Dissecting the Translation of Olfactory Information Processing into Approach Behavior using Orco and Octopamine Signaling in *Drosophila melanogaster*

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, 2014

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Date of oral examination: 23.01.2015

Note: The Data sets for the Figure 20B, Figure 23A and Figure 24A have been published prior to this Thesis in Schneider et al., 2012. The figures have been modified to suit the format of this thesis.

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wo wir sie suchen!

Abstract

Environmental stimuli present animals with input that needs to be processed and judged according to relevance. The animal is challenged with the decision to act and if, how to act in response. How components of the olfactory machinery function in odor processing with hindsight to behavioral output is not well understood. Neurotransmitters such as octopamine might be modulating behavioral output and decision making but how and what behaviors are affected is not known in detail. This thesis attempts to further the understanding of these processes.

The general organization of the olfactory system in insect olfaction is similar to that of vertebrates. Both express one olfactory receptor (OR) gene per olfactory sensory neuron (OSN). In contrast to vertebrates, insects have an additional almost ubiquitously present olfactory co-receptor (Orco) in OSNs encoded by the *orco* gene. Orco has been proposed to be essential for insect olfaction. Still how Orco contributes to the relevant content of the olfactory information for the live animal is not known in detail. In this thesis the function of the Orco is dissected using Orco loss-of-function mutants (*Orco*^{1}) in olfactory preference experiments. In general complex odor mixtures are more attractive for adult *Drosophila melanogaster* than single odors. Previously *Orco*^{1} mutants were shown to be unable to distinguish between similar complex odor mixtures. It is shown here, that *Orco*^{1} mutants can distinguish between two similar complex odor mixtures but in an odor concentration dependent manner. Furthermore, Orco is required for olfactory preference at low attractive odorant concentrations, while aversive behavior is mostly Orco independent at high odorant concentrations. In addition, olfactory preference for ethyl acetate and acetic acid is shifted to higher concentrations. These findings suggest that Orco increases odor sensitivity for olfactory preference behavior. Odor specific receptors are thought to give odors identities and have been proposed to be mis-localized in the absence of Orco. Here it is shown that *Orco*^{1} mutants are not anosmic and can sense and prefer single odors. To address whether Orco plays a functional role in the perception of odor identity two similar attractive single odors were tested against each other as single odors and in a complex odor mixture background. Indeed Orco mutants fail to distinguish between the two odors and odor containing mixtures. This provides evidence that Orco is not essential for odor perception and plays a role in odor identity assignment. Taken together the findings here indicate that Orco is not essential but probably aids odor sensing for olfactory preference and odor identity assignment by increasing odor sensitivity.

OA has been associated as a reward reinforcer and DA with punishment and negative reinforcement in behavior in Drosophila melanogaster. Furthermore, OA has been associated with response selection and decision making. Schneider *et al.* (2012) previously showed that activating OA/TA neurons with a sequence of 40Hz and 8Hz frequency in an optogenetic approach could induce site-preference in *Drosophila melanogaster*. However, whether this specific OA/TA neuron activation frequency is needed to induce site-preference and if activation of DA neurons can induce site-aversion is unclear. Here the blue light sensitive cation channel Channelrhodopsin-2 was expressed in DA and OA neurons to allow

optogenetic activation in the site-preference assay. It is shown here that activation of DA neurons can promote site-aversion, probably due to the negative reinforcement properties of DA. In addition, OA/TA neuron activation with different activation frequencies failed to induce site-preference indicating that site-preference promotion requires a specific OA/TA neuron activation frequency. OA/TA mediated response selection for site-preference and possible other behaviors might be activation frequency dependent. Site-preference and aversion involves targeted movement, a form of locomotion. Therefore it could be that the same set of OA/TA neurons also modulates locomotion. In addition, OA has been proposed to play a role in locomotion behavior. To investigate locomotion behavior an optogenetic locomotion setup was developed. Direct activation of DA neurons has been shown to promote increases in locomotion. To validate the setup it is shown that activation of DA neurons increases the locomotor output. To address whether the same set of OA/TA neurons that regulate site-preference also influence locomotion behavior OA/TA neurons were activated with different activation frequencies. Depending on the OA/TA neuron activation frequency, increased or prolonged locomotion as well as no change in behavior could be observed. These findings indicate that locomotor output is modulated by OA/TA neurons and changes in locomotion can be induced by specific OA/TA neuron activation frequencies. Finally, employing the GRASP system, cellular contact between OA/TA neurons and OSNs can be detected in the AL providing a possible site for modulation of the olfactory information processing.

This study provides insights into the function of Orco from a behavioral output point of view. Furthermore, it is shown that behavioral output can differ and be regulated by activating the same population of OA/TA neurons with different frequencies. This indicates that response selection might be regulated by neuronal activity/spiking patterns and not simple on and off of neurons.

Zusammenfassung

Tiere sind umgeben von Umweltreizen welche prozessiert und je nach Bedeutung bewertet werden müssen. Das Tier muss entscheiden ob es auf den gegebenen Reiz reagiert und wenn ja, auf welche Weise. Wie Verhaltensantworten durch einzelne Komponenten des olfaktorischen Systems beeinflusst werden ist noch nicht gut verstanden. Es könnte sein, dass Neurotransmitter wie z.B., Oktopamin (OA) Entscheidungen und Verhaltensantworten modulieren. Auf welche Weise und welche Verhaltensantworten moduliert werden ist noch wenig untersucht. Diese Arbeit soll dazu beitragen ein tieferes Verständnis dieser Prozesse zu erlangen.

Die generelle Organisation des olfaktorischen Systems ist ähnlich in Insekten und Vertebraten. In beiden wird pro olfaktorisch sensorischem Neuron (OSN) ein olfaktorisches Rezeptorgen (OR) exprimiert. Im Gegensatz zu Vertebraten besitzen Insekten jedoch einen zusätzlichen fast in allen OSNs vertretenden olfaktorischen Co-Rezeptor (Orco) der von dem *orco* Gen kodiert wird. Orco wurde als essenziell für die die olfaktorische Wahrnehmung in Insekten postuliert. Welchen Beitrag Orco zum Inhalt der olfaktorischen Information für das lebende Insekt hat, ist noch nicht im Detail bekannt. In dieser Arbeit wurde die Funktion von Orco, anhand von Mutanten mit nicht funktionellem Orco (*Orco*^{1}), in olfaktorischen Präferenz-versuchen untersucht. Für ausgewachsene *Drosophila melanogaster* Fliegen sind komplexe Duftgemische generell attraktiver als einzelne Düfte. In vorangegangenen Studien konnte gezeigt werden, dassOrco1 Mutanten nicht zwischen ähnlich komplexen Duftgemischen unterscheiden können. Hier konnte gezeigt werden, dass Orco Mutanten in Abhängigkeit der Duftkonzentration zwischen ähnlich komplexen Duftgemischen unterscheiden können. Des Weiteren ist Orco notwendig für olfaktorische Präferenz für niedrig konzentrierte attraktive Düfte, während aversives Verhalten zu hoch konzentrierten Düften hauptsächlich Orco unabhängig ist. Zusätzlich ist die olfaktorische Präferenz für Ethylacetat und Essigsäure auf höhere Konzentrationen verschoben. Diese Ergebnisse weisen darauf hin das Orco die Duftsensitivität für olfaktorisches Präferenzverhalten erhöht. Es wird vermutet, dass Duft spezifische Rezeptoren für die Identität von Düften zuständig sind und dass diese ohne Orco nicht mehr korrekt lokalisiert sind. Hier wird gezeigt dass *Orco*^{1} Mutanten nicht Duftblind sind und Einzeldüfte wahrnehmen und präferieren können. Um zu untersuchen ob Orco für die Wahrnehmung von Duftidentitäten eine Rolle spielt, wurden zwei ähnlich attraktive Einzeldüfte einzeln und mit einem komplexen Dufthintergrund gegeneinander getestet. In der Tat, *Orco*^{1} Mutanten scheitern daran zwischen den beiden Düften und Duft beinhaltenden Duftgemischen zu unterscheiden. Diese Ergebnisse liefern Beweise dafür, dass Orco nicht essenziell für die Duftwahrnehmung ist und eine Rolle in der Zuweisung von Duftidentitäten spielt. Zusammengenommen weisen die Ergebnisse darauf hin, dass Orco nicht essenziell für die Duftwahrnehmung für olfaktorische Präferenz und Duftidentifikation ist sondern wahrscheinlich eine unterstützende Funktion durch Erhöhung der Duftsensitivität einnimmt.

In Drosophila melanogaster wurde OA mit Belohnungsverstärkung und Dopamin (DA) mit Bestrafungs- und negativer Verstärkung für Verhalten assoziiert. Des Weiteren soll OA eine Rolle in Entscheidungsfindung und der Selektion von Verhaltensantworten spielen. Schneider et al. konnten zeigen, dass in *Drosophila* Seiten-Präferenz durch optogenetische

Aktivierung von OA/TA Neuronen mit einer Sequenz aus 40Hz und 8Hz Frequenzen induziert werden kann. Ob diese die Aktivierung von OA/TA Neuronen mit dieser Aktivierungsfrequenz notwendig ist um Seiten-Präferenz zu induzieren, und ob die Aktivierung von DA Neuronen Seiten-Aversion auslösen kann, ist noch nicht bekannt. In dieser Arbeit wurde der Blaulicht sensitive Kationen Kanal, Channelrhodopsin-2, in OA/TA und DA Neuronen exprimiert, um so optogenetisch Neurone im Seiten-Präferenz Versuch aktivieren zu können. Tatsächlich konnte Seiten-Aversion durch die Aktivierung von DA Neuronen induziert werden. Dieser Effekt ist wahrscheinlich auf die Funktion von DA als negativer Verstärker zurückzuführen. Des Weiteren führte die Aktivierung von OA/TA Neuronen mit unterschiedlichen Frequenzen nicht zu Seiten-Präferenz. Dieses Ergebnis deutet darauf hin, dass für die Induktion von Seiten-Präferenz eine spezifische Frequenz notwendig ist. Die OA/TA Neuron induzierte Entscheidung für Seiten-Präferenz und vielleicht auch anderen Verhaltensantworten könnte von der Aktivierungsfrequenz abhängen. Seiten-Präferenz- und Aversionsverhalten beinhalten Ziel gerichtete Bewegungen, was eine Form von Lokomotion ist. Aus diesem Grund könnte es sein, dass die gleiche Gruppe von Neuronen auch Lokomotion moduliert. Außerdem wird angenommen, dass OA eine Rolle in Lokomotionsverhalten spielt. Um Lokomotionsverhalten zu untersuchen wurde ein Setup zur optogenetischen Untersuchung von Lokomotion entwickelt. In einer vorangegangenen Studie wurde gezeigt, dass direkte Aktivierung von DA Neuronen eine Erhöhung von Lokomotion induzieren kann. Aktivierung von DA Neuronen in diesem Setup führte zu einer erhöhten Lokomotion, was die Funktionsfähigkeit des Setups bestätigt. Um zu untersuchen ob die gleiche Gruppe von OA/TA Neuronen, welche Seiten-Präferenz reguliert, auch Lokomotion beeinflusst, wurden OA/TA Neurone mit unterschiedlichen Frequenzen aktiviert. Es zeigte sich das je nach Aktivierungsfrequenz entweder eine Erhöhung, eine ausdauernde oder keine Veränderung der Lokomotion induziert werden konnte. Diese Ergebnisse deuten darauf hin, dass Lokomotion von Oktopamin moduliert wird und dass je nach OA/TA Neuron Aktivierungsfrequenz unterschiedliche Veränderungen in der Lokomotion bewirkt werden können. Im letzten Punkt konnte mit der GRASP Methode in neuroanatomischen Versuchen gezeigt werden, dass OA/TA Neurone und OSNs zellulären und möglicherweise synaptischen Kontakt im Antennallobus haben. Dieses Ergebnis liefert einen Hinweis darauf, an welcher Stelle die olfaktorische Informationsverarbeitung möglicherweise im modulieret werden könnte.

Diese Studie gibt Einsichten in die Funktionsweise von Orco aus der Sicht der Verhaltensantwort. Des Weiteren wird gezeigt, dass die Aktivierung der gleichen Gruppe von OA/TA Neuronen mit unterschiedlichen Frequenzen unterschiedliche Verhaltensantworten auslösen kann. Diese Ergebnisse deuten darauf hin, dass neuronale Aktivitätsmuster und nicht einfach ein an oder aus Status von Neuronen die Entscheidung zwischen Verhaltensantworten reguliert.

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1 Introduction

1.1 The olfactory system of *Drosophila melanogaster*

In *Drosophila melanogaster* the odors are perceived by olfactory receptors (ORs) localized in dendrites of about 1300 olfactory sensory neurons (OSNs) housed in sensillae, sensory hairs on the antennae and the maxillary palps (Fig.1; Buck and Axel, 1991; Shanbhag *et al.*, 2000; Vosshall *et al.,* 2000; Carlson, 2001; Larsson *et al.*, 2004). Three major morphological types of sensillae have been identified namely basiconic, trichoid and coeloconic. Basiconic and trichoid sensillae can each contain the dendrites of up to four OSNs. Each OSN is exclusively expressing a single species of the 62 known conventional odor receptor genes (Clyne *et al.,* 1999; Gao and Chess, 1999; Vosshall *et al.,* 1999 and 2000; Mombaerts, 2004; Goldman *et al.*, 2005). These ORs are highly specific or broadly tuned to wide range of odors (Hallem and Carlson, 2006). In addition, in coeloconic sensilla acid sensing ionotropic glutamate like-receptors (IRs) that are not co-expressed with ORs have been described (Benton *et al.*, 2009; Silbering *et al.*, 2011). The OSNs expressing the same ORs converge onto the same specific glomeruli of the 56 present in the first major odor stimulus processing center the antennal lobes (AL; Laissue *et al.*, 1999; Vosshall *et al.*, 2000; Gao *et al.*, 2000; Larsson *et al.*, 2004; Tanaka *et al.*, 2012). Here they synapse with second-order neurons (Vosshall *et al.*, 2000; Gao *et al.*, 2000), such as inhibitory GABAergic and excitatory glutamatergic local neurons (iLNs and eLNs; Okada *et al.*, 2009; Chou *et al.*, 2010; Tanaka *et al.*, 2012; Liu and Wilson, 2013). In addition they project onto and projection neurons (PNs) in a roughly 50 OSNs on three PNs ratio (Buck and Axel, 1991; Stocker *et al.*, 1990 and 1994; Vosshall *et al.*, 2000; Tanaka *et al.*, 2004). It is thought the LNs and PNs form local circuits within a glomeruli and across glomeruli (Ng *et al.*, 2002; Wilson and Laurent, 2005; Wilson *et al.*, 2004). In flies iLNs mediating pre- and postsynaptic inhibition and cholinergic eLNs have been described (Buchner, 1991; Jackson *et al.* 1990; Wilson and Laurent 2005; Shang *et al.* 2007; Olsen et al., 2007; Olsen and Wilson, 2008; Root *et al.*, 2008; Huang et al., 2010; Liu and Wilson, 2013). The olfactory information is further transmitted by around 150 PNs in bundles of three through four AL-tracts called the AL-mPN1, ALmlPN2, lALT and tALT into two higher brain centers the lateral horn (LH) and the calyces of the mushroom body (MB; Stocker *et al.*, 1990; Marin *et al.*, 2002; Lai *et al.*, 2008; Tanaka *et al.*, 2012). The MB is involved in olfactory learning and memory (Heisenberg *et al.*, 1985; Heisenberg, 2003) and the

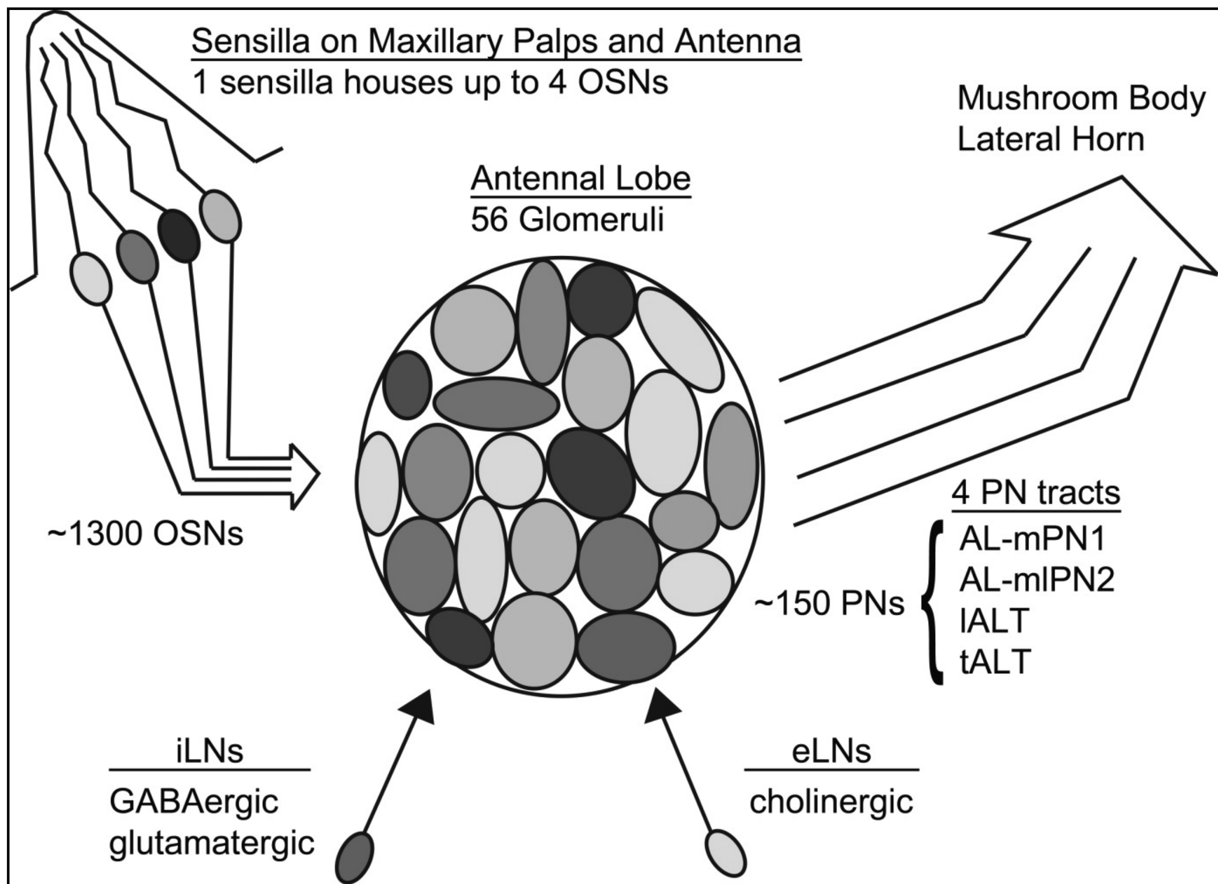


Figure 1. Scheme of the basic olfactory pathway in *Drosophila melanogaster*. The olfactory organ consists of porous sensillae located on the maxillary palps and the antennae. Each sensilla contains the dendrites of up to four OSNs. Around 1300 OSNs express one type of 62 known olfactory receptors on the dendrites that are exposed to the environment. OSNs project with their axons to the antennal lobe. Here OSN expressing the same type of receptor connect onto the same specific glomeruli, depicted in varying shades of grey (size, form and organization not anatomically correct). In the AL the OSN connect onto projection neurons and inhibitory and excitatory lateral neurons. From the AL the olfactory information is further transmitted through four antennal lobe tracts (PN tracts) to higher brain centers that are the LH and MB calyces.

LH mediates innate olfactory responses (Heimbeck *et al.*, 2001; Parnas *et al.*, 2013). Comparison of the insect olfactory systems including the system of *Drosophila* with vertebrates shows that insects olfactory neurons contain an olfactory receptor co-receptor (Orco) present in up to 80% of the OSNs not present in vertebrates (Vosshall *et al.*, 1999, 2000; Elmore *et al.*, 2003; Krieger *et al.*, 2003; Pitts *et al.*, 2004; Patch *et al.*, 2009).

1.1.1 The function of the olfactory receptor co-receptor Orco

The Orco (previously named Or83b by Vosshall *et al.*, 1999) is a highly conserved molecule and shares 70% amino acid sequence identity with its homologues in other insect species (Hill *et al.*, 2002; Krieger *et al.*, 2003; Pitts *et al.*, 2004). Orco is co-

expressed with conventional ORs in most OSNs in a variety of insect species including the hawk moth (*Manduca sexta*) and *Drosophila melanogaster* (Vosshall *et al.*, 1999, 2000; Elmore *et al.*, 2003; Krieger *et al.*, 2003; Pitts *et al.*, 2004; Patch *et al.*, 2009; List of Orco orthologues in Stengl and Funk, 2013). Orco is not present in OSNs that express IRs or in gustatory receptor (GR) expressing neurons and therefore appears to be OR containing neuron specific (Larsson *et al.*, 2004). The Orco and ORs belong to the 7 transmembrane domain G-protein coupled receptors (GPCR). In insects ORs and Orco have an inverted membrane topology resulting in an extracellular C-terminus normally required for G-protein binding in vertebrates (Benton *et al.*, 2006; Wistrand *et al.*, 2006; Lundin *et al.*, 2007; Smart *et al.*, 2008; Guo and Kim, 2010; Tsitoura *et al.*, 2010). Furthermore, odor molecules normally bind to their specific odor receptors. However, Orco does not bind odor molecules directly giving Orco an indirect role in odor perception (Dobritsa *et al.*, 2003; Elmore *et al.*, 2003; Hallem *et al.*, 2004a, b; Nakagawa *et al.*, 2005; Neuhaus *et al.*, 2005; Sato *et al.*, 2008; Wicher *et al.*, 2008; Jones *et al.*, 2011; Nichols *et al.*, 2011; Pask *et al.*, 2011; Chen and Luetje, 2012). It was shown that ORs and Orco form homo- and heteromeres in heterologous expression systems with so far unknown stoichiometry in the membrane (Neuhaus *et al.*, 2005; Benton *et al.*, 2006; German *et al.*, 2013). *In vivo* studies reported that upon loss-of Orco (*Orco*^{1} mutant; Larsson *et al.*, 2004) proper dendritic localization of the specific OR was disrupted in the dorsal organ of larvae as well as antennae of adult flies that could be rescued by expression of Orco (Benton *et al.*, 2006). This led to the assumption that Orco might act as a chaperone for ORs for correct protein folding, dendritic localization and stability in the membrane (Larsson *et al.*, 2004; Benton *et al.*, 2006). Indeed the electrical responses of OSNs are eliminated in flies carrying the *Orco*^{1} mutation or RNAi knockdown of Orco (Larsson *et al.*, 2004; Neuhaus *et al.*, 2005). In addition, Orco mutant larvae were unable to perform properly in chemotaxis experiments (Larsson *et al.*, 2004). Therefore it was assumed that Orco is a vital element for functional odor perception and thus olfaction. Although there are contradictory opinions in the field it is most agreed upon that Orco is a cyclic nucleotide gated non-selective cation channel that is metabotropically regulated by cAMP (Fig.2; Wicher *et al.*, 2008). Orco has been shown to have five protein kinase C (PKC) phosphorylation sites controlling cAMP sensitivity (Sargsyan *et al.*, 2011). Furthermore gating of Orco was abolished when the phosphorylation sites were mutated, indicating that Orco activation via cAMP is

PKC phosphorylation dependent (Sargsyan *et al.*, 2011; Getahun *et al.*, 2013). The topmost model for odor perception in insects states that the conventional ORs are GPCRs that activate G-proteins upon odor ligand binding leading to the formation of cAMP. cAMP in turn binds as a second messenger to Orco. This binding results in cation flux over the membrane and generates the I_m current (metabotropic current) and therefore results in stimulus dependent OSN activation (Wicher *et al.*, 2008). In support of this model several studies have shown that G-protein signaling is likely involved in the olfactory stimulus transduction. It could be shown e.g., that deletion of $G_{\alpha q}$ disrupted odorant responses in flies and that the adenylyl cyclase converging ATP to cAMP via G-protein activation is involved in olfactory signal transduction (Kain *et al.*, 2008; Deng *et al.*, 2011). Still other studies working with single sensillum recordings and heterologous expression systems found no evidence for coupling of the OR-Orco complex to G-proteins (Sato *et al.*, 2008; Smart *et al.*, 2008; Yao and Carlson, 2010). Another Orco connected current was also reported; a fast ionotropic current termed I_I that is independent of G-protein signaling and might be induced almost directly by conformational changes of the ligand binding OR (Wicher *et al.*, 2008; Smart *et al.*, 2008; Sato *et al.*, 2008). Still, the ionotropic current could so far

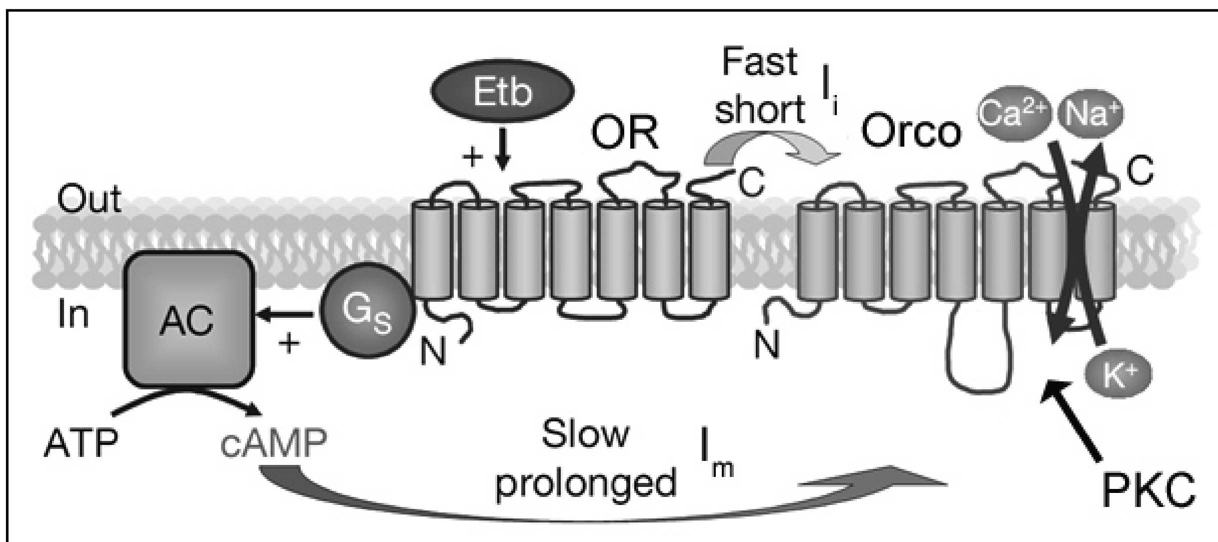


Figure 2. Model depicting signal transduction of the OR-Orco complex in odor perception. Shown are the OR and Orco in a theoretical complex with 7 transmembrane domains, extracellular C-termini and intracellular N-termini in a membrane. Binding of an odor molecule (here ethyl butyrate, EtB) to the OR elicits a conformational change leading to a direct opening of the Orco channel resulting in the fast less sensitive I_i current. In addition, G-proteins are activated which in turn activate adenyly cyclases (AC) which then convert ATP to cAMP. Phosphorylation of Orco by protein kinase C (PKC) sensitizes the Orco to cAMP which leads to a channel opening of Orco via cAMP and thus the slow and sensitive I_m current. Modified from Wicher *et al.*, 2008.

only be shown in heterologous expression systems and it remains to be seen if ORs and Orco even form heterodimers *in vivo* and I_i currents can be recorded.

An interesting aspect is a third current that is supposedly a leak current. Ca^{2+} dependent non-specific spontaneous activity could be recorded when Orco from different insect species was expressed alone in heterologous expression systems (Sato *et al.*, 2008; Wicher *et al.*, 2008; Jones *et al.*, 2011; Sargsyan *et al.*, 2011; Nolte *et al.*, 2013). Such spontaneous activity was diminished in Orco mutants *in vivo* marking the Orco leak current as a possible pacemaker current leading hyperpolarized OSNs to the spike threshold (Larsson *et al.*, 2004; Benton *et al.*, 2007; Deng *et al.*, 2011). Use of synthetical Orco agonists like VUAA1 in *Manduca sexta* (hawk moth) *in situ* experiments showed that spontaneous activity and overall background activity between olfactory responses was raised suggesting a role for Orco as an amplifier (Jones *et al.*, 2011; Nolte *et al.*, 2013). In addition, the use of Orco agonist and antagonist in *Drosophila melanogaster* shape spontaneous activity of OSNs (Su *et al.*, 2012). Since spontaneous activity underlies oscillations it has been hypothesized that Orco is involved in temporal coding and might modulate odor response kinetics and threshold by increasing background and spontaneous activity in OSNs (Stengl, 2010; Nolte *et al.*, 2013). Thus Orco is likely a metabotropically regulated pacemaker channel involved in the kinetics and thresholding of olfactory responses in odor detection (Stengl, 2010; Getahun *et al.*, 2013; Nolte *et al.*, 2013; review: Stengl and Funk, 2013). Still, substitution or loss of the conventional OR also influenced spontaneous activity in *Drosophila* OSNs indicating that not only Orco is involved in spontaneous activity (Dobritsa *et al.*, 2003; Elmore *et al.*, 2003; Hallem *et al.*, 2004a). In consensus, heterologous expression of OR22a alone produced spontaneous activity (Wicher *et al.*, 2008). Notably expression of ORs from different insect species alone without co-expression of Orco was enough to evoke odor specific responses in heterologous systems although this could not be shown *in vivo* so far (Wetzel *et al.*, 2001; Sakurai *et al.*, 2004; Nakagawa *et al.*, 2005; Neuhaus *et al.*, 2005; Grosse-Wilde *et al.*, 2006; Smart *et al.*, 2008; Deng *et al.*, 2011). Relatively high odor concentrations with long exposure times in a range of seconds were required to elicit detectable responses. These findings indicate that Orco is not required for odor detection as long as the conventional OR is properly inserted into the membrane. Still, odor responses could be strengthened by co-expression with Orco (Nakagawa *et al.*, 2005; Neuhaus *et al.*, 2005; Smart *et al.*, 2008).

Overall, most of the results were obtained from analysis of Orco function in heterologous expression systems. Still, the key functions of Orco are likely sensitizing OSNs to incoming odors by mediating spontaneous activity, acting as a chaperone for proper dendritic localization of ORs and in mediating the I_m current as a cAMP sensitive cation channel. However little is known about the Orco function in natural odor processing.

1.1.2 Odor Processing

Natural environments present the fly and any other living beings with ever changing sensory stimuli that need to be perceived, processed and judged according to their relevance. Odors usually do not occur as single odors but odor mixtures with varying components and concentrations that can also rapidly fluctuate (Murlis *et al.*, 1992). For example fruit flies are attracted to volatile compounds like ethanol, ethyl acetate (EtOAc) and acetic acid (AA) emanating from fermenting fruits and even more attracted to blends of these odors (Zhu *et al.*, 2003; Becher *et al.*, 2010; Lebreton *et al.*, 2012). Such attraction can be viewed as olfactory preference. Olfactory preference can be divided in at least four general steps: (1) odor perception (2) odor evaluation (3) response decision (4) execution of movement toward the odor. Here the first two steps will be described with hindsight to the functional relevance.

Odor processing occurs already at the level of the receptor neurons. A single odor activates various OR types depending on broadly or narrowly tuned ORs and is therefore coded by the combined activity of activated OSN subsets (Hallem and Carlson, 2006). Usually, odors that induce high OSN spiking rates in one OSN do the same in other OSN types that can be activated by the specific odor and the converse principle can be applied for weak ligands (Haddad *et al.*, 2010; Luo *et al.*, 2010; Olsen *et al.*, 2010). In addition to excitatory effects most odors also inhibit OSNs resulting in suppression of the usually spontaneous activity of OSNs (de Bryne *et al.*, 1999, 2001; Hallem *et al.*, 2004; Yao *et al.*, 2005; Hallem and Carlson, 2006; Schuckel *et al.*, 2009; Silbering *et al.*, 2011; Nagel and Wilson, 2011). Increases in odor concentration lead to increases in firing rates of single OSNs and additional OSNs might be recruited (Hallem and Carlson, 2006). The speed of the change in odor concentration also influences the OSN spiking (Kim *et al.*, 2011). Thus differences in odor concentrations are encoded by the OSN firing response (Nagel and Wilson, 2011). In addition, the odor sensitivity of the OSN decreases with strong

stimuli and increases with weak stimuli (Wark *et al*., 2007). Prolonged odor exposure of one minute can lead to reversible OSN sensory adaptation for up to 10 minutes with simultaneous reduced odor avoidance (Störtkuhl *et al*., 1999; Zufall and Leinders-Zufall, 2000). The onset and decay rates of OSN transduction depend on the OR type and the odor ligand meaning that the kinetics differ between OR and ligand combinations and ORs giving rise to odor coding at the receptor level (Nagel and Wilson, 2011; Wilson, 2013). Thus the OSN odor response depends on the odor ligand OR combination and its specific kinetics and the combination of activated OSN types most likely encoding the odor identity (Wilson, 2013). This can also be viewed as a “receptor code”. Odor concentration and changes in concentration are probably encoded by the OSN firing rates.

