 11C third panel). Activation of the neurons with a 2s 20Hz 19s 8Hz pattern resulted in site attraction in the experimental group (Figure 11C fourth panel). Stimulation with an average frequency of 18s 11.5Hz had no effect on the behavior (Thomas Giang, 2014; Figure 11C fifth panel), while constant light at 720 lux elicited site aversion in the experimental group (Thomas Giang, 2014; Figure 11C last panel).

Taken together, site attraction induced by the activation of *Tdc2*-GAL4 targeted neurons is frequency dependent. The single parts of the activation pattern of 2s 40Hz 16s 8Hz are not sufficient to induce site attraction and only a combination of a short higher frequency part followed by a longer lower frequency part was able to induce the same site attraction as observed in Schneider et al. (2012).

3.1.2. Activation of neurons targeted by the *Feb15*-GAL4 driver did not elicit site attraction

The *Feb15*-GAL4 driver expresses the *UAS-ChR2* transgene in 31 Tbh positive neurons including the neurons in the G3a cluster, in the VMI-VMII cluster and in parts of the VMIII cluster (Schneider et al., 2012) (Figure 12A). Neurons required for Tbh dependent ethanol attraction are included within the 31 Tbh positive neurons, as expression of *UAS-Tbh* in *Feb15*-GAL4 dependent neurons restored the loss of attraction observed in Tbh^{nM18} mutants (Schneider et al., 2012).

Activation of the *UAS-ChR2* transgene using a 2s 40Hz 16s 8Hz pattern was not sufficient to elicit site attraction. Both, the control and the experimental group were undecided (Figure 12B left panel). Substituting the 40z with 20Hz led to site aversion of the experimental group towards the blue light illuminated food odor trap (Figure 12B right panel). So activation of these neurons is not sufficient to elicit site attraction.

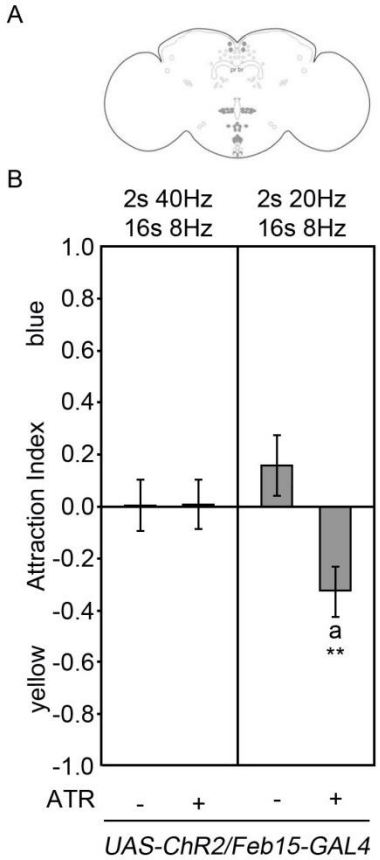


Figure 12: Activation of *Feb15*-GAL4 driven neurons with a reduced frequency led to site aversion when using the *UAS-ChR2* transgene

(A) The *Feb15*-GAL4 line drives expression in 31 neurons in the SOG and AL in the adult fly brain. The white cells are Tbh positive cells and the grey cells indicate a co-localization of Tbh and GFP (Schneider et al., 2012). (B) Light activation of *Feb15*-GAL4 driven neurons via expression of *UAS-ChR2* did not led to site attraction with a 2s 40Hz 16s 8Hz pattern (AIs of for control and experimental group: -0.00 ± 0.1 and 0.01 ± 0.1 respectively; n = 33, 34). But activation with a 2s 20Hz 16s 8Hz pattern elicited site aversion (AIs of for control and experimental group: -0.16 ± 0.12 and -0.33 ± 0.1 respectively; n = 22, 26). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. $^{**}P < 0.01$.

3.1.3. Activation of neurons targeted by the *fru*-GAL4 driver elicited site attraction

The *fru*-GAL4 driver targets three tyraminergic/octopaminergic neurons in the SOG (OA-VPM1, OA-VPM2 and OA-VUM) (Certel et al., 2010) (Figure 13A) in addition to other non tyraminergic/octopaminergic neurons (Lee et al., 2000; Billeter and Goodwin, 2004). To address whether these neurons might be involved in mediating site attraction, the *UAS-ChR2* was expressed in a *fru*-GAL4 dependent manner.

Activation of these neurons with a 2s 40Hz 16s 8Hz pattern in male flies elicited site attraction in the control group, but not in the experimental group and both groups were not significantly different from each other (Figure 13B left panel). The *fruitless* gene is a neural sex determination factor (Ryner et al., 1996) and the P-element insertion of the GAL4 into the second intron of the *fruitless* gene disrupted its function (Kimura et al., 2005). Therefore it might have an influence on the behavior of the male flies. Thus also female flies were tested. Here, activation of the *fru*-GAL4 targeted neurons induced site attraction to the blue illuminated food odor trap in the experimental group (Figure 13B right panel). Hence, it is possible that the three tyraminergic/octopaminergic neurons in the SOG mediate site attraction.

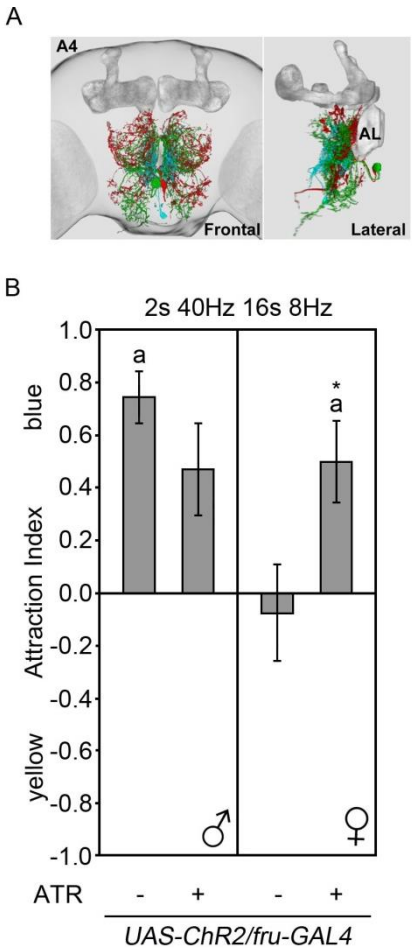


Figure 13: Activation of *fru*-GAL4 dependent neurons resulted in site attraction when using the *UAS-ChR2* transgene

(A) The *fru*-GAL4 line drives expression amongst others in three octopaminergic neurons in the SOG in the male adult fly brain (Certel et al., 2010). (B) Activation of *UAS-ChR2* in a *fru*-GAL4 dependent manner with a 2s 40Hz 16s 8Hz pattern elicited attraction in female flies (AIs of control and experimental groups are -0.08 ± 0.18 and 0.50 ± 0.15 respectively; n = 19, 20). In male flies, both groups showed an attraction towards the blue illuminated trap (AIs of control and experimental groups are 0.74 ± 0.1 and 0.47 ± 0.17 respectively; n = 20, 18).). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. $^{*}P < 0.05$.

3.1.4. Site aversion elicited by *6.2-Tbh*-GAL4 targeted neurons is light intensity and frequency dependent

The *6.2-Tbh*-GAL4 driver targets amongst other neurons, three tyraminergic/ octopaminergic neurons in the SOG: the VUMa4 neurons in the VMI-III clusters (Stefanie Hampel, 2007; Schneider et al., 2012; Figure 14C). In the two odor choice paradigm expression of *UAS-Tbh* in a *6.2-Tbh*-GAL4 dependent manner was not sufficient to restore the loss of ethanol attraction in Tbh^{nM18} mutants to control level (Schneider et al., 2012). To address whether the VUMa4 neurons are involved in mediating site attraction, the *UAS-ChR2* was expressed in a *6.2-Tbh*-GAL4 dependent manner.

Activating these neurons with a 2s 40Hz 16s 8Hz pattern and a light intensity of 1700 lux resulted in site aversion in the experimental and the control group towards the blue illuminated food odor trap (Figure 14A first panel). This effect could be due to a too high light intensity, thus the intensity was reduced to 1500 lux. Again, site aversion in both groups was observed (Figure 14A second panel). The same effect occurred at 1300 lux (Figure 14A third panel). The light intensity was further reduced to 1200 lux, which finally showed a distinct result. Activation of *6.2-Tbh*-GAL4 driven Tbh positive neurons is sufficient to elicit aversive behavior of the flies towards the blue light illuminated food odor trap when stimulated with a 2s 40Hz 16s 8Hz pattern (Figure 14A fourth panel). Activation with a light intensity of 1000 lux was not sufficient to cause site aversion, as both groups were undecided (Figure 14A last panel). These results show that the neurons targeted by the *6.2-Tbh*-GAL4 driver line, other than the *Tdc2*-GAL4 targeted neurons (Figure 11B), are light intensity sensitive.

To analyze whether the activation pattern influences site aversion, different patterns were used to activate *6.2-Tbh*-GAL4 dependent neurons (Figure 14B). 2s 40Hz was not sufficient to elicit site aversion (Figure 14B left panel). 16s 8Hz was sufficient to induce site aversion towards the blue illuminated trap again (Figure 14B middle panel). This activation pattern was not sufficient to elicit site attraction in Tbh positive neurons which are targeted by the *Tdc2*-GAL4 driver line (Figure 11C), but seems to be sufficient to cause site aversion in *6.2-Tbh*-GAL4 targeted neurons. 2s 20Hz 16s 8Hz is not sufficient to induce site aversion when using the *6.2-Tbh*-GAL4 driver (Figure 14B right panel), although this stimulation frequency was able to elicit site attraction when using the *Tdc2*-GAL4 line (Figure 11C).

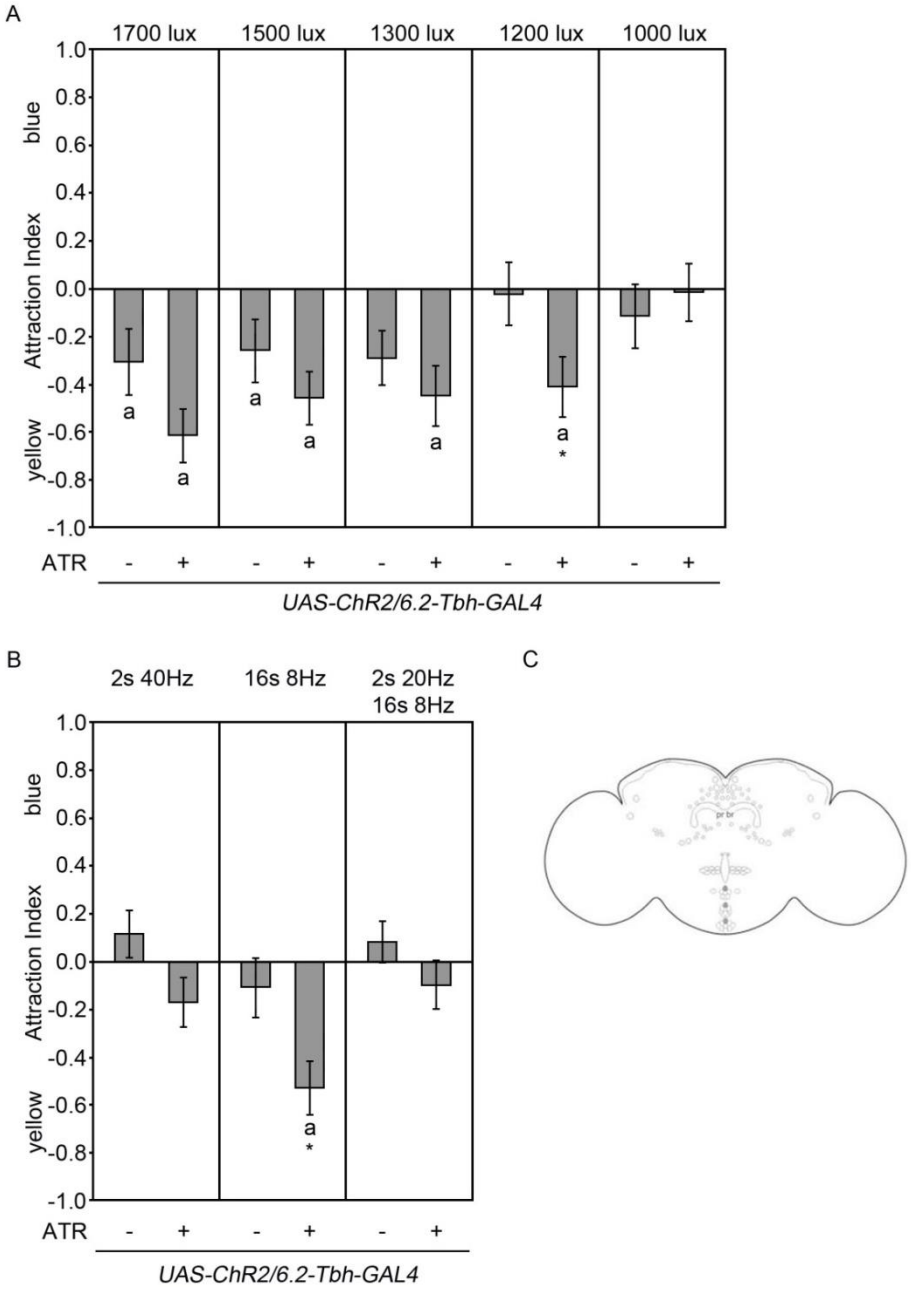


Figure 14: Site aversion elicited through activation of *6.2-Tbh*-GAL4 driven neurons is light intensity and frequency dependent using the *UAS-ChR2* transgene

(A) Only light activation of *6.2-Tbh*-GAL4 driven neurons expressing *UAS-ChR2* with an intensity of 1200 lux resulted in site aversion (AIs for the control group and the experimental group at 1700 lux: -0.31 ± 0.14 and -0.62 ± 0.11 respectively, n = 20, 16; AIs at 1500 lux: -0.26 ± 0.13 and 0.46 ± 0.11, n = 23, 19; AIs at 1300 lux: -0.29 ± 0.11 and -0.45 ± 0.12, n = 19, 16; AIs at 1200 lux: -0.02 ± 0.13 and -0.41 ± 0.13, n = 22, 19; AIs at 1000 lux: -0.12 ± 0.13 and -0.02 ± 0.12, n = 21, 19). (B) Activation of *norpA*^{1}*; UAS-ChR2/+; UAS-ChR2/6.2-Tbh*-GAL4 flies resulted in site aversion when using a 16s 8Hz pattern (AIs for the control group and the experimental group at 2s 40Hz: 0.11 ± 0.1 and -0.17 ± 0.11 respectively, n = 28, 28; AIs at 16s 8Hz: -0.11 ± 0.12 and -0.53 ± 0.11, n = 20, 14; AIs at 2s 20Hz 16s 8Hz: 0.08 ± 0.09 and -0.1 ± 0.1, n = 40, 36). (C) The *6.2-Tbh*-GAL4 line drives expression in three neurons in the SOG in the adult fly brain. The white cells are Tbh positive cells and the grey cells indicate a co-localization of Tbh and GFP (Schneider et al., 2012). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. *P < 0.05.

These site aversion results were unexpected, as the small subset of three VUMa4 neurons targeted by the *6.2-Tbh*-GAL4 driver line is part of the larger set of neurons targeted by the *Tdc2*-GAL4 line, which induced site attraction (Schneider et al., 2012).

There are now two possible explanations of these results. Firstly, it is possible that OA or TA are also able to mediate site aversion, but the influence of the subset of the three VUMa4 neurons is overwritten by the larger set of neurons targeted by the *Tdc2*-GAL4 driver line. Thus, a smaller subset of neurons, which is part of a bigger subset, can have the opposite effect than the larger subset. Or secondly, the *6.2-Tbh*-GAL4 line targets also other neurons, which are not Tbh positive but maybe dopaminergic or cholinergic. So it was investigated, whether the *6.2-Tbh*-GAL4 line also drives expression of *UAS-ChR2* in dopaminergic or cholinergic neurons by introducing *TH*-GAL80 or *Cha*-GAL80, respectively.

3.1.5. Site attraction and site aversion are mediated by a subset of acetylcholine co-expressing Tbh positive neurons

The *Cha*-GAL80 driver blocks the expression of *UAS-ChR2* in the cholinergic neurons (Kitamoto, 2002) and therefore was used to further characterize the subsets of site attraction and site aversion mediating neurons.

The *Tdc2*-GAL4*; Cha*-GAL80 driver reduces the expression of *UAS-ChR2* from 41 to 16 neurons in the G3a/AL2 cluster, the G3b cluster and the VMI-III cluster of the adult fly brain (Schneider et al., 2012). The neurons targeted by this driver line are not involved in olfactory ethanol attraction, as expression of *UAS-Tbh* in a *Tdc2*-GAL4*; Cha*-GAL80 dependent manner was not sufficient to restore the loss of ethanol attraction in Tbh^{nM18} mutants (Schneider et al., 2012). Activation of *UAS-ChR2* in *Tdc2*-GAL4*; Cha*-GAL80 targeted neurons with a 2s 40Hz 16s 8Hz pattern was not sufficient to induce site attraction (Figure 15A). Therefore cholinergic tyraminergic/octopaminergic neurons are sufficient to mediate site attraction. These neurons are probably located in the VMI-III cluster and are cholinergic as well as Tbh positive.

Elimination of cholinergic neurons targeted by the *fru*-GAL4 driver in male flies abolished site attraction (Figure 15B). Therefore, the three tyraminergic/ octopaminergic neurons in the SOG, which elicited site attraction, are also cholinergic and are probably part of the *Tdc2*-GAL4 targeted neurons inducing site attraction.

Thus, site attraction is mediated by neurons which are not only tyraminergic/ octopaminergic, but also cholinergic. To check for putative cholinergic neurons involved in site aversion the *Cha*-GAL80 driver was combined with the *6.2-Tbh*-GAL4 driver. Activation of *norpA¹; UAS-ChR2/+; UAS-ChR2/6.2-Tbh*-GAL4, *Cha*-GAL80 neurons with a 2s 40Hz 16s 8Hz pattern did not elicit site aversion (Figure 15C). Thus, the site aversion inducing Tbh positive VUMa4 neurons are also cholinergic.

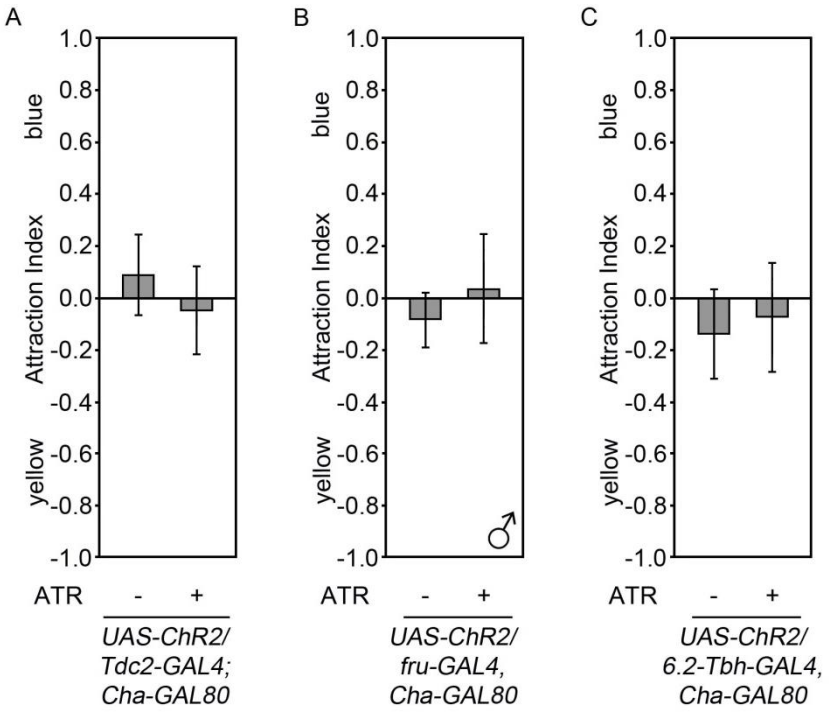


Figure 15: Elimination of the cholinergic neurons by using the *Cha*-GAL80 driver abolished site attraction and site aversion

(A) Activation of *norpA¹; UAS-ChR2/Tdc2*-GAL4*; UAS-ChR2/ Cha*-GAL80*UAS-ChR2* abolished site attraction (AIs for control and experimental group: 0.08 ± 0.16 and -0.05 ± 0.17 respectively; n = 25, 23). (B) Activation of male *norpA¹; UAS-ChR2/+; UAS-ChR2/fru*-GAL4, *Cha*-GAL80 flies abolished site attraction (AIs of control and experimental groups are -0.09 ± 0.11 and 0.03 ± 0.21 respectively; n = 8, 8). (C) Activation of *norpA¹; UAS-ChR2/+; UAS-ChR2/6.2-Tbh*-GAL4, *Cha*-GAL80 flies abolished site aversion (AIs for the control group and the experimental group: -0.14 ± 0.17 and -0.08 ± 0.21 respectively, n = 13, 11). For all experiments an activation pattern of 2s 40Hz 16s 8hz 2s 0Hz was used. Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups.