In the AL around 50 cholinergic OR specific OSNs form synapses on roughly three PNs per glomeruli and PN spiking in response to OSN input is more stable than in the OSNs and thus believed to represent odor stimuli more precisely (Stocker *et al*., 1990, 1994; Tanaka *et al*., 2004; Bhandawat *et al*., 2007; Kazama and Wilson, 2008, 2009). The probability of vesicular release and short-term depressions at the OSN-PN synapse can affect the PN activity and might vary depending on the activated OSNs (Kazama and Wilson, 2008; Wilson, 2013). PN responses peak earlier and decay faster as OSN responses and are more sensitive to incoming OSN stimuli at low firing rates but less sensitive at high firing rates (Bhandawat *et al*., 2007; Wilson *et al*., 2004; Olsen *et al*., 2010; Wilson, 2013). PNs can excite sister PNs in the same glomeruli and also excite LNs (Fig.3; Ng *et al*., 2002; Wilson *et al*., 2004; Kazama and Wilson, 2008; Yaksi and Wilson, 2010).

The information flow is modulated by GABAergic and glutamatergic iLNs and cholinergic eLNs. GABAergic iLNs show strong presynaptic inhibition to OSNs and have weak inhibition on PNs (Olsen and Wilson, 2008; Yaksi and Wilson, 2010). Blocking of GABA receptors leads to a prolonged PN response and it is therefore believed that GABAergic iLNs influence the PN response duration and act as a gain control (Wilson and Laurent, 2005; Olsen and Wilson, 2008; Olsen *et al*., 2010; Root *et al*., 2008). This means that higher OSN firing rates are necessary to saturate the PN firing rate (Wilson, 2013). Furthermore, iLN inhibition increases with increasing stimulus intensity and iLNs can also weakly inhibit other iLNs keeping the odor input in a working range for processing (Silbering and Galizia, 2007; Silbering *et al*., 2008; Olsen *et al*., 2010; Huang *et al*., 2010; Yaksi and Wilson, 2010). Generally most iLNs

provide global inhibition over the AL but a small part innervates only subsets of glomeruli (Stocker *et al.*, 1990; Wilson and Laurent, 2005; Shang *et al.*, 2007; Das *et al.*, 2008; Lai *et al.*, 2008; Okada *et al.*, 2009; Chou *et al.*, 2010; Seki *et al.*, 2010).

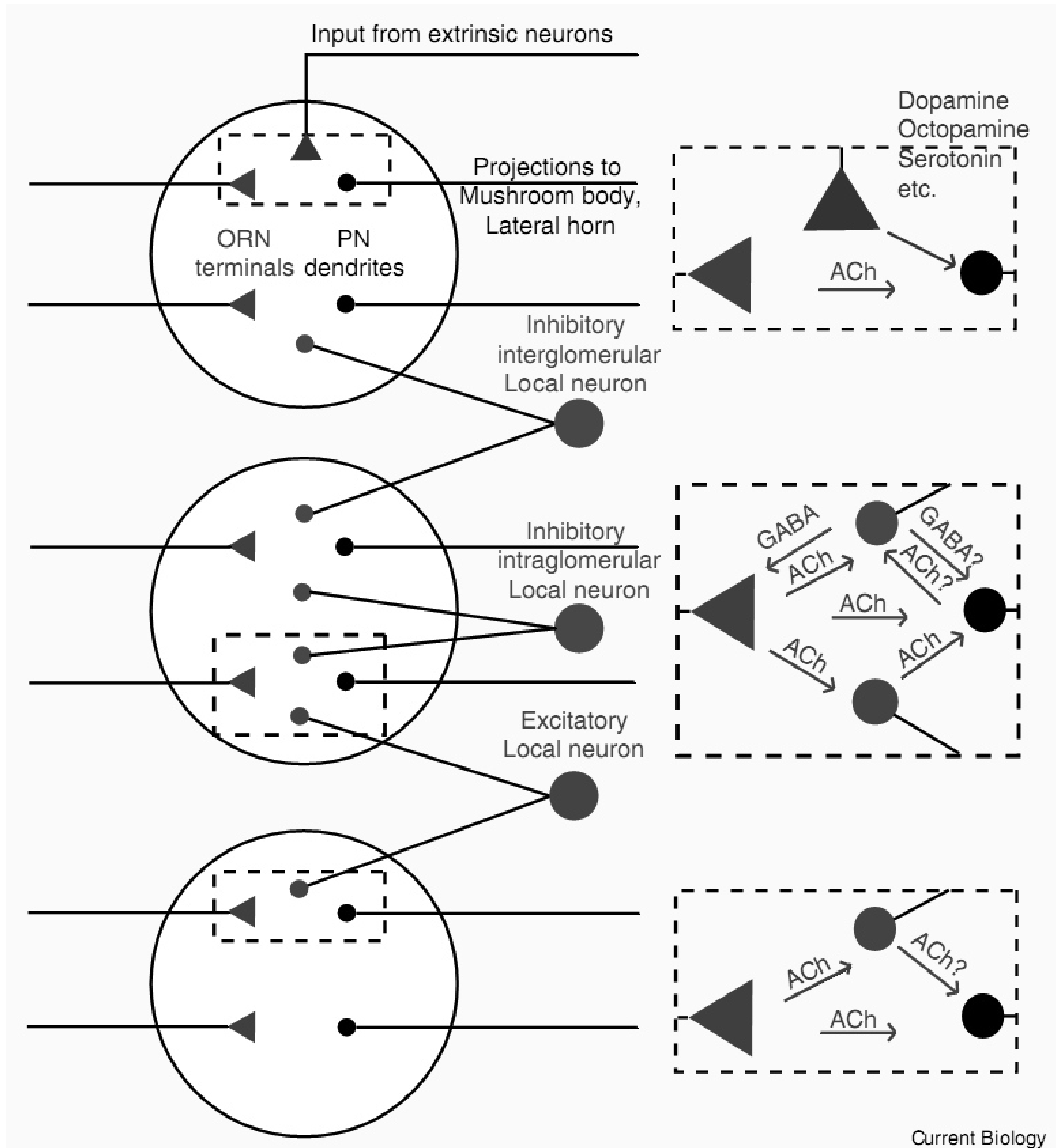


Figure 3. Olfactory processing in the antennal lobe. OSN terminals connect to PNs in glomeruli of the antennal lobe which then transmit the information to higher brain centers. Inhibitory and excitatory LNs modulate OSN and PN activity within glomeruli and across the glomerular population. Boxes with broken lines highlight the neurotransmitters involved in the transmission. Excitatory input might be mediated by acetylcholine (ACh) or as recent studies suggest via electrical synapses. Biogenic amines such as octopamine, serotonin or dopamine may act as neuromodulators on the olfactory processing. Picture taken from Masse *et al.*, 2009.

iLNs are mostly broadly tuned to odors but iLN mediated glomerular inhibition is still reported to be odor specific (Ng *et al.*, 2002; Wilson and Laurent, 2005; Chou *et al.*, 2010). This might be because glomeruli differ in their sensitivity to LN inhibition, likely due to variations in the level of GABA receptor expression (Root *et al.*, 2008; Olsen *et al.*, 2010). Overall it is thought that lateral inhibition boosts odor discrimination and identification over a wider range of concentrations because it regulates OSN synaptic transmission and thus PN saturation (Luo *et al.*, 2010; Olsen *et al.*, 2010). Thus eLNs provide a means to further regulate activity patterns in the AL. Another influence on olfactory processing in the AL is lateral excitation by eLNs. eLNs receive likely cholinergic input from OSNs as well as PNs. Excitation by eLNs in one glomeruli can inhibit or excite activity in other glomeruli regulating the glomerular activity pattern and thus PN responses (Olsen *et al.*, 2007; Silbering and Galizia, 2007; Root *et al.*, 2007; Shang *et al.*, 2007). iLNs might also be excited by eLNs.

Overall the previous receptor code gives rise to a “glomerular code” in the AL which is modified by a network of inhibitory and excitatory LNs most likely adding gain control of the olfactory input to broaden and control the odor concentration range for processing. Odor concentration is thought to be encoded by the activity in the PN responses while odor valence by activity in certain subsets of glomeruli and PNs (Bhandawat *et al.*, 2007; Silbering *et al.*, 2008; Semmelhack and Wang, 2009; Knaden *et al.*, 2012). Two glomeruli show activity at low and attractive apple-cider vinegar concentrations while an additional glomerulus is recruited at higher aversive concentrations. Thus specific subsets of PNs are activated by attractive odors depending on the concentration and a separate PN subset by aversive odors (Knaden *et al.*, 2012).

The PNs further transmit the information to higher brain centers, the LH and the MB (Tanaka *et al.*, 2004). The kenyon cells in the MB calyces are thought to translate the incoming odor information possibly as an odor identity in a “sparse code” where each odor stimulates a specific set of Kenyon cells that show only weak activity upon stimulation (Wang *et al.*, 2004). Similarly, in the LH specific segregated zones show activity to categories of odors for example fruity odors or pheromones (Jefferis *et al.*, 2007; Lin *et al.*, 2007).

Apart from the internal processing mechanism environmental factors also affect olfactory responses. For example environmental temperature changes can modulate the olfactory response at the receptor level and overall olfactory related

behavior as a means to adapt to environmental changes (Riveron *et al.*, 2009; Martin *et al.*, 2011). This means that the conditions in behavioral experiments need to be tightly controlled if consistent results are to be possible.

1.1.3 Odor mixture processing

Even though a great deal is known about the components of the odor processing machinery and the general influence they exhibit, it is still not clear and predictable how the information affects the actual behavior especially in case of complex odor mixtures.

As described above, simultaneous applied odors as is the case for odor mixtures influence each other through receptor and OSN inhibition on the first perceptual level and thus silence olfactory responses to components of the mixture (Schuckel *et al.*, 2009; Hillier and Vickers, 2011; Su *et al.*, 2011, 2012; Deisig *et al.*, 2012; Pregitzer *et al.*, 2012; Münch *et al.*, 2013). Thus odor mixtures evoke activation of OSNs in a combinatorial pattern due to the receptor code (Touhara, 2002; Yao *et al.*, 2005; Hallem and Carlson, 2006). At the same time one odor of the mixture might suppress certain glomeruli that are normally activated by another odor present in the mixture (Silbering and Galizia, 2007; Olsen *et al.*, 2010). In addition, it is possible that the same odor excites or dis-inhibits glomeruli that respond to odors not present in the applied odor mixture (Olsen and Wilson, 2008; Asahina *et al.*, 2009). This means that predicting an odor response by the mixture components alone would only be possible by analyzing all the inhibitory and excitatory inputs related to each odor compound with respect to the odor concentration.

Two principles to predict olfactory responses to odor mixtures have been described, the “elemental” and the “configural” coding (Review: Lei and Vickers, 2008). Elemental coding is based on predicting olfactory responses by the single components of the odor mixture where ultimately the response mimics the combined response characteristics of the individual odors. Configural coding takes odor mixture interactions into account on the receptor level and in the AL including inhibition and excitation leading to suppression of certain OSN types, recruitment of additional glomeruli and silencing of other glomeruli as described in the previous chapter. Therefore, the configural code does not represent a combination of the odor mixture component evoked activity but a novel activity pattern dependent on the odor mixture interactions. Both models have been shown to be present in the rat, honey bee as

well as the fruit fly (Galizia *et al.*, 1999; Linster and Cleland, 2004; Hallem and Carlson, 2006; Silbering and Galizia, 2007; Münch *et al.*, 2013). Odor molecules can be mapped into a multidimensional odor space according to their molecular characteristics (Kreher *et al.*, 2008). Odors that are clearly apart from each other in the odor space often show clearly separate glomerular activation patterns with no inhibitory or excitatory effects on each other (Kreher *et al.*, 2008). Olfactory responses to such odor mixtures can be predicted by the components by elemental coding because the masking effects of one over the other odor are very small. Odors that are more similar in the odor space might have overlapping glomerular activation patterns and the olfactory response is more likely modified and not as readily predictable by the single components. In addition, odors with a small physiochemical distance might be grouped into functional behaviorally meaningful groups aiding in predicting the olfactory response (Niewalda *et al.*, 2011). Even though understanding these codes would aid in understanding odor activity representations and why it is sometimes altered and at other instances not, it does not wholly explain the resulting behavioral output.

For example supporting the elemental coding, activity of single processing channels correlates to innate odor guided behavior (Semmelhack and Wang, 2009; Ai *et al.*, 2010; Knaden *et al.*, 2012; Min *et al.*, 2013; Dweck *et al.*, 2013; Ronderos *et al.*, 2014). Furthermore, in binary odor mixtures the valence of the single components could be used to predict the behavioral response (Thoma *et al.*, 2014). Binary mixtures of attractants were more attractive than the individual components alone and similarly binary mixtures of repellents less attractive than the constituent repellents. In addition, mixtures of repellents and attractants result in diminished attractiveness compared to the attractants alone. This would mean that the valence weight of the components decide the behavior. Still many odors cannot be defined as attractive or aversive since they are attractive at low and aversive at high concentrations and the activated glomerular pattern also depends on the concentration (Schlief and Wilson, 2007; Suh *et al.*, 2007; Semmelhack and Wang, 2009). An effect termed component dominance shown in the honey bee where the activated glomerular pattern is most similar to the most salient odor in a mixture argues for configural coding (Deisig *et al.*, 2006). In the sacred datura (*Datura wrightii*) an odor mixture of nine floral odor cues elicited foraging behavior but only as a mixture and not as single components or mixtures with only some of the

components (Riffel *et al.*, 2009). All nine odor cues alone elicited robust neural activity. These results point out that odor mixture components somehow interact and might results in a new odor percept that carries meaning but only when all the key components are present to give input and shape the neural activity pattern. It becomes quite clear that odor driven behavior is shaped by an array of factors that have not yet been conceived enough, especially in combination, to formulate rules to reliably predict olfactory behavior.

1.2 The amine Octopamine

Octopamine (OA) acts as neurohormone, neuromodulator and neurotransmitter in invertebrates and can be found in traces in vertebrates as well but with unknown functions. It is viewed as the homolog of norepinephrine in vertebrates (Roeder, 1999; Chentsova *et al.*, 2002; Roeder *et al.*, 2003; Gruntenko *et al.*, 2004; Roeder, 2005).

OA is synthesized in two catalytic steps from the aminoacid tyrosine. Tyrosine is decarboxylated by the tyrosine decarboxylase (TDC) to tyramine and tyramine (TA) in turn is hydroxylated by the OA rate limiting enzyme tyramine β-hydroxylase (TβH) to octopamine (Fig.4; Roeder, 2005). Two types of the TDC enzyme are present in the fruit fly *Drosophila melanogaster*, type 1 and 2 (Cole *et al.*, 2005).

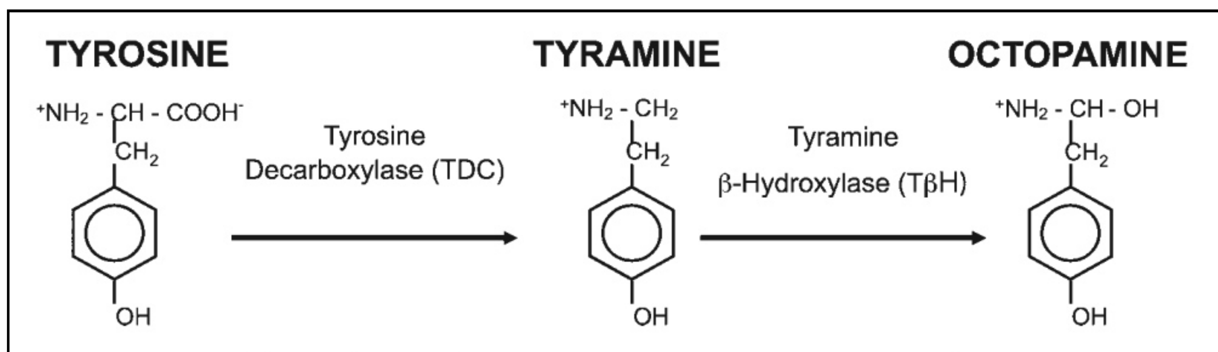


Figure 4. Octopamine synthesis. Tyrosine is decarboxylated by the tyrosine decarboxylase to tyramine. Tyramine is hydroxylated by the octopamine synthesis rate-limiting enzyme tyramine β-hydroxylase to octopamine (From Hardie *et al.*, 2007).

TDC1 is expressed and acts in non neuronal tissues in the periphery, while TDC2 is expressed and has functions in neuronal tissues. Mutations of both *TDC2* and *TβH* genes have been heavily utilized to study OA function in invertebrates (Monastirioti *et al.*, 1996; McClung and Hirsh, 1999; Scholz *et al.*, 2000; Chentsova *et al.*, 2002). Promotor elements of both genes were used in GAL4 constructs to drive expression

in OA and TA expressing neuronal cells to study the morphology of the respective neuronal systems (Cole *et al*., 2005; Busch *et al*., 2009; Schneider *et al*., 2012). Studies using the *TDC2*-GAL4 line face the problem, that TA and OA synthesis depend on TDC2 and therefore the analyzed neuronal function could potentially depend on OA, TA or both together. Therefore the neurons targeted by the TDC2-GAL4 line are referred to as OA/TA neurons.

OA can be found all over the nervous system of adult *Drosophila melanogaster* (Monastirioti *et al*., 1995; Busch *et al*., 2009). About 108 OA immunoreactive cells have been described with dense dendritic arborizations in most brain regions organized in clusters (Monastirioti *et al*., 1995; Sinakevitch and Straussfeld, 2006; Busch *et al*., 2009). Three clusters are located in the subesophageal ganglion (SOG) and are named the ventral median mandibular (VMmd), maxillary (VMmx) and labial (VMlb) cluster (Fig.5; Busch *et al*., 2009; Busch and Tanimoto, 2010).

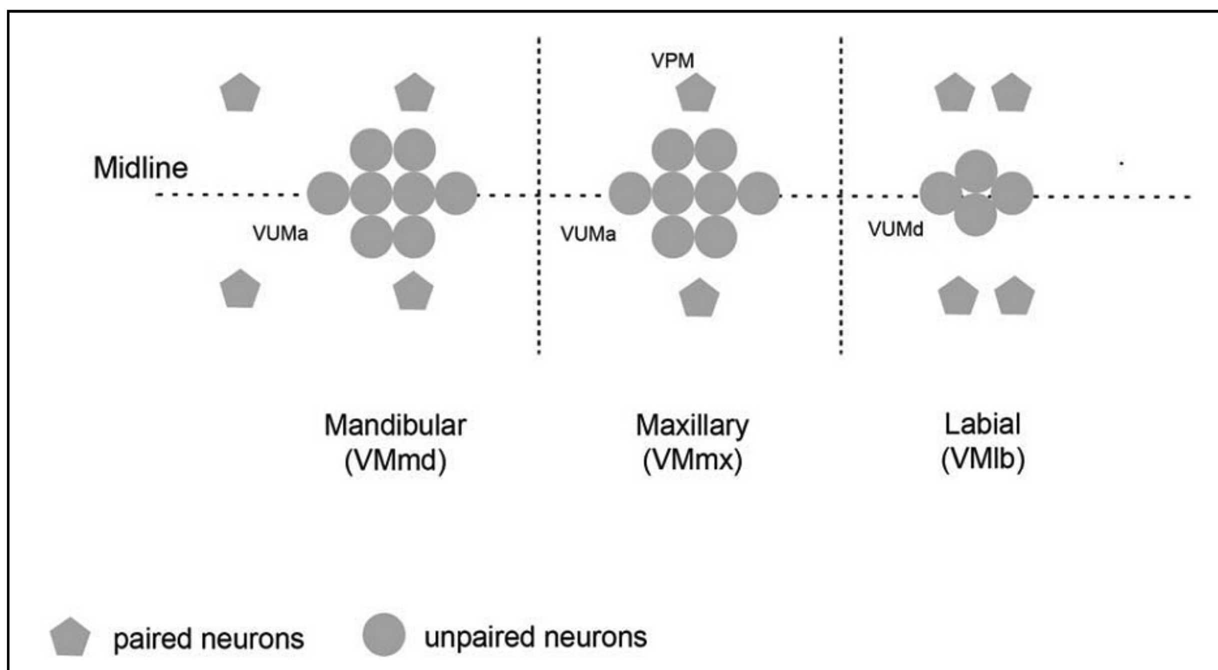


Figure 5. Cellular organization of the octopaminergic ventral median cluster in the SOG. Three clusters in the SOG have been investigated using the *TDC2*-GAL4 driver line and contain paired and unpaired neurons. They are located ventrally in the SOG and are called ventral median mandibular (VMmd; 12 cells), maxillary (VMmx; 10 cells) and labial (VMlb; 8 cells) cluster. The horizontal broken line indicates the midline from dorsal to ventral (left to right). The vertical broken lines indicate the borders between the depicted clusters (From Busch and Tanimoto, 2010).

They have been shown to be involved in various behaviors like decision making, olfactory behavior (honeybee) and aggression (Hammer, 1993; Certel *et al*., 2007, 2010; Andrews *et al*., 2014). They contain paired and unpaired neuronal cells called

ventral median unpaired (VUM) and ventral median paired (VPM) neurons. These neurons innervate the thoracic ganglion as well as prominent structures in the central nervous system (CNS) such as the AL, MB calyces, fan-shaped body (FB) of the central complex (CC) and LH implicated in various forms of behavior (Busch *et al.*, 2009).

1.2.1 Octopamine function in behavior

OA has been shown to play a role in ethanol preference and tolerance formation, where flies without OA ($T\beta H^{nM18}$ mutants) develop reduced ethanol tolerance and loss of olfactory ethanol preference while the ethanol sensitivity is not affected (Scholz *et al.*, 2000, 2005; Schneider *et al.*, 2012). It is also involved in the immune response (Adamo, 2010), various forms of stress (heat, starvation, mechanical stress; Chentsova *et al.*, 2002; Gruntenko *et al.*, 2004), ovulation (Lee *et al.*, 2003; Monastirioti, 2003; Lim *et al.*, 2014), wakefulness (Crocker and Seghal, 2008), visual processing (Suver *et al.*, 2012), appetite and feeding (Zhang *et al.*, 2013); sucrose responsiveness (Scheiner *et al.*, 2014) aggression and aggression associated pheromone sensing (Baier *et al.*, 2002; Potter and Luo, 2008; Hoyer *et al.*, 2008; Zhou *et al.*, 2008; Andrews *et al.*, 2014), regulation of heartbeat (Johnson *et al.*, 1997), behavioral choice (Certel *et al.*, 2007), associative learning and appetitive reinforcement and learning (Heisenberg *et al.*, 1985; de Belle and Heisenberg, 1994; Davis, 1996; Schwaerzel *et al.*, 2003; Kim *et al.*, 2007, 2013). TA has been less well studied but is thought to have opposing or balancing functions to OA (Lange, 2009). In ovulation for example TA contracts the oviduct muscles while OA relaxes them (Hardie *et al.*, 2007). Both TA and OA are involved in regulation of flight speed and maintenance, sucrose responsiveness and modulation of synaptic activity at the neuromuscular junction (NMJ) in larval body wall muscles among other behaviors (Nishikawa and Kidokoro, 1999; Scheiner *et al.*, 2002, 2014; Brembs *et al.*, 2007; Nagaya *et al.*, 2002).

OA has also been implicated to play a role in locomotion in *Drosophila* (Yellman *et al.*, 1997; Monastirioti *et al.*, 1996; Winther *et al.*, 2006). Modulation of the neuromuscular junction of larval body wall muscles is mediated by OA (Monastirioti *et al.*, 1995; Nishikawa and Kidokoro, 1999; Nagaya *et al.*, 2002; Dasari and Cooper, 2004; Shakiryanova *et al.*, 2011). Initiation and maintenance of larval motor pattern generation is affected by OA and stimulation of about 40 OA neurons

of the larval ventral nerve cord (VNC) can trigger locomotion (Fox *et al*., 2006; Selcho *et al*., 2012). Adult flies that have reduced levels of TA and OA, e.g. *inactive* mutant flies carrying a *tdc* gene knockdown, display low motor activity (O´Dell, 1993; Monastirioti *et al*., 1996; McClung and Hirsh, 1999; Chentsova *et al*., 2002 ;Saraswati *et al*., 2004; Cole *et al*., 2005). OA also modulates escape jumping, the startle response to ethanol and nicotine and regulates flight initiation and maintenance (Zumstein *et al*., 2004; Scholz, 2005; Brembs *et al*., 2007; Fuenzalida-Uribe *et al*., 2013). Furthermore, the ellipsoid body of the central complex implicated in motor activity control is innervated by OA immunoreactive processes (Strauss and Heisenberg, 1993; Sinakevitch and Straussfeld, 2006). Still no direct evidence has been shown on how exactly OA modulates locomotor behavior in adult flies in contrast to evidence from the larval system.

OA also affects the olfactory system. In cockroaches (*Periplaneta americana*) OA is secreted in the antenna and in moth (*Bombyx mori* and *Heliothis virescens*) OA receptors can be found in the antenna (Pass *et al*., 1988; von Nicksch-Rosengk *et al*., 1996). Furthermore, OA has been shown to enhance the olfactory response to pheromones but not to general odors (Pophof, 2002). In honeybees (*Apis melifera*) OA influences olfactory memory but not odor discrimination (Farooqui *et al*., 2003). In 1993 Hammer reported that sugar reward could be substituted by stimulation of the octopaminergic VUMmx1 neuron in olfactory conditioning experiments in honeybees (Hammer and Menzel, 1998). The VUMmx1 neuron showed a characteristic activity pattern when sugar reward was offered to the bee. The firing frequency pattern translates to 2s 40Hz followed by 16s 8Hz and 2s no activity. Since stimulation of this neuron using this "Hammer frequency" could substitute for the sugar reward it could be that this frequency pattern is the reward firing code. The VUMmx1 neuron has a similar innervation pattern as the VUMa2 neuron in *Drosophila*, both innervating olfaction related structures such as the AL, MB and LH (Hammer, 1993; Busch *et al*., 2009). In *Drosophila* appetitive reinforcement and olfactory conditioning is also modulated by OA (Schwaerzel *et al*., 2003; Schroll *et al*., 2006; Gervasi *et al*., 2010). One possible site for modulation of the olfactory information processing is the AL and MB. Indeed octopamine mushroom body receptors (OAMB) can be found in both neuronal structures in honeybees and *Drosophila* (Sinakevitch *et al*., 2013; Rein *et al*., 2013). In the fruit fly OA receptors could be detected on mPNs and GABAergic iLNs but not on OSNs and uPNs. Therefore it is thought that OA modulates inhibitory

neurons in the AL. There is no evidence for further direct modulation on neurons of the olfactory pathway so far.

1.3 The light sensible cation-channel Channelrhodopsin-2 (ChR2)

Non-invasive methods to induce activity in neuronal cells have been extensively used to study neuronal functions in intact animal systems (Lima and Miesenböck, 2005; Schroll *et al.*, 2006; Zhang *et al.*, 2007; Pulver *et al.*, 2009, 2011; Bellmann *et al.*, 2010; Schneider *et al.*, 2012). For example, among the genetically encoded tools are heat inducible TRPs (transient receptor potential) that allow to depolarize neurons upon exposure to a specific temperature. In 2002 in the photosynthetic algae *Chlamodymonas reinhardtii* a light gated proton channel belonging to the seven transmembrane domain proteins was identified named Channelrhodopsin-1 (Nagel *et al.*, 2002). This channel was modified to have peak excitation specifically at around 470nm wavelength and allows non-selective cation conductance and is called channelrhodopsin-2 (ChR-2; Fig.6; Nagel *et al.*, 2003). The light receptive chromophore *all-trans* retinal (ATR) has to be bound to ChR-2 for the channel to be functional. Upon light exposure of 470 nm wavelength ChR-2 changes conformation from the closed to the open state through ATR and thus allows cation conductance. In neurons cation flow over the membrane depolarizes neurons which then leads to action potentials and thus neuronal activity. Many modified versions of ChR-2 have emerged that change the channel kinetics and also wavelength excitability (Gunaydin *et al.*, 2010).

The channel conductance of ChR-2 is less than the common channels found in neuronal cells and is estimated to be around 50-250 fs (femtosiemens; Nagel *et al.*, 2003; Bamann *et al.*, 2008; Feldbauer *et al.*, 2009; Lin *et al.*, 2009). The speed of the membrane potential change is dependent on the intrinsic membrane properties of the cell as well as the channel kinetics. To achieve reliable excitation a high number of ChR-2s have to be present in the membrane. Using strong expression systems is therefore critical for neuronal activation. Although it has been shown that too strong expression leads to intracellular aggregates and thus reduced effectiveness of membrane depolarization while comparably lower expression of ChR-2 to good transition of the protein into the membrane (Lin *et al.*, 2009; Tsunoda and Hegemann, 2009). In addition, over-expression can effect membrane properties and also be toxic. Another important factor is desensitization. Repetitive pulsed or

continuous strong illumination leads to a response decay of ChR-2 of about 80% from the peak response at physiological pH (Nagel *et al.*, 2003; Ishizuka *et al.*, 2006; Lin *et al.*, 2009). This desensitized response can completely recover after 20 seconds without illumination (Lin *et al.*, 2009a). The light intensity has to be adjusted depending on the needs for the experimental assay. High light intensity for example leads to a rapid channel opening rate and a moderate closing rate (Nagel *et al.*, 2003; Isihizuka *et al.*, 2006; Lin *et al.*, 2009a). These characteristics can change depending on the light intensity.

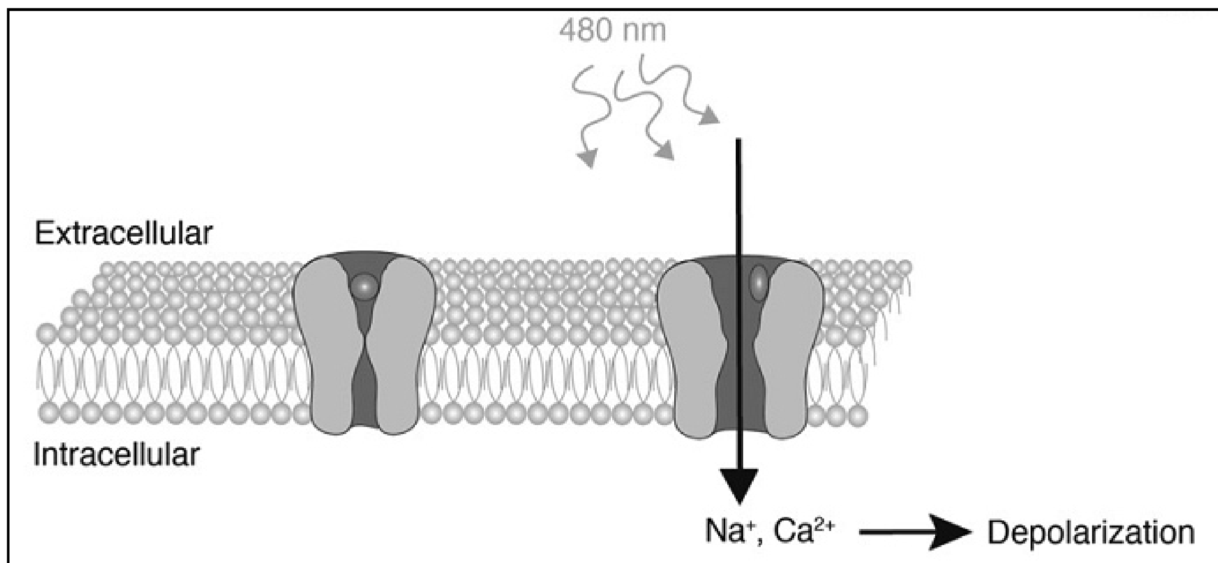


Figure 6. Conformational states of the light sensitive cation channel Channelrhodopsin-2. Depicted are the open (left) and closed (right) states of ChR-2 in a membrane in the presence of the chromophore *all-trans* retinal (blue sphere). Upon exposure to light with 480 nm wavelength ChR-2 converts from the closed to the open channel state and cat ions can pass the pore of the channel over the membrane resulting in a depolarization of the cell (from Fiala *et al.*, 2010).

ChR-2 has been used successfully in behavioral studies such as pain responses, appetitive and aversive learning in *Drosophila* larva or proboscis extension responses, escape reflex and locomotion in adult *Drosophila* flies (Lima and Miesenböck, 2005; Schroll *et al.*, 2006; Zhang *et al.*, 2007). It could be shown that photo-stimulation of dopaminergic neurons through expression of *ChR-2* under the control of the *Th*-GAL4 driver line leads to increased locomotion and also changes in the locomotion pattern in an arena (Lima and Miesenböck, 2005; Zhang *et al.*, 2007). Thus ChR-2 is a well suited tool to investigate behavior in adult *Drosophila melanogaster.*

1.4 Aims

Olfaction is one of the primary senses in the interaction of an individual with its environment to e.g. identify food sources or locate mating partners. Insect olfaction and the machinery and processing that produces olfactory related behavior is not well understood. Furthermore, neurotransmitters and modulators such as octopamine (OA) and dopamine (DA) have been shown to affect behavioral decision making and motor programs but how they influence these behaviors is not known in depth (Certel *et al.*, 2007; Kong *et al.*, 2010; Schneider *et al.*, 2012). Two main aspects were analyzed in this thesis to address these issues.

The first aspect of this thesis was to dissect the function of the insect unique olfactory co-receptor (Orco) in olfactory preference behavior in *Drosophila melanogaster*. Previous studies aimed to dissect the role of Orco on the cellular level (among others: Wicher *et al.*, 2008, Smart *et al.*, 2008). But how Orco affects olfactory related behavioral output has not been investigated in detail. To investigate this, behavioral experiments using the olfactory binary two choice trap assay were performed. Orco has been shown to be required for ethanol preference (Schneider *et al.*, 2012). To test whether Orco is required in the olfactory sensory neurons to mediate olfactory preference Orco was expressed in olfactory sensory neurons in *$Orco^1$* mutants. Previous studies proposed that Orco is essential for olfactory perception and olfactory behavior in *Drosophila melanogaster* (Larsson *et al.*, 2004). In contrast Schneider *et al.* (2012) could show that *$Orco^1$* mutant flies prefer food odor or ethanol over water. To determine if *$Orco^1$* mutants can sense and prefer odors apart from ethanol, three different odors were tested in addition to ethanol against water in olfactory preference experiments. Orco has been suggested to amplify odor stimuli and reduce the olfactory sensory neuron spike threshold (Stengl, 2010; Stengl and Funk, 2013; Getahun *et al.*, 2013). Therefore loss-of Orco might reduce and not abolish odor sensitivity. To test this hypothesis the olfactory preference and aversion to four different odors in complex odor mixtures were tested in dose dependency experiments. Odor specific receptors are thought to determine the identity of an odor and as such if the odor is preferred over another odor (Hallem and Carlson, 2006; Knaden *et al.*, 2012). To investigate whether Orco plays a role in odor identity processing, similar attractive odors were tested against each other as single odors and in a complex mixture background. Usually more complex odor mixtures are more

attractive than less complex mixtures (Zhu *et al.*, 2003). To determine how Orco affects odor complexity sensing single odors were tested against mixtures of two or three odors.

The second aspect was to investigate the role of OA and DA in modulation of site-preference and locomotion behavior. DA neurons have been implicated in negative and OA neurons in reward reinforcement (Schwaerzel *et al.*, 2003; Claridge-Chang *et al.*, 2009). Optogenetic activation of octopaminergic/tyraminergic (OA/TA) neurons with a specific frequency, a combination of 40Hz and 8Hz termed, has been shown to promote site-preference (Schneider *et al.*, 2012). Whether activation of DA neurons can induce site-aversion and if activation of OA/TA neurons with a specific activation frequency is needed to induce site-preference is unclear. To address these questions, the blue light sensitive cation channel Channelrhodopsin-2 was expressed in DA or OA/TA neurons to allow optogenetic activation in the site-preference assay. To determine whether OA/TA neuron mediated site-preference is dependent on a specific activation frequency, OA/TA neurons were activated with different frequencies. DA and OA have been shown to be involved in locomotion (Winther *et al.*, 2006; Brembs *et al.*, 2007; Kong *et al.*, 2010). Direct activation of DA neurons can even promote locomotion. To investigate the role of OA/TA and DA neurons in locomotion, an optogenetic locomotion setup and assay was conceived and established. To validate the functionality of the setup DA neurons were activated and the locomotor output analyzed. OA/TA neurons were to be activated with different activation frequencies to determine if OA/TA neurons modulate locomotion and if a specific activation frequency is necessary. Finally, immunohistochemical studies using the GRASP system were used to determine possible sites for synaptic interactions between OA/TA and DA neurons as well as OA/TA neurons and Orco positive OSNs.

2 Material & Methods

2.1 Material

2.1.1 Fly strains

Unless otherwise noted all fly strains were out-crossed for up to 10 generations with the w^{1118} background.

Fly strains	Origin
w^{1118}*; Tdc2-GAL4*	Cole *et al.*, 2005
w^{1118}*; Th-GAL4;* +	Friggi-Grelin *et al.*, 2003
w^{1118}; *UAS-Chr2; UAS-ChR2*	Schroll *et al.*, 2006
$y^{1}w^{-}$*; P{UAS-mCD8::GFP.L}LL5*	Lee and Luo, 2001
w^{*}*; P{Orco*-GAL4.*W}11.17*; +	Vosshall, 2008
w^{*}*; P{UAS-Orco.L}13.20A;* +	Vosshall, 2009
w; +; Or83b*^{1}	Larsson *et al.*, 2004
w^{1118}	Lindsey and Zimm, 1992
w;+;Tdc2-LexA/*TM3*^{sb,e}	Burke *et al.*, 2012
w^{1118}*; +; UAS-CD4::spGFP1-10*	Gordon and Scott, 2009
w; +; LexAop-myr-mCherry	Diegelmann *et al., 2008*
w; lexAop-CD4::spGFPII/CyO; TM2/TM6b	Gordon and Scott 2009
w^{1118}*; orco*-LexA*::VP16;* +	Lee and Luo, 2006
norpA^{-}*; UAS-ChR2; UAS-ChR2*	Nuwal, 2010

2.1.2 Flyholding

All *Drosophila melanogaster* strains were raised and held on ethanol and yeast free but otherwise standard cornmeal-agar medium. The flies were stored in medium or big sized vials in acclimatized environments of either 18°C for long time storage or 25° for amplification and experimental purposes. All storage rooms or devices provided 65% humidity and a light/dark rhythm of 12h/12h.

2.1.3 Chemicals

Chemical	Molecular formula	Concentration	Supplier
Acetic Acid (AA)	C2H4O4	pure	AppliChem
Acetophenone (AP)	C_8H_8O	100%	Sigma-Aldrich
All-trans Retinal (ATR)	$C_{20}H_{28}O$	pure	Sigma-Aldrich
Apple-mango juice	-	-	Alnatura
di-Sodium hydrogen phosphate	Na_2HPO_4	pure	Merck
Ethanol (EtOH)	C_2H_6O	≥99%	VWR
Etyl Acetate (EtOAc)	$C_4H_8O_2$	pure	AppliChem
Formaldehyde (FA)	CH_2O	37%	AppliChem
Fetal Calf Serum (FCS)	-	100%	Sigma-Aldrich
Glycerol	$C_3H_8O_3$	100%	Merck
Magnesium cholride	$MgCl_2$	pure	AppliChem
Potassium cholride	KCl	pure	Merck
Potassium dihydrogen phosphate	KH_2PO_4	pure	Merck
Sodium chloride	NaCL	pure	AppliChem
Tween-20	$C_{58}H_{114}O_{26}$	-	Sigma-Aldrich
Triton x-100	$C_{14}H_{22}O(C_2H_4O)_n$	-	Merck
VECTASHIELD mounting medium	-	-	VECTOR Laboratories inc

2.1.4 Solutions

Solution	Concentration	Component
All trans Retinal solution (ATR)	250mM ATR in 100% EtOH	
Drosophila-Ringer (1x)	110mM	NaCl
	4.7mM	KCl
	20mM	MgCl
	0.74mM	KH2PO4
	0.35mM	Na2PO4
	in 1 liter, pH 7.4	
PBS(10x)	27mM	KCl
	20mM	KH2PO4
	100mM	Na2HPO4
	137mM	NaCl
	in 1 liter, pH 7.4	
PBST (1x)	1xPBS + 0.5% Triton x-100	

2.1.5 Primary/Secondary Antibodies

Antibody	Clonality	Antigene	Dilution	Provider
anti-GFP chicken	polyclonal	GFP	1:1000	Invitrogen
anti-nc82 mouse	monoclonal	*bruchpilot*	1:10 - 1:20	DSHB
Goat anti-chicken Alexa 488			1:200	Invitrogen
Goat anti-mouse Cy3			1:100	Jackson Immuno Research

2.2 Methods

2.2.1 Immunohistochemistry

2.2.1.1 Wholemount antibody staining of the adult Drosophila CNS

Adult 3-5 day old male flies were anesthetized on ice prior to the dissection. The CNS was dissected in 1x standard Drosophila Ringer. The cuticle and all other tissue surrounding the CNS were removed. The brains were then transferred into 1x PBS. The brain tissue was fixated with 3.7% Formaldehyde diluted in 1x PBS (phosphate buffered saline) for 30min on a shaker at room temperature (RT). To stop the fixation and remove all remaining traces of the fixation solution the brains were rinsed 3 times with 1x PBST (washing buffer; 1xPBS + 0.5% Triton x-100) and then washed 3 times 15min with 1x PBST on a shaker at RT. To block unspecific antibody binding sites the brains were incubated in blocking solution containing 5% FCS (fetal calf serum) diluted in 1x PBST for 1h at RT on a shaker. The blocking solution was removed and the primary antibody mix added. The primary antibody mix contained the non-fluorescent antibodies specific for the respective antigen diluted in blocking solution. The brains were then put on 4°C on a shaker over night. At lower temperatures the binding of antibody and antigene is slowed down but also considered to result in increased binding. The primary antibody mix was removed and the brains rinsed 3 times with 1x PBST. To remove traces of the primary antibody mix the brains were

washed 3 times 15min with PBST on a shaker at RT. The washing solution was removed and secondary antibody mix added. In the secondary antibody mix were fluorescently labeled antibodies with binding specificity to the primary antibody by antibody host diluted in 1x PBST. To prevent bleaching of the fluorescent signal in all further steps the brains were exposed to light as little as possible. The brains were incubated in the mix for 2-3h on a shaker at RT. The secondary antibody mix was removed and the brains rinsed 3 times with 1x PBST. To remove traces of the secondary antibody mix the brains were further washed 3 times 15min with PBST on a shaker at RT. The brains were then transferred into 50% Glycerol diluted in 1xPBS and incubated on a shaker for 30min. Glycerol treatment renders the tissue almost transparent allowing better fluorescent signal detection. The brains were mounted in VECTA-shield (VECTASHIELD mounting medium H-1000) on microscope slides and sealed with colorless nail polish. Vectashield is a mounting medium that reduces quenching and improved preservation of fluorescence in the specimen.

2.2.1.2 GFP Reconstitution Across Synaptic Partners (GRASP)

In order to identify possible synaptic connections between different neuronal populations in the adult Drosophila CNS the GRASP method was used (Feinberg *et al.*, 2008; Gordon and Scott, 2009). LexA/LexAop and UAS/GAL4 expression systems were utilized in one fly to separately drive line the expression of $GFP_{1\text{-}10}$ and GFP_{11} the two parts of a GFP protein as described by Gordon and Scott in 2009. Separately the GFP1-10 as well as the GFP11 fragment cannot be excited to emit fluorescence. Reconstitution of the two fragments results in a GFP protein capable of emitting fluorescence (Fig.7). The GFP fragments are linked to membrane targeting protein sequence that localizes the GFP fragments to membranes. Close proximity of both GFP fragments by membrane proximity of the two cells expressing the two fragments allows reconstitution of the GFP protein.

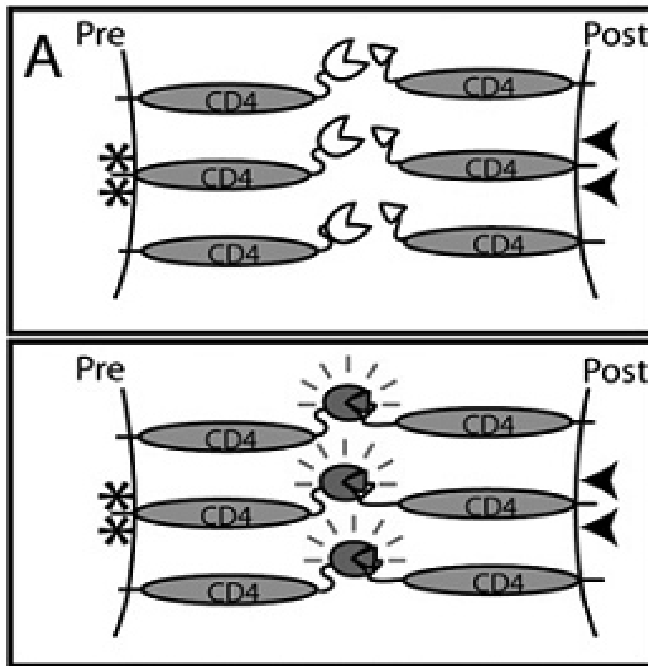


Figure 7. The GFP reconstitution across synaptic partners (GRASP) system. Pre- and Post synaptic membranes of two different cells at close proximity presenting each CD4 linked non-fluorescent complementary fragments of the GFP protein to the extracellular space (top). Close proximity of the two cellular membranes allows reconstitution of the GFP protein and regained fluorophore excitability (bottom). Modified from Feinberg *et al*., 2008.

2.2.1.3 Confocal laser scanning microscopy

The samples were analyzed with a Zeiss LSM 510 Meta confocal laser scanning microscope using Zeiss LSM 510 meta software. For fluorophore excitation an Argon laser (488 nm; intensity set to 50%) and Helium-Neon (543 nm; intensity set to 25%) laser were used. Pinhole width was set to 70-80µm and gain as well as offset adjusted depending on the immunohistochemical quality of the specimen. 10x, 20x and 63x (oil) objectives were used for magnifications as well as digital zoom ranging from 1-3x. The Slice interval for z-stacks was always set to 1µm.

The confocal z-stacks were further processed to z-projections using Fiji imaging software (National Institute of Health). Minor adjustments in color balance and contrast were made if it served better visibility of the detected fluorescent signal.

2.2.2 Behavioral methods

All flies used for behavioral assays were raised on 25°C and 65% humidity with a 12h dark/ 12light cycle. Genetic crosses were set up with a 1:2 ratio of male to female virgin flies in big food vials for preference experiments and in medium sized vials for locomotion experiments. Preference experiments were conducted in acclimatized rooms set to 25°C and 65% humidity. Locomotion experiments were conducted at 21°C and with no humidity control.

2.2.2.1 Olfactory Preference assay

To determine the olfactory preference for particular odors or odor mixtures a two-choice assay (Fig.8) was used as described in Ogueta *et al.*, 2010. Just briefly, 2-3 day old flies were anesthized with CO2 and 80 to 83 male flies were collected for each test. To remove any traces of CO_2 anesthesia, the flies were put into food containing medium sized vials for two days on 25°C prior to testing. The 4 to 5 day old male flies were then transferred into a closable glass beaker containing two odor traps filled with the odors of interest (Fig.8). Odors were diluted in water or apple-mango juice and the traps each filled with 1.5ml of the odor solutions. Flies could gain entry into the traps via a pipette tip with an opening diameter of 1.8mm. The glass beakers were then placed on cold-white illuminated plates for at least 16h. Beakers in which more than ten flies did not choose any of the two odor traps were discarded as invalid. The Preference Index (PI) was determined as described by the formula (Fig.8). A positive PI indicates preference, while a negative PI aversion with a total PI range from 1 to -1.

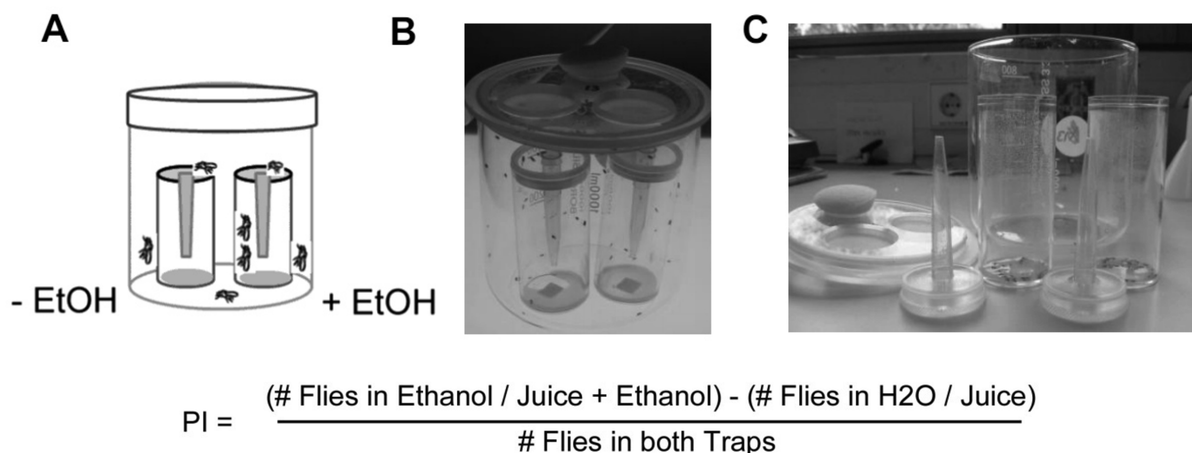


Figure 8. The olfactory binary choice preference trap assay. A Schematic drawing of the setup. **B** and **C** Live image of the assembled and disintegrated setup. The formula used to calculate the PI is shown in the bottom of the figure. Picture from Schneider, 2011.

2.2.2.2 Optogenetic assays

Optically impaired flies (*norpA[1]*) expressing Channelrhodopsin-2 (ChR-2) under the control of a GAL4-driver line (*Th*-GAL4 for DA neurons; *Tdc2*-GAL4 for OA/TA neurons) were used for all the optogenetic experiments. Control flies were raised on ethanol and experimental flies on 250mM ATR containing food medium vials under light exclusive conditions. For optogenetic preference experiments big sized food

vials and for optogenetic locomotion experiments medium sized food vials were used. The food medium was perforated by poking small holes into it to increase resorbation and mixing of ethanol and ATR with the food medium. The vials containing the perforated food were then treated with 150µl (medium sized vials) or 300µl (big sized vials) of either ethanol or 250mM ATR soluted in ethanol. All genetic crosses were set up in food vials treated this way. 2 day old adult male offspring were collected under CO2 anesthesia and transferred into fresh food vials treated with either ethanol or ATR as described above. The flies were then collected without any form of anesthesia for the optogenetic experiments after feeding on the treated food medium for two more days.

2.2.2.2.1 Olfactory optogenetic site-preference setup and assay

The optogenetic site-preference setup was used as described in Schneider *et al.*, 2012 (Fig.9). Just briefly, four to eight day old male flies were used in the experiments. Two traps each containing 1.5ml of apple-mango juice were placed in the bottom of the testing chamber and can be illuminated by either intense blue light (465-485nm; Cree, Germany) or warm-white light (Cree, Germany) LED diodes with a blue light filter (510nm; HEBO, Deutschland) located in the chamber lid (Fig.9B). Pipette tips cut at the tip to a 1.8mm opening were used as point of entry for the traps. Each diode is exclusively illuminating one of the two traps during the experiment. The diode heads are connected to a diode head controller through which the diode light intensities can be adjusted. The diode head controller in turn is connected to a computer running the custom made program LPTfreq (described in Schneider, 2011). The program can be used to set light pulse frequencies for the diodes. Three frequencies can be set in sequence with changeable duration of each frequency. In addition the number of consecutive repeats of the set sequence of frequencies can be set in cycle repeats. In contrast to the site-preference experiments conducted by Schneider *et al.*, 2012, the experimental setup was not placed on cold-white light plates for bottom up illumination. For the experiment the diode light intensities were set to 1800lx. The cycle length was always 20s and the illumination frequencies used for the experiments were 0hz, 8hz, 11.5Hz, 40hz or constant light. 80 to 83 male flies were inserted into the testing chamber and allowed to choose one of the odor traps for at least 16h. The PI was then calculated as described for the olfactory preference assay (see Fig.8).

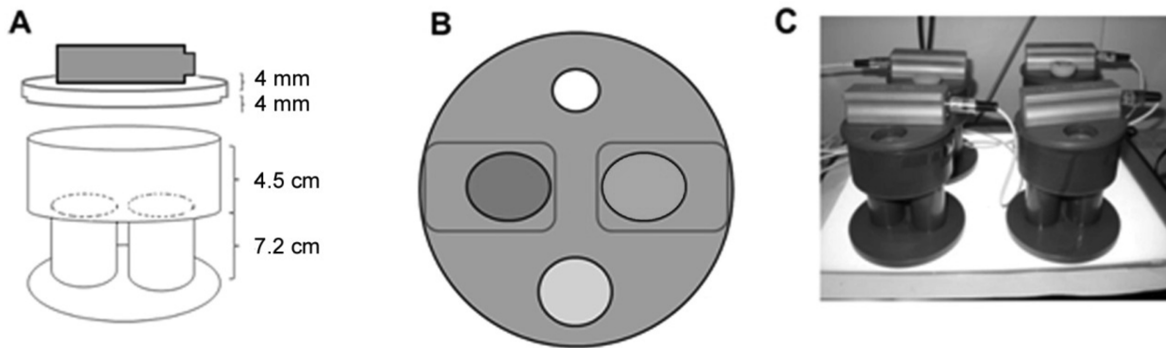


Figure 9. Optogenetic Two-Choice Preference Trap assay. A Schematic drawing of the testing chamber. **B** Schematic drawing of the lid containing a blue light (blue) and a warm white light diode modified by a blue light filter (yellow). **C** Live image showing four testing chambers on a cold white light illuminated plate during an experiment. From Schneider, 2011.

2.2.2.2.2 Optogenetic Locomotion Setup and assay

Two versions of the optogenetic locomotion setup were developed and used in this thesis, an early version (Fig.10) and an improved version (Fig.11). The early version was conceptualized and developed by T. Giang and H. Scholz and the improved version by A. Klein, T. Giang and H. Scholz. Both consist of a circular test arena with a diameter of 3.6cm placed on a glass plate which is illuminated by four blue light LED diodes (465-485nm; Cree, Germany) from four directions to minimize shaded areas in the arena (Fig.10 and 11). In the early version the test arena was illuminated by two diodes from above and two diodes from below located on diode heads while in the improved version by four freely movable and adjustable diodes only from below. Otherwise the setups are generally similar and the following description is for the improved version. In contrast to the early version, the improved version was installed as a fixed setup and allowed more stable illumination and thus increased consistency for experiments. The glass plate with the arena can be fitted into a height adjustable lift. The diodes a located on swan necks fastened each on one of the four support pillars of the setup that allow adjustments of the illumination direction and distance to the test arena. Three different lenses can be fitted to the diodes (diffusor lens used here; Cree) that allow either light beam spread or focus. The diodes are connected to a custom made diode head controller for a maximum of eight connected diodes where the light intensity can be adjusted. For all experiments the light intensity measured at the testing arena by a light-meter (5052, PeackTech) was set to 3000 lx. The diode head controller in turn is connected to a standard computer via a scat adapter. A camera (DCR-TRV950 Sony Network Handcam) is positioned perpendicular to the arena to document the movement of the fly in the test arena

from above. The camera is connected via a firewire cable to the computer to store the acquired videos for further processing. Videos were recorded with 25 frames per second. To increase the contrast between fly and background a cold white light plate with a blue light filter is placed beneath the arena. This is necessary because the arena would be otherwise completely dark during blue light illumination pauses which would render tracking of the flies movement impossible. The diode illumination pattern is controlled by the custom made program LPTfreq. The functions and interface of the program are described in the diploma thesis of A. Schneider, 2012 in detail. The program was developed for windows XP and adjusted to run on windows 7 for optogenetic locomotion experiments.

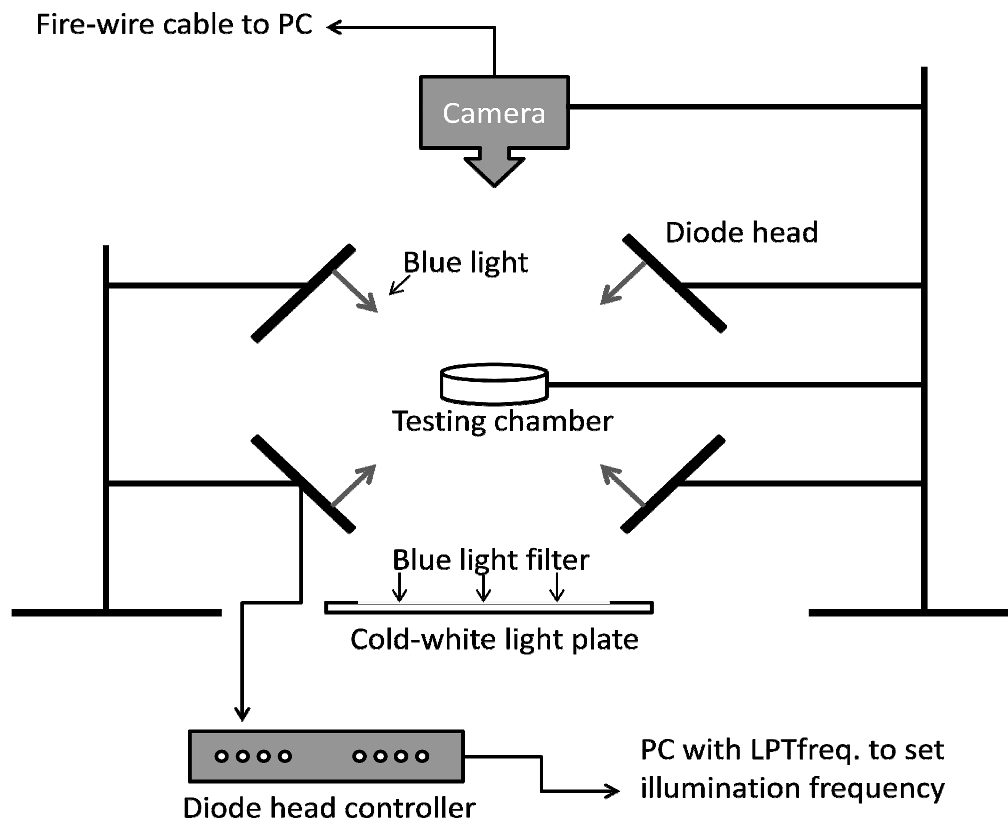


Figure 10. Early version of the optogenetic locomotion setup. Four diode heads are located above and below a testing chamber. Intense blue light diodes are located on the diode heads and directed at the testing chamber. A cold-white light plate with a blue light filter illuminates the arena from below. The diode heads are connected to a diode head controller that allows adjusting the light intensity of the diodes. The diode head controller is connected to a computer where the emitted light pulses can be set via the LPTfreq software. A camera is mounted above the testing chamber for recording of the movement of the fly.

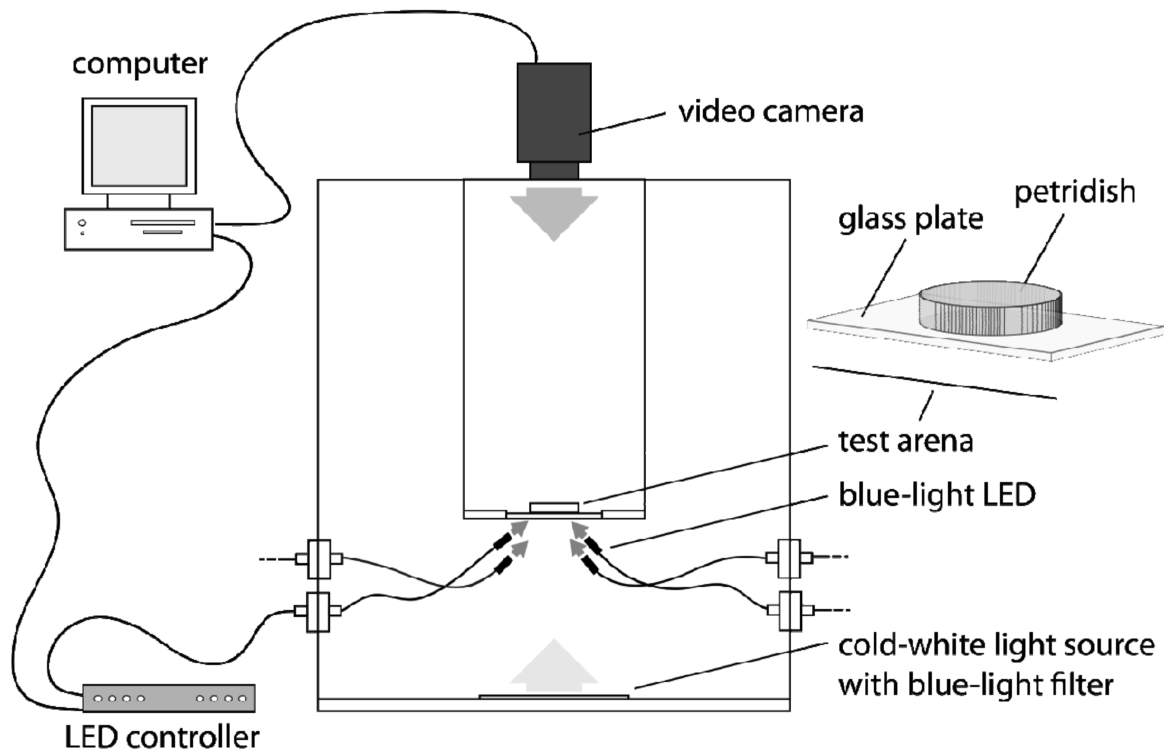


Figure 11. Improved version of the optogenetic locomotion setup. Four intense blue light diodes fastened to swan necks illuminate a circular test arena from below. From A. Klein, 2013.

Experiments were performed in the morning from 9am to 1pm and 2pm to 5pm because the flies have natural activity peaks before 9am, during midday and in the evening after 5pm. Single 4 to 7 day old male flies were placed in test arenas illuminated by cold-white light plates with a blue light filter for 20 min at 21°C to allow the flies to settle down, exploit the test arena and adjust to the room temperature and illumination. Blue light illumination frequencies and sequences of frequencies were set to 20s per sequence. The arenas were then placed into the setup for the experiment. The flies were recorded for a total of 3 min: 1min without blue light illumination (Pre-illumination), 1min during blue light illumination (Illumination) and 1min after the illumination (Post-illumination). During the illumination phase the picked sequence was repeated three times consecutively equaling 1min. Only flies that moved <5cm during the Pre-illumination phase were taken for analysis to equalize the pre-activity state of the flies and thus comparability between the flies pre activity state. The flies were tested alternating between control and experimental flies.

The acquired videos were stored for later analysis. Each video was cut into three 1min segments according to pre-illumination, illumination and post- illumination

phase using the video processing tool Virtual Dub (Freeware freeware available from Microsoft) and stored in the ".avi" video format. The video colors were split into green, blue and red channels using the imaging software Fiji (open source program available at: *http://fiji.sc*, Schindelin *et al.*, 2012) and all but the red channel were discarded. This was done to remove the blue color from the video and thus increase the contrast between background and fly body during blue light illumination. Since only the area of the testing arena is important for later fly tracking, the background surrounding the test arena was cropped. The video was than stored as ".avi".

The movement of the fly in the arena was automatically tracked using the tracking-software "Tracker" (Freeware available at: *http://www.cabrillo.edu*). To that end the video segments were loaded into the program and the following settings adjusted (for some of the following settings see Fig.12):

-Placement of x/y-coordinates at center of the arena

-Setting the diameter of the arena as 3.6cm

-Mark the fly by placing a point of mass on the position of the fly (ctrl + shift +left click on mouse)

-Set "automark" to 6 (determines the necessary similarity between the template -picture and following picture of the fly to be considered a match for the tracking; A new template is created as an overlay of old template and following picture match)

-Set "evolution rate" to 25% (determines the opacity of the overlay)

The flies movement was then automatically tracked according to the applied settings. The coordinate system results in x- and y-values giving information about the position of the fly in the arena. The number of traceable positions is determined by the frame rate of the video. 25 frames per second mean that 25 positions of the fly in the arena can be determined per second and thus 1500 in the one min video. The distance between the position of the fly from one frame to the next is assumed as equal to the distance traveled in 0.04s. Using these values the total distance the fly moved during

the video and also the velocity can be calculated. The acquired data was transferred to Microsoft excel for further calculations and analysis.