Taken together, site attraction and site aversion are mediated by acetylcholine co-expressing tyraminergic/octopaminergic neurons.

To rule out the possibility of dopaminergic neurons targeted by the *6.2-Tbh*-GAL4 driver line being responsible for the observed site aversion, a combination of *TH*-GAL80 and *6.2-Tbh*-GAL4 was used to activate the *UAS-ChR2* expressing neurons. Activation of the neurons in *norpA[1]; UAS-ChR2/TH*-GAL80*; UAS-ChR2/6.2-Tbh*-GAL4 flies with a 2s 40Hz 16s 8Hz pattern still led to site aversion (Figure 16 left panel). This indicates, that the former observed site aversion is not due to dopaminergic neurons, as they were eliminated by the *TH*-GAL80. This was also confirmed by an immunohistochemical staining, which revealed that there is no co-localization of dopaminergic neurons with the neurons targeted by the *6.2-Tbh*-GAL4 driver line (Sarah Salamon, data unpublished). An activation of only 16s 8Hz does not result in site aversion (Figure 16 right panel), which is contrary to the results of the *6.2-Tbh*-GAL4 driver line (Figure 14B) but matches the results of the *Tdc2*-GAL4 driver line (Figure 11C). These results indicate, that not only dopamine is able to induce aversive behavior in *Drosophila melanogaster* (Schwaerzel et al., 2003; Aso et al., 2012). OA or TA might be as well able to mediate aversive behavior.

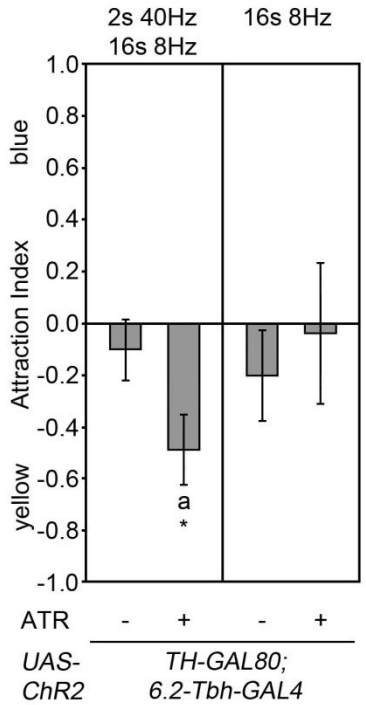


Figure 16: Elimination of the dopaminergic neurons from the *6.2-Tbh*-GAL4 targeted neurons abolished site aversion

Light activation of *norpA[1]; UAS-ChR2/TH*-GAL80*; UAS-ChR2/6.2-Tbh*-GAL4 flies only with a 2s 40Hz 16s 8Hz pattern resulted in site aversion (AIs for the control group and the experimental group at 2s 40Hz 16s 8Hz: -0.1 ± 0.12 and -0.49 ± 0.14 respectively, n = 14, 14; AIs at 16s 8Hz: -0.21 ± 0.18 and -0.04 ± 0.27, n = 13, 8). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. *P < 0.05.

3.1.6. Activation of other neurons in the SOG is not sufficient to elicit site attraction

To further localize possible neurons responsible for site attraction, the FlyLight data base (https://flweb.janelia.org/cgi-bin/flew.cgi) was screened for GAL4 driver lines that express in the SOG. Three GAL4 driver lines were picked: GMR29H06, GMR47A10 and GMR51D07. Blue light activation with a 2s 40Hz 16s 8Hz 2s 0Hz pattern at 1000 lux of neurons driven by all three driver lines did not result in attraction or aversion (Figure 17). Therefore neuronal activity of theses neurons might not be sufficient to induce attraction.

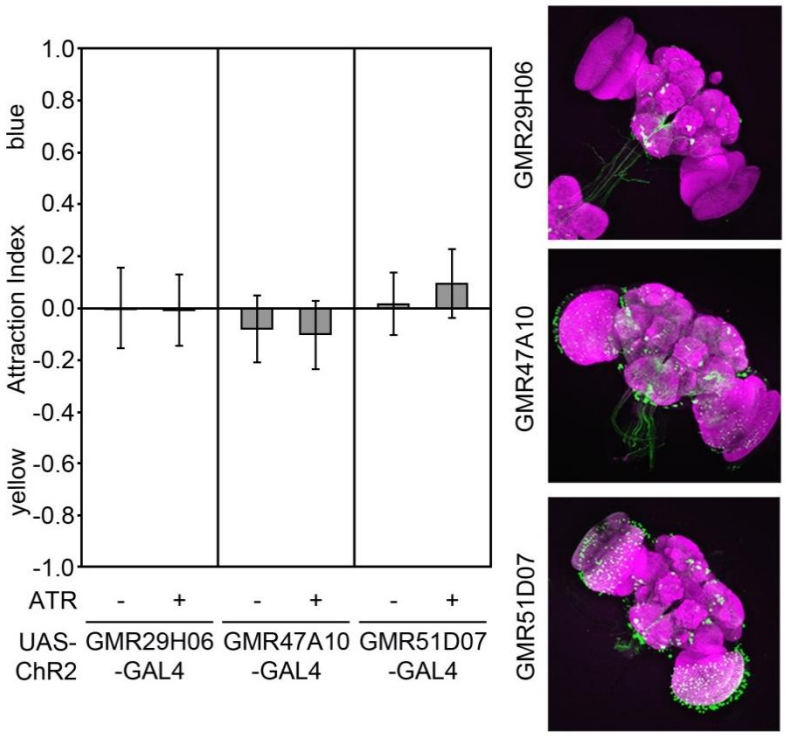


Figure 17: Activation of these neurons in the SOG is not sufficient to elicit site attraction

Light activation of *norpA[1]; UAS-ChR2/+; UAS-ChR2/GMR29H06* flies had no effect (AIs for the control group and the experimental group: -0.00 ± 0.15 and -0.01 ± 0.14 respectively, n = 23, 20). Activation of *norpA[1]; UAS-ChR2/+; UAS-ChR2/GMR47A10* flies had also no effect (AIs for the control group and the experimental group: -0.08 ± 0.13 and -0.1 ± 0.13 respectively, n = 36, 33). Activation of *norpA[1]; UAS-ChR2/+; UAS-ChR2/GMR51D07* flies also had no effect (AIs for the control group and the experimental group: -0.02 ± 0.12 and -0.1 ± 0.13 respectively, n = 30, 23). For all experiments and activation pattern of 2s 40Hz 16s 8Hz was used. Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. Pictures of immunohistochemical stainings were taken from FlyLight (https://flweb.janelia.org/cgi-bin/flew.cgi).

3.1.7. OA is required to switch the behavioral outcome/response

To investigate whether OA is able to reinforce the positive association with one of the food odor traps, 5% ethanol were added to one of the traps. The experiments were conducted with *norpA*^{1}*; UAS-ChR2/Tdc2*-GAL4*; UAS-ChR2/+* flies, which were stimulated with a 2s 40Hz 16s 8Hz pattern (Figure 18A). To test if OA could increase an already existing attraction, 5% ethanol were added to the blue light illuminated food odor trap. Both groups showed attraction towards the ethanol containing trap and the attraction of the experimental group was not significantly stronger than in the control group, so the reinforcers were not adding, suggesting a saturation value. (Figure 18A left panel). To analyze if OA is capable to switch a normal pre-existing ethanol attraction to the site of blue light activation, 5% ethanol were added to the food odor trap illuminated with yellow light (more precisely: warm white light with a blue light filter). Thus, the flies were confronted with two positive reinforcers. Here, the activation of Tbh positive neurons significantly suppressed the ethanol attraction in the experimental group, which was undecided, while the control group preferred the ethanol containing food odor trap (Figure 18A right panel). Taken together, neuronal light activation and ethanol odor are similar reinforcing and do not function in additive manner and OA is able to shift the attraction between two stimuli. Furthermore, the suppression of ethanol attraction through activation of these Tbh positive neurons indicates that OA is not directly involved in the attraction behavior, but mediates the switch between two choices.

This assumption is supported by the ethanol pre-feeding experiment in w^{1118} control flies and in Tbh^{nM18} mutants (Figure 18B). Feeding 10 % ethanol for 30 min and giving the flies a recovery phase of 3.5 h restored the loss of attraction towards ethanol in Tbh^{nM18} mutants. Ethanol causes oxidative stress (Sun et al., 2001; Wu and Cederbaum, 2003; Albano, 2006) and it is known that stress activates Tbh (Scholz et al., 2005; Châtel et al., 2013; Manuela Ruppert, 2013). Therefore OA signaling is probably increased in the ethanol pre-fed Tbh^{nM18} mutants. The w^{1118} control flies showed a non-, but almost significant reduced attraction towards alcohol containing food odors, due to their ability to adapt to a new situation. But the pre-fed Tbh^{nM18} mutants displayed an attraction index on naïve control level, which suggest that they are unable to adapt to a new situation. After pre-feeding they are now able to show attraction to ethanol as they have the innate attraction and also execute this behavior, but they are not able to shift their

behavioral response in a new situation. This is conform to the results of Brembs et al. (2007) and Thomas Kell (2017). In their studies, it was shown that the *Tbh*^{nM18} mutants are not impaired in a behavior itself, but have defects in initiation and termination of a behavior.

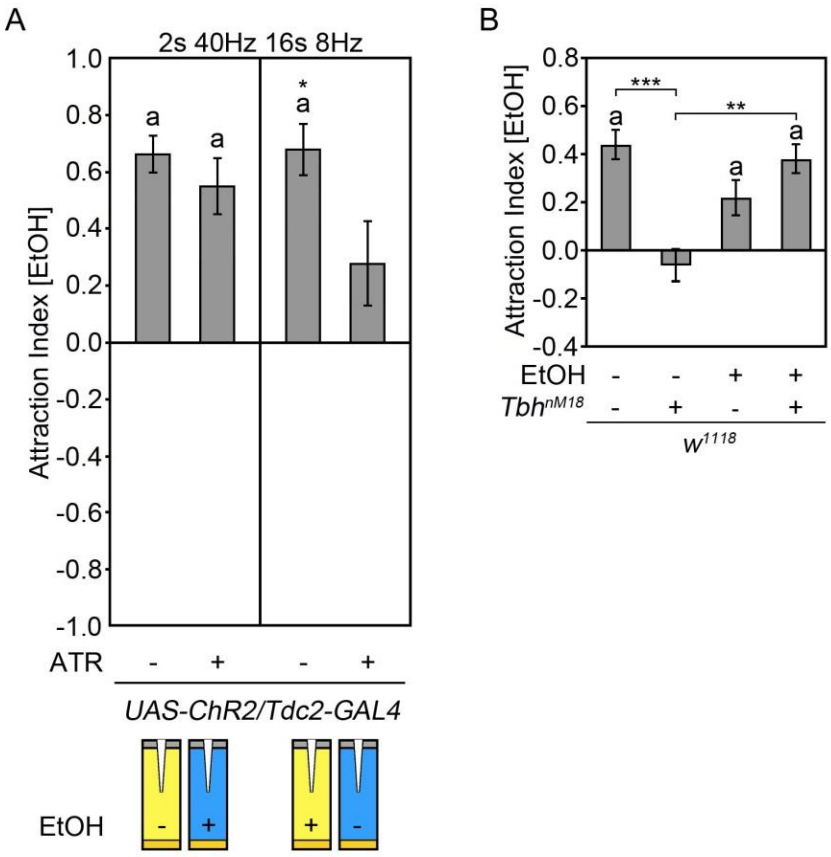


Figure 18: OA biases the behavioral outcome

(A) OA mediates the switch between two equally attractive stimuli. Activation with a 2s 40Hz 16s 8Hz pattern of *Tdc2*-GAL4 dependent neurons via *UAS-ChR2* with added ethanol to the blue light illuminated trap led to attraction in both groups (AIs for the control group and the experimental group: 0.66 ± 0.07 and 0.55 ± 0.1 respectively, n = 25, 17). Adding ethanol to the yellow illuminated trap led to attraction in the control group and to an indecisiveness in the experimental group (AIs for the control group and the experimental group: 0.68 ± 0.09 and 0.28 ± 0.15 respectively, n = 20, 18). (B) Feeding 10% ethanol rescued the loss of attraction to ethanol containing food odors in *Tbh*^{nM18} mutants to naïve control level, while the pre-fed *w*^{1118} control has a reduced attraction (AIs for *w*^{1118}: 0.44 ± 0.06 and with ethanol: 0.21 ± 0.08; for *w*^{1118}, *Tbh*^{nM18}: -0.07 ± 0.07 and with ethanol: 0.38 ± 0.06; n = 33, 31, 17, 20). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. The ANOVA post hoc Tukey test was used to determine difference between more than two groups. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

3.1.8. *norpA*^{1} mutants are impaired in olfaction

Experiments with the *UAS-ChR2* transgene were conducted in the *norpA*^{1} mutant background. The norpA phospholipase C is not only involved in phototransduction, but also in one olfactory organ in *Drosophila*, so there is an overlap between vision and olfaction in the fruit fly (Riesgo-Escovar et al., 1995). Thus it is possible, that the tested flies carrying the *norpA*^{1} mutation are impaired in olfaction. To test this and to therefore confirm the results of Figure 18, the *norpA*^{1}*; UAS-ChR2; UAS-ChR2* flies were tested against *w*^{1118} flies in the olfactory two odor choice paradigm. Both genotypes were able to distinguish juice from juice supplemented with 5% ethanol and thus showed an attraction towards the ethanol containing food odor trap (Figure 19). The *norpA*^{1}*; UAS-ChR2; UAS-ChR2* flies depicted a significantly reduced attraction for the ethanol-juice-mixture compared to the *w*^{1118} flies, but they were still able to distinguish different odors and to recognize the ethanol in a food odor mixture.

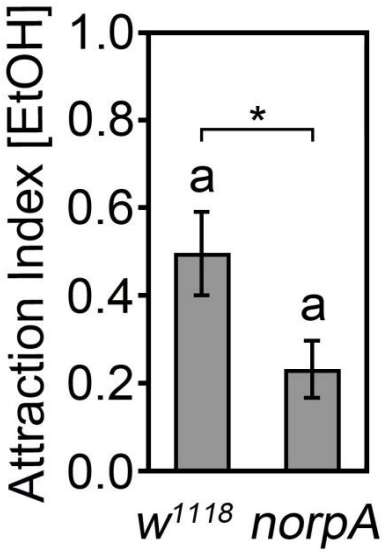


Figure 19: *norpA*^{1} mutants displayed a reduced attraction towards ethanol

norpA^{1}*; UAS-ChR2; UAS-ChR2* flies showed a reduced attraction towards 5% ethanol compared to *w*^{1118} flies (AIs for *w*^{1118} and *norpA*^{1}*; UAS-ChR2; UAS-ChR2*: 0.5 ± 0.1 and 0.23 ± 0.07 respectively, n = 26, 48). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. *$P < 0.05$.

From all these results we can conclude that neuronal activation using *UAS-ChR2* is light intensity and frequency dependent, with variation for different GAL4 driver lines. Furthermore, site attraction is mediated by neurons which are octopaminergic/ tyraminergic and cholinergic. A smaller subset of *6.2-Tbh*-GAL4 targeted tyraminergic/ octopaminergic/cholinergic neurons, which is part of the bigger site attraction mediating *Tdc2*-GAL4 driven subset, can also mediate site aversion. The behavioral

outcome is dependent on the subset and combination of activated neurons. Whether the observed site attraction and/or aversion is caused by OA or TA or by a combination of both neurotransmitters cannot be concluded from these results, so further experiments need to be done. But OA seems to be the neurotransmitter gating the switch between the two behaviors.

3.2. Octopamine is sufficient and required for site attraction and aversion

To further investigate whether OA or TA is the responsible neurotransmitter mediating site attraction or aversion, the *UAS-ChR2* transgene was crossed into the Tbh^{nM18} mutant background to investigate the behavior of flies lacking OA, but having increased levels of TA (Monastirioti et al., 1996) (Figure 20).

First it was tested, whether the observed site attraction through activating *Tdc2*-GAL4 driven neurons (see Figure 11B) is still inducible in OA lacking flies by expressing *UAS-ChR2* under the control of the *Tdc2*-GAL4 driver in the Tbh^{nM18} mutant background. Activation of w^{1118}*;* Tbh^{nM18}*; UAS-ChR2/Tdc2*-GAL4*; UAS-ChR2* flies did elicit the opposite behavior – site aversion – in the experimental group, no matter if a stimulation pattern of 2s 40Hz 16s 8Hz (Figure 20A left panel) or just 16s 8Hz (Figure 20A right panel) were used. These results lead to the conclusion that OA is indeed the neurotransmitter mediating site attraction and that TA might be the neurotransmitter responsible for site aversion, as TA is the neurotransmitter still present in Tbh^{nM18} mutants. These antagonistic effects of TA and OA have been observed before in *Drosophila* larvae (Saraswati et al., 2003) and adults (Brembs et al., 2007). The involvement of TA might be also an explanation, why here an activation of 16s 8Hz is enough to elicit a behavior. It is possible that the release of vesicles containing TA is differently regulated than the release of vesicles packed with OA and therefore do not need the same activation pattern. But eliminating the cholinergic neurons from the *Tdc2*-GAL4 driver line by using the *Tdc2*-GAL4, *Cha*-GAL80 line abolishes the site aversion (Figure 20B).

Based on this result, the Tbh positive neurons which are also cholinergic are not only involved in mediating attraction behavior but also in mediating aversion behavior. But so far it cannot be distinguished whether TA or acetylcholine is the site aversion mediating neurotransmitter. But as both behaviors – attraction and aversion – can be eliminated by removing the same cholinergic neurons, it is likely that both behaviors are mediated by the same set of acetylcholine co-expressing tyraminergic/octopaminergic neurons and that OA is definitely the neurotransmitter mediating site attraction.

To test whether the observed site aversion with the *Tdc2*-GAL4 driver in the Tbh^{nM18} mutant background could also be elicited by activation of *fru*-GAL4 dependent neurons, *UAS-ChR2* was again activated with a 2s 40Hz 16s 8Hz pattern in the mutant background in both, male and female flies (Figure 20C). Surprisingly, in this experiment the control groups and experimental groups of the female flies (Tbh^{nM18}/Tbh^{nM18} (middle panel) or Tbh^{nM18}/FM7 (right panel)) showed a tendency to site attraction (Figure 20C). Testing the male flies had no effect in both groups (Figure 20C left panel).

These results were rather unexpected. Firstly, this is not conform to the results of the *UAS-ChR2* in the non-mutant background, where the female flies provided the more reliable results and the male control group showed site attraction just like the experimental group. Thus the results cannot really be compared to each other. Secondly, the expected site aversion was not achieved. So the three targeted octopaminergic neurons are probably not cholinergic and maybe also not tyraminergic, because TA is not necessarily packed into vesicles for neurotransmitter release. Thus, they presumably do not belong to the subset of neurons capable of mediating site aversion. But removing the cholinergic neurons by using the *Cha*-GAL80 driver line in the $norpA^{1}$ background eliminated the site attraction, which suggested that the *fru*-GAL4 targeted tyraminergic/octopaminergic neurons are also cholinergic.

TA and OA can have opposing effects on the behavior in *Drosophila* (Saraswati et al., 2003; Brembs et al., 2007). To further analyze, if the site aversion behavior observed with the *6.2-Tbh*-GAL4 driver line (Figure 14) is caused by OA or TA, the *UAS-ChR2* transgene was expressed in a *6.2-Tbh*-GAL4 dependent manner in the Tbh^{nM18} mutant background. Stimulation of the w^{1118}, Tbh^{nM18}; *UAS-ChR2/+; UAS-ChR2/6.2-Tbh*-GAL4 flies with a 2s 40Hz 16s 8Hz pattern did not elicit site aversion (Figure 20D).

Taken together, OA is mediating site attraction and site aversion and not TA, as both behaviors can be eliminated by removing OA. Thus OA is not only the so far thought positive reinforcer, but can also act as a negative reinforcer. Earlier it was believed, that DA is involved in aversive behavior and OA is responsible for attraction/appetitive behavior (Schwaerzel et al., 2003), but this must be redefined now.