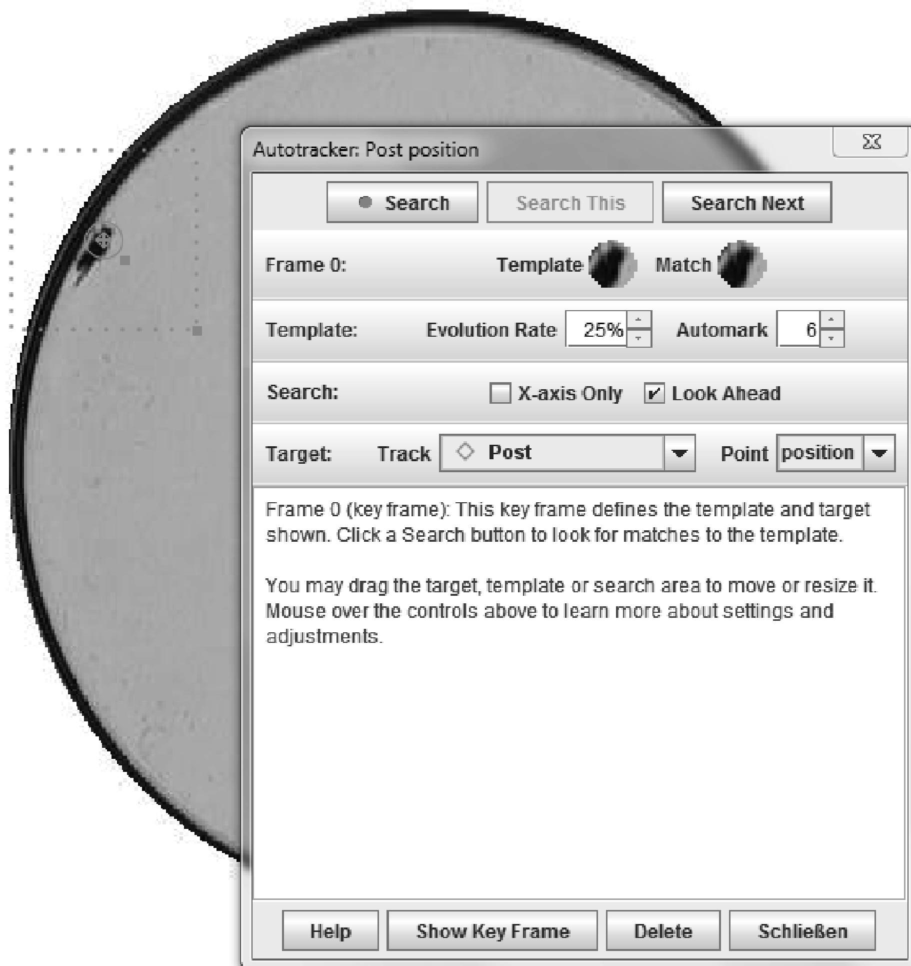


Figure 12. Screenshot of the tracking interface showing "automark and evolution rate" settings. Arena in grey with the fly marked by a point of mass (dotted green square and circle). From A. Klein, 2013.

2.2.3 Statistics

Mathematical calculations of mean value, standard deviation, standard error of the mean on all data sets were performed using Microsoft Office Excel 2007 (Microsoft)

Statistical analysis of the behavioral experimental data was performed with STATISTICA 9 (StatSoft Inc.). For statistical comparison of two normally distributed dependent data sets the student *t*-test was used ($P<0.05$). Comparison of more than two normally distributed dependent data sets was done with ANOVA post-hoc tukey HSD ($P<0.05$). For comparisons of two independent non-parametric data sets the Mann-Whitney U-test was used ($P<0.05$). For comparison of one normally distributed groups with Value=0 the one sample *sign* test was used ($P<0.05$).

3 Results

3.1 The Orco Function in Olfactory Preference

3.1.1 Orco is required for ethanol preference but not aversion

Flies prefer 5 % ethanol containing food odors over plain food odors when offered two similar odor traps (Schneider *et al*. 2012). Preference is measured in an olfactory binary choice assay consisting of two odor traps. Flies were allowed to choose between the traps for 16h and flies in each trap were counted and the preference index (PI) was calculated. The preference index ranges from -1 to 1 and is defined as preference when the value is positive and aversion when negative. We have previously shown that *Orco*^{1} mutants (*Orco* loss-of function; Larsson *et al*., 2004) do not prefer ethanol containing food odors (Schneider *et al*., 2012). However, to confirm that the observed behavioral changes are indeed due to loss-of *Orco* function within the OSNs, *Orco* function was restored in *Orco*^{1} mutants using the *Orco*-GAL4 driver line (Larsson *et al*., 2004; Fig.13A).

The P-element insertion of the used transgenes did not influence preference, since the UAS-*Orco* transgene and the GAL4 construct developed preference and did not interfere with the loss of preference phenotype of *Orco*^{1} mutants. The

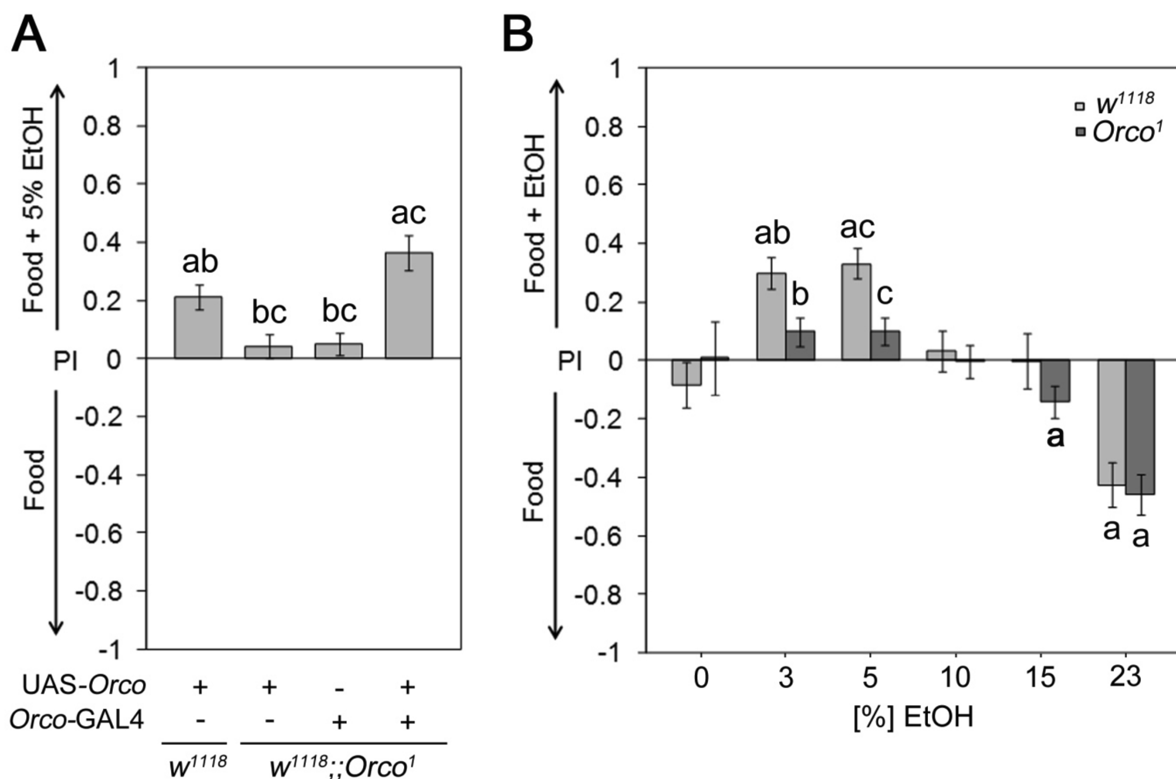


Figure 13. Ethanol preference depends on Orco mediated olfaction. A PI`s for food versus food plus 5% ethanol for positive (*UAS-Orco* in w^{1118} background) and negative controls (UAS/GAL4 transgene in $Orco^1$ mutant background) and for flies carrying both transgenes in the $Orco^1$ mutant background are shown. Negative transgene controls in the $Orco^1$ mutant background show no preference while the positive control shows preference for 5% ethanol in food. *Orco*-GAL4 dependent expression of *Orco* in OSNs restores preference for 5% ethanol in food in $Orco^1$ mutants (PI´s are w^{1118}; *UAS-Orco* 0.21 ± 0.04, w^{1118}; *UAS-Orco*; $Orco^1$ 0.04 ± 0.04, w^{1118}; *Orco*-GAL4;$Orco^1$ 0.05 ± 0.03, and w^{1118};*UAS-Orco*/*Orco*-GAL4;$Orco^1$ 0.36 ± 0.06. N=43, 37, 27, 28). **B** w^{1118} flies show preference for low ethanol concentrations (3 and 5%) and aversion at high concentration (23%) while $Orco^1$ mutants show only aversion at high ethanol concentrations (23%; PI´s listed in suppl. Table 1). Significant differences to PI=0 are indicated by "**a**" (one sample-sign test; $P<0.05$) and between w^{1118} and $Orco^1$ pairs by **b** and **c** (ANOVA *post-hoc* tukey HSD; $P<0.05$) respectively.

expression of UAS-*Orco* in $Orco^1$ mutants using the *Orco*-GAL4 line restored ethanol preference ($P < 0.001$). This indicates that ethanol preference is indeed dependent on *Orco* mediated olfaction in the Orco expressing receptor neurons.

$Orco^1$ mutants prefer 5% ethanol and plain food odors over water (Schneider *et al*., 2012) suggesting that single and complex odors can still be sensed. It has been proposed that *Orco* mediates OR sensitivity and therefore allow odor detection over a wider concentration range (Getahun *et al*., 2013). In order to test, whether the loss of preference in $Orco^1$ mutants is due to reduced of odor sensitivity, w^{1118} and $Orco^1$ mutant flies were offered the choice between food odor containing increasing ethanol concentrations (0-23%) and plain food odor (Fig.13B). As expected, w^{1118} control flies showed ethanol dose dependency as has been previously shown (Ogueta *et al*., 2010). In contrast to w^{1118} flies $Orco^1$ mutants failed to show preference for ethanol at any given concentration. However aversion at high ethanol concentration (23%) was still observed to a similar degree (PI = -0.4 to -0.5) indicating an *Orco* independent mechanism for aversive olfactory behavior to ethanol. Taken together, olfactory ethanol preference depends on *Orco* function in olfactory receptor neurons independent of ethanol concentration while aversion to high ethanol concentrations is *Orco* independent.

3.1.2 $Orco^1$ mutants distinguish between similar complex odor mixtures in a concentration dependent manner

Orco could be a potential ethanol receptor or alternatively the ethanol specific receptor is mislocalized since heterodimerization with *Orco* does not occur (Benton *et al*., 2006). In order to investigate whether Orco acts in ethanol detection specifically three additional odors, namely ethyl acetate (EtOAc), acetic acid (AA) and

acetophenone (AP) were presented in food odor in increasing concentrations versus plain food odor (Fig.14). EtOAc is a metabolite of AA and like ethanol can be found in fermenting fruits attractive to *Drosophila melanogaster* (Zhu *et al.*, 2002; Becher *et al.*, 2010).

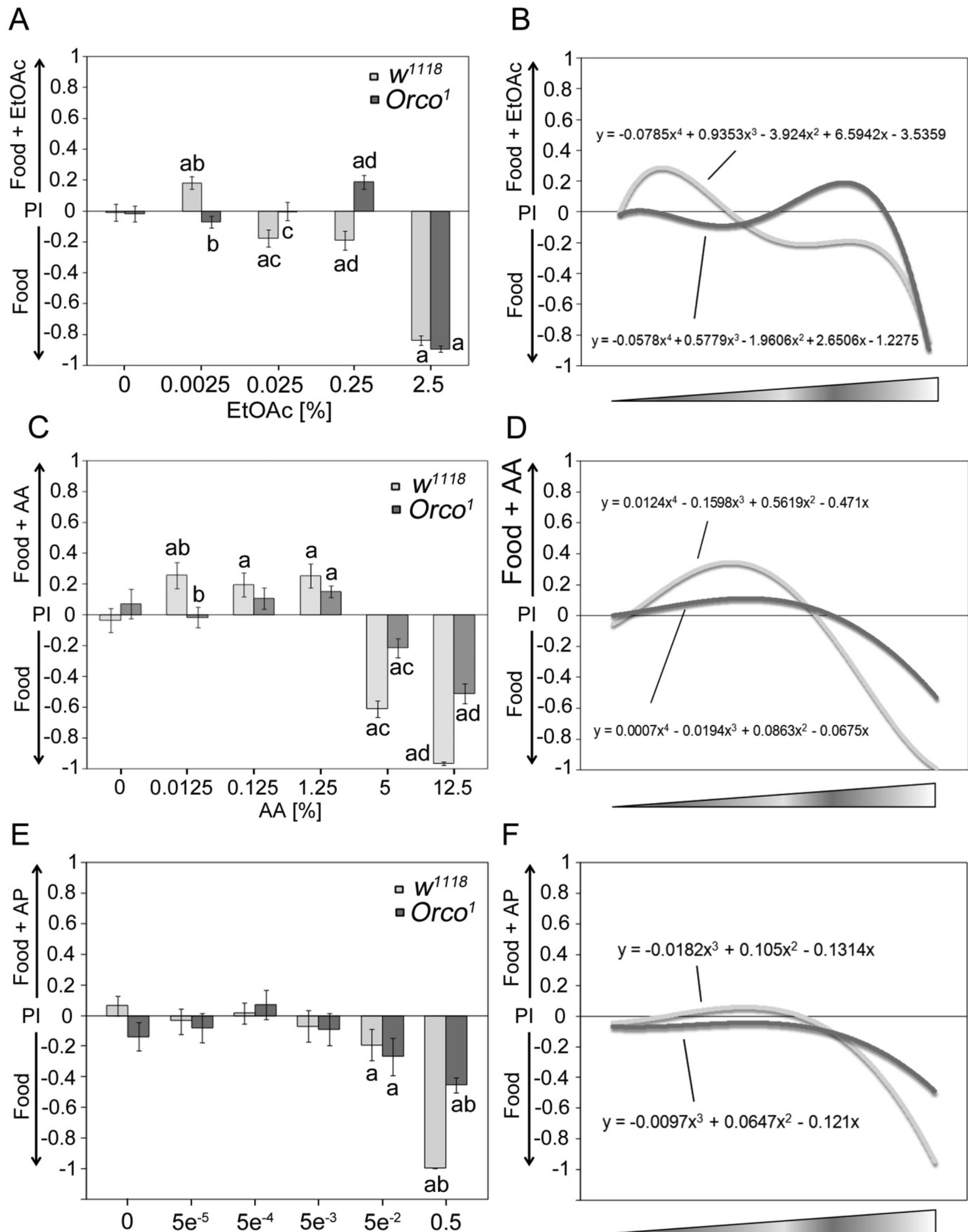


Figure 14. EtOAc, AA and AP preference is odor concentration and Orco dependent. Dose-dependency for different odor mixtures. **A** w^{1118} flies show a dose-dependent to EtOAc. $Orco^1$ mutants show a shift in preference to higher concentrations (w^{1118}/$Orco^1$ from low to high concentration N=26/29, 48/40, 27/28, 30/35, 19/17). **B** Polynomic analysis of EtOAc preference for w^{1118} and $Orco^1$ mutant flies (mathematical functions as indicated). $Orco^1$ mutant flies have a shift to the right in the EtOAc preference response profile. **C** w^{1118} flies show dose-dependency responses to AA. $Orco^1$ mutants show a shift in preference to higher concentrations and significantly reduced aversion (P < 0.01; w^{1118}/$Orco^1$ N=11/11, 20/21, 19/13, 20/20, 20/19, 10/19) **D** w^{1118} flies show a u-shaped concentration response curve. $Orco^1$ mutant flies have a flattened and right shifted AA response profile. **E** w^{1118} and $Orco^1$ mutant flies show no preference at all but aversion at high AP concentrations. Aversion to 0.5% AP was significantly higher in w^{1118} flies (w^{1118}/$Orco^1$ N=22/18, 24/23, 24/21, 25/24, 27/16, 13/10). **F** $Orco^1$ mutant flies have a right shifted AP response profile (PI´s listed in suppl. Table 1) Significant differences to PI=0 are indicated by **a** (one sample-sign test; *P*<0.05) and between w^{1118} and $Orco^1$ pairs by **b**, **c** and **d** (ANOVA *post-hoc* tukey HSD; *P*<0.05) respectively. **A** reevaluated dataset from S. Belaidi, 2012, **C** Pooled Dataset from S. Demirkol and TG.

AP is a volatile compound present in mango fruits and elicits aversive behavior in an olfactory two choice paradigm (Knaden *et al.*, 2012). Specific ORs have been shown to be responsive to EtOAc and AP (Hallem *et al.*, 2006), while AA perception is thought to be mediated via IRs (Benton *et al.*, 2009; Silbering *et al.*, 2011).

w^{1118} flies showed dose dependent EtOAc attraction and aversion (Fig.14A). $Orco^1$ mutant flies preferred EtOAc at a concentration already aversive for w^{1118} control flies (0.25%) and aversion at high a EtOAc concentration (2.5%) to a similar degree as the w^{1118} control flies (PI = -0.8). Polynomic analysis of the data showed a shift of the $Orco^1$ mutant EtOAc preference to higher concentrations and a normal aversion (Fig.14B). This indicates that Orco increases sensitivity to EtOAc in complex odor mixtures but not aversion consistent with the idea that two receptors for EtOAc exist (Kreher *et al.*, 2008). OR42b is responsive at low and OR42a at high EtOAc concentrations. One explanation could be that OR42a is Orco independent and thus $Orco^1$ mutants can show preference at high EtOAc concentrations. Similarly, w^{1118} flies showed dose dependent AA attraction and aversion (Fig.14C). $Orco^1$ mutants preferred higher AA concentrations (1.25%) than w^{1118} flies. In addition, $Orco^1$ mutants have a significantly lower aversion for high AA concentrations (5% and 12.5%) compared to the w^{1118} control flies ($P < 0.01$). Polynomic analysis showed a shift in $Orco^1$ mutant AA preference to higher concentrations that partly overlap with the w^{1118} controls but $Orco^1$ mutant flies have a flattened curve and reduced aversion (Fig.14D). This indicates that Orco increases sensitivity and aversion to AA in complex odor mixtures. IRs function without Orco and could therefore be responsible for preference at higher AA concentrations and the reduction in aversion in $Orco^1$ mutants.

Unlike the other tested odors AP was never preferred for both *w*^{1118} control and *Orco*^{1} mutant flies in confirmation of previous studies stating AP as aversive (Fig.14E; Hallem *et al.*, 2006). Aversion to 0.5% AP was significantly lower in *Orco*^{1} mutant flies compared to the *w*^{1118} flies ($p < 0.001$). Polynomic analysis showed a flattened profile with a reduced aversion for *Orco*^{1} mutant flies (Fig.14F). This indicates that Orco increases sensitivity to AP in complex odor mixtures affecting aversion.

Taken together, Orco increases sensitivity to odors in complex odor mixtures for preference and also aversion to odors apart from ethanol. Orco independent odor perception through possibly OR42a and IRs might be responsible for olfactory preference formation for EtOAc and AA. Since aversion can still be observed at least one Orco independent but also complemented odor sensing mechanism has to be present.

3.1.3 *Orco*^{1} mutant flies sense single odors

In general most odors are thought to have unique and separate identities due to odor specific reception on the receptor level reflected in also specific glomerular odor induced activation patterns in the antennal lobe (Vosshall *et al.*, 2000; Wang *et al.*, 2003; Hallem *et al.*, 2006;). One possibility is that in doses response curves odors are not sensed due to loss-of odor specific receptors. To determine whether *Orco* mutants can still sense single odors the preference of *w*^{1118} and *Orco*^{1} mutant flies to monomolecular odors at concentrations most likely resulting in similar preferences were tested. (Fig.15). In general flies choose between a single odor and water.

w^{1118} flies showed similar preference for the single odors AP, AA, ethanol and EtOAc diluted in water when the other choice was water. (PI =0.5 to 0.35; Fig.15). Interestingly 0.25% EtOAc induced aversive behavior in *w*^{1118} flies when presented in a food odor mixture background (Fig.14A) but elicited preference when offered alone highlighting the significance of the context of odor presentation. This finding hints either at a change in attractiveness due to the odor presentation context or that single molecules are perceived, but not the identity of the single odors. *Orco*^{1} mutants showed preference similar to the *w*^{1118} flies for all tested odors but AP and 0.0025% EtOAc where they showed no preference (Fig.15A and E).

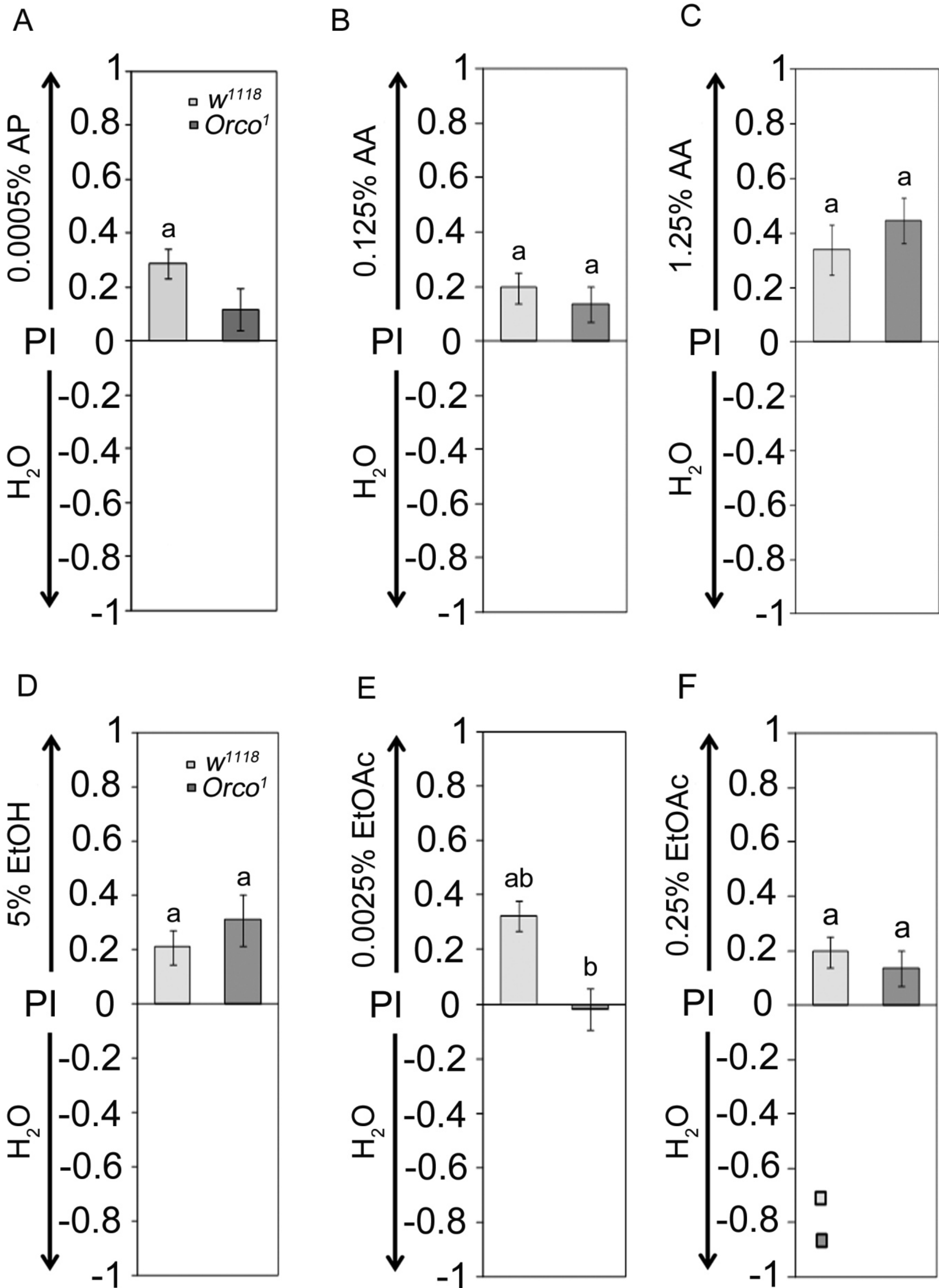

A
B
C
D
E
F
0.0005% AP
0.125% AA
1.25% AA
5% EtOH
0.0025% EtOAc
0.25% EtOAc
PI
H_2O
w^{1118}
$Orco^1$
a
ab
b
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Figure 15. w^{1118} and *Orco*^{1} mutant flies are similar attracted to single odors in a certain concentration range. Single odor presentations of AP, AA, ethanol and EtOAc diluted in water at indicated concentrations versus water. **A**-**F** w^{1118} flies preferred the single odor over water to a similar degree for most tested odors and concentrations (PI = 0.2-0.35). *Orco*^{1} mutant flies showed preference similar to the w^{1118} flies except for AP (**A**) and 0.0025% EtOAc **(E)** where no preference could be observed (PI´s are for w^{1118} / *Orco*^{1}: **(A)** 0.29 ± 0.05 / 0.12 ± 0.08, N=28,26, (**B**) 0.29 ± 0.06 / 0.24 ± 0.06, N=28,37, (**C**) 0.34 ± 0.09 / 0.45 ± 0.08, N=13,13, (**D**) 0.21 ± 0.06 / 0.31 ± 0.09, N=15,14, (**E**) 0.32 ± 0.05 / -0.02 ± 0.08, N=16,15, (**F**) 0.20 ± 0.06 / 0.14 ± 0.07, N=27,19). **C** 10-fold increase in AA concentration increased the preference slightly but not significantly compared to the lower AA concentration (**B**). **F** 100 fold decrease of EtOAc concentration abolished preference for *Orco*^{1} mutant flies. Significant differences to PI=0 are indicated by **a** (one sample-sign test; $P<0.05$) and between w^{1118} and *Orco*^{1} pairs by **b** (student *t*-test; $P<0.05$).

100-fold increase in EtOAc concentration recapitulated preference for EtOAc in *Orco*^{1} mutants (Fig.15F). This indicates that single odors are perceived and Orco increases sensitivity to single odors.

Taken together Orco increases sensitivity to single odors but single odor perception is not impaired. In addition, the preference level is comparable between w^{1118} and *Orco*^{1} mutant flies. One interpretation could be that the odor molecules are perceived but not the odor identity.

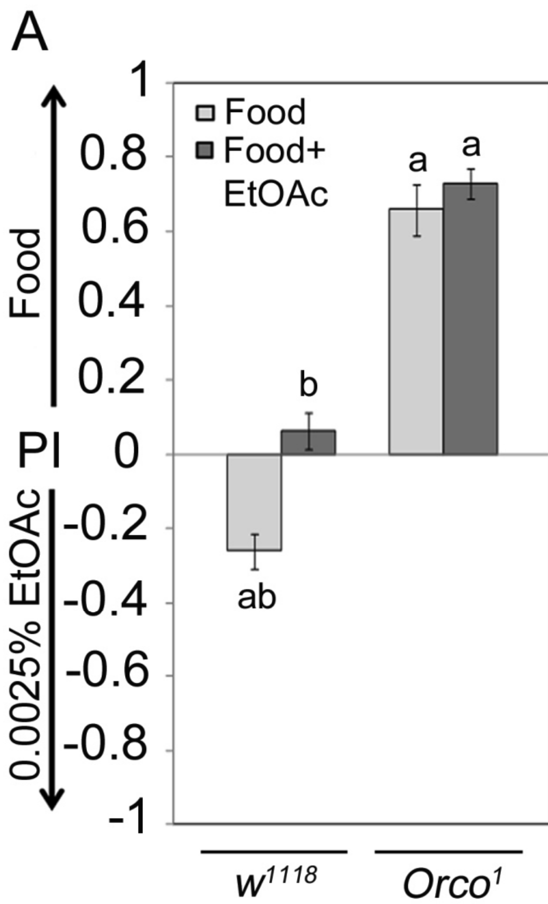
3.1.4 *Orco*^{1} mutants cannot distinguish between odors of similar complexity

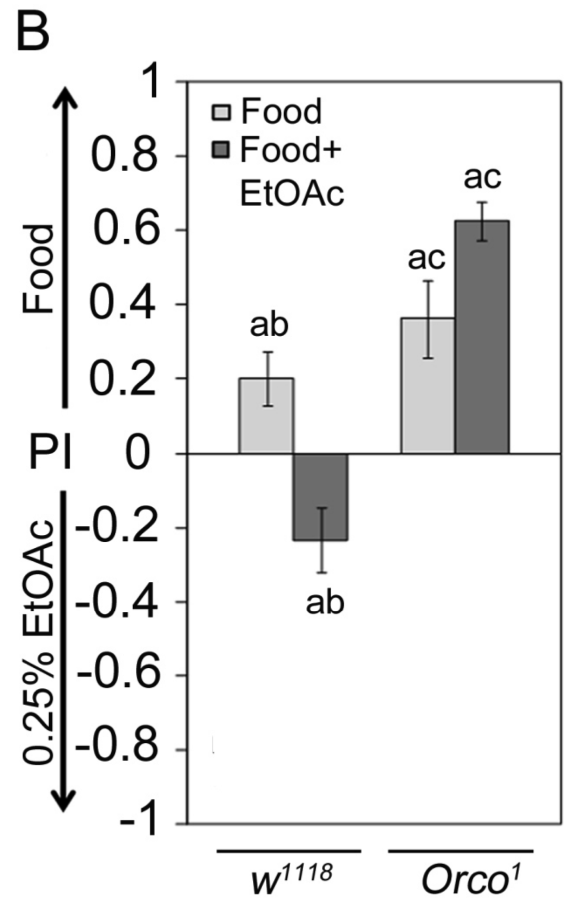
To analyze whether in general single odors can be recognized in a complex odor mixture flies were presented food odor or food odor containing either EtOAc or AA versus the same concentration of AA or EtOAc respectively (Fig.16). Two concentrations were tested for both odors.

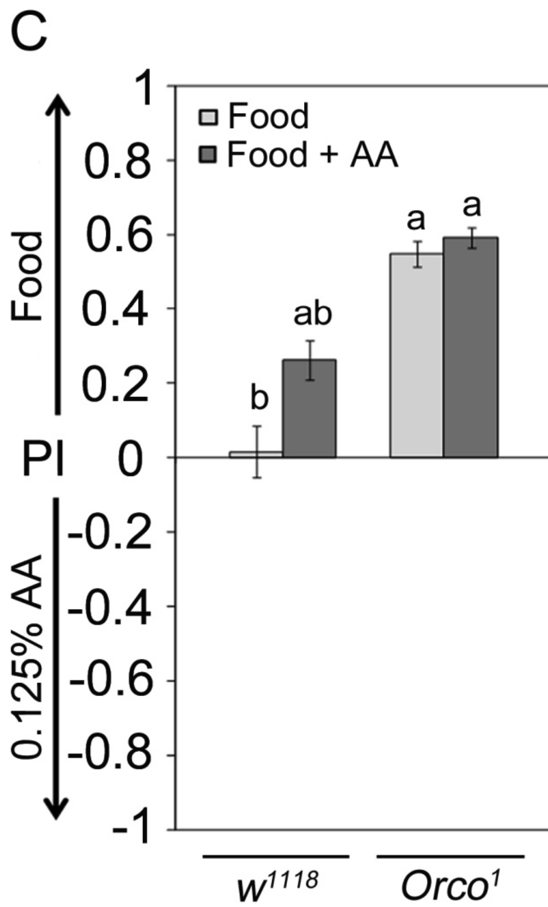
w^{1118} flies preferred the single odor EtOAc over the complex food odor (Fig.16A). Addition of EtOAc to the food odor mixture resulted in no preference. This means that w^{1118} flies can distinguish between the single odor and the complex odor but in contrast to previous studies (Zhu *et al*., 2003; Schneider *et al*., 2012) the single odor can be more attractive. In addition, it indicates that the single odor EtOAc is as attractive alone as in the complex background at the used concentration meaning that the odor presentation background is negligible depending on the single odor concentration. *Orco*^{1} mutants preferred the food odor and food odor plus EtOAc over EtOAc alone to a similar degree. This indicates that increases in odor complexity cannot be sensed in the absence of Orco. w^{1118} flies showed preference for the food odor mixture over EtOAc alone at 100-fold increase in EtOAc concentration and

addition of EtOAc to the food odor lead to aversion to the food odor mixture (Fig.16B). This indicates that the EtOAc concentration is most likely aversive which is increased in aversion by the food odor background. This also means that the odor presentation background gains or looses impact depending on the EtOAc concentration. *Orco*[1] mutant flies preferred food odor over EtOAc and showed an increase in preference with addition of EtOAc to the food odor. This shows that distinguishing between similar complex odor mixtures is Orco independent but dependent on the EtOAc concentration. One explanation could be that OR42a responsible for sensing EtOAc at higher concentrations is Orco independent and responsible for the increase in preference. Furthermore it might be that sensing of the lower EtOAc concentration (Fig.16A) depends on OR42b in an Orco dependent manner.

w^{1118} flies showed no preference for food odor or AA but preferred food odor plus addition of AA over AA significantly ($P < 0.05$; Fig.16C). This shows that odor complexity can be sensed and that the more complex food odor is preferred over the less complex food odor. In contrast, *Orco*[1] mutants strongly preferred the food odor mixtures over the single odor. No increase in preference for the more complex odor mixture by addition of AA could be observed (Fig.16C). This indicates that *Orco*[1] mutants cannot distinguish between similar complex odor mixtures even though sensing AA through IRs should still be functional. It might be that the ability to distinguish between food odor and food odor plus AA is AA concentration dependent as is the case for EtOAc. Therefore the AA concentration was 10-fold increased (Fig.16D). w^{1118} flies preferred food odor over AA and showed no preference with addition of AA to the food odor. This is similar to the phenotype observed with EtOAc at 0.0025%. It indicates that at this AA concentration AA as a single odor matters and not the food odor background or odor complexity. *Orco*[1] mutants preferred food odor and food odor with AA over AA alone to a similar degree. This shows that even at higher AA concentration the *Orco*[1] mutants cannot distinguish between similar complex odor mixtures indicating that Orco mediates sensitivity to AA in complex odor mixtures. In addition, it also shows that AA perception through IRs cannot facilitate discerning between similar complex odor mixtures. It is unclear whether Orco mediated olfaction is the primary pathway and IRs are working in complementation or if IRs do not play a role here at all.


A
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Food
PI
0.0025% EtOAc
Food
Food+
EtOAc
a
a
b
ab
w1118
Orco1


B
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Food
PI
0.25% EtOAc
Food
Food+
EtOAc
ac
ac
ab
ab
w1118
Orco1


C
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Food
PI
0.125% AA
Food
Food + AA
a
a
ab
b
w1118
Orco1

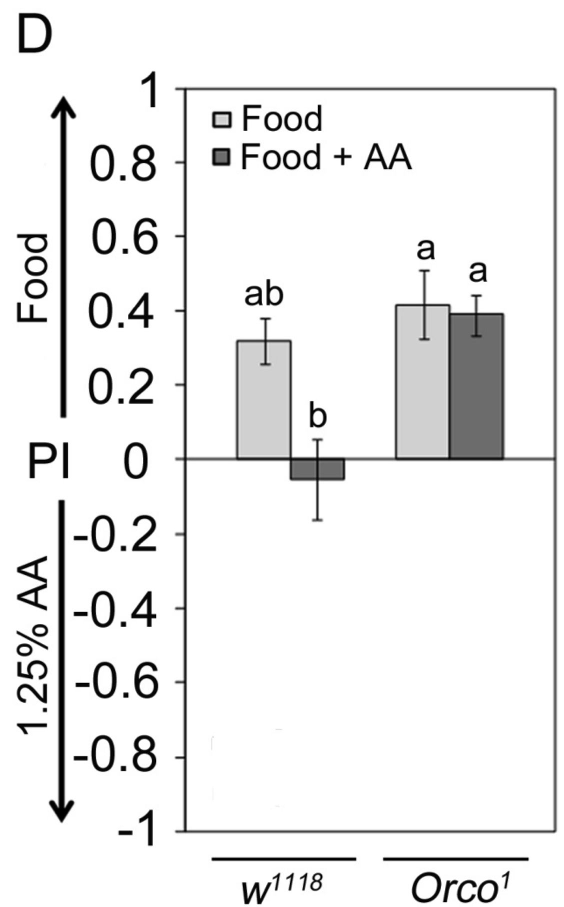

D
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Food
PI
1.25% AA
Food
Food + AA
a
a
ab
b
w1118
Orco1

Figure 16. Discerning between similar complex odor mixtures is Orco dependent. Comparison of food or food plus either EtOAc (**A**: 0.0025%, **B**: 0.25%) or AA (**C**: 0.125%, **D**: 1.25%) versus the respective concentration of EtOAc or AA in water for w^{1118} and $Orco^{1}$ mutant flies. **A** w^{1118} flies prefer 0.0025% EtOAc alone over food. Addition of EtOAc to food significantly changes the preference to no preference. $Orco^{1}$ mutant flies prefer food and food containing 0.0025% EtOAc over 0.0025% EtOAc alone and show no preference change with addition of EtOAc to food (PI´s are from left to right: -0.26 ± 0.05, 0.06 ± 0.05, 0.66 ± 0.07, 0.73 ± 0.04, N=23, 23, 22, 22). **B** 100 fold increase in EtOAc concentration leads to preference for food alone in w^{1118} and addition of EtOAc to food changes the preference to aversion. $Orco^{1}$ mutants prefer food alone and food plus EtOAc over EtOAc alone. Preference for food containing EtOAc is significantly higher than for food alone (p<0.05; PI´s: 0.20 ± 0.07, -0.23 ± 0.09, 0.36 ± 0.10, 0.63 ± 0.05, N=26, 19, 19, 18). **C** w^{1118} flies prefer food containing 0.125% AA but not food alone. $Orco^{1}$ mutant flies have a significant higher preference for both conditions of the complex odor mixture and prefer both conditions equally (PI´s: 0.02 ± 0.07, 0.26 ± 0.05, 0.55 ± 0.03, 0.59 ± 0.003, N=14, 21, 13, 17). **D** 10-fold increase in AA concentration results in preference for food alone and a significant change to no preference when AA is added to food in w^{1118} flies (p<0.05). $Orco^{1}$ mutants prefer food and food plus AA over AA alone. They show no difference in preference between the two conditions (PI´s: 0.32 ± 0.06, -0.05 ± 0.11, 0.42 ± 0.09, 0.39 ± 0.06, N=19, 13, 16, 15). Significant differences to PI=0 are indicated by **a** (one sample-sign test; *P*<0.05) and between w^{1118} and $Orco^{1}$ by **b** and **c** (ANOVA *post-hoc* tukey HSD, *P*<0.05).

Taken together, the ability to perceive single odor component changes in a complex odor mixture is bound to the presence of Orco for AA but in case of EtOAc it also depends on the single odor concentration in the mixture. This might be due to OR42a responsible for sensing higher EtOAc concentrations in a possibly Orco independent manner. Sensing of AA in complex odor mixtures cannot be achieved by IRs alone and it could be that likely no other Orco independent means of perception have an influence. Furthermore it becomes clear that the single odor gains or looses prominence over the odor background dependent on the single odor concentration. However, outside of that particular concentration range it is not clear whether the odor mixture is perceived as a different odor by adding another component or if the added component can still be perceived and judged in relation to the odor context.

3.1.5 Odor preference is not changed by increased odor complexity

To address whether changes in odor complexity can increase preference, three different odor mixtures were tested against the single odor AP diluted in water (Fig.17). The concentrations of each odor elicited comparable preference when tested against water and can therefore be sensed (see Fig.15). If odor complexity increases olfactory preference then mixtures of these odors should be preferred over a single odor alone.

First AP was tested against ethanol and EtOAc (Fig.17A). w^{1118} flies did not prefer one over the other indicating that this binary mixture does not increase

preference. This could be because of repressing or masking effects of these two odors over each other or AP over one odor of the binary mixture. It has been shown that AP has no masking effects over EtOAc but could potentially mask ethanol (Kreher *et al.*, 2008). If that is true AP would be matched against EtOAc which could account for the no preference phenotype. It could also be that AP is simply as attractive as the combination of ethanol and EtOAc. This is rather unlikely because AP has been reported to be only aversive in a two-choice paradigm before (Hallem *et al.*, 2006; Knaden *et al.*, 2012). Similarly, *Orco*^{1} mutant flies showed no preference which is consistent with Orco increasing sensitivity.

To test whether the combination of odors is responsible, ethanol and AA were tested against AP (Fig.17B). Still *w*^{1118} flies did not show any preference which could still be due to AP masking ethanol but would essentially mean that EtOAc is as attractive as AA.

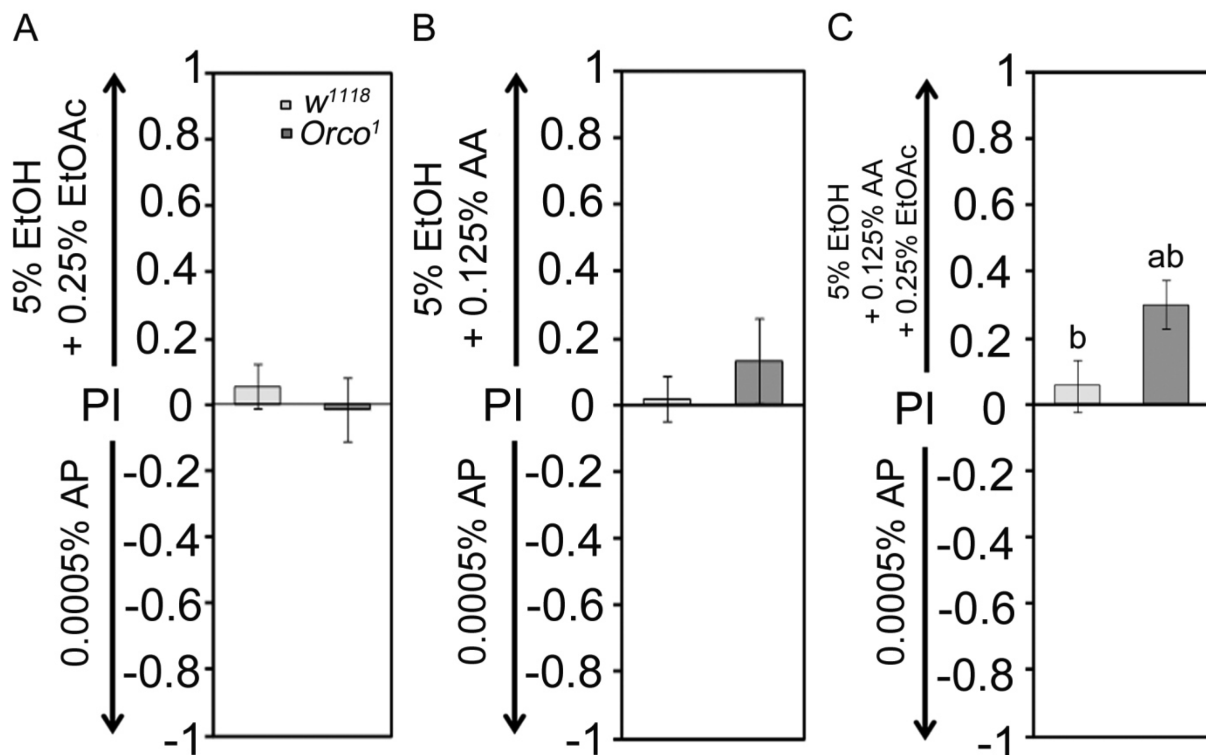


Figure 17. Odor complexity does not influence olfactory preference. A *w*^{1118} and *Orco*^{1} mutants fail to distinguish between the binary odor mixture (5% ethanol and 0.25% EtOAc) and the single odor AP 0.0005% (PI´s are for *w*^{1118} / *Orco*^{1}: 0.05 ± 0.07 / -0.01 ± 0.10, N=23,14). **B** *w*^{1118} and *Orco*^{1} mutants show no preference when offered the binary odor mixture of 5% ethanol and 0.125% AA and the single odor AP (PI´s are for *w*^{1118} / *Orco*^{1}: 0.02 ± 0.07 / 0.13 ± 0.13, n=28,15). **C** *w*^{1118} control flies show no preference while *Orco*^{1} mutant flies prefer the odor mixture of ethanol, AA and EtOAc over the single odor AP to a significant degree (p<0.05; PI´s are for *w*^{1118} / *Orco*^{1}: 0.06 ± 0.08 / 0.30 ± 0.07, n=21,18; Significant differences to PI=0 marked by **a** (one sample sign test; *P*<0.05;) and between *w*^{1118} and *Orco*^{1} by **b** (student *t*-test; *P*<0.05*)*.

That aside another explanation could be that binary mixtures are not more attractive than single odors. *Orco*^{1} mutants also showed no preference maybe due to the same reason. Further increases in odor complexity might lead to a shift in preference for the mixture. In order to test this ethanol, EtOAc and AA were tested against AP (Fig.17C). w^{1118} flies showed no preference indicating that odor complexity by itself does not lead to preference. Furthermore masking effects of AP over ethanol are rather unlikely since EtOAc and likely AA are not being masked as suggested by the binary mixture experiments. Surprisingly, *Orco*^{1} mutant flies showed preference for the odor mixture. This suggests that Orco might repress preference for certain odor mixtures. Given that Orco interacts with odor specific receptors, it could be that Orco is a negative regulator for channels mediating olfactory preference.

Taken together, odor mixtures of two or three attractive odors are not preferred over a single odor in w^{1118} flies indicating that odor complexity is not the primary aspect in olfactory preference. In addition Orco might play a role as a negative regulator for olfactory preference most likely in combination with specific olfactory receptors.

3.1.6 *Orco*^{1} mutants fail to distinguish between odor identities

Ethanol and AA are present in rotting fruits and the smell of these odors signal oviposition sites, putative availability of mating partners and food sources (Zhu *et al.*, 2002; Joseph *et al.*, 2009; Becher *et al.*, 2010). This suggests that odors have a specific identity apart from being positive or negative. Still it is quite possible that every odor that is perceived as good leads to an approach behavior without further specific meaning for the fly prior to arriving at the target location and vice versa. In order to analyze whether flies can distinguish between single odors diluted in water at concentrations perceptible for both w^{1118} and *Orco*^{1} mutant flies (see Fig.15) AP, EtOAc, AA and ethanol were tested against each other (Fig.18). w^{1118} flies always preferred one of the presented odors over the other odor significantly ($p<0.05$) indicating that single attractive odors can be distinguished from each other. Under the assumption that the preference level for the tested odor concentration when tested against water alone is a measure for attractiveness these results indicate that odor identity decides preference and not initial similar levels of attractiveness. The most attractive odor for w^{1118} flies was AP followed by AA and EtOAc while ethanol was

the least attractive in contrast to previous studies and our data (see Fig.14) showing that AP is only neutral and aversive (Hallem *et al*., 2006; Knaden *et al*., 2012).

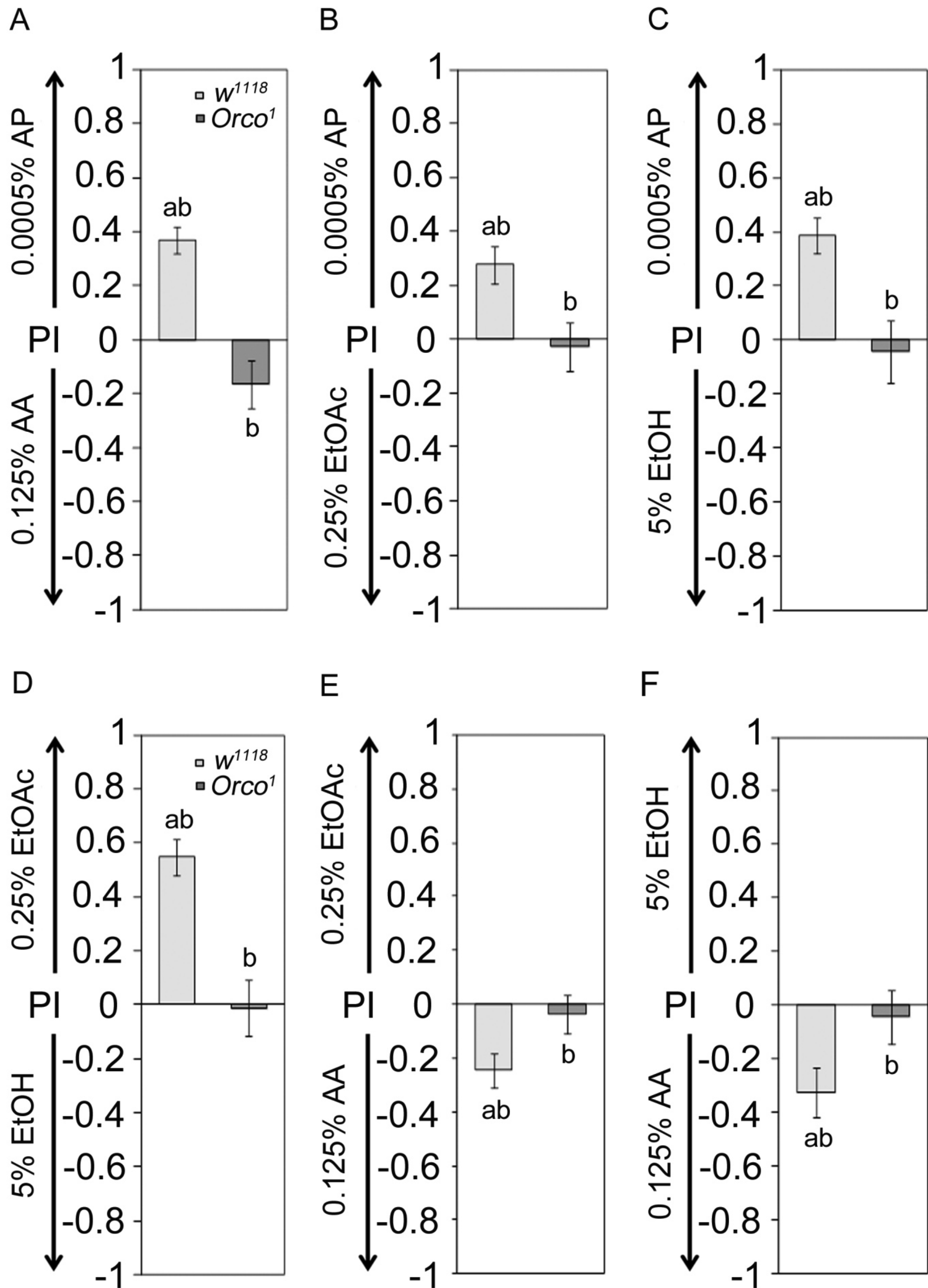


Figure 18. Orco plays a role for odor identity. Single odor comparisons of AP, EtOAc, ethanol and AA at indicated concentrations. **A-F** *w*^{1118} flies distinguish and show preference for one over the other odor in all odor pairings. **A-C** AP was preferred over all other single odors. **C,D,F** Ethanol was in no case preferred. **B,D,E** EtOAc was preferred only when tested against ethanol (D). **A,E,F** AA was only preferred when tested against EtOAc and ethanol. **A-F** *Orco*^{1} mutants fail to distinguish between the odors regardless of the tested odor pairing (PI´s are for *w*^{1118} / *Orco*^{1}: (A) 0.37 ± 0.05 / -0.16 ± 0.09, N=27,19, (B) 0.28 ± 0.07 / -0.03 ± 0.09, N=22,16, (C) 0.39 ± 0.07 / -0.04 ± 0.11, N=18,17, (D) 0.55 ± 0.0 / -0.01 ± 0.10, N=15,13, (E) -0.24 ± 0.06 / -0.04 ± 0.07, N=20,25, (F) -0.33 ± 0.09 / -0.04 ± 0.10, N=12,12). The order of preference of *w*^{1118} flies for the tested odors is: AP>AA>EtOAc>ethanol. Significant differences to PI=0 marked by **a** (one sample sign test; *P*<0.05;) and between *w*^{1118} and *Orco*^{1} by **b** (student *t*-test; *P*<0.05*)*.

Still it could be that stimulation of AP receptor expressing OSNs leads to inhibition of receptor neurons mediating perception of the other tested odors. But it has been shown that AP odor stimulation does not lead to inhibition of EtOAc sensing OSNs and vice versa indicating that a model of inhibition is unlikely (Hallem and Carlson, 2006; Kreher *et al.*, 2008). For AA and ethanol this might still be possible. Another explanation could be that odor identity is linked to attractiveness, although if this is predetermined or shaped by prior exposure is difficult to dissect. If it is predetermined, it is surprising that although ethanol is emitted from food sources ethanol was the least attractive odor. Since 5% ethanol is seldom found in nature 3% ethanol was tested against 5% ethanol and 0.125% AA separately (suppl. Fig.1). Interestingly no preference for *w*^{1118} could be observed when 3% ethanol was tested against 5% ethanol and 3% ethanol was tested against 0.125% AA. The results suggest that odor concentrations of the same odor in a narrow range are perceived as identical while compared to a different odor exhibit a changed attractiveness. This shows that odor identity and odor concentration shape olfactory preference. In contrast *Orco*^{1} mutant flies did not prefer one odor over the other in any of the pairings and differed significantly from the *w*^{1118} flies (p<0.05; Fig.18). This indicates that *Orco* dependent olfaction is necessary to distinguish between two single odors at attractive concentrations. Furthermore it could be that the loss of *Orco* results in a loss or mis-localisation of odor specific receptors and the flies therefore fail to recognize the odor identity although the tested odors alone could be perceived as shown before (see Fig.16). This shows that single odor preference depends on odor intensity and identity.

Taken together, *w*^{1118} control flies can distinguish between odors of similar attractiveness while the preference is clearly concentration dependent. The choice is likely due to the ability to distinguish between odor identities. *Orco*^{1} mutants fail at

this task, which might be due to miss-localized odor specific receptors and thus the odors have likely no distinguishable identity anymore. However, the odor value or identity might be dependent on the odor background which is complex in natural environments.

3.1.7 Odor recognition within food odor mixtures depends on food odor concentration

Single odors usually do not exist in nature. Therefore differences in complex odor mixtures might influence odor evaluation and identification. In order to test if odor mixtures influence the recognition of single odors in an odor mixture by odor identity, food odor containing EtOAc, AA or AP were tested against ethanol in food odor (Fig.19A-C). If the odor background holds no meaning, ethanol should not be preferred.

w^{1118} control flies showed no preference when ethanol was tested against AA, or AP in the food odor background (Fig.19A and B), indicating that maybe the odor complexity is more important than the single odor identity. The single odors can clearly be sensed in food odor as can be seen in the dose dependency experiments (Fig.13C and Fig.14). This means that the odor complexity gains prominence over the odor identity if it is identical in odor component number on both sides. As expected *Orco*^{1} mutants showed no preference since they are likely unable to discern odor identities and thus the odors are perceived as identical. Surprisingly w^{1118} flies preferred the ethanol containing food odor mixture over the EtOAc containing mixture (Fig.19C). This finding conflicts with the idea that the odor complexity decides the preference. It could mean that depending on the added odor the resulting mixture has a different overall identity and that the ethanol containing mixture identity is more attractive. In contrast *Orco*^{1} mutants showed no preference further supporting the necessity of Orco to distinguish odor identities.

Represented as single odors EtOAc was strongly preferred over ethanol (see Fig.18D). Reduction of the food odor intensity while keeping the single odor concentrations stable should result in a switch of preference from ethanol to EtOAc. To that end ethanol and EtOAc were tested against each other with 10-fold, 100-fold and 1000-fold reduced food odor concentration on both sides (Fig.19D).

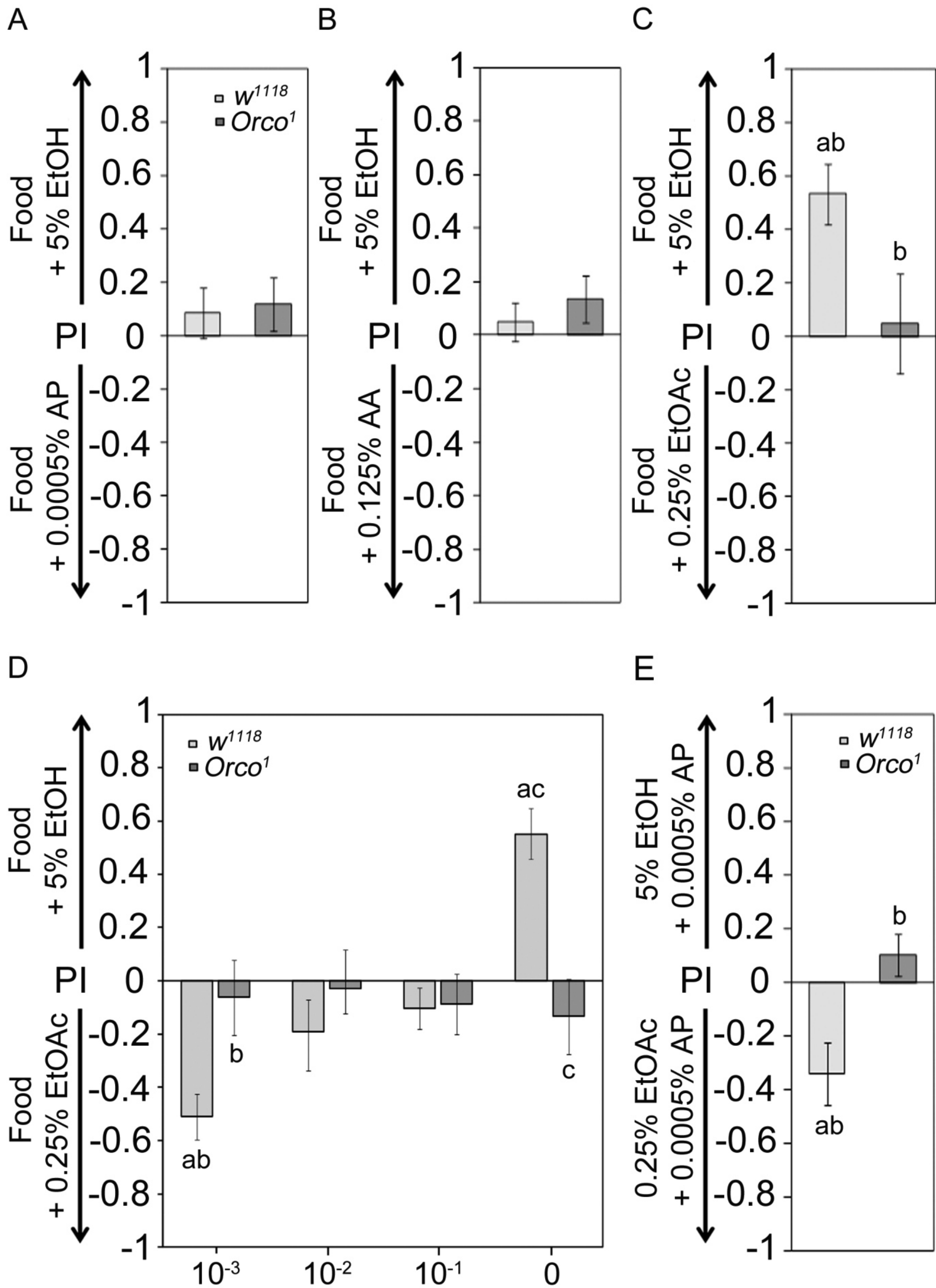

A
B
C
D
E
Food + 5% EtOH
Food + 0.0005% AP
Food + 0.125% AA
Food + 0.25% EtOAc
5% EtOH + 0.0005% AP
0.25% EtOAc + 0.0005% AP
PI
w1118
Orco1
ab
b
ac
c
10-3
10-2
10-1
0

Figure 19. Ethanol perception within a food odor mixture depends on the concentration of the food odor. **A-C** w^{1118} flies can distinguish between food containing ethanol and food containing EtOAc but not when food containing AP or AA is offered. *Orco*^{1} mutants fail to distinguish between odor mixtures regardless of the tested odor pairing (w^{1118} / *Orco*^{1}: **(A)** 0.09 ± 0.09 / 0.12 ± 0.10, N=21,20, (**B**) 0.05 ± 0.07 / 0.14 ± 0.09, N=40,28, (**C**) 0.53 ± 0.11/ 0.05 ± 0.19, N=14,9). **D** w^{1118} flies show preference for food containing 5% ethanol over food containing 0.25% EtOAc. Dilution of food odor leads to no preference at 10- and 100-fold dilution (10^{-1} and 10^{-2}) and aversion at 1000-fold dilution (10^{-3}). *Orco*^{1} mutants show no preference for either ethanol or EtOAc regardless of the food odor dilution (w^{1118} / *Orco*^{1}: (**10^{-3}**) -0.50 ± 0.08 / -0.06 ± 0.14, N=11,10, (**10^{-2}**) **-**0.19 ± 0.12, -0.03 ± 0.14, N=20,19, (**10^{-1}**) **-**0.10 ± 0.07 / -0.08 ± 0.11, N=21,20, (**0**) 0.55 ± 0.10 / -0.13 ± 0.14, N=18,14). **E** w^{1118} flies show aversion for 5% ethanol mixed with $5e^{-4}$% AP when tested against 0.25% EtOAc mixed with $5e^{-4}$% AP. *Orco*^{1} mutants show no preference (w^{1118} / *orco*^{1}: -0.50 ± 0.06, 0.47 ± 0.08, N=23,21). Significant differences to PI=0 marked by **a** (one sample sign test; $P<0.05$;) and between w^{1118} and *Orco*^{1} by **b** (student *t*-test; $P<0.05$).

w^{1118} flies preferred the ethanol containing food odor mixture over the EtOAc containing mixture. 10- and 100-fold dilution of the food odor resulted in no preference for either odor mixture. 1000-fold food odor dilution resulted in aversion for the ethanol containing food odor mixture. These findings show that preference can be switched to aversion by reducing the food odor background intensity. The aversion mimics the result of the single odor comparison of ethanol and EtOAc (see Fig.18). This indicates that the single odor identity and thus the attractiveness are increased by decreasing the odor mixture background but not the odor complexity. Or phrased differently the complex odor mixture might loose identity with decreased concentration and therefore the single odor gets more prominent. As expected *Orco*^{1} mutant flies showed no preference regardless of the food odor dilutions presented most likely due to the inability to sense odor identity.

To investigate whether the observed phenotype depends on the food odor or if a single odor background yields similar change in preference from EtOAc to ethanol, food odor was substituted with AP (Fig.19E). w^{1118} flies preferred the mixture of EtOAc and AP over the mixture of ethanol and AP. This phenotype copies the preference behavior observed when ethanol and EtOAc were tested against each other as single odors (see Fig.18D), indicating that the single odor AP background does most likely not change the identity of ethanol or EtOAc or their binary mixture with AP. This means, that to change the odor identity either food odor and/or its inherent odor complexity is required or a certain level of complexity not reached by the single odor background. Expectedly, *Orco*^{1} mutants showed no preference.

Taken together, Orco is required to sense single odor differences in similar complex food odor mixtures most likely due to failing to distinguish between odor identities. Single odor identity possible changes to an odor mixture identity exhibiting a different attractiveness as the single odor alone. This odor mixture identity does not depend on complexity but odor mixture intensity.

3.2 The role of Dopamine and Octopamine in site-preference

3.2.1 Activation of dopaminergic neurons promotes site-aversion

We previously showed that activation with a 40Hz and 8Hz light stimulation sequence mimicking the firing pattern of the VUMmx1 neuron in the honeybee (Hammer, 1993) of octopaminergic/tyraminergic (OA/TA) neurons using the *Tdc2*-GAL4 line driving expression of the blue light activatable non-selective cation channel Channelrhodopsin-2 (ChR-2) resulted in site-Preference (Schneider *et al.*, 2012). This specific light activation sequence was termed the "Hammer frequency (Fig.20A). Dopamine (DA) has been shown to be involved in both punishment and reward reinforcement and reward for odor memory (Riemensperger *et al.*, 2005; Burke *et al.*, 2012; Liu *et al.*, 2012). Activation of these neurons might therefore lead to site-aversion behavior.

To test this hypothesis the *Th*-GAL4 driver line, containing a promotor element of the Tyrosinehydroxylase, was used to drive expression of ChR-2 in most DA neurons (Friggi-Grelin *et al.*, 2003). The flies also carried the “*no receptor potential A1*” (*norpA^1*) mutation which renders the flies visual behavior defective (Bülthoff, 1982). Flies raised on ethanol treated food were used as controls while flies raised on 250mM all-*trans* retinal (ATR), which acts as the light receptive chromophore in ChR-2, soluted in ethanol treated food were used as experimental flies. The flies were then tested in the optogenetic olfactory site-preference assay using the “Hammer frequency” as a light stimulation protocol (Fig.20B). The control flies showed no site-preference (PI) while the experimental flies showed site-aversion (Fig20B; PI ± SEM: control -0.075 ± 0.094, experimental -0.412 ± 0.099, P<0.05). Thus activation of neurons using the *Th*-GAL4 driver indeed mediates olfactory site aversion. However it is not clear if the observed site-aversion is “Hammer frequency” activation dependent. It is also not clear if site-preference induced by activation of OA/TA

neurons using the *Tdc2*-GAL4 driver line is dependent on the Hammer frequency or can be achieved by other activation frequencies.