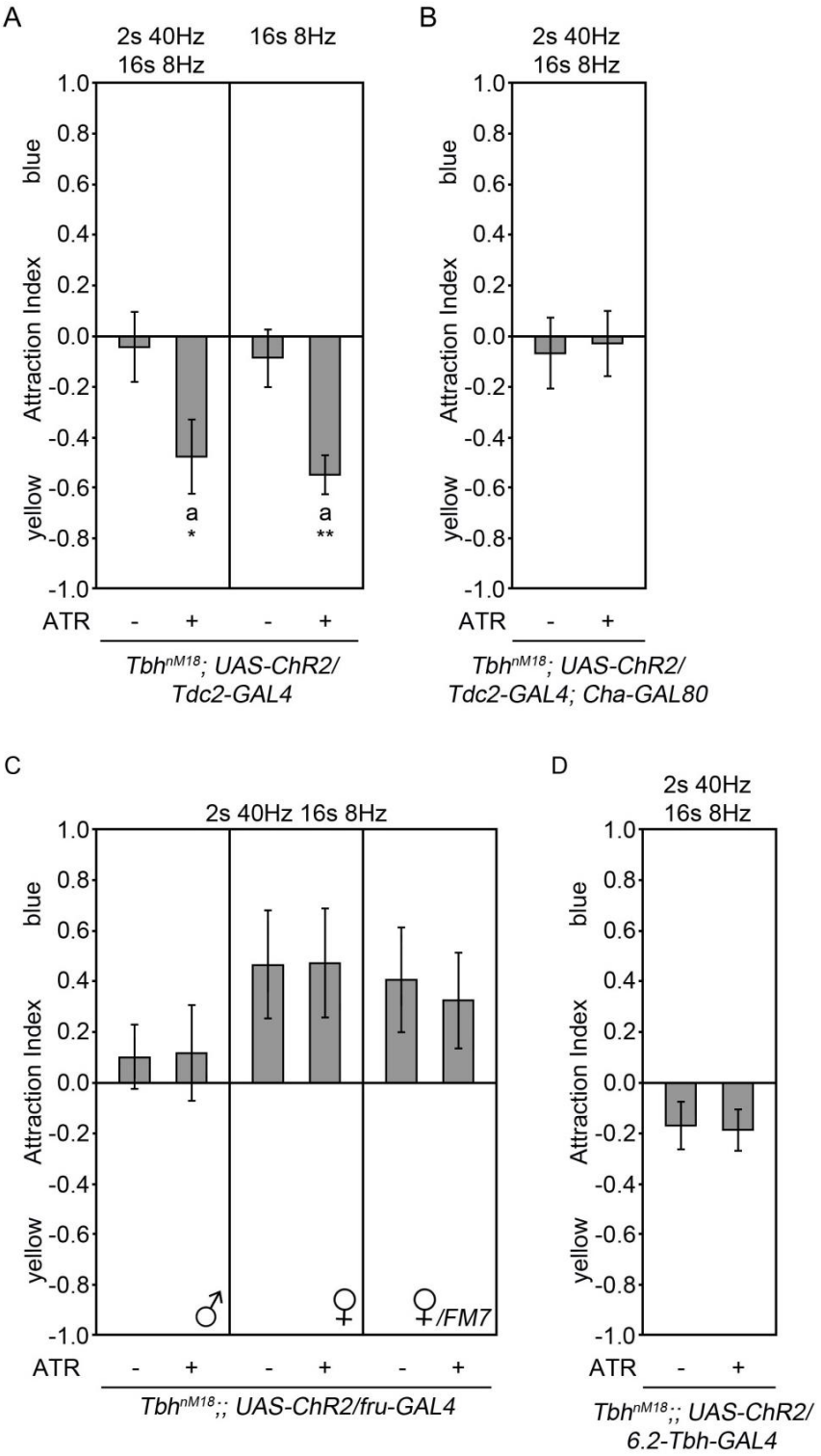


Figure 20: Activation of neurons in a *Tbh^{nM18}* mutant background eliminated the observed site attraction and site aversion in a non-mutant background

(A) Activation of *Tdc2*-GAL4 dependent neurons via *UAS-ChR2* in a *Tbh^{nM18}* mutant background led to site aversion (AIs for the control group and the experimental group at 2s 40Hz 16s 8Hz: -0.05 ± 0.14 and -0.48 ± 0.15 respectively, n = 15, 12; AIs at 16s 8Hz: -0.09 ± 0.11 and -0.55 ± 0.08, n = 20, 19). (B) Light activation of *w^{1118}, Tbh^{nM18}; UAS-ChR2/Tdc2*-GAL4*; UAS-ChR2/Cha*-GAL80 flies with a 2s 40Hz 16s 8Hz had no effect (AIs for the control group and the experimental group: -0.07 ± 0.14 and -0.03 ± 0.13 respectively, n = 16, 16). (C) Activation of *UAS-ChR2* in a *fru*-GAL4 dependent manner in *Tbh^{nM18}* mutants with a 2s 40Hz 16s 8Hz pattern had no effect in male flies (AIs of control and experimental groups are 0.1 ± 0.13 and 0.12 ± 0.19 respectively; n = 11, 9). In female flies, both groups showed a tendency towards the blue illuminated trap (AIs of control and experimental groups for females are 0.46 ± 0.21 and 0.47 ± 0.22 respectively; n = 14, 13 and for females with the FM7 balancer are 0.41 ± 0.21 and 0.32 ± 0.19 respectively; n = 13, 11). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. (D) Activation of *6.2-Tbh*-GAL4 dependent neurons via *UAS-ChR2* in a *Tbh^{nM18}* mutant background with a 2s 40Hz 16s 8Hz pattern had no effect (AIs for the control group and the experimental group: -0.17 ± 0.1 and -0.19 ± 0.08 respectively, n = 20, 19). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. *$P < 0.05$ and **$P < 0.01$.

3.3. Site attraction and aversion induced by neuronal light activation are transgene independent

3.3.1. Penetrance of blue and red light through the flies' cuticle

To further investigate, whether the observed effect of site attraction and site aversion indeed depends on neuronal activation and not on the kinetics of the transgene, other channelrhodopsins were tested. Red light is less scattered and absorbed by the cuticle of a fly and thus penetrates the fly better and also reaches deeper brain regions (Inagaki et al., 2014 and Figure 21). Therefore it was decided to test red light activatable channelrhodopsins: Chrimson (Klapoetke et al., 2014) and ReaChR (Lin et al., 2013).

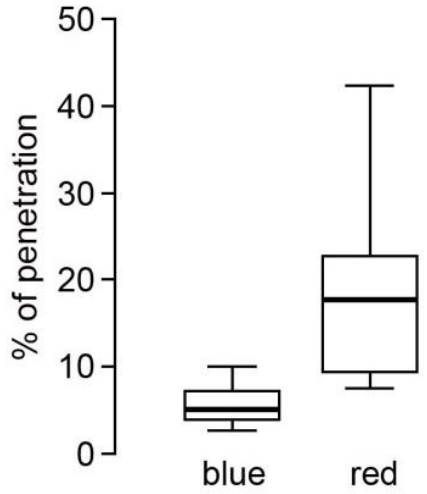


Figure 21: Penetration of blue and red light through the cuticle of a fly head

Blue light had a penetration rate through the flies' head cuticle of 5.57% ± 2.6, while red light was able to penetrate the cuticle by 18.71% ± 11.56. Errors are STDV.

3.3.2. The *UAS-Chrimson* transgene is not suitable for the optogenetic site attraction assay

The first tested channelrhodopsin was Chrimson, a yellow/red light activatable channelrhodopsin with a spectral peak at 590 nm (Klapoetke et al., 2014). Therefore the diodes in the optogenetic site preference assay were exchanged. Instead of the blue light diode with 465-485 nm, which led to activation of *UAS-ChR2*, a red light diode was used (620-630 nm) for activation. The warm white light diode with a blue light filter was substituted by an amber (585-595 nm) or a green light diode (520-535 nm). Flies expressing the *UAS-Chrimson* under the control of the *Tdc2*-GAL4 driver were stimulated with an activation pattern of 2s 40Hz 16s 8Hz 2s 0Hz. It was started with a light intensity of 300 lux, as our red light diode penetrates the fly's cuticle much better than our blue light diode (Figure 21) and so a lower intensity should be enough for activation of the neurons. The *norpA*^{1} mutation was not crossed into the *UAS-Chrimson* genotype, since flies do not see red light (Paulk et al., 2012).

Activation with a green and a red light diode induced site aversion in the experimental group when illuminated with a light intensity of 300 lux, while the control group also showed a strong tendency for site aversion but was not significant different form zero due to a low number of experiments (Figure 22A left panel). Increasing the intensity to 500 or 800 lux resulted in indecisiveness of both groups (Figure 22A middle panels). Activation at 1800 lux elicited site attraction in the control and the experimental group (Figure 22A right panel). Using the red and the amber light diode, the experimental

group showed site aversion at light intensities of 300, 500 and 800 lux and was almost significantly different from zero at 1800 lux (Figure 22B). The control group displayed site aversion and 800 and 1800 lux and showed a tendency for site aversion at 300 and 500 lux, which was not significantly different form zero (Figure 22B). Testing the red light diode at 300 lux against darkness, the experimental group showed site attraction, while there was only a tendency in the control group (Figure 22C). The not significant differences of the control groups are due to the low number of experiments.

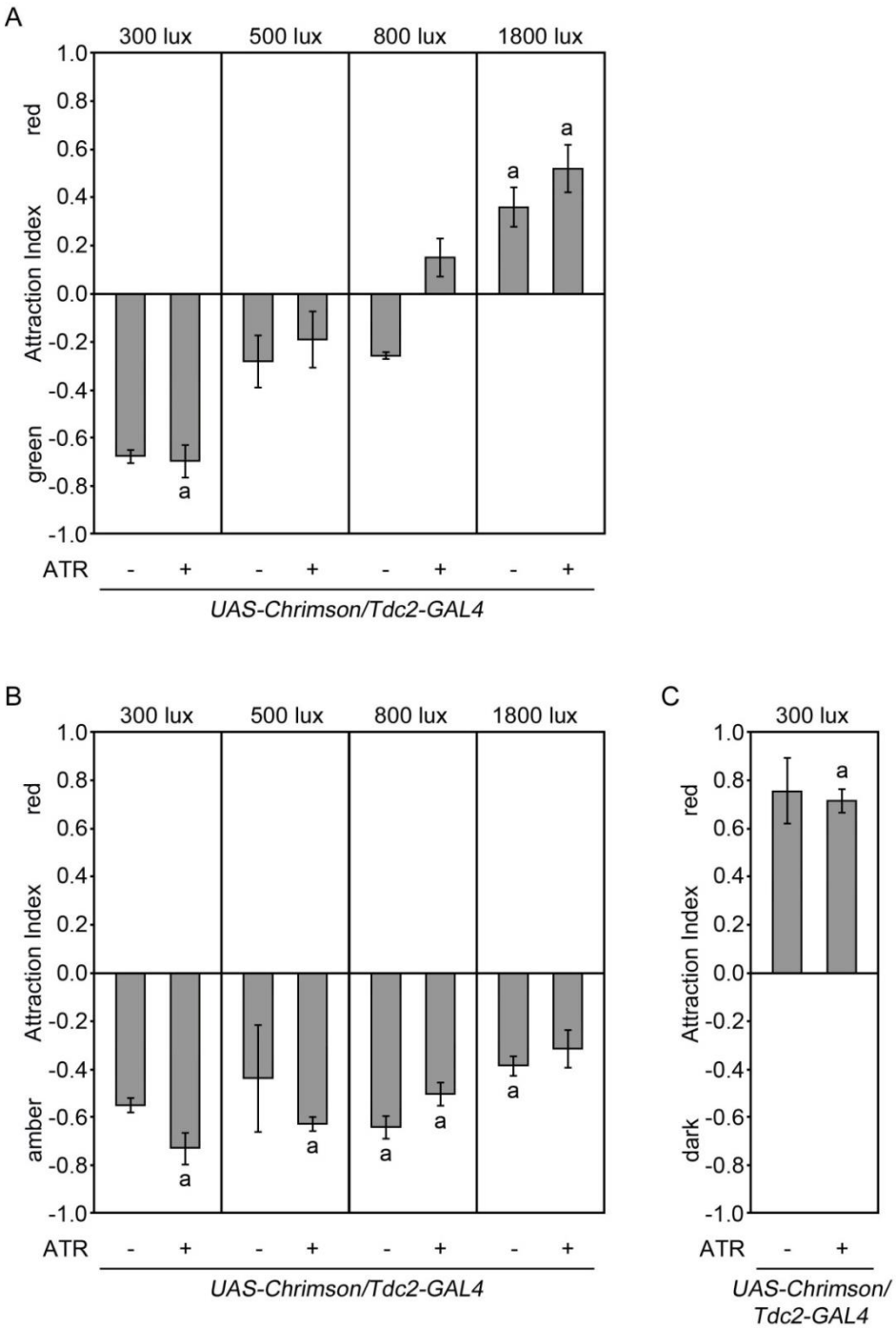


Figure 22: Red light activation of *UAS-Chrimson* did not result in site attraction

The optogenetic site preference assay was used to test the *UAS-Chrimson* transgene under the control of the *Tdc2*-GAL4 driver to find the suitable diodes and light intensity. (A) Using a red and a green light diode leads to site aversion in the control group and the experimental group at a low light intensity (AIs of control and experimental group at 300 lux: -0.68 ± 0.03 and -0.7 ± 0.07 respectively, n = 4, 10). Using medium light intensities of 500 and 800 lux causes indecisiveness in both groups (AIs for the control group and the experimental group at 500 lux: -0.28 ± 0.11 and -0.19 ± 0.12 respectively, n = 4, 8; AIs at 800 lux: -0.26 ± 0.02 and -0.15 ± 0.08, n = 2, 8). Using a high intensity of 1800 lux elicits site attraction in both groups (AIs for the control group and the experimental group at 1800 lux: -0.36 ± 0.08 and -0.52 ± 0.1 respectively, n = 7, 6). (B) Using a red and an amber light diode leads to site attraction towards the amber diode in the control group and the experimental group at all light intensities (AIs of control and experimental group at 300 lux: -0.55 ± 0.03 and -0.73 ± 0.07 respectively, n = 2, 9; AIs at 500 lux: -0.44 ± 0.22 and -0.63 ± 0.03, n = 2, 8; AIs at 800 lux: -0.64 ± 0.05 and -0.51 ± 0.05, n = 6, 7; AIs at 1800 lux: -0.34 ± 0.04 and -0.32 ± 0.08, n = 7, 5). (C) When the red light diode was tested against darkness both groups showed site attraction (AIs of control and experimental group at 300 lux: 0.76 ± 0.14 and -0.71 ± 0.05 respectively, n = 7, 24). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups.

All these results must be considered with caution, as the control group had a very high mortality rate during the experiment (Figure 23A-C). While the experimental group showed normal decision rates of 81.8-100%, the control group displayed decision rates of only 18.2-75%, independent of the diode combination used for activation. Surprisingly, the flies were not only undecided, they also died during the time of a normal experimental round in the pipette tips or in the testing area, which never happened in this extent to any other genotype. Therefore it was decided to test another red light activatable channelrhodopsin.

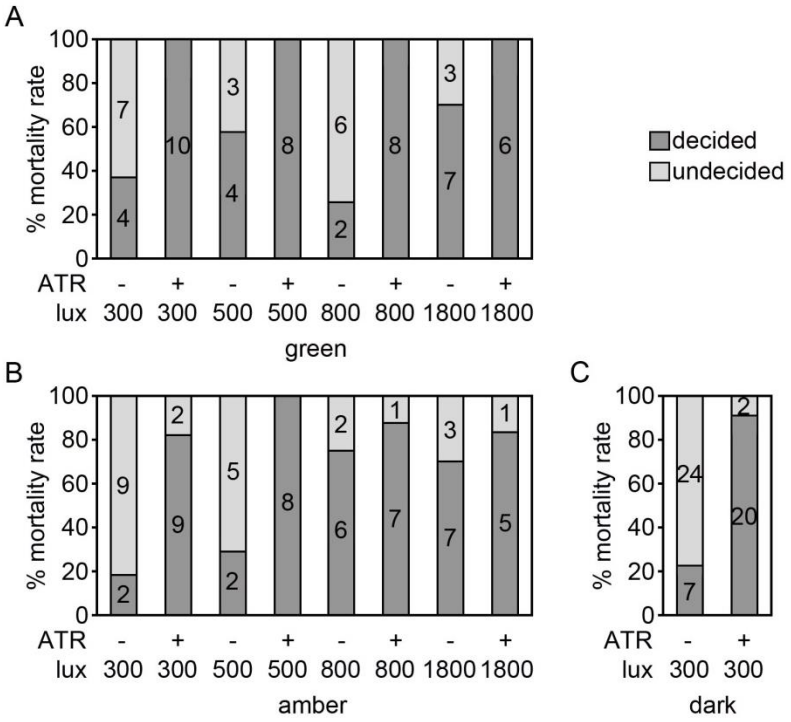


Figure 23: The control group displayed a high indecisiveness and a high mortality rate

Activation of *UAS-Chrimson* in *Tdc2*-GAL4 driven neurons led to a high indecisiveness in the control group. (A) The control group showed decision rates of 25-70% while the experimental group displayed a decision rate of 100% when using a green and a red light diode (Percentage of decided flies for the control group and the experimental group at 300 lux: 36.4% and 100% respectively, n = 11, 10; Percentage at 500 lux: 57.1% and 100%, n = 7, 8; Percentage at 800 lux: 25% and 100%, n = 8, 8; Percentage at 1800 lux: 70% and 100%, n = 10, 6). (B) The control group showed decision rates of 18,2-75% while the experimental group displayed a decision rate of 81.8-100% when using an amber and a red light diode (Percentage for the control group and the experimental group at 300 lux: 18.2% and 81.8% respectively, n = 11, 11; Percentage at 500 lux: 28.6% and 100%, n = 7, 8; Percentage at 800 lux: 75% and 87.5%, n = 8, 8; Percentage at 1800 lux: 70% and 83.3%, n = 10, 6). (C) The control group showed decision rates of 25.9% while the experimental group displayed a decision rate of 92.3% when the red light diode (300 lux) was tested against darkness (n = 31, 22).

3.3.3. Red light activation is not suitable for *UAS-ReaChR*

The other tested red activatable channelrhodopsin is ReaChR with an activation optimum of 590-630 nm (Lin et al., 2013). Flies expressing the *UAS-ReaChR* under the control of the *Tdc2*-GAL4 driver were stimulated with an activation pattern of 2s 40Hz 16s 8Hz 2s 0Hz. The following diode combinations in different light intensities were tested: red and amber, red and green, red and darkness. Again, the *norpA*^{1} mutation was

not crossed to the *UAS-ReaChR* transgene, as the red light is not visible for flies (Paulk et al., 2012).

Activation with a green and a red light diode led to site aversion in the control group at 1800 lux, while the experimental group was undecided and to site aversion in the experimental group at 1200 lux, where the control group was undecided (Figure 24A right and middle panel). Using a light intensity of 300 lux resulted in a tendency of site aversion in both groups (Figure 24A left panel). Activation with an amber and red light diode at light intensities of 1200 and 1800 lux elicited site aversion in both groups (Figure 24B). Testing the red light diode at an intensity of 300 lux against darkness, both groups showed site attraction towards the red illuminated food odor trap (Figure 24C).

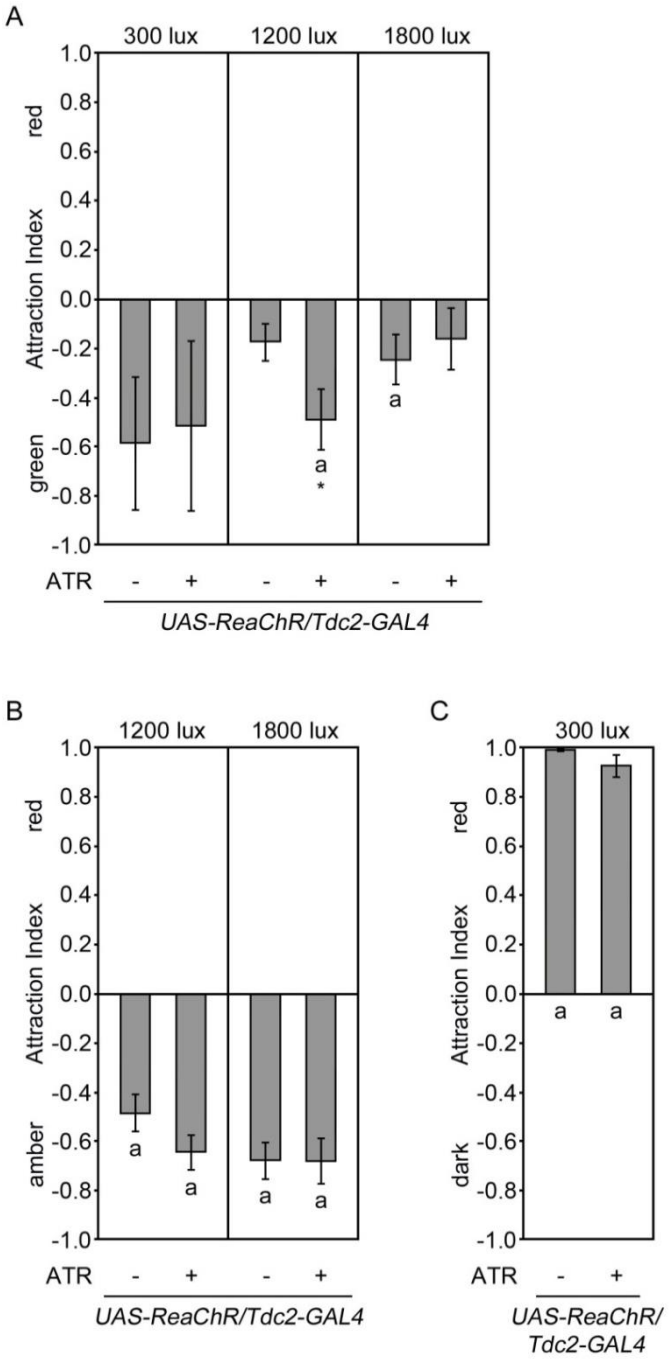


Figure 24: Red light is not a suitable wavelength for activation of neurons via the *UAS-ReaChR* transgene

The optogenetic site preference assay was used to test the *UAS-ReaChR* transgene under the control of the *Tdc2*-GAL4 driver to find the suitable diodes and light intensity. (A) Using a red and a green light diode led to site aversion in the control group and the experimental group at a low light intensity (AIs of control and experimental group at 300 lux: -0.59 ± 0.27 and -0.52 ± 0.35 respectively, n = 7, 5). Using a light intensity of 1200 lux caused attraction towards the green illuminated food odor trap (AIs for the control group and the experimental group at 1200 lux: -0.17 ± 0.08 and -0.49 ± 0.12 respectively, n = 16, 12). Using a high intensity of 1800 lux elicited site attraction in the control group but not in the experimental group (AIs for the control group and the experimental group at 1800 lux: -0.25 ± 0.1 and -0.16 ± 0.13 respectively, n = 27, 20). (B) Using a red and an amber light diode led to site attraction towards the amber diode in the control group and the experimental group at all light intensities (AIs of control and experimental group at 1200

lux: -0.49 ± 0.08 and -0.64 ± 0.07 respectively, n = 15, 11; AIs at 1800 lux: -0.68 ± 0.07 and -0.68 ± 0.09, n = 16, 11). (C) When the red light diode was tested against darkness both groups showed site attraction (AIs of control and experimental group at 300 lux: 0.99 ± 0.005 and -0.93 ± 0.04 respectively, n = 21, 11). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. **P* < 0.05.

Red light is not a suitable wavelength for activating ReaChR, since the flies never showed site attraction towards the red light illuminated food odor trap (Figure 24). Therefore the wavelength for activation was altered. The red light diode was replaced with an amber diode and since the green LED light is to close in the light spectrum, a blue light diode was chosen as the second light source. Surprisingly, there was no interfering effect of the blue light with the not blind flies. Therefore the *norpA*^{1} mutation was not introduced in the following experiments.

3.3.4. Site attraction elicited by amber light activation in *Tdc2*-GAL4 dependent neurons is intensity dependent and frequency independent

Activation of *Tdc2*-GAL4 driven neurons expressing the blue light activatable *UAS-ChR2* induced site attraction towards the blue light illuminated food odor trap. This behavior was not intensity but frequency dependent. Now it was investigated, whether these results are reproducible by using the alternative red/amber light activatable *UAS-ReaChR* transgene.

The new used LED combination of amber and blue light illumination was tested with an activation pattern of 2s 40Hz 16s 8Hz 2s 0Hz. It was started with a light intensity of 800 lux, because this was the light intensity which still caused reliable results with *UAS-ChR2* but did not interfere with the optical system of the fly. And as amber light penetrates the cuticle of a fly about four times as well as blue light (Inagaki et al., 2014), this should be sufficient to activate the *UAS-ReaChR* transgene. But when using this light intensity, both groups are undecided (Figure 25A left panel). As a light intensity of 800 lux obviously did not work here and using the red and green diode with 1200 lux resulted in a significant difference between the two groups, this was the intensity tested next. This seemed to be a suitable intensity, as the control group was undecided and the

experimental group was significantly different from random choice and to the control group (Figure 25A right panel). This attraction behavior result is comparable to the one obtained with *UAS-ChR2* under the control of *Tdc2*-GAL4 (Figure 11B), but now the activation seems to be intensity dependent.

The combination of a blue and an amber light diode used as an activation wavelength for *w^{1118}; UAS-ReaChR/Tdc2*-GAL4*; UAS-ReaChR/+* with an activation pattern of 2s 40Hz 16s 8Hz also resulted in site attraction behavior like it was observed with the blue light activatable *UAS-ChR2*. Furthermore the amber light diode is as suitable to the activation spectrum of *UAS-ReaChR* as a red light diode would be, so it was decided to further work with this diode combination. Stimulation of the blue light activatable *UAS-ChR2* in *Tdc2*-GAL4 dependent neurons revealed a frequency dependence of either site attraction or site aversion (Figure 11C and 14B). Now it should be investigated, whether this frequency dependence is also present in the *UAS-ReaChR* transgene in *Tdc2*-GAL4 driven neurons. Therefore different stimulation patterns were used to activate ReaChR.

Activation of *Tdc2*-GAL4 dependent neurons via *UAS-ReaChR* with a 2s 20Hz 16s 8Hz pattern or only with a 16s 8Hz pattern also results in site attraction of the experimental group (Figure 25B left and middle panel). These results indicate that the amber light activatable *UAS-ReaChR* is more robust and less frequency dependent than the blue light activatable *UAS-ChR2*. Again a smaller number of n was needed and an activation pattern of 16s 8Hz was able to elicit site attraction, which was not sufficient in the experiments with the blue light activatable *UAS-ChR2*. Stimulation with constant light elicited site aversion (Figure 25B right panel), which is also conform to the results of ChR2.

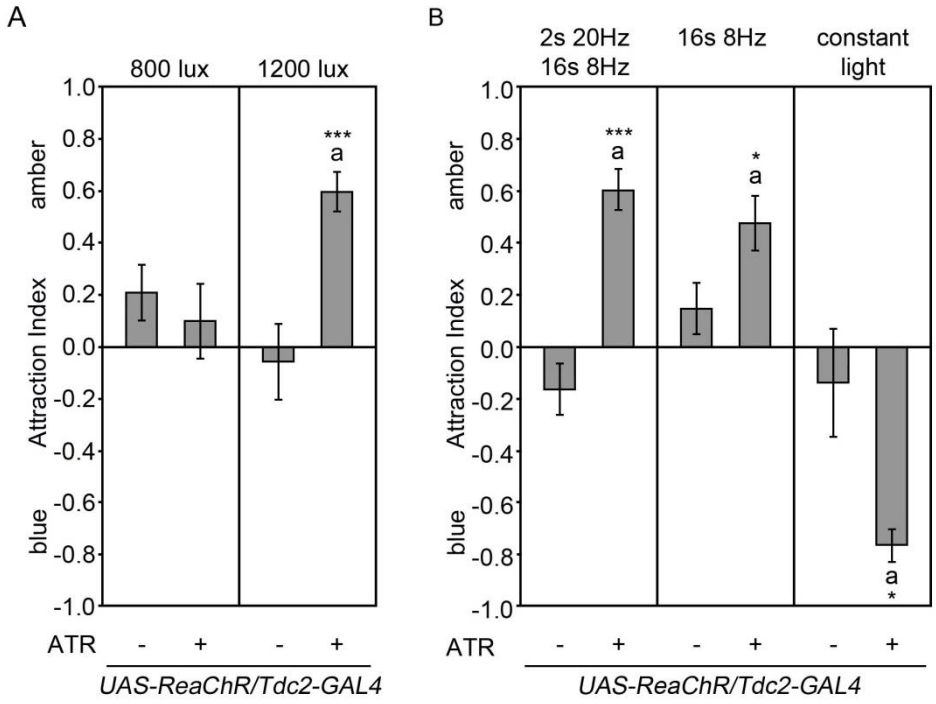


Figure 25: Activation of *Tdc2*-GAL4 dependent neurons via the *UAS-ReaChR* transgene is light intensity dependent but frequency independent

(A) Activation of *UAS-ReaChR* in *Tdc2*-GAL4 driven neurons with an amber light diode at 800 lux with 2s 40Hz 16s 8Hz had no effect (AIs for the control group and the experimental group: 0.21 ± 0.11 and 0.1 ± 0.14 respectively, n = 14, 5). But using a light intensity of 1200 lux led to site attraction (AIs for the control group and the experimental group: -0.06 ± 0.15 and 0.6 ± 0.08 respectively, n = 18, 18). (B) Activation of *UAS-ReaChR* in *Tdc2*-GAL4 targeted neurons with a 2s 20Hz 16s 8Hz or a 16s 8Hz pattern induced site attraction (AIs for the control group and the experimental group at 2s 20Hz 16s 8Hz: -0.17 ± 0.1 and 0.6 ± 0.08 respectively, n = 13, 7; AIs at 16s 8Hz: 0.15 ± 0.1 and 0.47 ± 0.11, n = 12, 8). Activation with constant light induced site aversion (AIs for the control group and the experimental group: -0.14 ± 0.21 and -0.77 ± 0.06 respectively, n = 14, 10). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. **P* < 0.05 and ****P* < 0.001.

From this, five things can be concluded: 1. the results received with the *UAS-ChR2* transgene are not transgene dependent, but due to neuronal activation. 2. *UAS-ReaChR* is a functional a proper alternative transgene for neuronal activation in the optogenetic site attraction assay. 3. The combination of an amber and blue light diode is suitable for neuronal activation. 4. The activation using the *UAS-ReaChR* transgene seems to be more robust than the *UAS-ChR2* transgene as a smaller number of n is needed to achieve comparable AIs. 5. Differences in cuticle penetration between blue and red light do not account for loss of neuronal activation, since a light intensity of 800 lux was enough to activate the *UAS-ChR2* transgene, but not sufficient to activate the *UAS-ReaChR* transgene.

3.3.5. Activation of neurons targeted by the *Cha*-GAL4 driver using ReaChR is not suitable for inducing site attraction or aversion

It was shown in the optogenetic experiments with the blue light activatable *UAS-ChR2* that the neurons that mediate site attraction and site aversion are not only tyraminergic/octopaminergic, but also cholinergic (Figure 15). To address the function of acetylcholine, neurons in a *Cha*-GAL4 dependent manner were activated with amber light using *UAS-ReaChR*. However, expression of the transgene under the control of *Cha*-GAL4 resulted in a reduced number of offspring. Thus only a small number of experiments could be conducted (n = 3, 3). Activation of *UAS-ReaChR* in a *Cha*-GAL4 dependent manner with an activation pattern of 2s 40Hz 16s 8Hz led to a tendency of the control group to site attraction and to a tendency of site aversion in the experimental group (Figure 26).

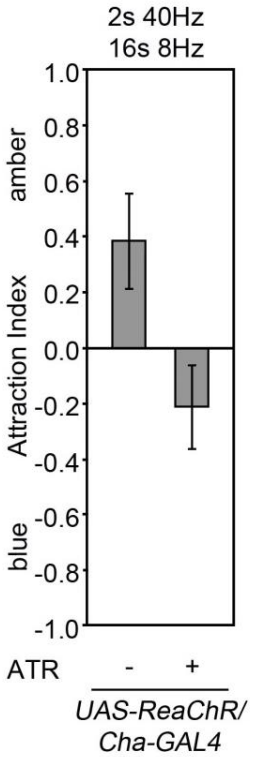


Figure 26: Expression of *UAS-ReaChR* in a *Cha*-GAL4 dependent manner is not suitable for eliciting site attraction or site aversion

Activation of *UAS-ReaChR* in *Cha*-GAL4 driven neurons did not result in a clear outcome (AIs for the control group and the experimental group: 0.38 ± 0.17 and -0.21 ± 0.15 respectively, n = 3, 3). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups.

The small number of hatched flies with the right genotype could be due to a lethality of the flies expressing *UAS-ReaChR* under the control of *Cha*-GAL4. The choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine, is common in a high number of cells in *Drosophila melanogaster* (Yasuyama and Salvaterra,

1999) and therefore a high number of neurons expressed *UAS-ReaChR*. *UAS-ReaChR* is activatable by a broader spectrum and more sensitive than *UAS-ChR2* and thus it might be possible that the targeted neurons were already activated by very low light influences during the development. And as such a high number of neurons were affected, it could be that there appeared some defects in the animals. All pupae hatched, so it is likely that the defect already occurred in the larval stages or even before and the eggs did not develop into larvae or the larvae did not pupate. So maybe in this case it would have been better to express *UAS-ChR2* in a *Cha*-GAL4 dependent manner.

3.3.6. Activation of neurons targeted by the *fru*-GAL4 driver elicited site aversion

Activation of *fru*-GAL4 driven neurons elicited site attraction in the experimental group of female flies when using the blue light activatable *UAS-ChR2*, while in male flies both groups showed site attraction (Figure 13). It was tested, if this result could also be repeated using the amber light activatable *UAS-ReaChR* transgene.

Activation of *UAS-ReaChR* in *fru*-GAL4 driven neurons with an activation pattern of 2s 40Hz 16s 8Hz in male and female flies led to an unexpected result. The experimental group of the male flies showed site aversion, while the control group was undecided (Figure 27 left panel). In the experiment with the female flies, both groups showed aversion towards the amber illuminated food odor trap (Figure 27 right panel) and were not significantly different from each other.

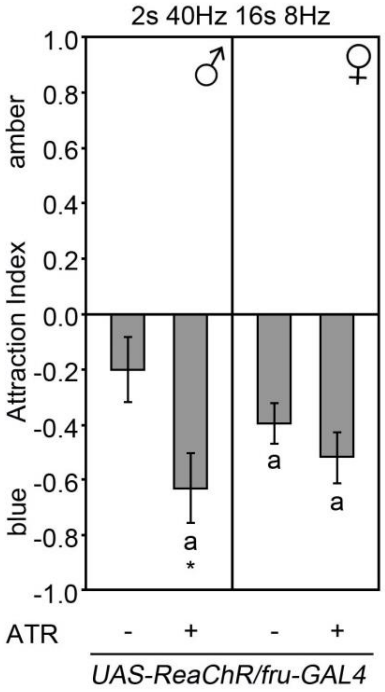


Figure 27: Activation of *fru*-GAL4 dependent neurons with the *UAS-ReaChR* transgene elicited site aversion

Activation of *UAS-ReaChR* in a *fru*-GAL4 dependent manner in a 2s 40Hz 16s 8Hz pattern elicited site aversion in male flies (AIs of control and experimental groups are -0.21 ± 0.12 and 0.64 ± 0.13 respectively; n = 12, 10). In female flies, both groups showed an aversion towards the blue illuminated trap (AIs of control and experimental groups are -0.4 ± 0.07 and 0.52 ± 0.09 respectively; n = 20, 12). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. **P* < 0.05.

These results were surprising compared to the ones obtained with the blue light activatable *UAS-ChR2* (Figure 13). Here, the male flies were the ones which provided analyzable results and in the females both groups showed site aversion. This is the opposite result as observed before. So both results cannot be really compared to each other. Even more striking was the fact that activation of *fru*-GAL4 driven neurons elicited site attraction with *UAS-ChR2*, but site aversion when *UAS-ReaChR* is expressed.

3.3.7. Site aversion elicited by amber light activation in *6.2-Tbh*-GAL4 dependent neurons is intensity dependent and frequency independent

Activation of the blue light activatable *UAS-ChR2* in *6.2-Tbh*-GAL4 targeted neurons elicited site aversion towards the blue light illuminated food odor trap (Figure 14). This behavior was intensity and frequency dependent. Now it should be examined, whether these results are reproducible by testing the alternative red/amber light activatable *UAS-ReaChR* transgene.

Activation of *UAS-ReaChR* in *6.2-Tbh*-GAL4 targeted neurons with a 2s 40Hz 16s 8Hz pattern at 1200lux (a suitable intensity for *UAS-ReaChR*/*Tdc2*-GAL4 and for *UAS-ChR2*/*6.2-Tbh*-GAL4) led to a tendency to site aversion towards the amber illuminated food odor trap in both groups, which is not significant due to the low number of experiments (Figure 28A left panel). This aversion was probably caused by a too high intensity, so the light intensity was reduced to 800 lux (a suitable light intensity for *UAS-ChR2*/*Tdc2*-GAL4, but not for *UAS-ReaChR*/*Tdc2*-GAL4). Activation of *UAS-ReaChR* in a *6.2-Tbh*-GAL4 dependent manner with an activation pattern of 2s 40Hz 16s 8Hz at 800 lux resulted in the expected site aversion of the experimental group (Figure 28A right panel). Hence, the achieved site aversion probably mediated by OA is real and not an artefact of the blue light activatable *UAS-ChR2*. Furthermore, it also showed the already observed light intensity dependence of the combination of the target gene and the GAL4 driver line.

Inducing of site attraction through activation of *UAS-ReaChR* in *Tdc2*-GAL4 driven neurons was in comparison to *UAS-ChR2* activation not frequency dependent. Thus the *UAS-ReaChR* transgene is less selective, which should be verified by examining a putative frequency dependence in w^{1118}*; UAS-ReaChR/+; UAS-ReaChR/6.2-Tbh*-GAL4 flies. Here, light activation with a pattern of 2s 20Hz 16s 8Hz or only 16s 8Hz elicited even higher site aversion towards the amber illuminated food odor trap in the experimental group (Figure 28B). Again, activation with the *UAS-ReaChR* transgene is not frequency dependent. The activation pattern of 2s 20Hz 16s 8Hz, which was not sufficient for the blue light activatable *UAS-ChR2*, was able to elicit site aversion. This supports the assumption, that the *UAS-ReaChR* is less selective and maybe more sensitive than the *UAS-ChR2*.

The site aversion obtained with an activation pattern of 16s 8Hz is the highest achieved so far and reaches a value of almost double as high as with an activation pattern of 2s 40Hz 16s 8Hz. Probably the 40Hz sequence attenuates the aversion and might activate neurons which maybe even would elicit an attraction behavior, but which is overwritten by the neurons activated through the 8Hz.

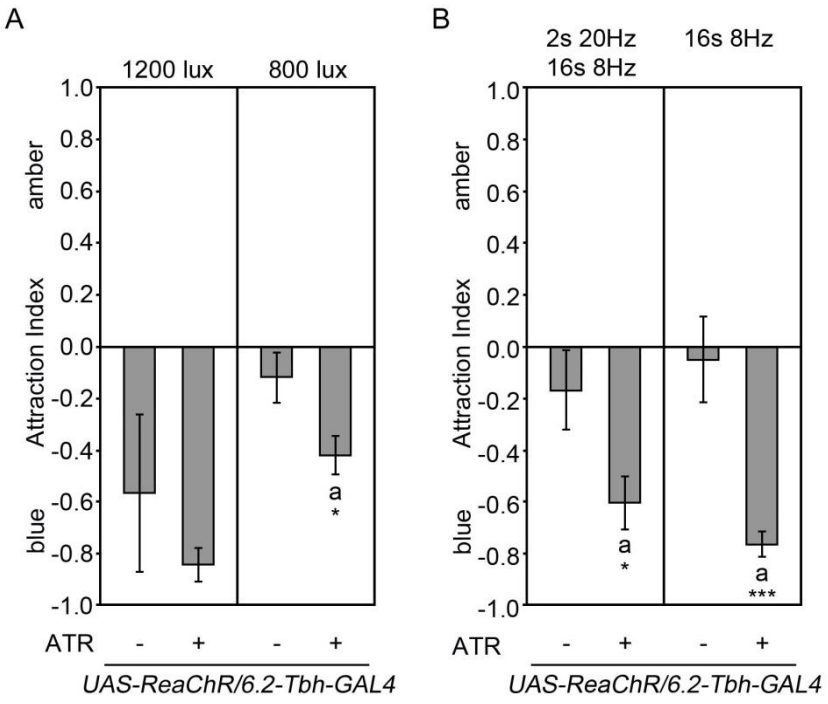


Figure 28: The elicited site aversion via activation of *6.2-Tbh*-GAL4 driven neurons using ReaChR is light intensity dependent but frequency independent

(A) Activation of *6.2-Tbh*-GAL4 dependent neurons via *UAS-ReaChR* with a 2s 40Hz 16s 8Hz pattern at 800 lux resulted in site aversion (AIs for the control group and the experimental group at 1200 lux: -0.57 ± 0.3 and -0.84 ± 0.06 respectively, n = 4, 3 and AIs at 800 lux: -0.12 ± 0.1 and -0.42 ± 0.08, n = 23, 20). (B) Activation of *6.2-Tbh*-GAL4 dependent neurons via *UAS-ReaChR* with a 2s 40Hz 16s 8Hz pattern or a 16s 8Hz pattern resulted in site aversion (AIs for the control group and the experimental group at 2s 20Hz 16s 8Hz: -0.17 ± 0.15 and 0.61 ± 0.1 respectively, n = 23, 16 and at 16s 8Hz: -0.05 ± 0.17 and 0.77 ± 0.05, n = 18, 17). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between the two groups. *$P < 0.05$ and ***$P < 0.001$

Taken together, the neuronal activation of the different neurons results in site attraction or site aversion and this is not due to the use of different transgenes. The theory of frequency dependence proposed for the blue light activatable *UAS-ChR2* cannot be confirmed with the amber light activatable *UAS-ReaChR*, as a lot more frequencies elicited the same behavior when using the *UAS-ReaChR* transgene for activation. Therefore the postulated frequency dependence might be due to the kinetics of the different transgenes.