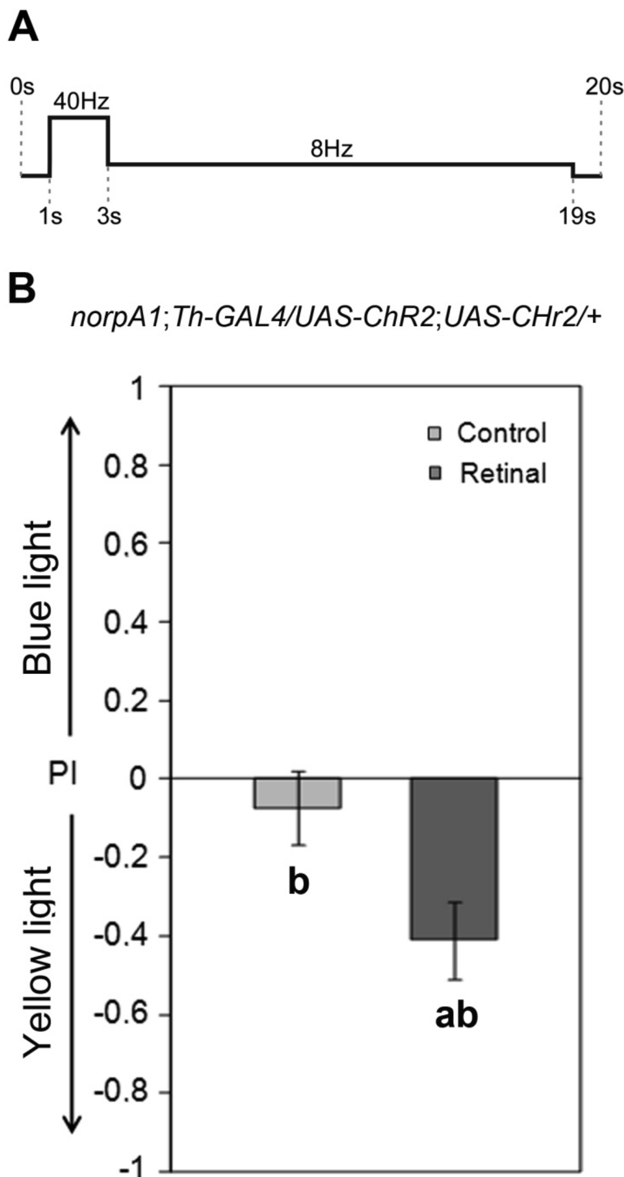
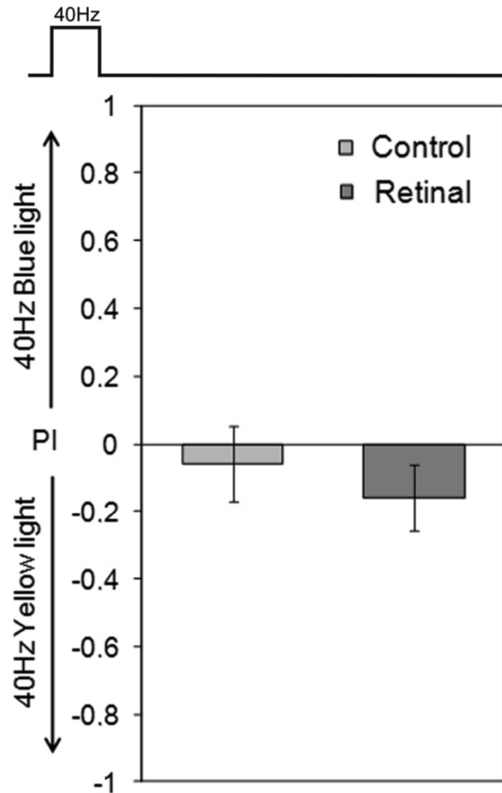


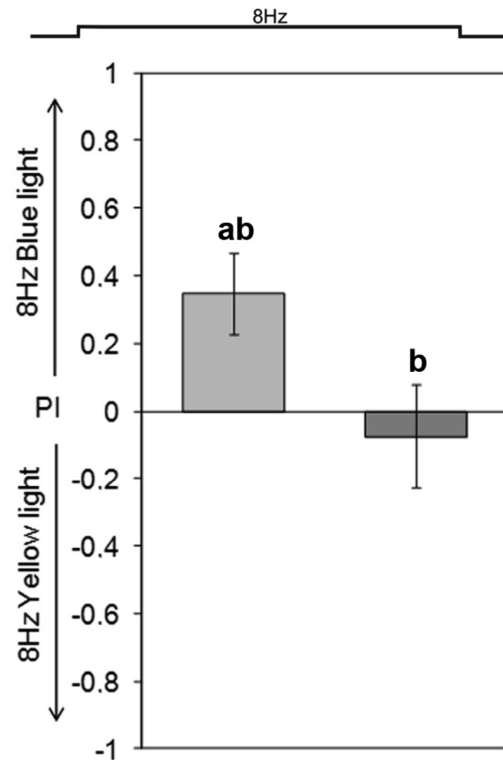
Figure 20. Olfactory site-aversion is mediated by dopaminergic neurons. **A** Depicted is one cycle of the light stimulus sequence (total length: 20s) of the “Hammer frequency”. Base line represents 0Hz and changes in height the frequency switch. **B** *Th*-GAL4 was used to express ChR-2 in dopaminergic neurons. *norpA*[1] mutant background was used to render the flies optically impaired. Control flies (green) showed no site-preference (norpA1;Th-GAL4/UAS-ChR-2;UAS-ChR-2/+: -0.075 ± 0.094, N = 35) while experimental flies (blue) showed blue light site-aversion significantly different to the control (norpA1;Th-GAL4/UAS-ChR-2;UAS-ChR-2/+: -0.412 ± 0.99, N = 20, $P < 0.05$). Significant differences to PI = 0 indicated by “**a**” (one-sample sign test; P<0.05). Significant differences between control and experimental flies indicated by "**b**" (student *t*-test; P<0.05). **B** published in Schneider *et al.*, 2012).

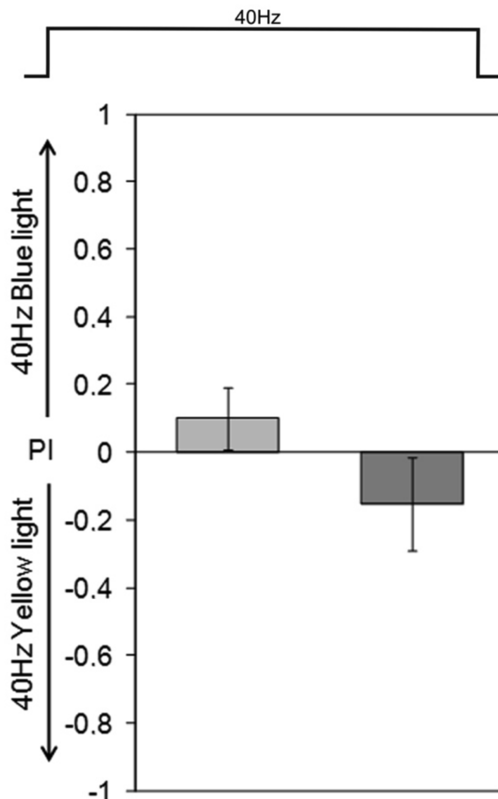
3.2.2 OA/TA neuron stimulation with segments of the “Hammer frequency” fail to induce site-preference

Firing patterns can vary in between neurons or networks of neurons. To test if the observed site-preference behavior by activation of OA/TA neurons using the Tdc2-GAL4 driver line (Schneider *et al.*, 2012) is activation frequency dependent, different illumination frequencies were used in optogenetic site-preference experiments (Fig.21 and 22). The “Hammer frequency” consists of three parts, namely 2s 40Hz, 16s 8Hz and 2s no illumination. Therefore the first part (2s 40Hz) and second part

(16s 8Hz) were used for activation while still maintaining the cycle length of 20s (Fig.21A and B).







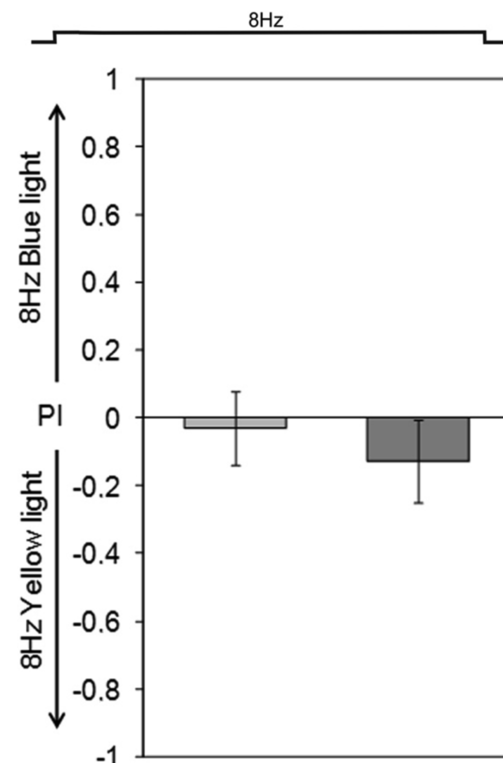


Figure 21. OA/TA neuron stimulation with 40Hz and 8Hz stimulation does not induce site-preference. site-preference in the optogenetic olfactory site-preference assay. The light stimulation sequence per cycle is depicted above the graphs (length: 20s). Genotype used depicted above. **A** Control and Retinal flies (experimental) showed no site-preference when stimulated with the 2s 40Hz frequency (control -0.059 ± 0.111, N = 20; Retinal -0.160 ± 0.098, N = 21). **B** Control flies showed site preference significantly different to experimental flies which showed no site-preference when activated with 16s 8Hz light frequency (control 0.348 ± 0.119, N = 22; Retinal -0.074 ± 0.151, N = 22). **C** Control and experimental flies showed no site-preference when stimulated with 18s 40Hz light frequency (control 0.100 ± 0.092, N = 29; Retinal -0.150 ± 0.138, N =24) experimental flies showed no site-preference (control -0.029 ± 0.109, N = 29; Retinal -0.128 ± 0.121, N = 20). Significant differences to PI=0 indicated by "**a**" (one-sample sign test; *P*<0.05). Significant differences between control and experimental flies indicated by "**b**" (student *t*-test; *P*<0.05).

2s 40Hz activation did not induce site-preference nor aversion for both control and experimental flies (Fig.21A). Extending the duration of the activation did not change the behavior (Fig.21C) indicating that frequency or duration of a 40Hz activation pattern is not sufficient for preference.

Surprisingly blue light illumination with 16s 8Hz elicited site-preference in control flies suggesting that the light by itself has an effect (Fig. 21B; $P < 0.05$). It could be that depending on the light stimulus frequency the light intensity needs to be adjusted to abolish effects on the control flies. The experimental flies showed no preference indicating that the activation frequency does not induce preference. Still if 16s 8Hz blue light illumination would normally induce preference without neuronal activation, then activation of neurons with this frequency reduced the preference to no preference. This reduction could possibly be aversion. Extending the duration of the 8Hz stimulation did not induce site-preference for both control and experimental flies (Fig.21D) indicating that the duration of 8Hz activation is not sufficient to induce site-preference.

Taken together the two frequencies contained in the Hammer frequency were in frequency and duration not sufficient to induce site-preference. This means that other activation characteristics have to hold the key for site-preference induction.

3.2.3 Site-preference mediated by OA/TA neurons depends on the activation frequency

Another possibility responsible for mediating site-preference could be the number of light pulses during the "Hammer frequency". To test whether this is true, 18s 11.5Hz activation was applied to *norpA^{1};Tdc2-GAL4/UAS-Chr2;UAS-ChR2/+* flies, since that frequency gives the same number of blue light pulses as the "Hammer frequency"

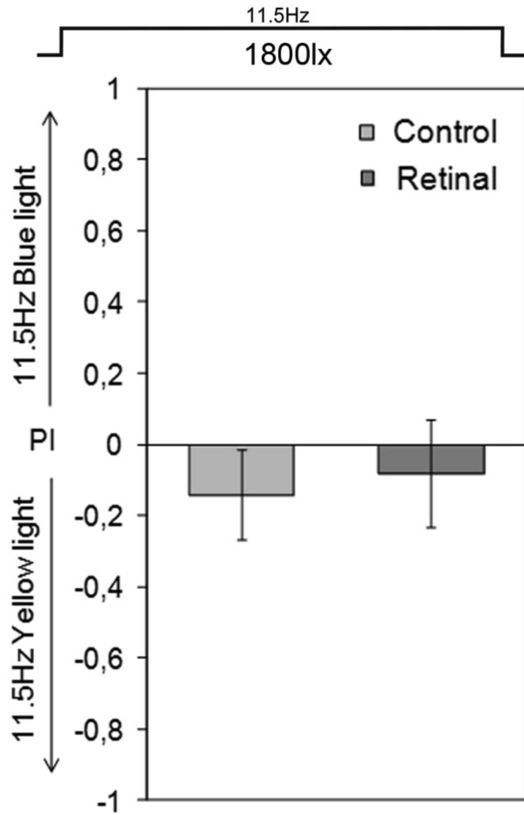
(Fig.22A). Both control and experimental flies showed no site-preference indicating that the number of pulses alone is not the key to drive site-preference behavior.

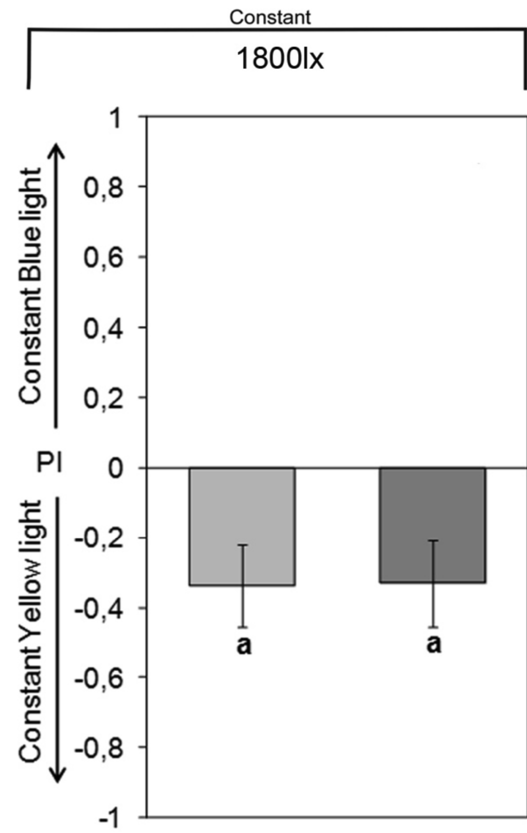
The important neurons might also be on and off neurons, meaning that no specific frequency is required apart from turning the OA/TA neurons maximally on or off. To test this, the flies were exposed to constant illumination without pause (Fig.22B). Control and experimental flies showed site-aversion to a similar degree indicating that constant blue light illumination by itself is aversive for the flies regardless of neuronal activation. Possible effects of neuronal activation on the preference behavior could be masked by the aversion phenotype.

The experimental setup allows only a 50% duty cycle for illumination, meaning that for e.g. with a 1Hz frequency there is 0.5s blue light illumination and 0.5s no illumination per second. This means that constant illumination results in more than double the amount of illumination since there is no 2s pause. In order to make the constant light experiments comparable to the frequency experiments, the light intensity has to be similar, meaning the energy delivered has to be the same. To that end the lux (lx) was adjusted from previously 1800lx to 720lx per diode.

The control flies did not show any preference while the experimental flies still showed aversion when 72lx light intensity was used ($p<0.05$; Fig.22C). This indicates that reduction of the light intensity could remove the blue light effect on the control flies. Since the experimental flies still show site-aversion, activation of OA/TA neurons with constant light leads to site-aversion. This indicates that a specific frequency is indeed needed for site-preference. However, why constant activation of OA neurons is aversive is unclear.

Taken together, none of the tested frequencies could elicit site-preference independent of frequency, duration, and number of pulses. Interestingly constant neuronal activation with the same intensity caused aversion. However the switch between frequencies as is the case form 40Hz to 8Hz in the Hammer frequency was not investigated here and could potentially be the important factor.


A norpA1;Tdc2-GAL4/UAS-ChR2;UAS-ChR2/+
11.5Hz
1800lx
Control
Retinal
11.5Hz Blue light
11.5Hz Yellow light
PI
1
0,8
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
-0,8
-1


B
Constant
1800lx
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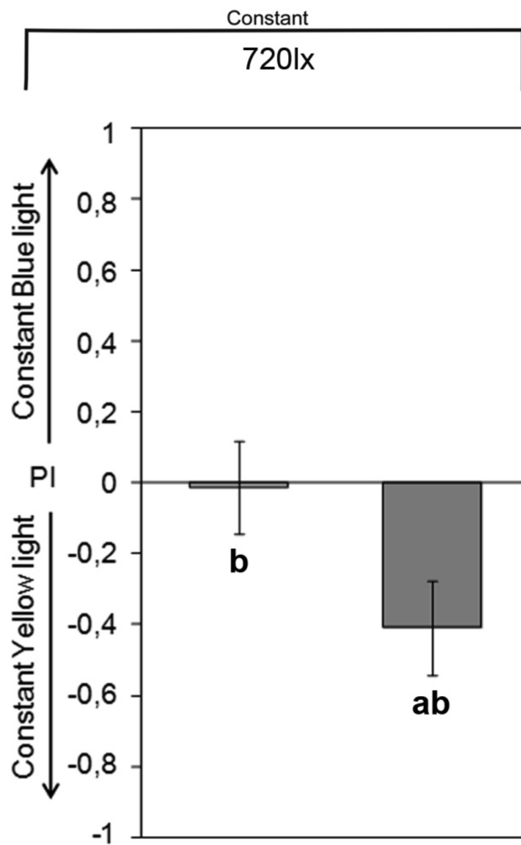
C
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720lx
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ab
1
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0,4
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0
-0,2
-0,4
-0,6
-0,8
-1

Figure 22. Constant activation and activating pulse number to induce OA mediated site-preference. A Control and experimental flies showed no site-preference when stimulated with 18s 11.5Hz light frequency (control -0.142 ± 0.127, N = 24, Retinal -0.081 ± 0.151, N = 16). **B**. Control and experimental flies showed site-aversion when stimulated with constant light at 180olx (control -0.339 ± 0.124, N = 16, Retinal -0.331 ± 0.134, N = 16). **C** Control flies showed no site-preference, while Retinal flies showed site-aversion when stimulated with constant light at 720lx (control -0.013 ± 0.129, N = 16, Retinal -0.408 ± 0.134, N = 16). Significant differences to PI=0 indicated by "**a**" (one-sample sign test; *P*<0.05). Significant differences between control and experimental flies indicated by "**b**" (student *t*-test; *P*<0.05).

3.3 The role of Dopamine and Octopamine in modulating locomotion

3.3.1 Optogenetic activation of dopaminergic neurons induces locomotion in adult *Drosophila melanogaster*

Dopaminergic neurons (DA) have been shown to innervate the central complex and more specifically the ellipsoid body (EB) a structure known to be involved in locomotion (Kong *et al*., 2010). Furthermore, direct activation of these neurons leads to changes in locomotion. To confirm that DA neurons are indeed involved in locomotion in the adult fruit fly and validate the optogenetic locomotion setup developed during the thesis an optogenetic approach was used. To drive expression of ChR-2 in most DA neurons throughout the adult CNS the *TH*-GAL4 driver line was utilized. The neurons were then depolarized through intense blue light exposure using the 2s 40Hz and 16s 8Hz blue light activation frequency ("Hammer Frequency") while the flies movement was documented. Control flies were not fed with ATR so ChR-2 cannot be activated.

Blue light exposure with the "Hammer Frequency" leads to an increase in locomotion in the control flies hinting at effects due to the blue light alone (Fig.23). This means that even though the flies are optically impaired (*norpA*^{1} mutation) they have other means to sense the blue light exposure. These could possibly be heat sensors like TRPs (transient receptor potential) or blue light sensitive photopigments involved in circadian rythmicity and arousal called cryptochromes (Cry; Emery *et al*., 2000; Kumar *et al*., 2012).

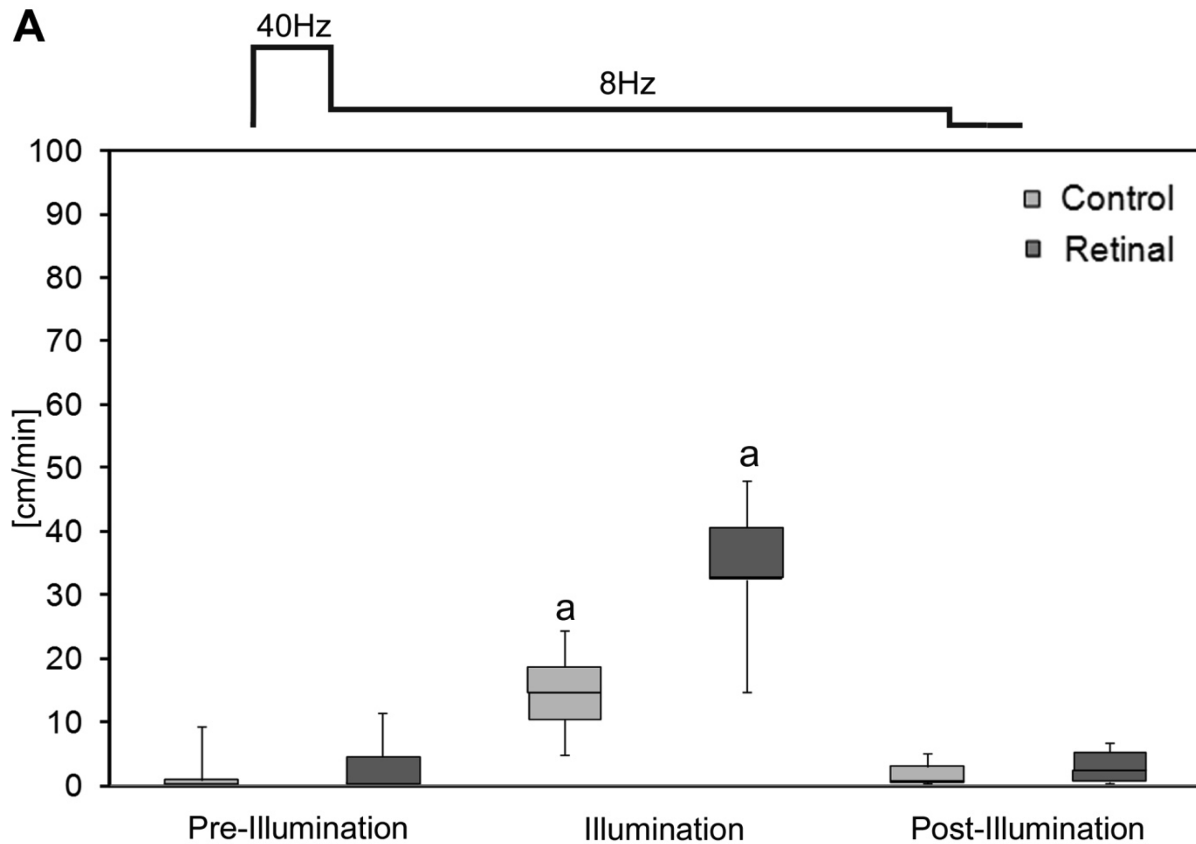


Figure 23. Neuronal activation of DA neurons with the "Hammer Frequency" increases locomotor output. A Male *norpA[1];UAS-ChR-2/Th*-GAL4*;UAS-ChR-2/*+ flies (control: green; experimental: blue) tested in the optogenetic locomotion setup. Three cycles of the "Hammer Frequency" (1 depicted above) were used to activate DA neurons using blue light. The flies movement was documented for 1min prior (Pre-Illumination) to blue light illumination, 1min during illumination and 1min after illumination (Post-Illumination). Locomotor output was measured in cm per min. Experimental flies have a significantly increased locomotor output during the Illumination phase compared to the control flies (*P*=0.007; Control/Retinal in cm/min Mean ± SEM: Pre 1.66 ± 3.43/3.04 ± 4.41, Illu. 14.60 ± 7.12/34.51 ± 4.00, Post 1.89 ± 2.11/3.05 ± 2.74, N=7/7). Significant differences between the control and experimental groups are indicated by "**a**" (Mann-Whitney U-test; *P*<0.05).

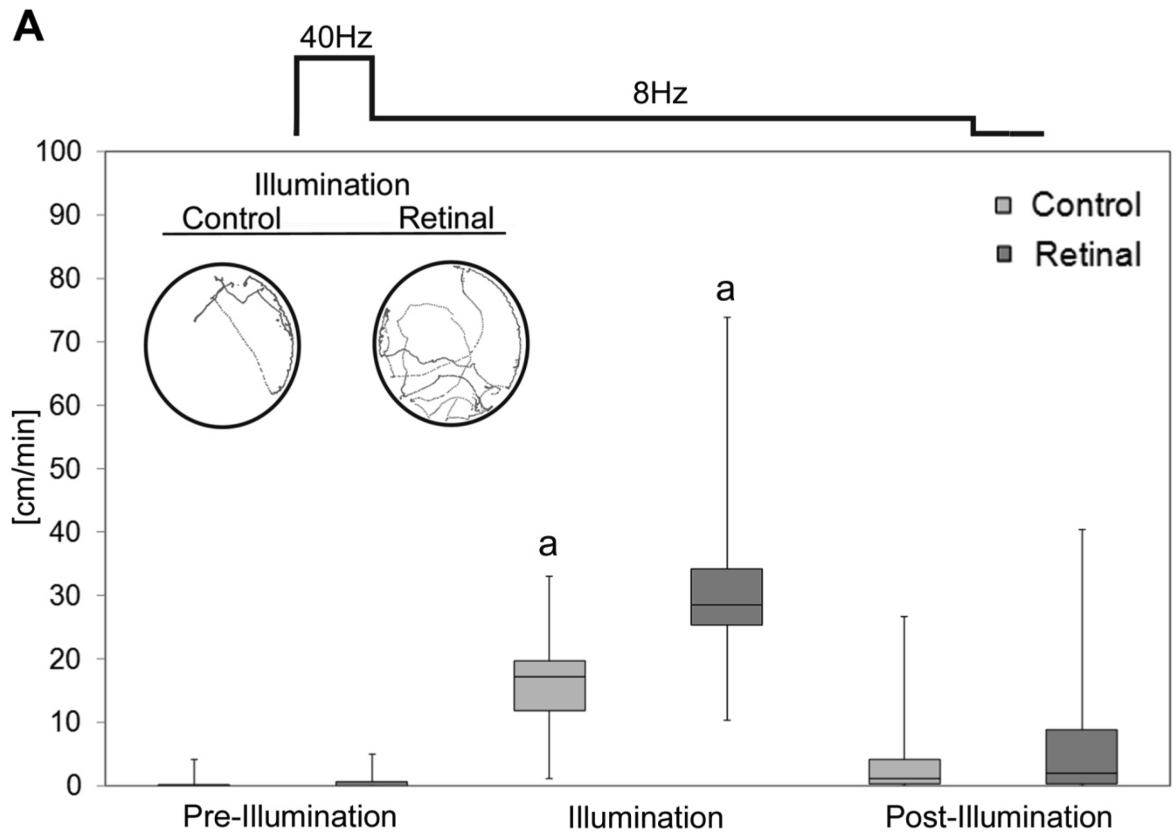
Photoactivation of DA neurons leads to a significant increase in locomotion during illumination in experimental flies compared to the control flies. This indicates that stimulation of DA neurons with the "Hammer Frequency" induces an increase in locomotion. Therefore DA neurons are indeed involved in locomotion. However it is not clear whether a specific activation frequency is needed to increase the locomotor output. Furthermore OA has been suggested to act functionally upstream of DA neurons in appetitive motivation and this might also be the case for locomotion (Burke *et al.*, 2012).

3.3.2 OA/TA neuron stimulation with the “Hammer Frequency” increases locomotor output

Preference behavior can be induced by the “Hammer Frequency” activation of OA/TA neurons (Schneider *et al.*, 2012). Since execution of directed movement is part of preference behavior and requires locomotion it is possible that activation of OA/TA neurons also induces locomotion. Furthermore it has been suggested, that OA neurons might act functionally upstream of dopaminergic neurons and there are DA neurons known to innervate the EB, a central complex structure involved in motor activity (Kong *et al.*, 2010; Burke *et al.*, 2012). Therefore it is possible that OA/TA neurons influence locomotion through DA neurons. To test whether activation of OA/TA neurons using three cycles of the “Hammer Frequency” influences locomotion the optogenetic locomotion assay was used with male *norpA^{1};UAS-ChR-2/Tdc2-*GAL4*;UAS-ChR-2/+* flies. Flies raised on food treated with ethanol are used as controls and flies raised on 250mM ATR treated food as experimental flies. Single flies are tested at a time and only flies that move less than 5cm during the Pre-Illumination phase are included in the data analysis.

The control flies show an increase in locomotion due to the blue light exposure indicating a neuronal activation independent of locomotion response as observed in the previous chapter (Fig.23A). In the Post-Illumination phase the locomotor output decreases to Pre-Illumination levels showing the blue light dependency of the increased locomotion.

Photoactivation of OA/TA neurons with the “Hammer Frequency” results in significantly increased locomotor output of the experimental flies (Retinal) compared to the control flies (P=0.001; Fig.24A). In addition, the increase in movement does not lead to prolonged locomotor activity in the Post-Illumination phase indicating short term modulation of the locomotor behavior. The walking path in the circular arena does not follow any specific pattern but rather random movement (Fig.24A).


A
40Hz
8Hz
Illumination
Control
Retinal
Control
Retinal
a
a
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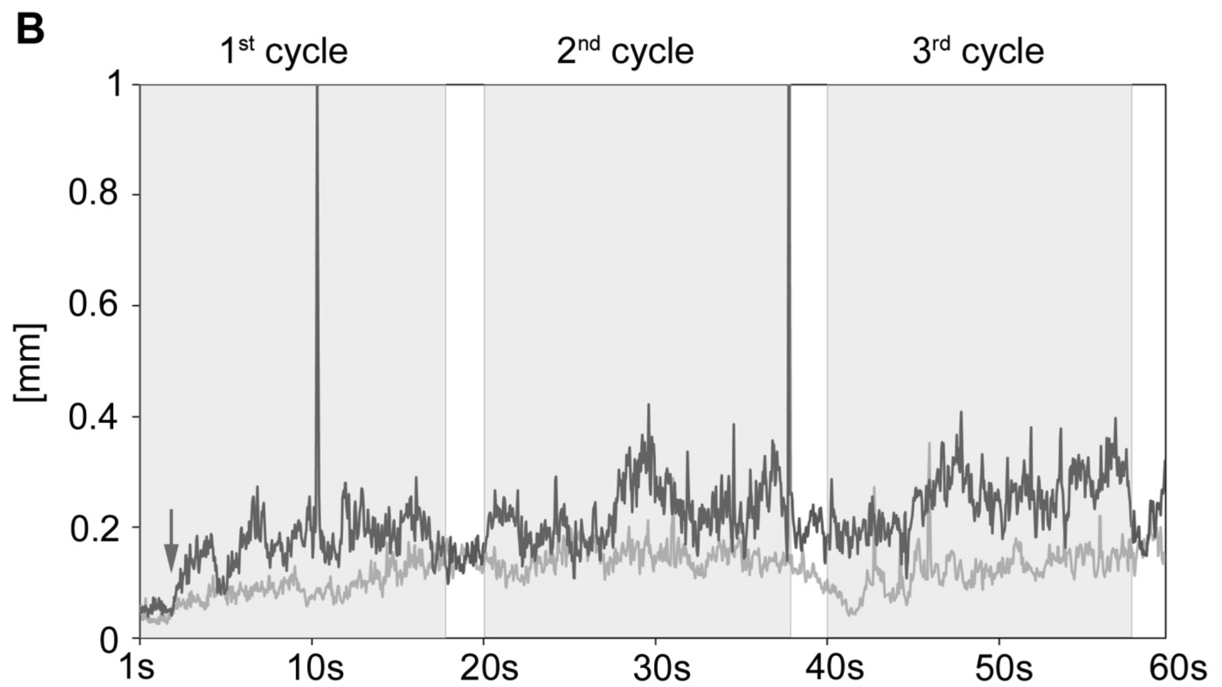
100
90
80
70
60
50
40
30
20
10
0
Pre-Illumination
Illumination
Post-Illumination


B
1st cycle
2nd cycle
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10s
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30s
40s
50s
60s

Figure 24. Neuronal activation of OA/TA neurons with the "Hammer Frequency" increases locomotor output. A Male *norpA[1];UAS-ChR-2/Tdc2*-GAL4*;UAS-ChR-2/*+ flies (control: green; experimental: blue) tested in the optogenetic locomotion setup. Three cycles of the "Hammer Frequency" depicted above the diagram (1 cycle shown) were used to activate OA/TA neurons using blue light. The flies movement was documented for 1min prior (Pre-Illumination), 1min during and 1min after to blue light illumination (Post-Illumination). Locomotor output was measured in distance covered per min (cm/min). Only flies with movement ≤5cm/min during the Pre-Illumination phase were included in the data. Exposure to 2s 40Hz and 16s 8Hz significantly increased the distance covered by experimental flies compared to the control flies during the Illumination phase (*P*=0.001; Control/Retinal in cm/min Mean ± SEM: Pre 0.32 ± 0.21/0.69 ± 0.33, Illu. 16.28 ± 1.71/30.68 ± 3.66, Post 3.92 ± 1.51/6.53 ± 2.71, N=19/16). Overall walking path during Illumination phase shown in the top left. **B** The mean distance covered in between time points (measured in mm every 40ms) during the 1min blue light Illumination phase for control and experimental flies (same color code as above). Experimental flies show higher distances covered in between time points during illumination. Slight decrease in movement is visible in the 2s without illumination of each cycle. Significant differences between the control and experimental groups are indicated by "**a**" (Mann-Whitney U-test; *P*<0.05). Pooled data from A. Klein (2013) and TG.