3.4. OA is required and sufficient for olfactory ethanol attraction

3.4.1. Activation of OA signaling restores the loss of attraction towards ethanol enriched food odors in *Tbh^{nM18}* mutants

Tbh^{nM18} mutants have a deletion in the *Tbh* gene and thus do not produce OA, but have six to eightfold increased TA levels (Monastirioti et al., 1996). Due to this mutation they fail to show attraction towards ethanol containing food odors (Schneider et al., 2012). To investigate whether the increased TA levels or the loss of OA is responsible for the loss of olfactory ethanol attraction in *Tbh^{nM18}* mutants, different pharmacologically active substances were fed to *Tbh^{nM18}* mutants and to their genetic control *w^{1118}* flies. The utilized substances were TA and OA, epinastine (an OA antagonist), clonidine and naphazoline (two OA agonists) and yohimbine (a TA antagonist) (Figure 29).

Feeding 57mM OA, a concentration that restored the egg laying defect in *Tbh^{nM18}* mutant females (Monastirioti et al., 1996), improved the sugar learning performance index in *Tbh^{nM18}* mutant males (Schwaerzel et al., 2003) and partially rescued the reduced carbohydrate intake in *Tbh^{nm18}* mutant (Jan Götz, 2018), restored the loss of attraction in *Tbh^{nM18}* mutants and had no effect on the control group (Figure 29A). This result indicates that OA is sufficient for mediating olfactory attraction behavior and that increased levels of OA do not influence this behavior.

To independently confirm that OA signaling is required for attraction, the OA receptor agonist epinastine was fed to *w^{1118}* control flies at a concentration of 3mM (Figure 29B), a concentration which effectively interrupted TfAP-2 induced hyperactivity in *Drosophila* (Williams et al., 2014). Feeding epinastine led to a loss of olfactory attraction towards ethanol containing food odors in *w^{1118}* flies mimicking the *Tbh^{nM18}* mutant phenotype. Thus it can be concluded, that OA is not only sufficient but also necessary for mediating olfactory attraction behavior in *Drosophila melanogaster*.

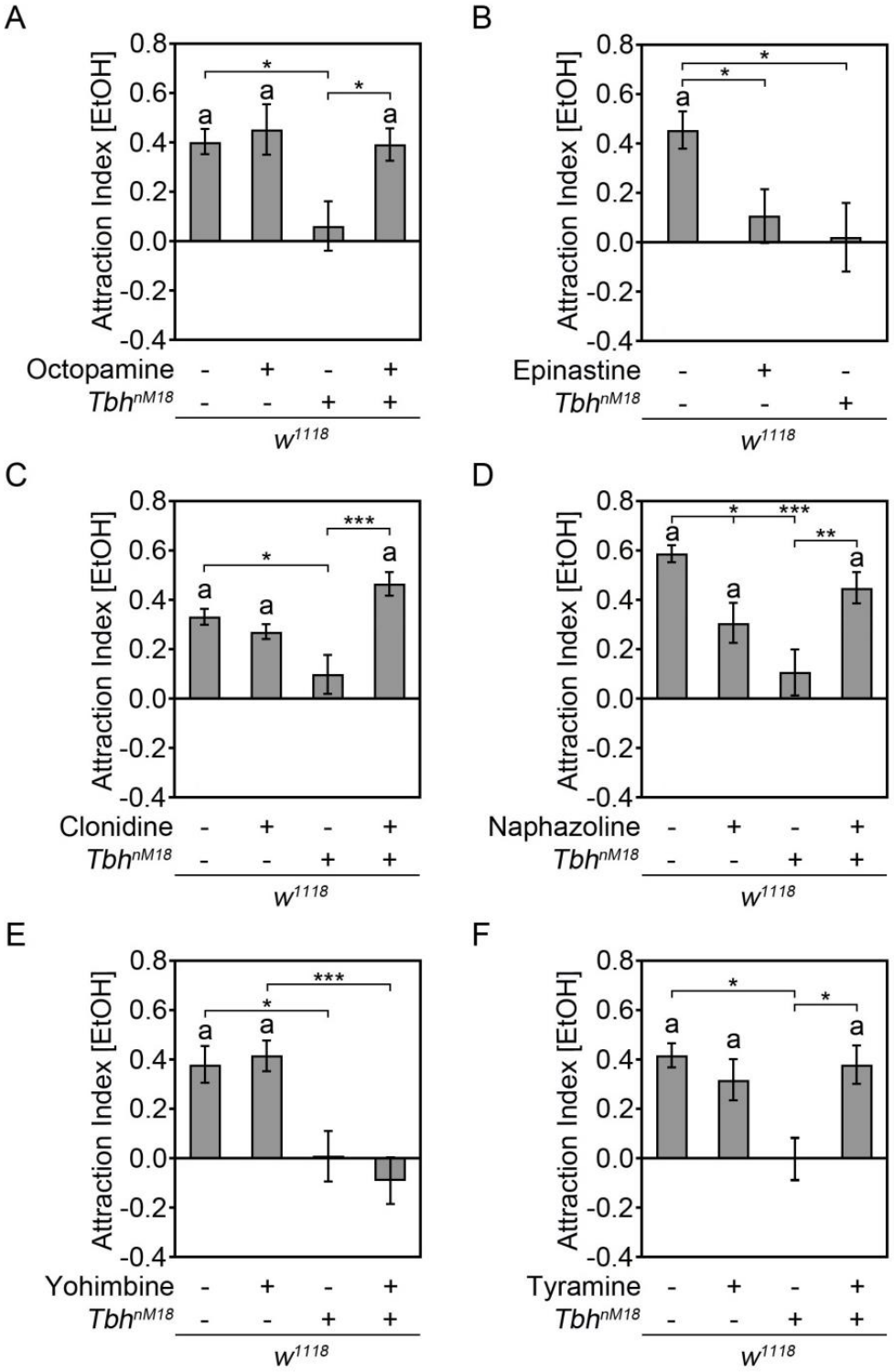

A
B
C
D
E
F
Attraction Index [EtOH]
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
a
*
***
**
Octopamine
Epinastine
Clonidine
Naphazoline
Yohimbine
Tyramine
Tbh^nM18
w^1118

Figure 29: The loss of attraction towards ethanol containing food odors in *Tbh^{nM18}* mutants is caused by the lack of OA.

The loss of attraction in *Tbh^{nM18}* mutants towards ethanol containing food odors could be restored by feeding OA or OA agonists. (A) Feeding 57mM OA restored the loss of attraction to ethanol containing food odors in *Tbh^{nM18}* mutants (AIs for *w^{1118}*: 0.4 ± 0.05 and with OA: 0.45 ± 0.11; for *w^{1118}, Tbh^{nM18}*: 0.06 ± 0.1 and with OA: 0.39 ± 0.07; n = 25, 21, 19, 22). (B) Blocking OA receptors by feeding 3mM Epinastine eliminated the attraction of control flies for ethanol containing food odors (AIs for *w^{1118}*: 0.45 ± 0.08 and with Epinastine: 0.11 ± 0.11; for *w^{1118}, Tbh^{nM18}*: 0.02 ± 0.15; n = 29, 28, 19). (C) Feeding 50mM Clonidine restored the loss of attraction to ethanol containing food odors in *Tbh^{nM18}* mutants (AIs for *w^{1118}*: 0.33 ± 0.04 and with Clonidine: 0.27 ± 0.03; for *w^{1118}, Tbh^{nM18}*: 0.1 ± 0.08 and with Clonidine: 0.47 ± 0.05; n = 30, 28, 29, 31). (D) Feeding 200nM Naphazoline restored the loss of attraction to ethanol containing food odors in *Tbh^{nM18}* mutants (AIs for *w^{1118}*: 0.59 ± 0.04 and with Naphazoline: 0.3 ± 0.08; for *w^{1118}, Tbh^{nM18}*: 0.1 ± 0.09 and with Naphazoline: 0.45 ± 0.07; n = 32, 33, 29, 33). (E) Blocking TA receptors by feeding 25mM Yohimbine did not alter the attraction to ethanol containing food odors (AIs for *w^{1118}*: 0.38 ± 0.08 and with Yohimbine: 0.41 ± 0.07; for *w^{1118}, Tbh^{nM18}*: 0.01 ± 0.11 and with Yohimbine: 0.09 ± 0.1; n = 40, 40, 21, 24). (F) Feeding 288mM TA restored the loss of attraction to ethanol containing food odors in *Tbh^{nM18}* mutants (AIs for *w^{1118}*: 0.42 ± 0.05 and with OA: 0.32 ± 0.09; for *w^{1118}, Tbh^{nM18}*: -0.005 ± 0.09 and with OA: 0.38 ± 0.08; n = 24, 27, 20, 20). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The ANOVA post hoc Tukey test was used to determine difference between more than two groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

OA agonists mimic the function of OA, thus feeding of the two OA agonists clonidine and naphazoline (Evans, 1981; Maqueira et al., 2005) should have a similar effect like feeding OA itself. Feeding 50mM clonidine rescued the mutant phenotype of the *Tbh^{nM18}* mutants and did not affect the behavior of *w^{1118}* control flies (Figure 29C). Feeding 200nM naphazoline was also suitable to restore the *Tbh^{nM18}* mutant phenotype back to control level (Figure 29D). Interestingly, naphazoline also altered attraction in the *w^{1118}* control flies suggesting that the right amount at the right time of a neurotransmitter is important or that other receptors might be affected.

To address the putative function of increased TA levels on the loss of olfactory attraction in *Tbh^{nM18}* mutants, the TA antagonist yohimbine was fed to both groups in a concentration of 25mM (Figure 29E), a concentration that rescued the *Tbh^{nM18}* mutant phenotype in flight initiation and maintenance (Brembs et al., 2007). Blocking TA signaling had no effect on the olfactory ethanol attraction, neither in the control flies nor in the *Tbh^{nM18}* mutants, thus TA is probably not involved in mediating olfactory attraction.

Feeding 288mM TA, a concentration that successfully reduced the carbohydrate intake in *w^{1118}* flies (Jan Götz, 2018), to *Tbh^{nM18}* mutants and *w^{1118}* control flies and thus

increasing TA levels restored the loss of ethanol attraction in *Tbh^{nM18}* mutants and had no effect on the *w^{1118}* control flies (Figure 29F). This indicates that TA might affect olfactory attraction behavior, but only if OA is missing.

From all these results it can be concluded that the loss of attraction towards ethanol containing food odors in *Tbh^{nM18}* mutants is caused by the loss of OA and not due to the higher levels of TA. OA is required for ethanol attraction and furthermore has to be present in the right amount to enable proper attraction behavior. The missing effect in *w^{1118}* control flies fed with OA could be due to a reverse synthesis of OA to TA, so that there is still a balance between these two neurotransmitters. Feeding the OA antagonist epinastine reveals that OA is not only sufficient, but also necessary for olfactory ethanol attraction. As the OA agonist naphazoline had an effect on the control group and the other OA agonist clonidine did not, it might be possible that the different agonists might bind to different OA receptors and thus have different effects. The surprising effect of TA, which was the same like feeding OA to the flies, is contradictory to the other findings. There is no balance between the two neurotransmitters and also a reverse synthesis is not possible, as the *Tbh^{nM18}* mutants lack the Tbh enzyme. Thus TA signaling must also be involved in olfactory attraction towards ethanol containing food odors, at least if OA is missing, as the *Tbh^{nM18}* mutants now show attraction. But this supports the assumption that the loss of attraction in *Tbh^{nM18}* mutants is not due to elevated TA levels, as *w^{1118}* control flies with increased TA still show ethanol attraction.

3.4.2. Altered levels of Tbh negatively influence the olfactory attraction while simultaneously altered levels of TA and OA might not influence olfactory attraction behavior

In the next experiments, the influence of different Tbh levels (and thus indirectly the ratio of OA to TA) in the fly on olfactory ethanol attraction was investigated.

In Figure 30A, three different *Tbh* mutants are compared to each other. The *Tbh^{nM18}* mutants have a deletion in the *Tbh* gene affecting the coding sequence of the second

exon (Monastirioti et al., 1996; Manuela Ruppert, 2013). Another tested *Tbh* mutant with no OA is the Tbh^{Del3} mutant, which carries a larger mutation and is missing the complete first and second exon (Manuela Ruppert, 2013). These two *Tbh* mutants, which both are lacking OA, should now be compared to see whether the larger deletion in the Tbh^{Del3} mutants has a more severe effect on olfactory attraction behavior than the smaller deletion in the Tbh^{nM18} mutants. To investigate the effect of higher *Tbh* levels, the P-element insertion line *d01344* exhibiting upregulated *Tbh* transcript expression (Manuela Ruppert, 2013), was simultaneously tested. None of the mutated *Tbh* alleles showed olfactory ethanol attraction. No matter if too low *Tbh* levels, like in Tbh^{nM18} mutants or in Tbh^{Del3} mutants, or too high *Tbh* levels like in *d01344* flies, both resulted in reduced attraction towards ethanol. This indicates that a certain amount of OA is important to develop normal ethanol attraction.

In Figure 30B the effect of simultaneously reduced TA and OA levels is shown. The $Tdc2^{RO54}$ mutant lacks both neurotransmitters (Cole et al., 2005) and reduction of TA and OA resulted in olfactory attraction. This suggests, that not only the presence of OA is necessary for developing olfactory ethanol attraction, but also that there might be an interplay between the two neurotransmitters TA and OA and that a certain balance between these two might be needed.

An upregulated *Tbh* transcript expression through the whole development resulted in a reduced olfactory ethanol attraction (Figure 30A). The effect of an upregulation of Tbh transcript in the cells is shown in Figure 30C. Expression of *UAS-Tbh* in *Tdc2-GAL4* targeted neurons eliminated the olfactory attraction towards the ethanol containing food odor trap. If an overexpression of *Tbh* actually leads to higher Tbh enzyme levels, this might cause a higher turnover rate from TA to OA and therefore to an imbalance between TA and OA. This supports the assumption, that the amount of available OA is essential for forming normal olfactory attraction. Too high *Tbh* expression has a similar effect as no or too less Tbh. Furthermore, the ratio or interaction of TA to OA could be involved in mediating attraction behavior.

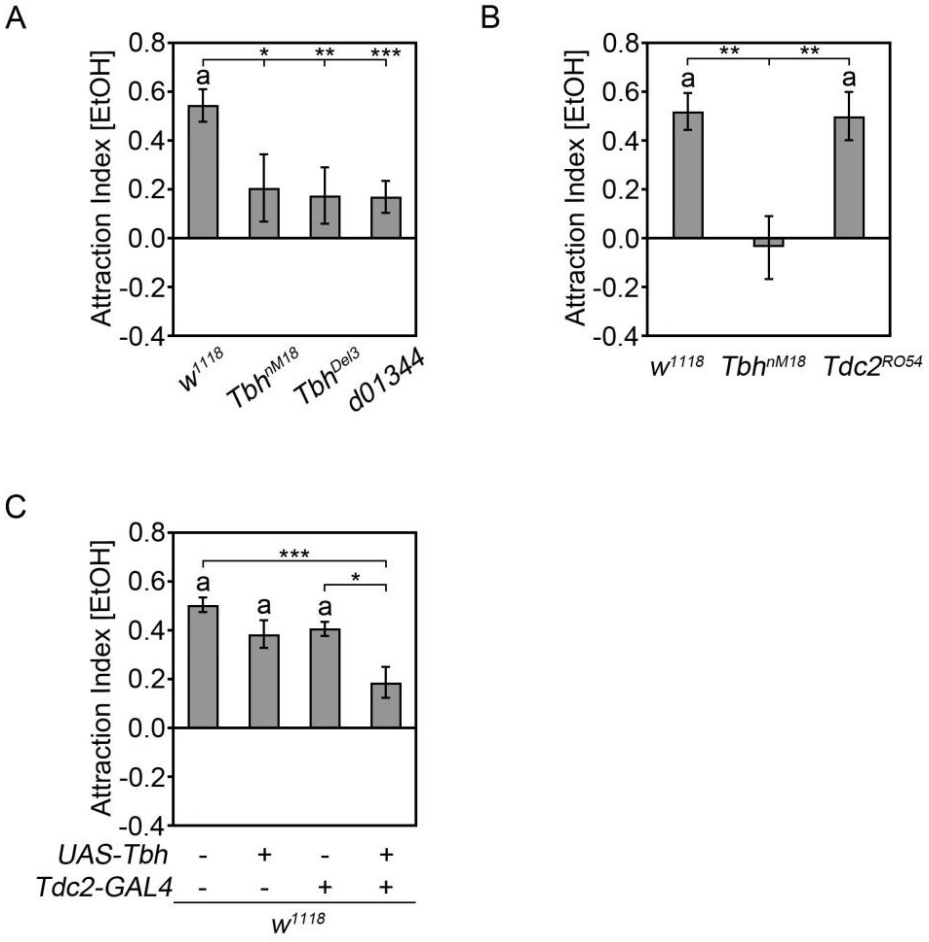


Figure 30: Altered levels of *Tbh* result in loss of attraction while simultaneously reduced TA and OA levels do not influence olfactory ethanol attraction

(A) Altered levels of Tbh in different mutants resulted in loss of attraction. All *Tbh* mutants showed no attraction to ethanol containing food odors (AIs for w^{1118}: 0.55 ± 0.07; for w^{1118}, Tbh^{nM18}: 0.21 ± 0.14, for w^{1118}, Tbh^{Del3}: 0.17 ± 0.12 and for w^{1118}, *d01344*: 0.17 ± 0.07; n = 27, 19, 16, 37). (B) Tbh^{nM18} mutants showed no attraction to ethanol containing food odors while $Tdc2^{RO54}$ mutants displayed control like preference behavior (AIs for w^{1118}: 0.52 ± 0.08; for w^{1118}, Tbh^{nM18}: -0.04 ± 0.13 and for w^{1118}; $Tdc2^{RO54}$: 0.5 ± 0.1; n = 29, 21, 30). (C) An overexpression of *Tbh* in *Tdc2*-GAL4 dependent neurons eliminated the attraction to ethanol containing food odors (AIs for w^{1118}: 0.5 ± 0.03; for w^{1118};; *UAS-Tbh*: 0.38 ± 0.06; for w^{1118}; *Tdc2*-GAL4: 0.41 ± 0.03 and for w^{1118}; *Tdc2*-GAL4;*UAS-Tbh*: 0.19 ± 0.06; n = 38, 24, 26, 28). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The ANOVA post hoc Tukey test was used to determine difference between more than two groups. *P < 0.05, **P < 0.01 and ***P < 0.001.

From the last three experiments it can be concluded, that a certain balance of TA and OA is needed for displaying normal olfactory ethanol attraction. Decreased levels of OA in combination with increased TA levels results in the same mutant phenotype as increased levels of OA and decreased levels of TA due to an overexpression of the Tbh enzyme. A reduction of both neurotransmitter levels resulted in wild type like olfactory ethanol attraction.