To investigate if there are specific activity bouts and how the 2s without illumination during the "Hammer Frequency" illumination affects the flies locomotor output, the mean distances traveled over the time course of the Illumination phase are plotted against time for control and experimental flies (Fig.24B). The locomotor output for both control and experimental flies increases after a delay of 2s past the beginning of blue light exposure (red arrow). The increase in locomotion is stronger in experimental than in control flies and the level of locomotor output is also mostly higher in experimental flies throughout all Illumination phases while the control flies seem to maintain their locomotion level. Control flies show no specifically timed strong decrease in locomotion after the Illumination phase which might be because their locomotion level throughout the experiment was overall lower. A strong decrease in locomotion of the experimental flies is visible at the end of the third cycle of illumination which could be due to the exhaustion following the forced increase in movement by the neuronal activation.

Taken together, blue light exposure by itself induces locomotion independent of neuronal activation maybe due to heat sensors or blue light sensitive photopigments. Neuronal activation of OA/TA neurons using the "Hammer Frequency" induces increased locomotion. The difference in overall locomotion between control and experimental flies is likely due to consistent higher levels of locomotion and not to short high activity bouts. Therefore OA/TA neurons firing with the "Hammer Frequency" are involved and can increase locomotor output. Still analysis of the locomotion during neuronal activation with the "Hammer Frequency"

with higher resolution could show when and how the difference in locomotor activity between control and experimental flies occurred.

To that end the locomotor activity during the Illumination phase is investigated in more detail (Fig.25). The sum of the distances traveled during each cycle of the "Hammer Frequency" illumination pattern separated in the blue light illumination (illu.; 18s) and pause (P.; 2s) segment is investigated (Fig.25A).

The control flies show roughly constant distance covered between the illumination segments and pause segments of the three cycles. This indicates that the response to the blue light exposure is constant for at least 1min. The experimental flies have significant higher locomotor output during all illumination phases but not during the pause phases compared to the control flies. This indicates that the observed increase in locomotion is due to the illumination phases and thus during neuronal activation.

To further increase the resolution of the analysis, the first "Hammer Frequency" illumination cycle (20s) was split in ten 2s bins allowing to visualize and compare the distances covered in smaller time fragments (Fig.25B). There is no significant difference between control and experimental flies in the first three bins up to 6 seconds after the beginning of the illumination. From that time point onward the experimental flies show significantly higher locomotor activity than the control flies. The increased activity of the experimental flies lasts until 16 seconds into the illumination phase from where on till the end of the cycle there is no significant difference to the control flies anymore. Overall the locomotor activity seems to increase in control flies over time, while the experimental flies have a locomotor activity pattern resembling an inverted u-shaped profile (polynomic curves in broken lines). This could be due to neuronal activity saturation or adaptation to the neuronal activation.

The observed increase in locomotion of experimental flies does not occur at the change from 40Hz to 8Hz illumination and neither does the switch from 8Hz to no illumination have any apparent effect. Rather the increase happens during the 8Hz stimulation segment and more precisely 4 seconds past the beginning of the 8Hz stimulation. This indicates that the increase in locomotion by neuronal activation is linked to the 8Hz stimulation in a delayed fashion and not as an immediate effect

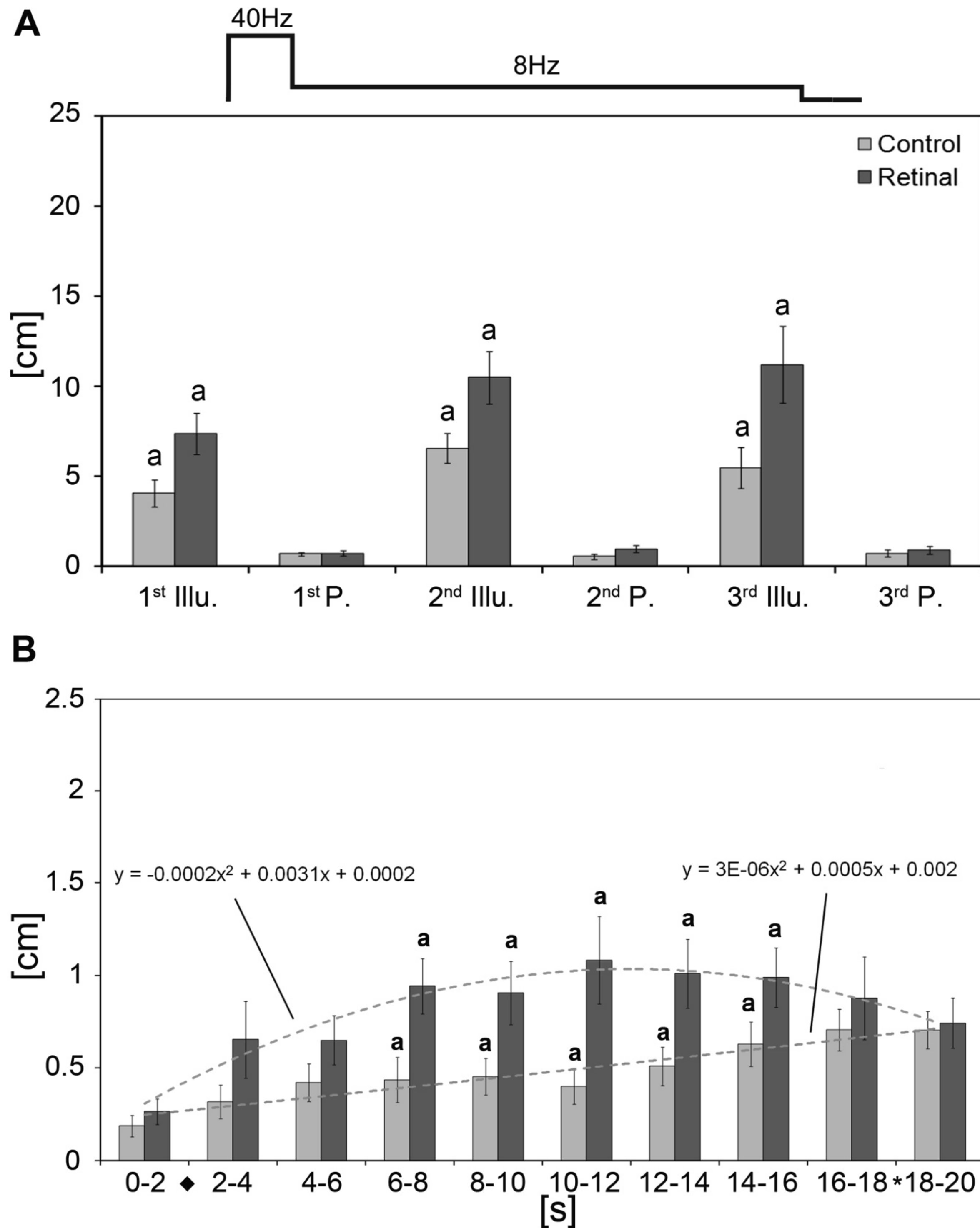


Figure 25. Locomotor activity increase is not stable. A Sum of the distances traveled in cm during the three cycles of the Illumination phase using the “Hammer Frequency”. Experimental flies have significantly increased locomotor output during the Illumination phases compared to control flies. **B** The mean distance the flies traveled in cm binned in two seconds each over the first illumination cycle. Experimental flies covered significantly more distance than the controls in the bins from 6 to 16 seconds of illumination (*P*<0.05; Data summarized in suppl. Table 2). Polynomic curves indicate the trend. Change from 40Hz to 8Hz indicated by diamond and from 40Hz to no illumination by asterisk. Significant differences between the control and experimental groups are indicated by "**a**" (Mann-Whitney U-test; *P*<0.05).

pointing to a modulatory role for OA/TA neurons in locomotion as has been suggested in previous studies (NMJ in larva; Monastirioti *et al.*, 1995). However whether the observed increase in locomotion is "Hammer Frequency" dependent as could be shown for site-preference or this phenotype can be induced using other frequencies or segments of the "Hammer Frequency" is unclear.

3.3.3 Locomotor output depends on OA/TA neuron activation with a specific frequency

It is unclear which part of the "Hammer Frequency" activation pattern leads to increased locomotion or if this phenotype depends on specific activation patterns at all. To investigate whether there is OA/TA neuronal activation pattern dependency for locomotion increase OA/TA neurons were activated with the frequency segments of the "Hammer Frequency" separately.

OA/TA neuron stimulation with three cycles of 2s 40Hz frequency followed by 18s no illumination each does not lead to increased locomotor output in experimental flies compared to the control flies (Fig.26A). There was no significant difference in locomotion in all phases of the experiment between control and experimental flies. This indicates that 2s 40Hz stimulation of OA/TA neurons is insufficient to induce an increase in locomotion or this segment of the "Hammer Frequency" does not play a role in locomotor activity induction. Both show an increase in locomotion during the Illumination phase although non-significantly different to the pre- and post-Illumination phases.

The activity pattern during the three cycles of the Illumination phase is also not different between control and experimental flies (Fig.26B). Clearly visible is the roughly 2s delay of the blue light induced locomotion increase. This indicates that the time period of 2s blue light illumination is likely too short to allow visible effects of neuronal activation, since movement could only be observed after this delay also in case of stimulation with the "Hammer Frequency" (see Fig.24B).

Control and experimental flies show a rapid burst of locomotor activity at the offset of the blue light stimulation with a peak activity at around 1-2s past the illumination. This activity decays more slowly back to pre illumination levels at around 8s past the illumination. This activity pattern followed all three illumination cycles to a

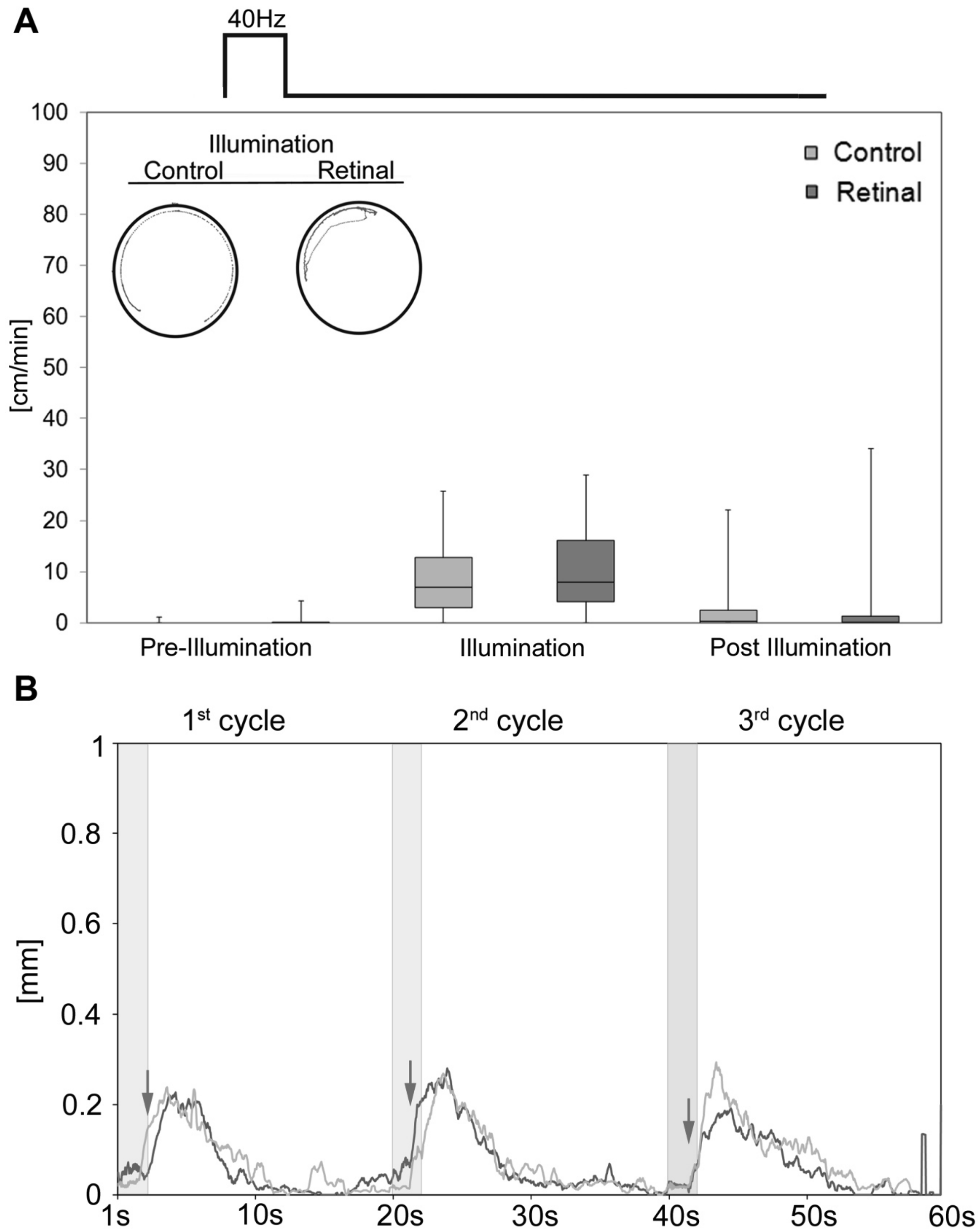


Figure 26. OA/TA neuron activation with 2s 40Hz frequency does not affect locomotor output. A *norpA¹;UAS-ChR-2/Tdc2*-GAL4*;UAS-ChR-2/+* flies tested in the optogenetic locomotion setup. OA/TA neurons were activated with three cycles of 2s 40Hz frequency (depicted above). Locomotor output was measured in distance covered per min (cm/min). 2s 40Hz frequency activation does not significantly alter the locomotor output between control and experimental flies (Control/Retinal in cm/min Mean ± SEM: Pre 0.16 ± 0.44 /0.53 ± 1.24, Illu. 10.48 ± 9.21/10.07 ± 8.41, Post 4.65 ± 10.22/4.73 ± 10.25, N=20/18). Overall walking path during Illumination phase shown in the top left. **B** The mean distance covered in between time points during the 1min blue light Illumination phase for control and experimental flies. Illumination induces both control and experimental flies movement to a similar degree with locomotor activity delay of about 2s (red arrows). Significant differences between the control and experimental groups analyzed by Mann-Whitney U-test (*P*<0.05).

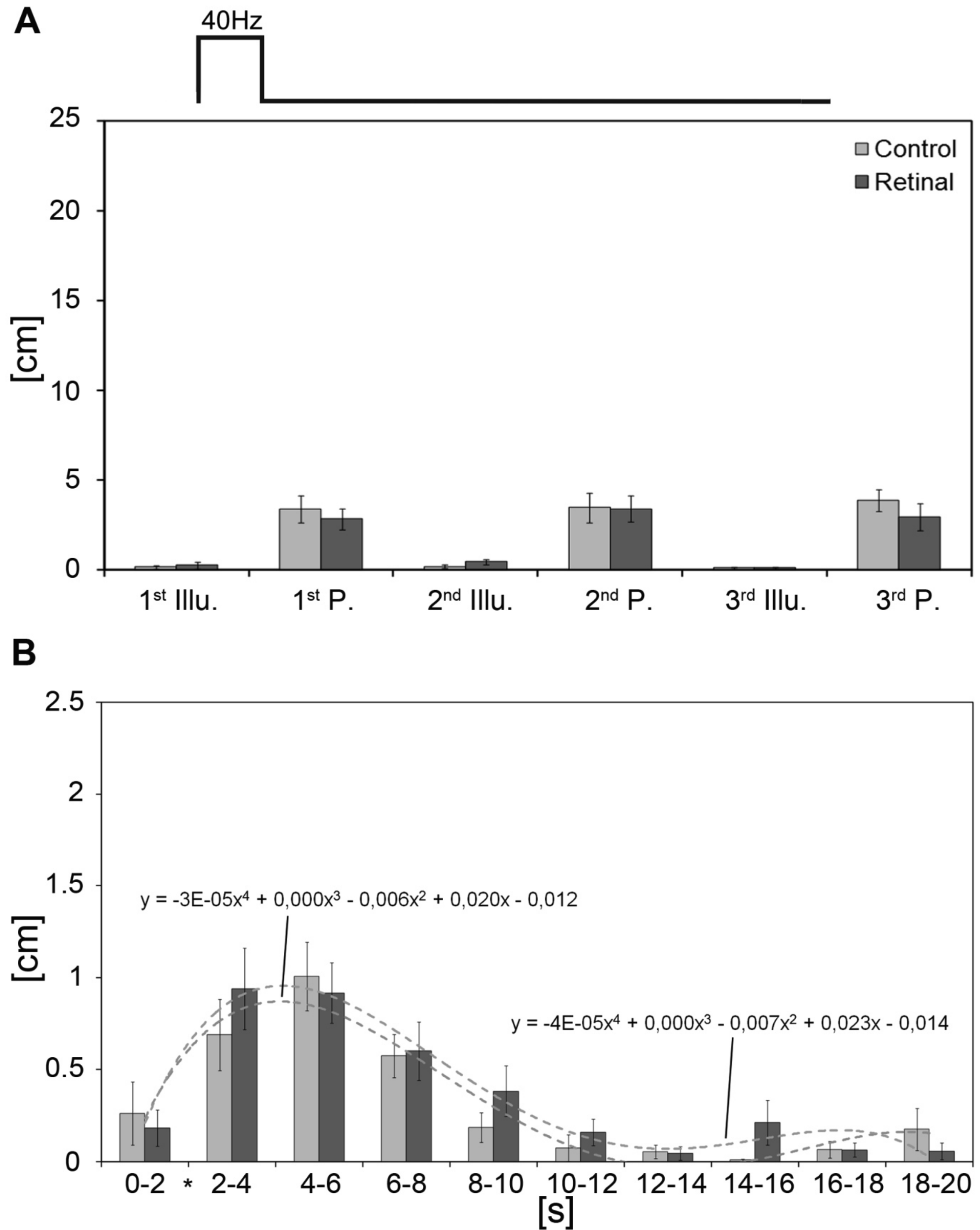


Figure 27. OA/TA neuron activation with 2s 40Hz frequency does not affect locomotion. A Sum of the distances traveled in cm during the three cycles of the Illumination phase using 2s 40Hz activation frequency. Control flies and experimental flies show no difference in locomotion in illumination and pause phases. **B** The mean distance the flies traveled in cm binned in two seconds each over the first illumination cycle. Control and experimental flies show no significant difference ($P<0.05$; raw Data shown in suppl. Table 3). Change from 40Hz to 0Hz indicated by a asteriks. Significant differences verified by Mann-Whitney U-test ($P<0.05$).

similar degree resembling a startle response to the blue light independent of neuronal activation.

The distances traveled during the 2s illumination and the pauses in illumination do not significantly differ between control and experimental flies (Fig.27A). As expected the locomotor activity in the pause phases is higher than in the illumination phase, since the activity burst started with the offset of the illumination. Analysis of the activity pattern in the first illumination cycle also reveals no difference between control and experimental flies (Fig.27B). These data clearly show the OA/TA neuron stimulation with 2s 40Hz frequency alone is not responsible for increases in locomotion.

OA/TA neuron stimulation with 16s 8Hz leads to significantly increased locomotion in experimental flies compared to control flies (Fig.28A). This indicates that OA/TA neuron stimulation with the 16s 8Hz frequency segment of the “Hammer Frequency” induces an increase in locomotion. The flies show base line locomotion levels during the Post-Illumination phase showing that the increase in locomotion is not a lasting effect. The walking pattern during the stimulation shows no specific pattern.

Control flies show the typical activity delay of around 2s upon the beginning of illumination (Fig.28B). In addition the increase in locomotor activity of control flies persists until the offset of the illumination where the locomotion decreased to a lower level after the first cycle and to base line levels after the second cycle. Still the level of locomotor activity is on similar level in all three cycles. This indicates that the response to the blue light is constant over the cycles and lasts until offset of the blue light illumination showing that the flies are not only startled. The experimental flies show a rapid increase in locomotion upon blue light exposure but with a shortened delay by roughly 1s at the beginning of the first illumination cycle (red arrow). The level of locomotion is comparable to that of the control flies but does not decrease as strongly during the phases of no illumination. The delay before the increase in locomotion is still visible even though the overall activity did not decrease as strongly as in the control flies. In addition the increase in locomotion is not as strong as in the control flies in between the cycles. This is likely due to the already higher levels of locomotor activity. Overall the locomotion levels of the experimental flies seem more constant at higher levels persisting through the no illumination phase for the experimental flies. The difference in overall distance traveled during the Illumination

phase is very slightly (*P*=0.03) and likely due to less decrease in activity during the pauses in illumination compared to the control flies. This indicates that OA/TA neuron stimulation with 16s 8Hz does not lead to increased locomotion during stimulation but rather to prolonged increases in locomotor activity after blue light exposure lasting for at least 4s.

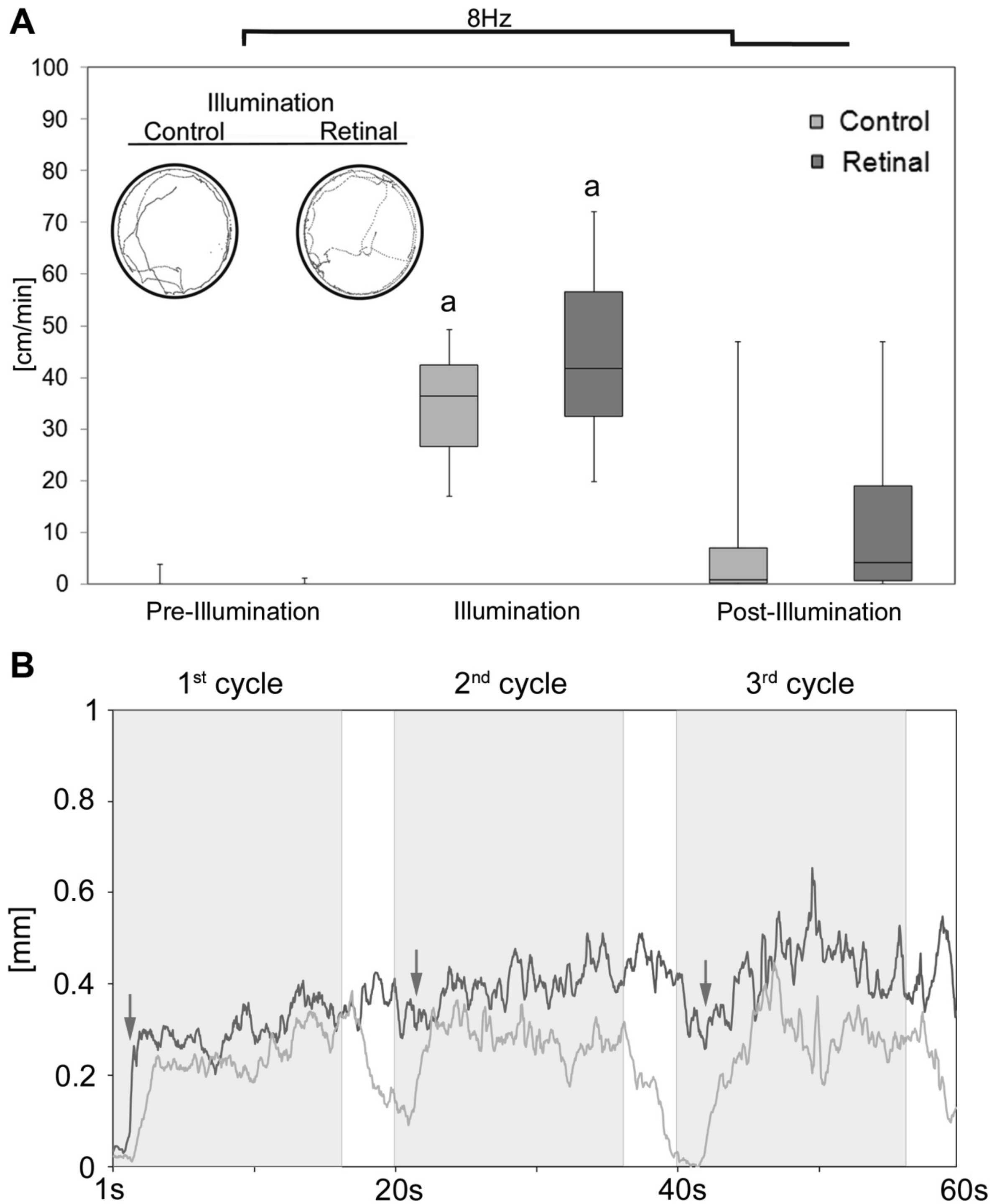
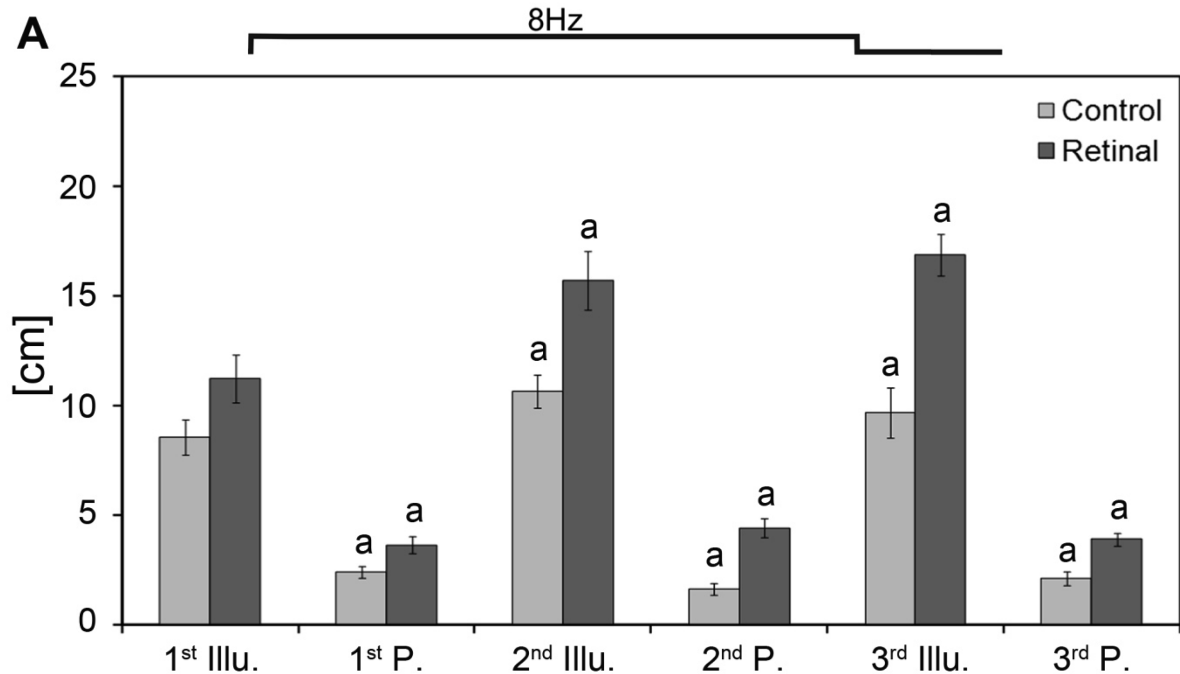


Figure 28. OA/TA neuron activation with 16s 8Hz frequency increases locomotor output. A *norpA[1];UAS-ChR-2/Tdc2*-GAL4*;UAS-ChR-2/*+ flies tested in the optogenetic locomotion setup. OA/TA neurons were activated with three cycles of 16s 8Hz frequency. The experimental flies have significantly increased locomotor output during Illumination phase compared to control flies (*P*=0.03; Control/Retinal in cm/min Mean ± SEM: Pre 0.39 ± 1.15 /0.12 ± 0.36, Illu. 35.13 ± 9.95/ 44.52 ± 16.58, Post 4.85 ± 6.58/11.61 ± 14.66, N=18/18). Overall walking path during Illumination phase shown in the top left. **B** The mean distance over time covered during the 1min blue light Illumination phase for control and experimental flies. Control flies show a 2s delay for locomotion upon blue light exposure and a strong decrease after blue light offset. Experimental flies have shortened delay and reduced decreases in locomotion between the cycles. Significant differences between the control and experimental groups are indicated by "**a**" (Mann-Whitney U-test; *P*<0.05).

The sum of the distances traveled during the blue light exposure phases and pauses of the three cycles individually stays constant and does not differ in control flies (Fig.29A). The experimental flies show similar activity as the control flies in the first illumination cycle and significant increases in locomotion in all pauses. This is expected since they have a reduced decrease of locomotor output during the pause of the illumination cycles. The distance traveled during the second and third blue light exposure is significantly increased in experimental flies. This indicates that the overall increase in locomotion is a combination of increased locomotion during the illumination phases as well as the pauses. Furthermore the effect of OA/TA neuron activation with 16s 8Hz is indeed not an instantaneous effect but increases with a delay arguing for modulatory effects of the stimulated neurons.

Analysis of the first illumination cycle shows that the locomotion pattern between control and experimental flies is mostly similar (Fig.29B). As expected the control flies show low locomotion during 0-2s and an increase in locomotion persisting over the blue light exposure phase and a strong reduction at the offset of blue light exposure. The locomotor activity persists until two seconds after the illumination phase and is strongly decreased in the last two seconds of the cycle. The experimental flies have a significantly higher locomotion in the first 0-2s quantitatively confirming the previous observation that the locomotion increase onset is reduced by 1s (see Fig.28B). The locomotor activity stays roughly constant throughout the blue light exposure phase and is significantly higher as in control flies during the 8-10s bin. Furthermore the experimental flies show significantly increased locomotion in the last bin. This confirms the previous observation that the increase in locomotor activity in experimental flies persists after blue light exposure into the illumination pauses. Thus OA/TA neuron activation with 16s 8Hz induces an increase in locomotion that can be attributed to a shortened locomotion increase onset, prolonged locomotion into the illumination pause phases of each cycle and increasing locomotion by repeated blue

light stimulation cycles. This indicates that 8Hz stimulation of OA/TA neurons might influence the startle response as has been suggested previously (Scholz, 2004) and possibly raises the arousal state of the flies for a prolonged time in a likely modulatory way. Modulatory, because the locomotion increases over time which argues for an indirect role on the locomotor system.



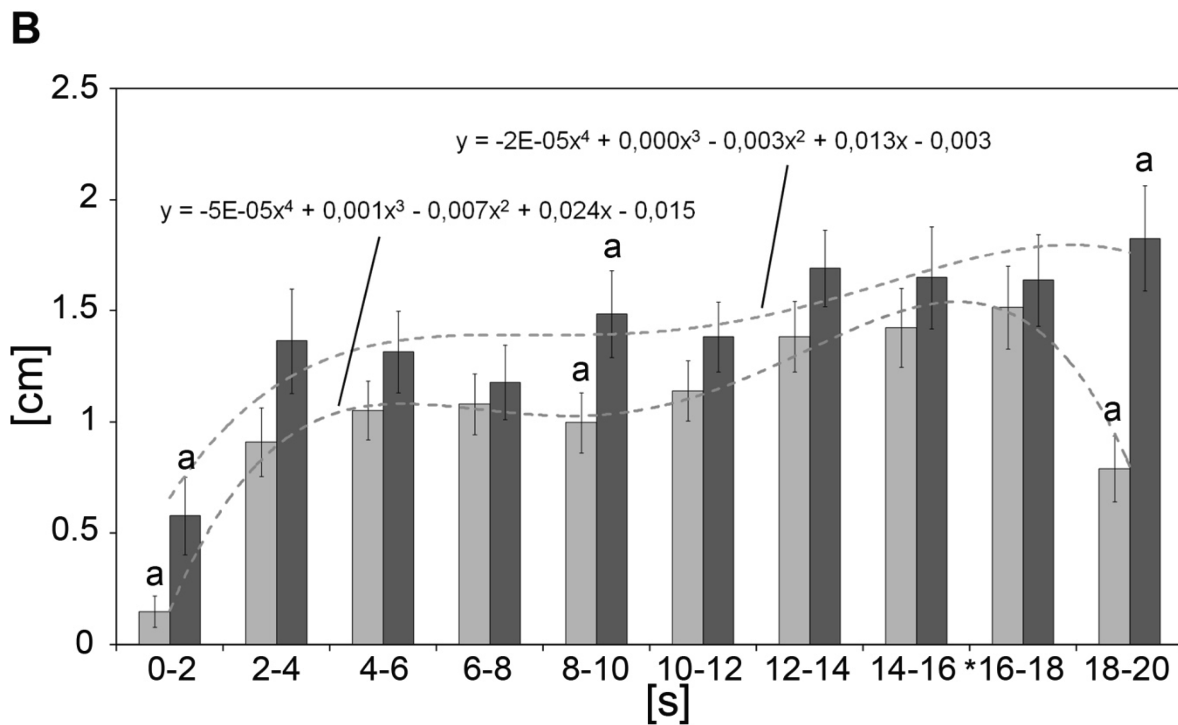


Figure 29. OA/TA neuron activation with 16s 8Hz increases locomotor over time. A Sum of the distances traveled during the three cycles of the Illumination phase using 16s 8Hz activation frequency. Experimental flies have significantly increased locomotor activity in all pauses and in the 2nd and 3rd illumination phases. **B** The mean distance the flies traveled in cm binned in two seconds each over the first illumination cycle. Experimental flies covered significantly more distance than the controls in the bins 0-2, 8-10 and 18-20s. (P<0.05; raw Data shown in suppl. Table 4). Change from 8Hz to 0Hz indicated by a asteriks. Significant differences indicated by "**a**" (Mann-Whitney U-test; P<0.05).