3.4.3. *Tbh*^{nM18} mutants showed no aversion against high concentrations of ethanol

Wild type flies prefer natural concentrations of alcohol like 5% (Ogueta et al., 2010; Schneider et al., 2012), but show aversive behavior towards higher concentrations of ethanol like 23% (Ogueta et al., 2010; Giang et al., 2017). Now it is interesting to know, if *Tbh*^{nM18} mutants also fail to show not only the attractive behavior, but also the aversive behavior towards higher concentrations of ethanol. If OA is the neurotransmitter mediating aversive behavior, than the *Tbh*^{nM18} mutants should show no or a reduced aversion towards the ethanol enriched food odor trap. The two odor choice paradigm was used to test juice against juice with 23% ethanol (Figure 31). The *w*^{1118} control flies displayed a significant aversion towards the 23% ethanol containing food odor trap, while the *Tbh*^{nM18} mutants had a non-significant tendency of aversion against the ethanol containing food odor trap, which was also not significantly different to the control group. The shown aversion of the control flies here (AI: -0.29 ± 0.07) is reduced compared to former results (AI: ~-0.4 for *w*^{1118}; Giang et al., 2107 and AI: ~-0.9 for Canton S; Ogueta et al., 2010). To achieve a significant result, further experiments should be conducted. Using a higher concentration of ethanol (30%) could lead to a higher aversion of the control group and therefore to a significant difference to the *Tbh*^{nM18} mutants. A lower concentration of ethanol (20%) could still lead to aversion in the control group but might not be enough for the *Tbh*^{nM18} mutants to sense the ethanol in a negative way. Thus, there might be a shift to the right in the dose-response-curve towards ethanol in *Tbh*^{nM18} mutants.

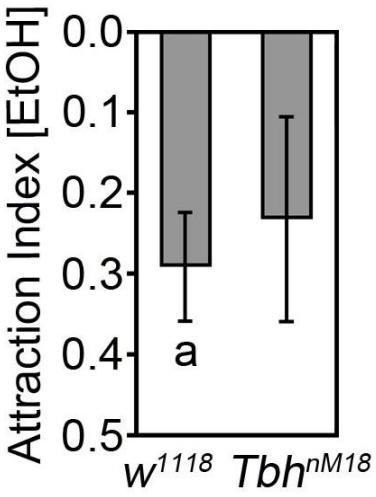


Figure 31: *Tbh*^{nM18} did not show aversion towards 23% ethanol

The control *w*^{1118} showed a significant aversion towards 23% ethanol in juice, while the *Tbh*^{nM18} mutants did not (AIs for *w*^{1118} and *Tbh*^{nM18}: -0.29 ± 0.07 and -0.23 ± 0.13 respectively, n = 26, 21). Errors are s.e.m. and the letter **a** indicate difference from random choice as determined by One-sample sign test. The Student's t-test was used to determine difference between more the two groups.

Hence, a lack of OA might weaken the aversive effect of high ethanol concentrations. This could be either due to the fact that OA mediates also aversive behavior or that TA is involved in aversion, but without OA it cannot be executed properly. But as feeding TA had no aversive effect, neither in the control group nor in the Tbh^{nM18} mutants, this contradicts the presumption that TA might be the neurotransmitter mediating aversion. Therefore, this points to OA as the neurotransmitter mediation attraction and aversion behavior.

From all these results it can be concluded, that OA is the neurotransmitter sufficient and necessary for mediating olfactory attraction towards ethanol containing food odors. Additionally, the right amount of OA and its ratio to TA is crucial for developing normal ethanol attraction behavior. Nevertheless, a role of TA in mediating attraction cannot be completely excluded. It is possible, that TA is involved but its effect cannot be seen as OA, which mediates the motivation, is missing and so the behavior cannot be executed. Only at low OA and high TA levels, the effect of TA is visible. OA is also involved in mediation of aversion, as Tbh^{nM18} mutants fail to show aversion towards high concentrations of ethanol. Again, the role of TA cannot be completely ruled out, as the executing neurotransmitter OA is missing. Furthermore, OA is responsible for the switch between attraction and aversion, as Tbh^{nM18} mutants fail to adapt to a new circumstance.

3.5. Knock down of OA receptors in olfactory sensory neurons did not result in a clear candidate for involvement in olfactory attraction

Now it is known, that OA is the responsible neurotransmitter for mediating the switch between site attraction and site aversion and for innate ethanol containing food odor attraction. But it is not known, which OA receptors are involved in the signaling pathway of these behaviors. To investigate this question, different OA receptors were knocked down in olfactory sensory neurons (OSNs) by using the *Orco*-GAL4 driver line (Thomas Giang, 2014) by using RNAi. There are four different OA receptors: OAMB, Octβ1R, Octβ2R and Octβ3R (Han et al., 1998; Maqueira et al., 2005). The OAMB is an α-

adrenergic-like receptor, while the other three are β-adrenergic-like receptors (Dudai and Zvi, 1984; Han et al., 1998; Evans and Maqueira, 2005). It has already been shown that the OAMB receptor is involved in appetitive learning and memory (Heisenberg et al., 1985; deBelle and Heisenberg, 1994; Davis, 1996; Kim et al., 2013), which makes him a possible candidate for attraction behavior.

The different OA receptors were knocked down in the OSNs. Elimination of the Octβ1R in *Orco*-GAL4 driven neurons leads to a non-significant reduction in the experimental group compared to the three control groups (Figure 32A). A knock down of the Octβ2R in the OSNs did also not result in a significant reduction in the experimental group (Figure 32B). Instead, the UAS- and GAL4 control group showed an untypical behavior, as they were not attracted towards the ethanol containing food odor, which they should have been. For the *UAS-Octβ2R-RNAi* control group it could be possible, that the UAS transgene might have an effect on the behavior. But for the *Orco*-GAL4 control group, which had the same genotype used as a control for the other UAS transgenes, it was expected to display attraction as it did in the other experiments. But also repeating this experiment, to exclude experimental mistakes, did not change the result. Ablation of the Octβ3R in the OSNs also led to a non-significant reduced attraction of the experimental group towards the ethanol containing food odor (Figure 32C). Elimination of the OAMB receptor – the most promising candidate – in *Orco*-GAL4 driven neurons had no effect on the experimental group (Figure 32D).

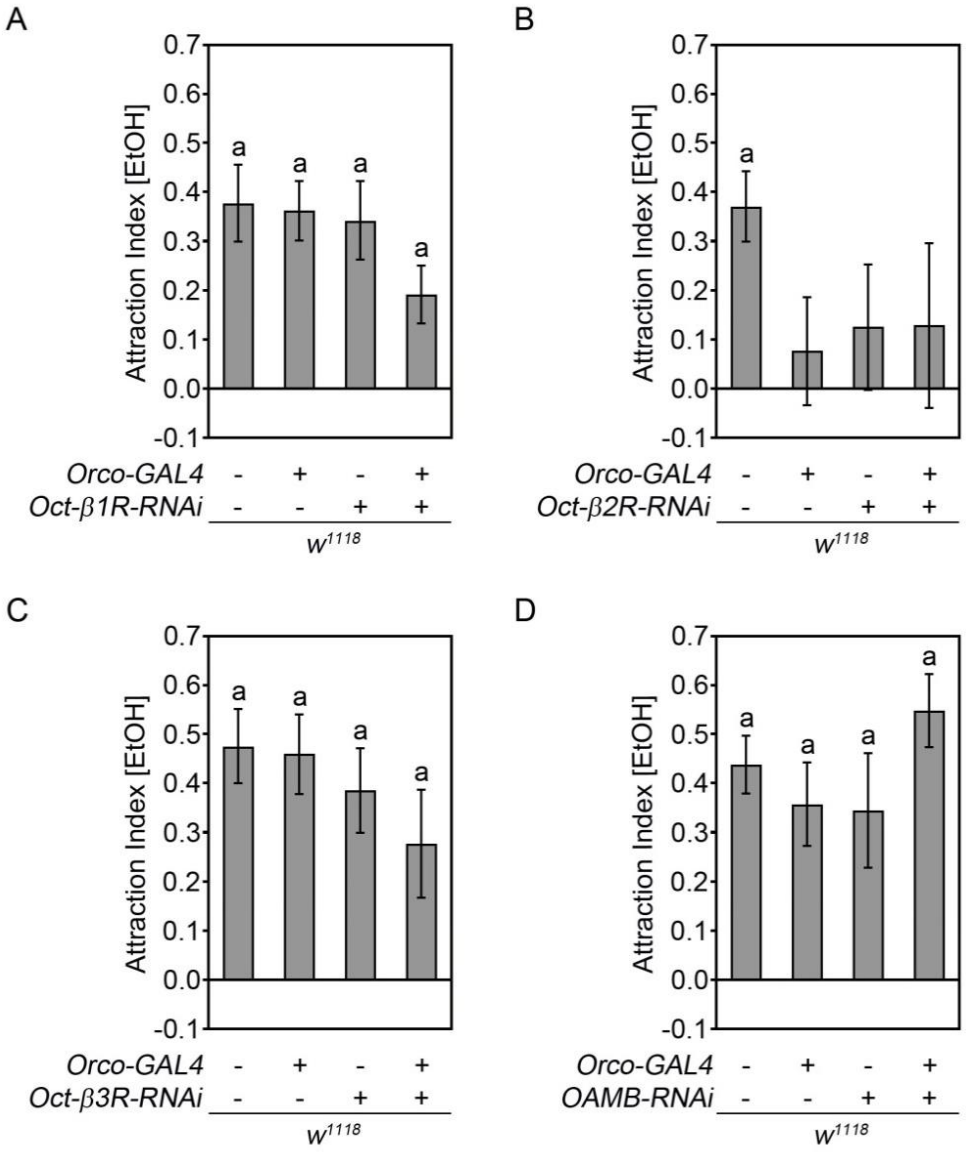


Figure 32: RNAi induced knock down of OA receptors in OSNs had no significant effect on olfactory attraction towards ethanol containing food odors

RNAi induced knock down of OA receptors in OSNs. (A) A knock down of the Oct-β1R receptor in OSNs using the *Orco*-GAL4 driver line resulted in a non-significant reduction of the olfactory attraction towards ethanol containing food odors in the experimental group (AIs for w^{1118}: 0.38 ± 0.08; for w^{1118}*; Orco*-GAL4: 0.36 ± 0.06; for w^{1118}*; UAS-Oct-β1R-RNAi* : 0.34 ± 0.08 and for w^{1118}*; Orco*-GAL4*; UAS-Oct-β1R-RNAi*: 0.19 ± 0.06; n = 29, 31, 29, 41). (B) A knock down of the Oct-β2R receptor in OSNs did not result in a significant reduction in the experimental group (AIs for w^{1118}: 0.37 ± 0.07; for w^{1118}*; Orco*-GAL4: 0.08 ± 0.11; for w^{1118}*; UAS-Oct-β2R-RNAi*: 0.13 ± 0.13 and for w^{1118}*; Orco*-GAL4*; UAS-Oct-β2R-RNAi*: 0.13 ± 0.17; n = 26, 28, 23, 20). (C) A knock down of the Oct-β3R receptor in OSNs had no effect on the experimental group (AIs for w^{1118}: 0.47 ± 0.08; for w^{1118}*; Orco*-GAL4: 0.46 ± 0.08; for w^{1118}*; UAS-Oct-β3R-RNAi*: 0.39 ± 0.09 and for w^{1118}*; Orco*-GAL4*; UAS-Oct-β31R-RNAi*: 0.28 ± 0.09; n = 33, 21, 30, 32). (D) A knock down of the OAMB receptor in OSNs had no effect on the experimental group (AIs for w^{1118}: 0.44 ± 0.06; for w^{1118}*; Orco*-GAL4: 0.36 ± 0.09; for w^{1118}*; UAS-OAMB-RNAi*: 0.35 ± 0.12 and for w^{1118}*; Orco*-GAL4*; UAS-OAMB-RNAi*: 0.55 ± 0.08; n = 32, 31, 30, 30). Errors are s.e.m., the letter "a" indicates random choice significantly different from zero as determined by One-sample sign test. The ANOVA post hoc Tukey test was used to determine difference between more than two groups.

From these results it cannot definitely be said if one of the OA receptors is involved in mediating the olfactory attraction towards ethanol in *Drosophila melanogaster*. The missing effect in the experiment could be due to an incomplete or non-functional knock down via the RNAi. But assuming that the RNAi is functional, the receptors Octβ1R, Octβ3R and OAMB can be ruled out in mediating olfactory ethanol attraction in OSNs. To further investigate the role of the OA receptors in olfactory attraction, ablation could be tested in other neurons, like the projection neurons or the lateral interneurons. Another possibility is to test not only flies with eliminated receptors in a certain set of neurons, but to test flies which lack the receptors in the whole body. These OA receptor mutants still have to be tested.

3.6. *Tbh* mutants display a defect in locomotion

Tbh^{nM18} mutants do not show attraction to ethanol containing food odors (Schneider et al., 2012). This could be due to either a sensory impairment (they do not sense the difference between the two offered food odor traps), to an affected motivation (they do sense the difference between the two offered food odor traps but cannot recognize an advantage of one of the traps) or to a defect in locomotion (they do sense the difference between the two offered food odor traps and recognize that one trap is more positive than the other, but are not able to execute the approach towards the more positive food odor source). Here the defect in locomotion was investigated by testing the three different *Tbh* mutants in a locomotion assay. The travelled distance and the walking speed were examined in more detail.

Regarding the distance covered during the one minute of filming, the Tbh^{nM18} mutants are not significantly different from their w^{1118} control group (Figure 33A), although they walk about 3.5 cm less than the w^{1118} flies, which is about 15% less covered distance compared to the control. The Tbh^{Del3} and *d01344* mutants are significantly different from the w^{1118} control group. Regarding the walking velocity, all *Tbh* mutants showed a significantly reduced speed compared to the w^{1118} flies (Figure 33B). These data suggest

an impaired locomotion phenotype of the *Tbh* mutants. But it was not tested if this defect could be improved with a positive or negative stimulus as a motivation and if there would be changes in covered distance and walking speed due to a stimulus. Thus the conclusion of a defect in locomotion must be considered with reservation, as it could still be a motivational problem.

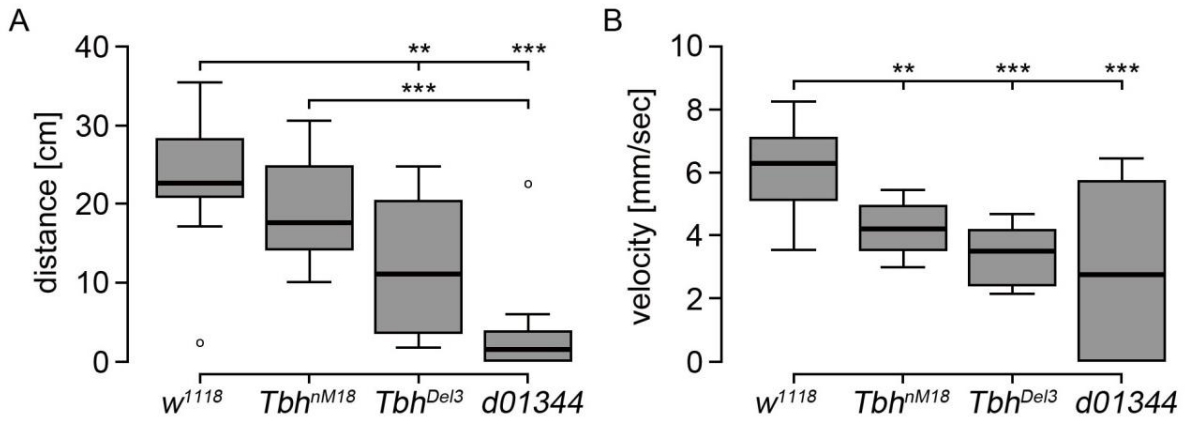


Figure 33: *Tbh* mutants display an impaired locomotion phenotype

(A) *Tbh* mutants walked less than their control group (distance for w^{1118}: 23.05cm ± 8.31; for Tbh^{nM18}: 19.46cm ± 7.11; for Tbh^{Del3}: 11.78cm ± 8.99 and for *d01344*: 4.23cm ± 7.19; n = 13, 15, 12, 9). (B) *Tbh* mutants move slower than their control group (velocity for w^{1118}: 6.06mm/sec ± 1.31; for Tbh^{nM18}: 4.21 mm/sec ± 0.79; for Tbh^{Del3}: 3.38 mm/sec ± 0.95 and for *d01344*: 2.68 mm/sec ± 2.65; n = 13, 15, 12, 9). Errors are STDV, ANOVA post hoc Tukey test $^{**}P < 0.01$ and $^{***}P < 0.001$.

4. Discussion

4.1. OA acts as a positive and as a negative reinforcer

A subset of tyraminergic/octopaminergic/cholinergic neurons is sufficient and necessary for mediating site attraction and site aversion but the behavioral outcome is dependent on the combination of activated neurons. A larger set of *Tdc2*-GAL4 targeted tyraminergic/octopaminergic/cholinergic neurons mediates site attraction, while the three *6.2-Tbh*-GAL4 dependent tyraminergic/octopaminergic/cholinergic VUMa4 neurons mediate site aversion. Thus OA is able to function as a positive and a negative reinforcer in *Drosophila*.

Until now it was thought that OA only acts as a positive reinforcer. A positive reinforcer strengthens a behavior, since it is added as a reward to the desired behavior shown by the organism (Skinner, 1953). The positive reinforcing effects of OA had been shown for example in *Apis mellifera* and *Drosophila melanogaster*. In honey bees, depolarization of the octopaminergic VUMmx1 neuron could substitute for the rewarding US (in that case sucrose) in olfactory conditioning and therefore functions as a positive reinforcer in learning and memory (Hammer, 1997). Replacing the US with an OA injection had the same effect and elicited the PER (Hammer and Menzel, 1998). The same effect of OA in appetitive associative learning was shown in *Drosophila* larvae. Light activation of tyraminergic/octopaminergic neurons with the blue light activatable ChR2 could substitute for the US (in this case fructose) (Schroll et al., 2006), while elimination of tyraminergic/octopaminergic neurons in *Tdc2*-GAL4/*UAS-shi^{ts}* flies abolished appetitive learning and memory (Honjo and Furukubo-Tokunaga, 2009). In the adult fly, the role of OA in positive reinforcement was demonstrated by the inability of *Tbh^{nm18}* mutants to form an appetitive memory (Schwaerzel et al., 2003) or by the impaired appetitive memory acquisition of OAMB receptor mutants with reduced OAMB expression in the MB (Kim et al., 2013). Heat activation in *Tdc2*-GAL4/*UAS-dTrpA1* flies established short-term appetitive memory, while elimination of tyraminergic/octopaminergic neurons in *Tdc2*-GAL4/*UAS-shi^{ts}* flies impaired short-term appetitive learning and memory (Burke et al., 2012). Comparable to my thesis, the reinforcing effect of OA in eliciting site

attraction towards the blue illuminated food odor trap with ChR2 expressed in *Tdc2*-GAL4 dependent neurons has been shown earlier in the optogenetic site attraction assay with a different light intensity (Schneider et al., 2012).

Tdc2-GAL4 dependent neurons mediate site attraction, independent of the used channelrhodopsins, ChR2 and ReaChR. Elimination of cholinergic neurons by introducing *Cha*-GAL80 abolished site attraction. Thus a subset of tyraminergic/ octopaminergic/cholinergic neurons mediates site attraction. The same subset of neurons was not able to restore olfactory ethanol attraction in Tbh^{nm18} mutants (Schneider et al., 2012), but is responsible for mediating aggression behavior in male and female *Drosophila* (Zhou et al., 2008). Attraction towards a food odor source and aggression towards another fly are both approaching behaviors and might thus be related somehow. In oviduct contraction, OA interacts with the neurotransmitter GABA (Rodriguez-Valentin et al., 2006), so it might be possible, that attraction behavior might also be regulated by more than only one neurotransmitter.

The neurotransmitter TA can be ruled out of being responsible of mediating site attraction, as *Tdc2*-GAL4 dependent neurons in the Tbh^{nm18} mutant background lack site attraction and display site aversion. The observed site aversion could be due to the increased levels of TA, since TA might also function as a neurotransmitter (Nagaya et al., 2002; Roeder et al., 2003). In the *honoka* mutant, a TA receptor mutant, the effects of TA (and not of OA) on neuromuscular junction potentials are abolished (Nagaya et al., 2002) and this mutant is slightly hyperactive and impaired in olfactory tasks (Roeder et al., 2003). This endorses the role of TA as an independent neurotransmitter, which might be able to mediate site aversion. Thus, OA and TA might function antagonistically by inducing site attraction and site aversion, respectively. These antagonistic effects of OA and TA were already investigated in *Drosophila*. In the locomotion of *Drosophila* larvae, feeding OA or yohimbine (TA receptor antagonist) partially restored the mutant phenotype of Tbh^{nm18} mutants (Saraswati et al., 2003). In flight initiation and maintenance, OA is necessary for flight maintenance while TA acts as an inhibitor for flight initiations (Brembs et al., 2007). And the increased levels of TA in Tbh^{nm18} mutants prohibit carbohydrate consumption on control level after feeding OA to the flies (Jan Götz, 2018), which might be caused by the fact, that OA receptors are also able to bind TA (Roeder, 2005, Bayliss et al., 2013; Ohhara et al., 2014) and thus the surplus of TA

might prevent OA binding to the OA receptors. Furthermore, OA and TA have opposing effects on cAMP levels in *Drosophila melanogaster* and in *Apis mellifera*. OA is able to either increase the intracellular cAMP or Ca^{2+} levels (Evans and Robb, 1993; Balfanz et al., 2005; 2014; Maqueira et al., 2005), while TA reduces the intracellular cAMP levels (Blenau et al., 2000; 2017). In the locust OA and TA regulate attractive and repulsive behavior, respectively. The α-adrenergic OA receptors mediate attraction in gregarious locusts and can switch from repulsion to attraction in solitary locusts, while the TA receptors mediate repulsion in solitary locusts and can switch from attraction to repulsion in gregarious locusts (Ma et al., 2015). But artificially further increased TA levels in Tbh^{nm18} mutants did not result in aversive olfactory ethanol behavior. Therefore TA can be either ruled out as the neurotransmitter mediating site aversion or the two behaviors site attraction and olfactory attraction are mediated through two different pathways.

The hypothesis, that the two different attraction behaviors might be mediated in two different pathways is supported by the results obtained through expression of ChR2 in a *Feb15*-GAL4 dependent manner. The *Feb15*-GAL4 driver was able to restore the loss of olfactory ethanol attraction in Tbh^{nm18} mutants (Schneider et al., 2012), but did not result in site attraction. The expression pattern of the *Tdc2*-GAL4 and the *Feb15*-GAL4 driver overlap in major parts, regarding the targeted Tbh positive neurons (Schneider et al., 2012). In the AL2 cluster, there is a 100% overlap of Tbh positive neurons in both driver lines, in the VUM I and VUM II cluster the *Feb15*-GAL4 targets eight out of nine and seven out of nine Tbh positive neurons of the *Tdc2*-GAL4, respectively. Only in the VUM III cluster, there is a bigger difference and only three out of nine Tbh positive neurons of the *Tdc2*-GAL4 driver line are targeted by the *Feb15*-GAL4 driver. Hence, it is possible, that these six non-targeted neurons in the *Feb15*-GAL4 driver line are involved in mediating site attraction. The induced site aversion caused by *Feb15*-GAL4 targeted neurons with a modified activation pattern could be due to the membrane properties of the targeted neurons or caused by alternative released neurotransmitters. To check this, the *Cha*-GAL80 driver and the *TH*-GAL80 driver should be combined with the *Feb15*-GAL4 driver to eliminate putative cholinergic and dopaminergic neurons, respectively, and to investigate the influence of OA, this driver line should be tested in the Tbh^{nm18} mutant background.

Expression of ChR2 in a *fru-GAL4* dependent manner resulted in contradictory findings. The site attraction of male flies in both groups could be due to the fact that the *fruitless* gene is a neural sex determination factor (Ryner et al., 1996) and the GAL4 P-element insertion disrupted its function (Kimura et al., 2005). *fru* expression is essential for the establishment of a male-typical network and induced neurons to form male-typical projections, which are eliminated in female flies by cell death. There are neuronal sex differences in two regions of the brain: in the optic lobes and a region next to the AL. The neurons in the optic lobes are male specific. The mAL (medial located just above the AL) neurons are sexually dimorphic. Male flies have a larger number of these neurons, which project bilateral and have dendritic branches shaped like a horse tail instead of neurons projecting contralateral with forked-shaped dendritic branches in female flies. In absence of *fru* the mAL neurons are completely feminized, so *fru* functions as a male-female switch in the CNS (Kimura et al., 2005). These neuronal differences could explain the differences in site attraction behavior of male and female flies, since female control flies were undecided and the experimental group showed site attraction. Furthermore, the *fru-GAL4* line targets a very high number of neurons (Lee et al., 2000; Billeter and Goodwin, 2004), of which only a few are tyraminergic/octopaminergic and thus the site attraction could also be mediated by different neurons. Elimination of the cholinergic neurons revealed indecisiveness in both groups of the male flies. This suggests, disregarded of the male/female problem, that the neurons mediating the site attraction contain one to three of the OA-VPM1, OA-VPM2 and OA-VUM neurons, which are octopaminergic and cholinergic. These three octopaminergic neurons are also involved in male-male aggression behavior, since elimination of *fru* in these neurons leads to male-male courtship (Certel et al., 2010). Attraction and aggression are both approaching behaviors, so it might be possible that similar neurons are involved in both behaviors. Expression of ChR2 in a *fru*-GAL4 dependent manner in the Tbh^{nM18} mutant background did not induce site attraction or aversion in male flies. This could be either due to the lacking OA, but is unlikely as the increased TA levels are still present, if these neurons are also tyraminergic and not only octopaminergic or do not release TA as a neurotransmitter. To test this, the experiment could be repeated in a $Tdc2^{RO54}$ mutant background, with lacking OA and TA levels (Cole et al., 2005).

The *6.2-Tbh*-GAL4 line was also not able to restore the mutant phenotype of Tbh^{nM18} flies in olfactory ethanol attraction back to control level (Schneider et al., 2012). So it was not really expected, that this driver line would have an effect on site attraction. But activation of neurons in a *6.2-Tbh*-GAL4 dependent manner, including the three Tbh positive VUMa4 neurons, induced site aversion. The small subset of three VUMa4 neurons targeted by the *6.2-Tbh*-GAL4 driver is included in the set of neurons targeted by the *Tdc2*-GAL4 driver, which induced site attraction (Schneider et al., 2012). There are now two possible explanations of these results. Firstly, it is possible that OA or TA are also able to mediate site aversion, but the influence of the subset of the three VUMa4 neurons is overwritten by the output of the larger set of neurons targeted by the *Tdc2*-GAL4 driver line. Therefore, a smaller subset of neurons, which is part of a bigger set, can have the opposite effect than the bigger set of neurons. These two different behavioral outcomes are probably mediated through two different pathways or different OA and TA receptors might be activated. But it is likely, that the site attraction mediating network is the dominant one, since site aversion can be overwritten by site attraction. This indicates, that the VUM neurons are not homogenous and are able to elicit opposite behaviors. A functional heterogeneity has also been shown for dopaminergic PAM neurons, which are also both capable of mediating appetitive or aversive behavior (Liu et al., 2012). Secondly, the *6.2-Tbh*-GAL4 driver might target also neurons, which release other neurotransmitters. Expression of ChR2 in *TH*-GAL4 dependent neurons induced site aversion (Schneider et al., 2012), so there might be dopaminergic neurons targeted by the *6.2-Tbh*-GAL4 driver. This is a very likely explanation, as dopaminergic neurons have a tonic firing rate of 2-10Hz in rats (Grace and Bunney, 1983; Grace et al., 2007), which reflects the aversion elicited by 16s 8Hz, an activation pattern which had solitary no effect in any other GAL4 driver line. But DA can be ruled out as a responsible neurotransmitter, since elimination of dopaminergic neurons using *TH*-GAL80 still resulted in site aversion. Elimination of cholinergic neurons using *Cha*-GAL80 abolished site aversion. Thus the three VUMa4 neurons are tyraminergic/octopaminergic and cholinergic. TA as a possible mediator for site aversion can be ruled out by the fact, that expression of a channelrhodopsin in *6.2-Tbh*-GAL4 dependent neurons in the Tbh^{nm18} mutant background did not induce site aversion. The increased levels of TA were not sufficient to induce site attraction, thus OA is also capable of mediating site aversion.

The neurotransmitter acetylcholine might also be a putative candidate for mediating site attraction and site aversion. Elimination of cholinergic neurons abolished site attraction induced by *Tdc2*-GAL4 or fru-GAL 4 driven neurons and also site aversion in the Tbh^{nm18} mutant background or induced by *6.2-Tbh*-GAL4 targeted neurons. This could be tested by expressing ChR2 in cholinergic neurons. But a co-expression of acetylcholine in tyraminergic/octopaminergic neurons might also be just a coincidence and acetylcholine might not be involved in site attraction or aversion behavior.

Thus, OA is sufficient and necessary to mediate site attraction and site aversion, but the behavioral outcome is dependent on the combination of activated neurons. The larger set of *Tdc2*-GAL4 targeted tyraminergic/octopaminergic/cholinergic neurons mediates site attraction, while the three *6.2-Tbh*-GAL4 dependent tyraminergic/octopaminergic/ cholinergic VUMa4 neurons mediate site aversion. The different or even contrary roles of tyraminergic/octopaminergic neurons have been described for feeding behavior of *Drosophila*. Activation of a subset of octopaminergic neurons called VPMs inhibited odor tracking, as OA acts as a rewarding neurotransmitter, while blocking the synaptic output of *Tdc2*-GAL4 driven neurons resulted in the same phenotype (Sayin et al., 2018). OA also sensitizes sugar and bitter taste neurons, which also supports the possibly opposite roles of octopaminergic neurons (LeDue et al., 2016; Wang et al., 2016). So it is possible, that OA can function as a positive and as a negative reinforcer.

Concordant to this finding in this thesis, recent studies revealed the negative reinforcing effect of OA in learning and memory in *Drosophila*, as Tbh^{nm18} mutants displayed an impaired phenotype in aversive learning compared to their control (Iliadi et al., 2017). The role of aversive reinforcement/learning so far has been ascribed to DA. In *Drosophila* larvae, acquisition of aversive learning was abolished by eliminating dopaminergic neurons in *TH*-GAL4/*UAS-*shi^{ts} larvae (Schwaerzel et al., 2003; Honjo and Furukubo-Tokunaga, 2009; Selcho et al., 2009). Light activation of dopaminergic neurons using *TH*-GAL4/*UAS-ChR2* flies was able to substitute the punishing US to induce aversive learning and memory in larvae (Schroll et al., 2006), while photoactivation of *TH*-GAL4/*UAS-*$P2X_2$ flies (Claridge-Chang et al., 2009) or heat activation of *TH*-GAL4/*UAS-dTrpA1* flies (Aso et al., 2012) had the same effect in adult flies. Furthermore, DA receptor mutants fail to acquire aversive memory in larvae (Selcho et al., 2009) and in adult *Drosophila* (Kim et al., 2007). But recent studies

revealed that DA is also involved in appetitive learning and can also function as a positive reinforcer. Impairment in appetitive learning and memory was observed, when dopaminergic neurons are eliminated in *TH*-GAL4/*UAS-shi*^{ts} larvae (Selcho et al., 2009). The DA receptor mutants do not fail to establish, but are impaired appetitive learning and memory in adult flies (Kim et al., 2007). In larvae, the effect was more severe (Selcho et al., 2009). The appetitive and aversive olfactory memories mediated by DA are probably located in the same MB neuropil (Schwaerzel et al., 2003; Kim et al., 2007). The paired posterior lateral (PPL1) neurons and the paired anterior medial (PAM) neurons are involved in aversive learning (Claridge-Chang et al., 2009; Aso et al., 2010 and 2012), while the PAM neurons are also involved in appetitive learning (Burke et al., 2012; Liu et al., 2012), which suggests a functional heterogeneity of the PAM cluster (Liu et al., 2012).

Furthermore it was shown, that octopaminergic and dopaminergic neurons interact in rewarding memories, since OA receptors expressed on dopaminergic neurons are essential for the OA signaling in appetitive learning and memory and octopaminergic neurons directly interact with dopaminergic neurons in gustatory reward (Burke et al., 2012; Huetteroth et al., 2015). So both neurotransmitters are able to function as a positive and negative reinforcer. DA and OA have the same origin amino acid – tyrosine (Nagatsu et al., 1964; Brandau and Axelrod, 1972). DA and norepinephrine, the mammalian homologue of OA (Roeder, 1999), both belong to the catecholamines (Nagatsu et al., 1964) and are processed by decarboxylases (Livingston and Tempel, 1983). Thus, it is likely, that these two neurotransmitters are also similar in their putative functions. It was also shown that the DA receptor AmDOP2 and the OA receptor AmOA1 have similar pharmacological properties and their structural properties related to their function are highly conserved (Beggs et al., 2011). This also indicates that there might be similar functions of the DA and the OA signaling pathways.

The results about OA mediating site attraction and site aversion obtained in this thesis are consistent with the "Orchestration Hypothesis", written by Sombati and Hoyle (1984). The hypothesis claims that there is a neuronal network for every set of behavior, which can be selectively activated or inhibited through the release of OA. This allows the suppression of opposing behaviors and therefore internal conflicts of the fly. This hypothesis was found on the contrary role of octopaminergic DUM neurons in the locust

on flight initiation and oviposition digging. The DUM neurons can function in two ways: Firstly, they promote a specific behavior through direct excitatory action of OA or through OA promoted inhibition of an inhibition. Secondly, they prevent the appearance of a specific behavior by enhancing inhibitory inputs to a neuronal circuitry generator. So the DUM neurons have a modulatory function and the behavioral outcome depends on the most active DUM neurons at a certain time point (Sombati and Hoyle, 1984).

4.2. OA is sufficient and necessary for olfactory ethanol attraction

Olfactory attraction towards ethanol is mediated by OA and the loss of olfactory attraction in Tbh^{nm18} mutants is due to the loss of OA and not caused by the increased levels of TA.

w^{1118} flies normally show attraction to food odor sources, which contain natural concentrations of ethanol (Ogueta et al., 2010; Schneider et al., 2012), while Tbh^{nm18} mutants lack this attraction behavior (Schneider et al., 2012). But this loss of attraction towards ethanol containing food odors is not due to a loss of odorant perception, as the Tbh^{nm18} mutants are still able to choose a food odor or ethanol odor over pure water (Schneider et al., 2012). This loss of ethanol attraction is due to the lack of OA, since feeding OA or OA agonists restored the olfactory attraction of Tbh^{nm18} mutants back to control level, blocking OA signaling in w^{1118} flies mimicked the Tbh^{nm18} mutant phenotype and blocking TA signaling had no effect on any of the groups. This substantiates the role of OA as a positive reinforcer in attraction behavior. The reduced olfactory ethanol attraction in naphazoline fed control flies suggests that a too high activation of OA receptors might result in an aversive effect or that different/additional receptors might be activated. This indicates towards a putative role of OA as a negative reinforcer. The effect of restored olfactory ethanol preference in TA fed Tbh^{nm18} mutants is explainable by two possibilities: Firstly, high enough levels of TA are able to mediate olfactory attraction in the absence of OA. Although OA receptors show a strong preference for OA over TA (Maqueira et al., 2005), they are also able to bind TA (Roeder, 2005, Bayliss et al., 2013; Ohhara et al., 2014). So the OA signaling pathway is activated

by TA. Secondly, TA binds to TA receptors and activates a TA signaling pathway, which somehow influences olfactory attraction behavior. It was already suggested by Brembs and colleagues (2007) that the role of TA is only effective when there are high TA and low OA levels.

All in all, these results reveal that OA is not only sufficient, but also necessary for attraction behavior. Restoring the mutant phenotype of Tbh^{nm18} mutants to the control level by feeding OA reassures the sufficiency and ablation of ethanol attraction in control flies by feeding the OA antagonist epinastine demonstrates the necessity of OA in olfactory attraction behavior.

Wild type flies like Canton S or w^{1118} flies do not only show an attraction towards natural concentrations of ethanol, but also an aversion towards too high concentrations (Ogueta et al., 2010; Schneider et al., 2012; Giang et al., 2017). But Tbh^{nm18} mutants do not show a significant aversion towards 23% ethanol containing food odors. This endorses the presumption, that OA and not TA is the neurotransmitter mediating aversion, as a lack of OA resulted in a loss of aversion. But as there is a tendency with a high variance of the Tbh^{nm18} mutants towards aversion to the 23% ethanol, an even higher concentration might result in a significantly different value from zero. This would also indicate that Tbh^{nm18} mutants are still able to execute a behavior, but they need a higher/stronger/ more negative stimulus to initiate the behavior, in this case a high enough concentration of ethanol. The need of an aversive stimulus was showed in larval locomotion, where the impaired phenotype of Tbh^{nm18} mutants could be improved by giving a negative stimulus like sodium chloride (Thomas Kell, 2017). A reduction of the ethanol concentration to for example 20% might result in a significant difference between the two groups, since w^{1118} flies might already show an aversion, while the Tbh^{nm18} mutants might be completely undecided. A further reduction of the ethanol concentration is not recommendable, as w^{1118} flies are indecisive towards 15% ethanol in juice (Giang et al., 2017).

4.3. Olfactory attraction is not mediated by the tested OSN OA receptors

The three different OA receptors Octβ1R, Octβ3R and OAMB expressed on the OSNs are not involved in mediating olfactory attraction towards ethanol containing food odors in *Drosophila melanogaster*.

The three β-adrenergic-like OA receptors (Octβ1Rs, Octβ2Rs and Octβ3Rs) are involved in a high number of octopaminergic functions (Ohhara et al., 2012) and have a strong binding affinity for OA over TA (Maqueira et al., 2005). For the OAMB receptor expressed in the MB, it was already shown that it is involved in appetitive olfactory learning in *Drosophila* (Kim et al., 2013). The involvement of the so far known OA receptors in olfactory ethanol attraction behavior could not be really clarified in this study, since elimination of the Octβ1R, Octβ3R and OAMB did not result in a significant phenotype. The Octβ2R gave no reliable results, as two out of three controls did not show attraction towards the ethanol containing food odor trap. There are five explanations for these results. Firstly, it is possible, that none of the tested receptors is involved in mediating olfactory ethanol attraction, which would be surprising as it was shown that OA is involved in innate olfactory ethanol attraction (Schneider et al., 2012). Secondly, the RNAi did not work or at least did not eliminate all of the OA receptors. Thirdly, the OA could have bound to other receptors, like the TA receptors or the Oct-Tyr receptors and thus provide the necessary signaling for olfactory attraction. This is possible, as TA or Oct-Tyr receptors have a higher affinity for TA, but are also activated by OA (Bayliss et al., 2013). Fourthly, the elimination of one OA receptor could have been compensated by another OA receptor, like it was shown for the female sterility and fecundity, where the loss of the Octβ2R could be (partly) compensated by expression one of the other three OA receptors (Lim et al., 2014). Fifthly, the OA receptors were eliminated in the wrong neurons. Other neurons could be tested by using other GAL4 driver lines, for example in the projection neurons or in the local neurons, both types of neurons are located in the AL and involved in the olfactory pathway in *Drosophila* (Anton and Homberg, 1999). As all OA receptors are highly expressed in the MB (El-Kholy et al., 2015), a GAL4 driver which eliminates OA receptor expression in the whole MB or in specific parts of the MB would be suitable. A knock down of an OA receptor in

the olfactory lobes should also be more promising, since the olfactory lobes are the brain region where the olfactory information is processed (Farooqui et al., 2003). Another way to address the question, which OA receptor is involved in olfactory ethanol attraction, is to test mutants of the respective receptor and then cause a knock down in different types of neurons with only one OA receptor type.

4.4. The interaction of OA and TA is important for the behavioral outcome

An interaction and probably a certain balance between the two neurotransmitters OA and TA is required to result in a proper behavioral outcome and the function of TA is only of importance if OA is missing and TA levels are increased.

Overexpression of the *Tbh* gene – with *UAS-Tbh* or in the *d01344* mutant – leads to the same loss of olfactory ethanol attraction phenotype like a lack of OA (Tbh^{nm18} or Tbh^{Del3} mutants). But the overexpression phenotypes have to be considered with more caution, as expression of the wild type *UAS-Tbh* leads to a functional Tbh protein. But for the *d01344* mutant this is still uncertain, as the increase of *Tbh* was only verified on a transcriptional level (Manuela Ruppert, 2013). This is also true for locomotion, where all *Tbh* mutants, no matter if Tbh deficient or upregulated *Tbh* expression, showed a decrease in travelled distance and walking speed.

This was already shown for other behaviors in *Drosophila*. Saraswati and colleagues (2003) showed that Tbh^{nm18} mutant larvae are severely impaired in locomotion. But this phenotype could be equally rescued by either feeding OA or blocking TA signaling through the TA antagonist yohimbine. Feeding OA and yohimbine simultaneously further improved the locomotion phenotype. So both neurotransmitters seemed to be involved in locomotion. Application of OA or TA on decapitated flies increased locomotion and hind leg grooming, whereas OA was more effective than TA (Yellmann et al., 1997). Brembs and colleagues (2007) represent the opinion, that not the relative levels of OA and TA are important, but that there has to be a concerted interaction

between both neurotransmitters, as they might have different sites of action and that the role of TA is only effective when there are high TA and low OA levels. *Tbh*^{nm18} mutants are impaired in flight initiation and maintenance, but feeding OA did not restore the mutant phenotype, while restoring Tbh levels did, which simultaneously increased OA levels and decreased TA levels. Blocking TA signaling in *Tbh*^{nm18} mutants was most effective at restoring the phenotype (independent from the OA levels), but blocking TA signaling in wild type flies had no effect (Brembs et al., 2007).

If OA and TA levels are reduced, like in the *Tdc2*^{RO54} mutant, again an olfactory attraction towards ethanol containing food odors was observed. This olfactory ethanol attraction is explainable by three different possibilities: Firstly, not only OA is important for proper olfactory attraction behavior, but also a balance of TA and OA is essential. This is conform to the results of the three different *Tbh* mutants, but contradictory to the results of TA and OA feeding. Secondly, the phenotype of the *Tdc2*^{RO54} mutants could be on wild type level, as the mutants were crossed to *w*^{1118} and thus are only heterozygous, because the *Tdc2*^{RO54} mutation is balanced over CyO and almost only balanced flies hatched. It was observed that flies carrying the CyO balancer are severely impaired in locomotion (Malvina Kuschmann, data unpublished) and therefore the effects of impaired locomotion during decision making were eliminated by replacing the CyO balancer with a wild type allele. Thirdly, the *Tdc2*^{RO54} mutant is not a null mutant and it should be verified whether the *Tdc2*^{RO54} mutant really is a mutant lacking TA and OA or if there might be any levels of TA and OA left. Otherwise it is possible that other neurotransmitters are able to substitute for these two neurotransmitters and mediate the decision of the flies to choose the ethanol containing food odor. One possible neurotransmitter is serotonin, which is also involved in mediation ethanol attraction (Xu et al., 2016). *Tdc2*^{RO54} mutants and *Tbh*^{nm18} mutants both display reduced starvation-induced hyperactivity, a phenotype that could be rescued by feeding TA. Thus the elevated TA levels in *Tbh*^{nm18} mutants are not responsible for the starvation-induced hyperactivity (Yang et al., 2015). Unlike *Tbh*^{nm18} mutants, *Tdc2*^{RO54} mutants have an overall reduced locomotor activity (Yang et al., 2015; Hardie et al., 2007). This indicates that TA is required for general motor behavior of flies, but OA is required for the starvation-induced hyperactivity and that OA and TA might interact and probably work in a synergistic way in regulating this behavior (Yang et al., 2015).

Another mutant suitable for testing the effects of reduced TA and OA levels is the *iav* mutant. *iav* mutants have a reduced Tdc activity and 2.5 fold lower levels of TA in the brain compared to wild type flies (McClung and Hirsh, 1999) and only 15% left of the OA wild type level (O'Dell, 1993). These mutants show a less severe phenotype in locomotion than the Tbh^{nm18} mutants, which also indicates towards the importance of balanced OA and TA levels and that both neurotransmitters contribute to the locomotion defect in Tbh^{nm18} mutants (Saraswati et al., 2003). Regarding ethanol induced behavior, it is also possible that tolerance development towards ethanol requires also a balance of both neurotransmitters. The *iav* mutant had an increased sensitivity and a decreased hyperactivity at the first exposure and developed normal tolerance, while the Tbh^{nm18} mutants displayed normal sensitivity, but an increased hyperactivity and a decreased tolerance (Scholz, 2005). So the altered levels of TA and its relation to the OA level seemed to affect the behavior towards ethanol exposure, especially the ethanol-activating effect on locomotion seems to be regulated by TA (Scholz, 2005). But the *iav* mutant was not tested for olfactory ethanol attraction. This could be done, as it would be interesting to see, what kind of phenotype they will show.

4.5. OA biases the behavioral outcome

OA mediates the switch between attraction and aversion behavior in *Drosophila*. A set of tyraminergic/octopaminergic neurons is sufficient to shift the attraction to a less attractive food odor and the induction of olfactory attraction for ethanol containing food odors also requires the function of OA.

From the obtained results it is now known that OA can act as a positive and as a negative reinforcer. But it is not solved how this is managed. It is possible that OA mediates the switch between a positive and a negative response, depending on the neurons which release OA upon activation. This hypothesis is supported by the finding that activation of tyraminergic/octopaminergic neurons shifts the attraction from olfactory ethanol attraction to indecisiveness in the optogenetic site attraction assay. The flies were confronted with two positive reinforcers: ethanol in the yellow trap and activation of

Tdc2-GAL4 driven neurons on the blue light illuminated side. Thus, there was no significant attraction to one of the food odor traps. This suppression of attraction towards the ethanol containing food odor trap caused by activation of tyraminergic/octopaminergic neurons implies that OA might not be directly involved in the behavior itself, but might mediate the switch between two different/opposing behaviors. The role of octopaminergic neurons in promoting behavioral switches was described recently in fruit flies and locusts. In locusts, TA and OA shift the behavior from attraction to repulsion and vice versa, respectively (Ma et al., 2015). In *Drosophila*, octopaminergic neurons mediate the switch between food seeking and stopping when the food source is found (Sayin et al., 2018). To further verify this assumption, the same optogenetic experiment done here could be repeated with *UAS-ChR2* in the Tbh^{nM18} background. If OA can act as a positive and a negative reinforcer and is mediating the switch between attraction and aversion, the experimental flies should not be undecided and still show the observed site aversion when tested without ethanol.

The role of OA in biasing the behavioral outcome is also endorsed by the results of pre-feeding ethanol in the olfactory two odor choice paradigm, which showed that OA is required to trigger approach behavior. The pre-fed Tbh^{nM18} mutants show an ethanol attraction on non-fed w^{1118} level, while the pre-fed w^{1118} flies show a non-significant reduction in their attraction. This indicates that Tbh^{nM18} mutants are still able to develop innate ethanol attraction and are also able to show this behavior. But they are not able to adapt to new situations. This was also shown for flight behavior by Brembs et al. (2007) and for larval locomotion by Thomas Kell (2017), where it was observed that Tbh^{nM18} mutants are not impaired in the behavior itself, but have defects in the initiation and termination of the behavior. In flight behavior, the basic function and morphology of the flight apparatus is still intact, but the initiation and maintenance are affected (Brembs et al., 2007). The reduced locomotion phenotype was investigated in Tbh deficient mutants (Tbh^{nM18} and Tbh^{Del3} mutants), and a *Tbh* overexpressing mutant (*d01344*). In adults, *d01344* mutants were more affected than the Tbh deficient mutants. The Tbh^{nM18} mutants were even not significantly different from the control group. In the larvae, the Tbh deficient mutants showed a more severe phenotype in the crawled distance, in their velocity and in the time spend crawling than the Tbh overexpressing mutant (Thomas Kell, 2017). Thus, the larval and adult data are conflictive to each other regarding the covered distance, while the observed speed phenotypes are comparable.

But when the Tbh deficient mutant larvae were given a negative stimulus, their crawling speed increased and a higher distance was covered, but there was no effect on the *d01344* mutant (Thomas Kell, 2017). This also indicates that the *Tbh* mutants are not impaired in the behavior itself (in this case locomotion), but that they are lacking the motivation to show this behavior. Comparing the crawled distance of larvae to find a place to pupate in the food vials, the Tbh^{nM18} and Tbh^{Del3} mutants covered a higher distance than the w^{1118} flies (Ruppert et al., unpublished). This is a hind towards impairment in initiation and termination of a certain behavior, but not in the behavior itself. Comparing the locomotion results to the olfactory attraction results, it can be observed that in both behaviors the *Tbh* mutants, not matter if less or missing Tbh or an upregulated Tbh expression, perform worse than the w^{1118} control flies. The question was asked, whether the loss of olfactory ethanol attraction is caused by a sensory impairment, by a motivational problem or by a defect in locomotion. The possibility of olfactory impairment can be ruled out, as it was shown that the Tbh^{nM18} mutants are able to distinguish between a simple (5% ethanol in water) and a complex odor (5% ethanol in juice) (Gerbera Claßen, 2011). And based on the larval locomotion experiment it is likely, that the defect in locomotion in adult flies is also caused by the lack of motivation. So the loss of olfactory ethanol attraction is probably also due to missing motivation, as it was shown that *Tbh* mutants are able to perform, when they are given the right stimulus (Thomas Kell, 2017).

4.6. Blue and amber light activatable channelrhodopsins are both suitable for neuronal activation to elicit site attraction and site aversion

The behavioral outcome elicited by expression of the blue light activatable ChR2 or the amber light activatable ReaChR transgene in different subset of neurons is not transgene dependent and thus is real and due to the neuronal activation. This neuronal activation is neither intensity nor frequency dependent and the observed differences are caused by the kinetics of the channelrhodopsin transgenes.

In this thesis, the blue light activatable ChR2 and the red light activatable ReaChR were used to activate Tbh positive neurons involved in mediating site attraction or aversion. Some differences in the optimal set up and also in some results occurred between these two different channelrhodopsins. Both channelrhodopsins derive from the same algae *Chlamydomonas reinhardtii* (Nagel et al., 2003; Lin et al., 2013), but the ReaChR is an altered version through different point mutations and was engineered by Lin and colleagues (2013). The third used channelrhodopsin Chrimson derives from the algae *Chlamydomonas noctigama* and is also a red-shifted channelrhodopsin (Klapoetke et al., 2014), but was not usable for my experiments. The advantage of using red light activatable channelrhodopsins is that red light is less absorbed and scattered by the cuticle of *Drosophila melanogaster* than blue light (Inagaki et al., 2014).

To activate Chrimson, Klapoetke and colleagues (2014) used three different wavelengths: 470nm (blue), 617nm (amber) and 720nm (red). Blue and amber light elicited action potentials at a much lower intensity and at only a tenth of pulse duration than red light did. This was surprising, as the spectral peak of Chrimson is at 590nm which is much closer to red light than blue light and the red light is less absorbed by the tissue than the blue light (Inagaki et al., 2014). But the results are conform with the results obtained in this thesis, as the flies which did not die during the experiment always showed attraction to the amber illuminated food odor trap. Due to the high mortality rate of the control group, Chrimson was neglected and ReaChR was used instead as a red light activatable channelrhodopsin.

For activation of ReaChR, Lin and colleagues (2013) used different wavelength and intensities, ranging from 410nm – 650nm and from 0.08mW/mm^2 – 11.75mW/mm^2. The most suitable wavelengths were amber and red ones (590nm – 630nm) and the best intensities were 4.09mW/mm^2 – 11.75mW/mm^2. With pulsed light stimulation of 10Hz no significant differences between pulse duration and light intensity were detected between amber (617nm) and red (627nm) light. Pulse with too weak intensity and too short duration led to an insufficient depolarization, while a too high intensity caused extra action potentials and a depolarization block due to insufficient repolarization (Lin et al., 2013). This observation is concordant to the results achieved in this thesis, as too high light intensities or constant light led to aversive behavior. Inagaki and colleagues (2014) used red (627nm), amber (590nm), green (530nm) and blue (470nm) light for

activation of ReaChR. When not normalized, green diodes show the strongest intensity and amber diodes the weakest intensity, therefore the flies often showed an even better scoring when illuminated with green light, although the maximum peak of ReaChR is around 590nm (Inagaki et al., 2014). But this does not explain the attraction of the flies in the experiments done here, as there the light intensity of the green and red diode were adjusted to each other. In the behavioral experiments done by Inagaki et al. (2014), blue and green light illumination often led to paralysis of the flies, while amber and red light illumination elicited the expected behavior. In their experiments, it was more or less negligible if an amber or red light diode was used, as always only one light source was used to activate the ReaChR. But for the optogenetic site attraction assay conducted in this thesis, two different light sources are needed: one to activate the channelrhodopsin and one to have no effect. The first tested combinations were red (620nm – 630nm) vs. green (520nm – 535nm) and red vs. amber (585nm – 595nm). Blue light (465nm – 485nm) was ruled out, as it is said that blue light interferes with the optical system of the fly (Paulk et al., 2012). Although it is called red-activatable channelrhodopsin and is optimally excited with orange to red light (590nm – 630nm) (Lin et al., 2013), the red light diode used in the experiments for this thesis was not a suitable light source. Current studies also state that it more sensitive to green light than to red light (Dawydow et al., 2014; Krause et al., 2017). The tested flies showed an attraction towards the green or even stronger towards the amber light illuminated food odor trap when combined with the red light diode. So it was decided to use the amber diode as an activation light source. But as the red and green diodes both are too close to the wavelength of the amber diode, it was tested if the blue light diode could be used as the "negative" light source, which was surprisingly suitable at a certain light intensity. In the experiments with the blue light activatable ChR2, the flies were in the *norpA*^{1} mutant background, which renders the fly blind (Bülthoff, 1982; Bloomquist et al., 1988) to eliminate interference of the blue light with the optical system of *Drosophila* (Paulk et al., 2012). So it was expected, that to high intensities of blue light would lead to site aversion in the tested *UAS-ReaChR* flies, as it had happened in experiments with *UAS-ChR2* in the *Tbh*^{nM18} mutant background. In that case, the intensity had to be reduced to avoid a negative influence of the blue light. But unexpectedly, there was no interference, even at light intensities which caused site aversion in *Tbh*^{nM18}*, UAS-ChR2/Tdc2-GAL4* flies. So maybe there is no interference between blue light and the optical system and

the observed site aversion to blue light was just a coincidence and the *norpA*^{1} mutation might not be a null mutation.

One striking phenotype of *UAS-ReaChR* was observed when it was expressed under the control of the *Cha*-GAL4 driver. Almost no males with the right genotype hatched, so there were not enough flies to achieve reliable results. But all larvae which pupated hatched, which indicates that there must be a disturbance earlier to the larval stage, maybe already from eggs to larvae. As it is published that the red light activatable ReaChR is more light sensitive than the blue light activatable ChR2 (Dawydow et al. 2014), it might be possible that there was non-constant darkness through the flies' development and thus the *UAS-ReaChR*/*Cha*-GAL4 was activated accidently to early and caused some severe dysfunctions. Thus it may be possible to obtain more flies with the correct genotype and therefore receive better results with expression of the less light sensitive *UAS-ChR2* under the control of the *Cha*-GAL4 driver line.

It was already shown that pulsed light is a better way to activate neurons via channelrhodopsins than constant light (Pulver et al., 2009). Therefore, next to constant light different activation patterns were tested. All of these frequencies derived from the original activation pattern 2s 40Hz 16s 8Hz 2s 0Hz, which was shown to be able to substitute for an unconditioned stimulus in appetitive reward learning in the honey bee (Hammer, 1993). This activation pattern was able to elicit attractive and aversive behavior, depending on the subset of neurons activated. This was true for the blue light activatable ChR2 and also for the red light activatable ReaChR. But other neuronal light activation experiments with *Drosophila* were not necessarily done with this kind of activation pattern. Instead other frequencies or even constant light were used (Schroll et al., 2006; Zhang et al., 2007; Crisp et al., 2008; Pulver et al., 2009). So it was tested, whether this activation pattern, which was successfully used by Schneider et al. (2012) and Xu et al. (2016), is really necessary for proper activation of the neurons. Activation of blue light activatable ChR2 indicated a frequency dependence, which is not only dependent on the tool but also on the activated neurons, as they might have different membrane properties. Also the released neurotransmitter might influence the needed frequency, as an activation pattern of 8Hz was enough to elicit behavior in the *Tbh*^{nM18} mutant background, but not for *UAS-ChR2* itself. OA and TA are both packed in synaptic vesicles (required for neurotransmitter release at the synapse) and in dense-core

vesicles (required for extra synaptic release) (Grygoruk et al., 2014). It is possible, that OA and TA each are packed in a different type of vesicle and therefore another activation pattern is needed. But so far, the contribution of the two vesicle types and their release are not known in specific behaviors (Grygoruk et al., 2014). Activation of amber light activatable ReaChR revealed no frequency dependence at all, thus it is likely, that to observed results with the blue light ChR2 are caused by the kinetics of the channelrhodopsin. The only requirement for ReaChR seems to be some kind of frequency. But as it is less selective than the ChR2, it would have been interesting to see, whether the short activation pattern of 2s 40Hz would have also been enough to elicit the attraction behavior. Constant light elicited site aversion in the experimental group for both channelrhodopsins. This could be due to either a hyperpolarization of the neurons after activation or to an initial constant activation of the neurons followed by no activation anymore as the reservoirs of neurotransmitters are all emptied due to the constant activation. To test this, ablation of the neurons via tetanus toxin, which eliminates synaptic transmission by inhibiting the neurotransmitter release through cleavage of the SNARE proteins (Sweeney et al., 1995), or via Kir (inwardly rectifying potassium channel), which hyperpolarizes the neurons and thus suppresses the release of neurotransmitters (Johns et al., 1999) could be performed.

Regarding the light intensity, there are contradictory findings in this thesis compared to the fact that red light penetrates the cuticle of *Drosophila* better than blue light (Inagaki et al., 2014) and that the red light activatable ReaChR is more light sensitive than the blue light activatable ChR2 (Dawydow et a., 2014). So it was assumed that red light activation needs a lower intensity of the diodes to have an effect on the behavior than the blue light. But this presumption was not verified and the ReaChR seems to be more light dependent than the ChR2, which is unexpected due to the better penetration properties of red and amber light. This was also already shown by Lin et al. (2013) and Inagaki et al. (2014). Both groups showed that amber (and blue) light need less intensity and shorter pulse durations to elicit a certain behavior than the red light. The differences of the used light intensity for the different GAL4 driver lines might be caused by the position of the targeted neurons in the fly brain. Overall, there has to be a minimum light intensity, which is sufficient to elicit a behavioral response, and a maximum light intensity, since higher intensities would induce site aversion behavior in the control group.

4.7. Closing remarks

OA mediates site attraction and site aversion and thus functions as a positive and negative reinforcer. Furthermore, it biases the behavioral outcome depending on the activated neurons. In addition, OA is sufficient and necessary for olfactory ethanol attraction and is also involved in olfactory aversion. Moreover, a certain interaction and probably a balance between OA and its precursor TA are needed for proper behavior. So OA is involved in two different behaviors and mediates the response towards an olfactory stimulus and direct activation of neurons. These results suggest that these two behaviors are regulated through the same or at least through two similar pathways.

5. List of Abbreviations

AI	Attraction Index
AL	Antennal Lobe
ASM	Anterior Superior Medial
ATR	All-*trans* Retinal
°C	Degree Celsius
Ca^{2+}	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate
ChAT/Cha	Choline Acetyl Transferase
ChR	Channelrhodopsin
ChR2	Blue light activatable Channelrhodopsin
cm	Centimeter
CNS	Central Nervous System
CS	Conditioned Stimulus
d	Days
DA	Dopamine
DAC	Dorsal Anterior Cluster
Dbh	Dopamine β-Hydroxylase
DMC	Dorsal Medial Cluster
DPC	Dorsal Posterior Cluster
dTRPA1	*Drosophila* Transient Receptor Potential A1
DUM	Dorsal Unpaired Median
Fru	Fruitless
g	Gramm
GABA	Gamma-Aminobutyric acid
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
h	Hours
Hz	Hertz
iav	Inactive
Kir	Inwardly Rectifying Potassium Channel
LED	Light Emitting Diode
LH	Lateral Horn
LP	Lateral Protocerebrum
MB	Mushroom Body
ml	Milliliter
mm	Millimeter

mm^2	Square Millimeter
mM	Millimolar
mW	Milliwatt
µl	Mikroliter
NA	Noradrenaline
NaCL	Sodium Chloride
nm	Nanometer
nM	Nanomolar
norpA-	No Receptor Potential A-
OA	Octopamine
OSN	Olfactory Sensory Neuron
PAM	Paired Anterior Medial
PER	Proboscis Extension Response
PPL1	Paired Posterior Lateral
ReaChR	Red activatable Channelrhodopsin
RNAi	Ribonucleic Acid Interference
RT-PCR	Real Time Polymerase Chain Reaction
s	Seconds
s.e.m.	Standard Error of the Mean
SOG	Suboesophageal Ganglion
SPG	Supraoesophageal Ganglion
STDV	Standard Deviation
TA	Tyramine
Tbh	Tyramine β-Hydroxylase
Tdc	Tyrosine Decarboxylase
TH	Tyrosine Hydroxylase
UAS	Upstream Activating Sequence
US	Unconditioned Stimulus
VL	Ventrolateral
VM	Ventromedial
VNC	Ventral Nerve Cord
VPM	Ventral Paired Median
VUM	Ventral Unpaired Median
VUMmx1	Ventral Unpaired Median Cell of Maxillary Neuromere 1
YFP	Yellow Fluorescent Protein

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Köln, 29.08.2018

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Gerbera Claßen