3.3.4 OA/TA activation pulse number affects locomotor output

In order to investigate whether the duration of the OA/TA neuron activation plays a role in locomotion, OA/TA neurons were stimulated with 16s 40Hz frequency (Fig.30). No difference in locomotor activity is visible during all phases of the experiment for control and experimental flies (Fig.30A). Both show similar levels of locomotor activity due to the blue light exposure and a strong decrease in locomotion in the post-Illumination phase. This indicates that 16s 40Hz frequency has no effect on the locomotor output. Furthermore it shows that the activation frequency matters, since the same activation duration using 8Hz activation frequency increases locomotion. The walking path in the experimental arena does not implicate any specificity.

The locomotion pattern over time during the Illumination phase is similar between control and experimental flies over the three cycles and shows the typical locomotion delay at the beginning of each cycle and a decrease in locomotion during the pauses in illumination (Fig.30B). This further confirms that 16s 40Hz does not affect locomotion.

The sum of the distances traveled during the illumination phases of the three cycles is similar for control and experimental flies and in between the three cycles (Fig.3A). Similarly, no difference in locomotion is visible in the pause phases. In addition the quantification of the distances traveled in the first cycle in higher resolution shows no significant differences between control and experimental flies (Fig.31B). These data further indicate that 16s 40Hz activation of OA/TA neurons does not alter locomotor output. One explanation could be that OA/TA neurons need a specific frequency to transfer the information necessary to increase or alter locomotion and 40Hz activation is not able to do so.

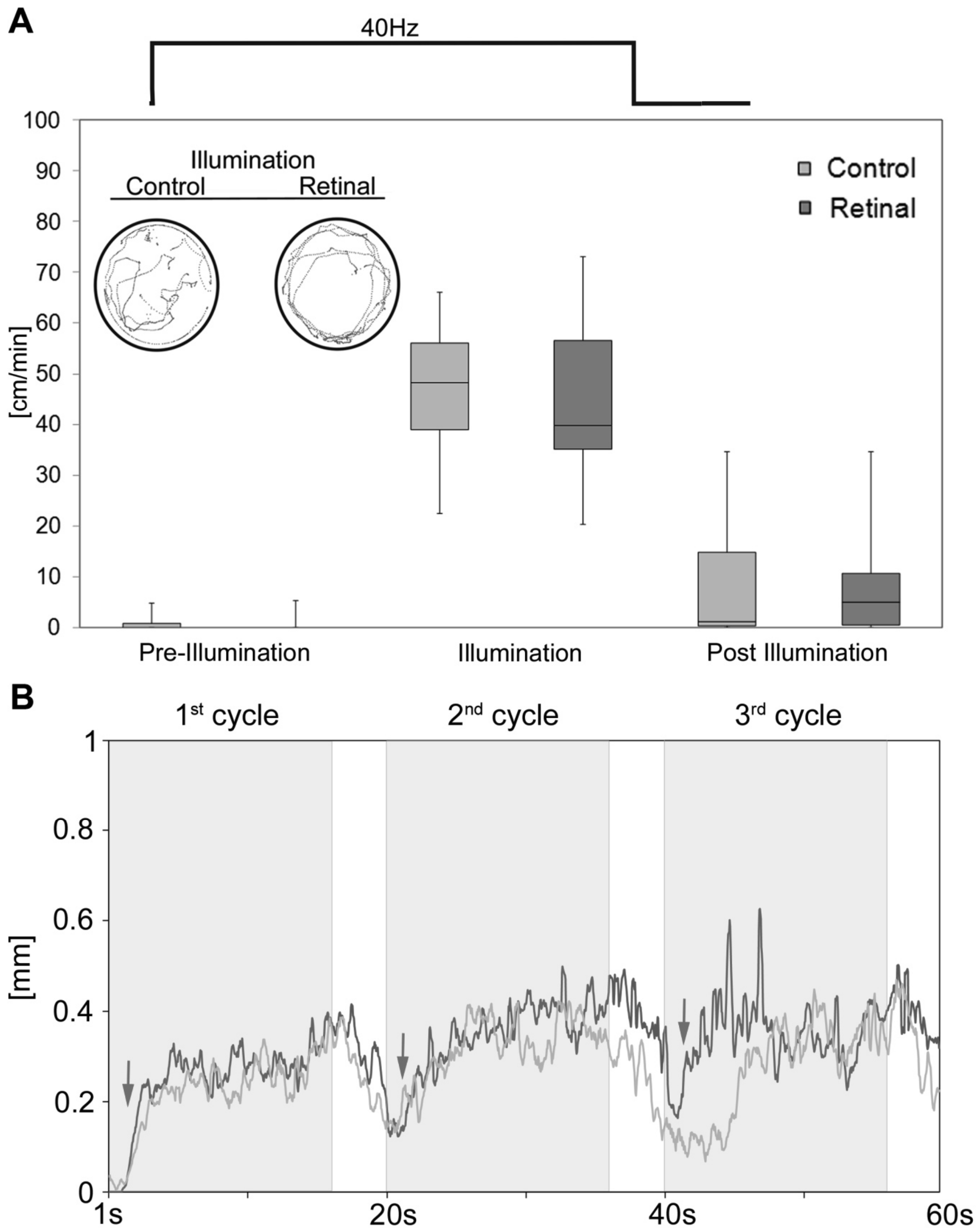


Figure 30. OA/TA neuron activation with 16s 40Hz frequency fails to affect locomotor output. A *norpA[1];UAS-ChR-2/Tdc2*-GAL4*;UAS-ChR-2/*+ flies tested in the optogenetic locomotion setup. OA/TA neurons were activated with three cycles of 16s 40Hz frequency. The locomotor output is not significantly altered between control and experimental flies (Control/Retinal in cm/min Mean ± SEM: Pre 0.74 ± 1.39/0.59 ± 1.49, Illu. 46.74 ± 13.08/44.49 ± 15.22, Post 8.71 ± 11.80/8.38 ± 11.50, N=18/21). **B** The mean distance covered in between time points during the 1min blue light Illumination phase for control and experimental flies. Locomotion is not different between control and experimental flies. Activity delays as indicated (red arrows). Decrease in movement is visible at around 1-2s past blue light exposure. Significant differences verified by Mann-Whitney U-test (*P*<0.05).

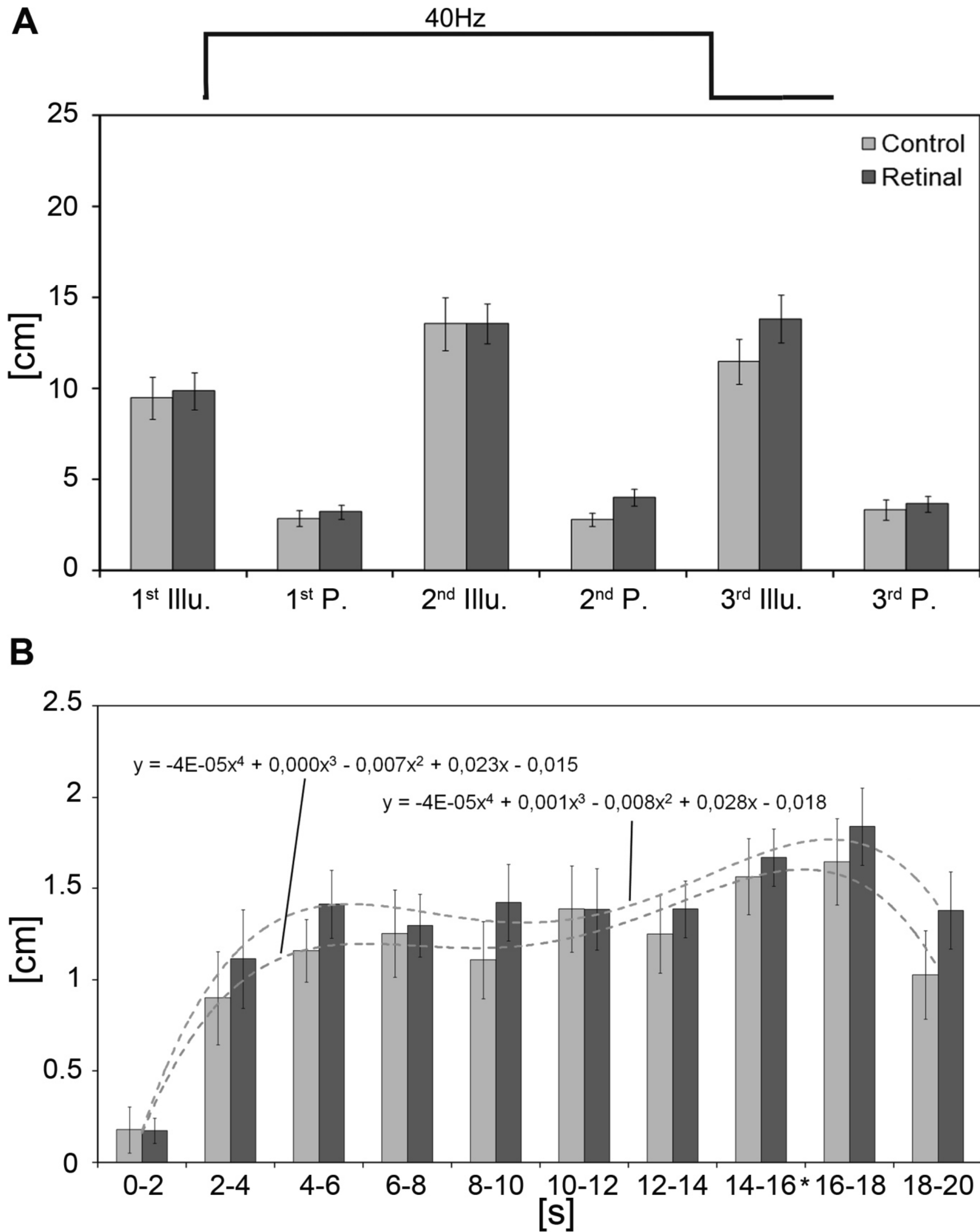


Figure 31. OA/TA neuron activation with 16s 40Hz does not affect locomotor output. A Sum of the distances traveled during three cycles of the Illumination phase with 16s 40Hz activation frequency. Control and experimental flies have similar locomotion during the illumination and the illumination pauses. **B** The mean distance the flies traveled binned in two seconds each over the first illumination cycle. Control and experimental flies show no difference in locomotion (raw Data shown in suppl. Table 5). Polynomic curves indicate the trend of locomotion. Change from 40Hz to 0Hz indicated by a asteriks. Significant differences verified by Mann-Whitney U-test (*P*<0.05).

The number of OA/TA neuron activating blue light pulses during the “Hammer Frequency” might play a role in mediating increases in locomotion. To test this OA/TA neurons were stimulated with 18s 11.5Hz activation frequency (Fig.32).

Control flies show an increase of locomotion during the Illumination phase and a strong decrease in locomotion in the Post-Illumination phase (Fig.32A). The experimental flies have a similar level of locomotor activity during the Illumination phase but significantly increased locomotion during the Post-Illumination phase in comparison to the control flies (*P*=0.02). This indicates that OA/TA neuron activation with 11.5Hz frequency does not alter locomotion during neuronal activation but prolongs locomotor activity. It could be that the arousal state of the flies is prolonged when OA/TA neurons are activated with this frequency. The walking path in the arena during the Post-Illumination phase also indicates that the experimental flies covered more distance but does not show any further specificity. As expected, the walking pattern over time during the three cycles of the Illumination phase shows no difference between control and experimental flies and the level of locomotion stays roughly constant during the blue light exposures (Fig.32B). In contrast to the previously tested frequencies, the locomotion during the illumination pauses is not decreased but rather decreases at the beginning of the following illumination cycle with a delay to increases in locomotion of roughly 2s. This is probably due to the illumination pause being 2s and not 4s as in the previous experiments. The shortened pause might also be the reason for the reduced decrease in locomotion in between the illumination phases of the three cycles.

As expected the sum of the distances during the blue light exposure and illumination pauses of the three cycles does not differ between control and experimental flies (Fig.33A). Similarly, control and experimental flies show no difference in the locomotor activity level in a more detailed analysis of the first illumination cycle (Fig.33B). This further suggests that OA/TA neuron activation with 18s 11.5Hz frequency does not influence locomotion upon stimulation. The increased locomotor activity in the Post-Illumination phase shows that the applied frequency prolongs locomotion but does not acutely increase it. This further argues for a modulatory role of OA/TA neurons in locomotion.

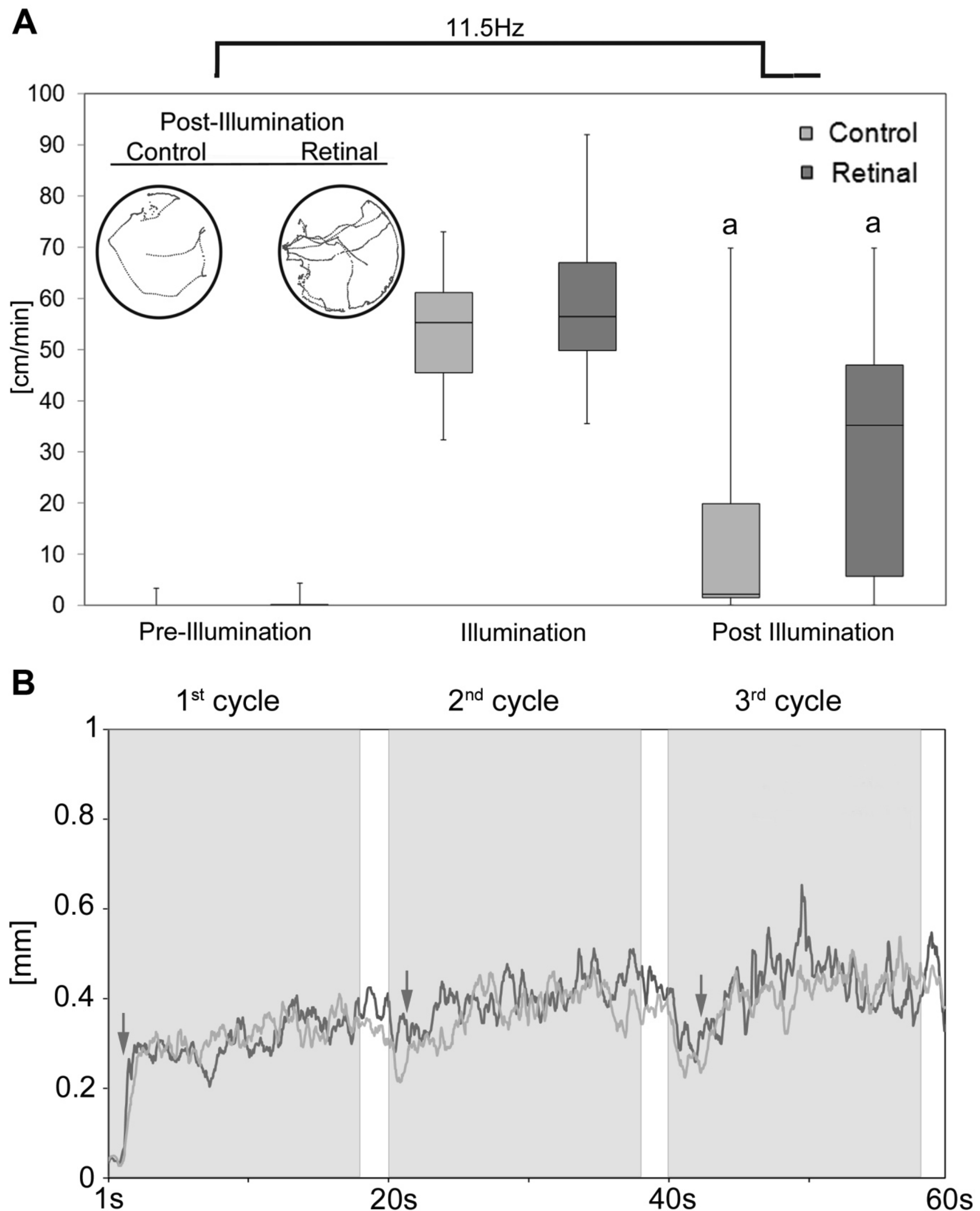


Figure 32. OA/TA neuron activation with 18s 11.5Hz frequency prolongs locomotor output. A *norpA[1];UAS-ChR-2/Tdc2*-GAL4*;UAS-ChR-2/*+ flies tested in the optogenetic locomotion setup. OA/TA neurons were activated with three cycles of 18s 11.5Hz frequency. The locomotor output is not altered during the Illumination phase. Experimental flies have significantly higher locomotor output in the Post-Illumination phase (*P*=0.02; Control/Retinal in cm/min Mean ± SEM: Pre 0.33 ± 0.90/0.42 ± 1.06, Illu. 54.26 ± 12.35/58.32 ± 13.26, Post 12.91 ± 15.44/30.35 ± 23.97, N=19/19). Overall walking path during the Post-Illumination phase shown in the top left. **B** The mean distance covered in between time points during the 1min blue light Illumination phase for control and experimental flies. Illumination induces both control and experimental flies movement to a similar degree with locomotor activity delay as indicated (red arrows). Significant differences are indicated by "**a**" (Mann-Whitney U-test; *P*<0.05).

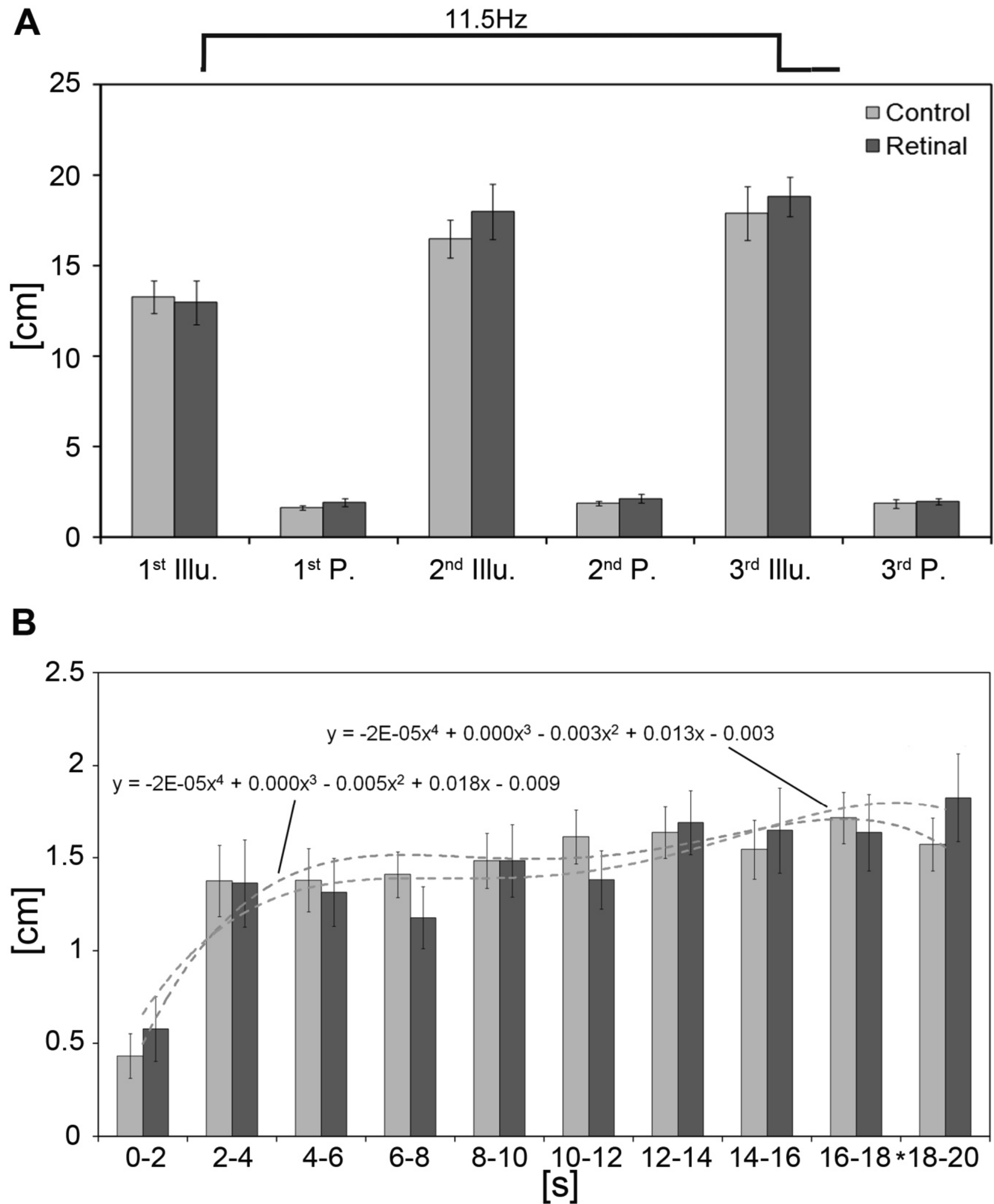


Figure 33. OA/TA neuron activation with 18s 11.5Hz frequency does not affect locomotion during the illumination cycles. A The Sum of the distances traveled during three cycles of 18s 11.5Hz activation frequency. Control and experimental flies show similar locomotor output during the illumination and the illumination pauses. **B** The mean distance the flies traveled binned in two seconds each over the first illumination cycle. Control and experimental flies show no significant difference in locomotion (raw Data shown in suppl. Table 6). Polynomic curves indicate a similar trend (polynomic functions as indicated). Change from 11.5Hz to 0Hz indicated by a asteriks. Significant differences verified by Mann-Whitney U-test ($P<0.05$).

Taken together these results indicate that the 16s 8Hz segment of the "Hammer Frequency" is the OA/TA stimulation frequency that acutely influences locomotion but in a modulatory way since increases in locomotion increase with repeated stimulation. Furthermore OA/TA neurons firing with 18s 11.5Hz can prolong locomotion probably to changes in the arousal state of the fly. OA/TA neuron stimulation with different durations of 40Hz frequency had no effect on the locomotion at all. This clearly shows that to alter locomotion the OA/TA neurons have to be stimulated with a specific frequency and that depending on the frequency locomotion can be altered in at least two different ways, namely short term increases and prolonged locomotion. Experiments using constant light exposure could further elucidate whether specific activation frequencies are indeed necessary to affect locomotion or if the OA/TA neurons in question can act as on and off neurons. Furthermore, a switch between frequencies during an illumination cycle as is the case in the "Hammer Frequency" has not been investigated so far and could potentially play an important role in modulating activity. For example a combination of 8Hz and 11.5Hz activation frequencies might increase locomotion during stimulation as well as lead to prolonged locomotion.

3.4 Neuroanatomical Studies

3.4 .1 OA/TA neurons form contact to DA neurons at the calyx neuropile

Neuronal activation of both DA and OA neurons leads to increased locomotion (Fig. 22 and 23). Dopaminergic neurons have been suggested to act downstream of octopaminergic neurons in reward signaling (Burke *et al.*, 2012). A possible site for such a connection has been proposed to be the anterior medial protocerebrum (ampr). Since TDC2-GAL4 driven OA/TA neurons do not innervate the EB it could be that OA/TA neurons influence locomotion through DA neurons (Busch *et al.*, 2009).

In order to investigate whether there is indeed a direct synaptic connection between OA and DA neurons the GRASP (GFP reconstitution across synaptic partners; Feinberg *et al.*, 2008;) system was employed. One membrane tagged GFP fragment was expressed in dopaminergic neurons using the *Th*-GAL4 driver line driving expression of *UAS-mCD4::spGFP1-10* The complementary GFP fragment was expressed in OA/TA neurons using the *Tdc2*-LexA driver line driving expression of *lexAop-mCD4::spGFP11*. Both fragments alone do not emit fluorescence while

reconstituted to a complete GFP the ability to emit detectable fluoresence is returned. Reconstituted GFP is possible at sites with close proximity like cell-cell contacts or synapses of the two neuronal populations expressing each one part of the GFP since CD8 is a membrane tag. Adult male flies were dissected and stained for nc82 (bruchpilot) a marker for active zones. Colocalistaion of both GFP and nc82 signal should mark synaptic contacts between OA/TA and DA neurons. No antibodies were used to detect the reconstituted GFP.

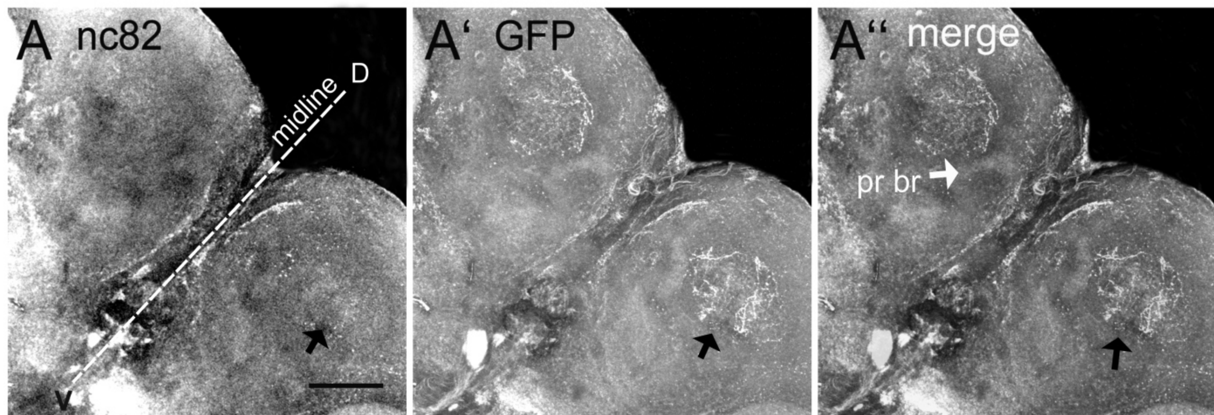


Figure 34. OA/TA and DA neurons have contact at the MB calyces. Male *w*[1118];*Th*-GAL4/*lexAop-mCD4::spGFP11; Tdc2*-LexA/*UAS-mCD4::spGFP1-10* CNS stained against nc82 (**A**; cy3, magenta). GRASP signal (**A'**; GFP, green) can be detected at the calyx neuropil indicated by black arrows located lateral to the protocerebral bridge (white arrow pr br). **A''** Merge of nc82 and GRASP signal. Broken line indicates midline from dorsal (D) to ventral (V). Scale bar, 50µm.

GRASP signal is only detected in the region of the calyx neuropil and not the ampr as has been suggested (Fig.34A-A''). No colocalization with nc82 could be detected. The GFP signal is either elongated in stripes or punctuate. Elongated regions might be membrane proximities of axons while punctuate signals synaptic contacts. No co-localisation of the GRASP with the nc82 signal could be detected. This indicates that GRASP signal represents cellular rather then synaptic contacts because nc82 labeles active zones. However, the nc82 antibody staining is very weak in the calyx neuropil area showing that it did not penetrate the tissue well. Co-localization might have been visibile with better tissue penetration of the nc82 antibody. Therefore it can only be concluded that OA/TA and DA neurons have cellular and possibly synaptic contact in the region of the calyx neuropil. The membrane tags for the GFP fragments are not specific for pre- and post-synapse and therefore the polarity of the possible connection cannot be investigated.

3.4.2 Orco positive OSNs and OA/TA neurons are in close proximity in the AL

In cockroaches OA has been shown to be secreted in the antenna and in honeybees OA receptors have been shown to be present in the antennae (Pass *et al.*, 1988; von Nicksch-Rosengk *et al.*, 1996). In *Drosophila* OA receptors could be detected on mPNs and inhibitory LNs but not OSNs (Sinakevitch *et al.*, 2013). But it is not known whether OSNs and OA neurons form direct synaptic contact. To investigate if OA/TA neurons and Orco positive OSNs are in close proximity to each other two expression systems were used, the UAS/GAL4 and LexA/LexAop system. mCherry was expressed in Orco positive neurons using the *Orco*-LexA driver line and GFP in OA/TA neurons using the *Tdc2*-GAL4 driver line (Fig.35). mCherry can be detected in the antennal nerve (AN), throughout the AL and in the in the connective between the antennal lobes (AC; Fig.35A and B). The expression pattern of *Tdc2*-GAL4 has been

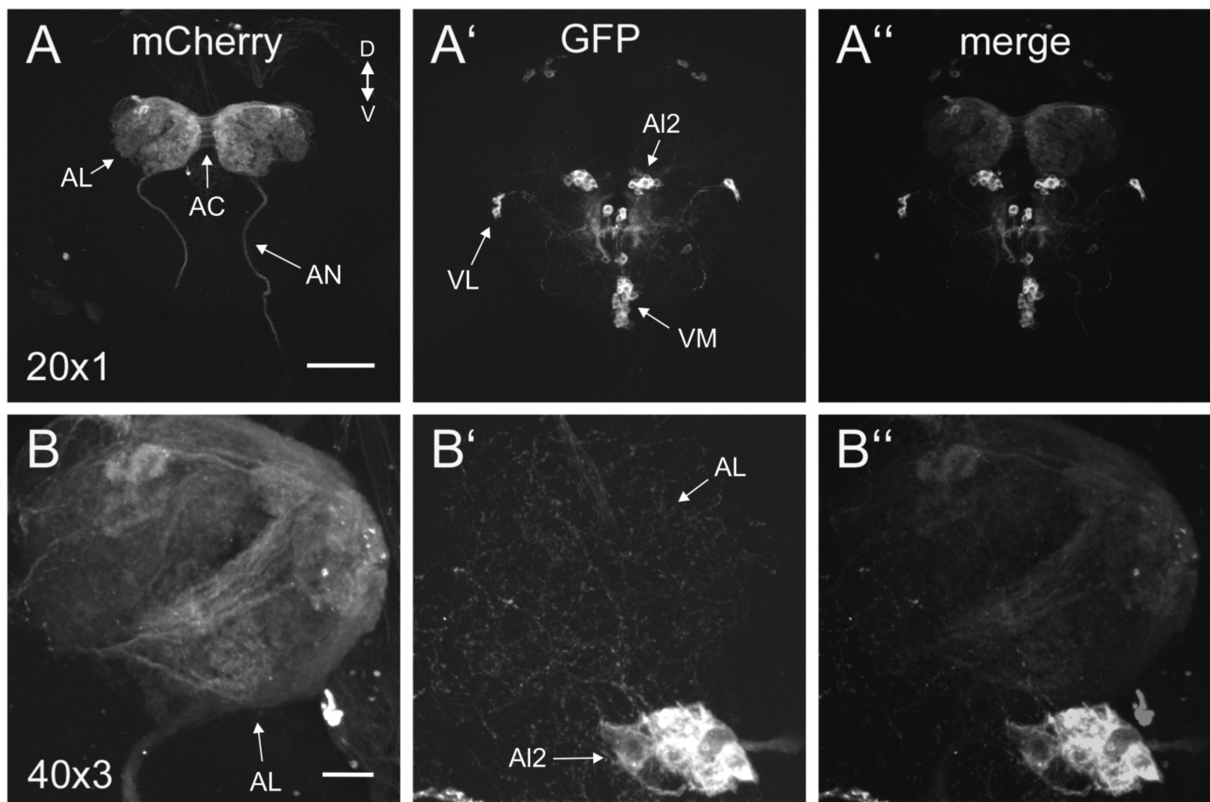


Figure 35. Orco positive OSNs and OA/TA neurons both innervate the AL. Adult male w^{1118}; *Tdc2*-GAL4/*UAS-mCD8::GFP; Orco*-LexA/*lexAop-mCherry* CNS were stained against GFP. **A-A''** Overview of the CNS showing Orco positive OSNs labeled by mCherry (**A**; magenta) and OA/TA neurons by GFP (**A'**; green) separately and as a merge of colors innervating the AL. **B-B''** Magnification of the left AL region. Magnifications as indicated. Scale bars, 50µm (**A-A''**) and 10µM (**B-B''**).

investigated in great detail before and detected GFP signal confirms most of the neurons (Fig.35A’ and B’). Fine arborizations are visible in the AL as has been previously shown for the *Tdc2*-GAL4 labeled VM neurons VUMa2, VUMa6 and VPM5 (Busch *et al.*, 2009). The merge of both fluoresences shows no co-localistaion but close proximity of mCherry and GFP (Fig.35A’’ and B’’). Thus Orco positive OSNs and OA/TA neurons are in close proximity in the AL.

3.4.3 Orco positive OSNs and OA/TA neurons form synaptic contact in the AL

To illuminate whether OA/TA neurons and Orco positive OSNs have synaptic contact in the AL the GRASP system was employed. *Tdc2*-GAL4 was used to drive expression of *UAS-CD4::spGFP1-10* in OA/TA neurons and *Orco*-LexA to drive expression of the *lexAop-CD4::spGFP11* in Orco positive OSNs. Synaptic or membrane contact should lead to detectable reconstituted GFP signal. An antibody against nc82 labels *bruchpilot* a marker for active zones and thus synapses. For detection of reconstituted GFP no antibody was used.

nc82 is visible as small dots representing active zones all over the CNS including the AL where it outlines the glomerular organization (Fig.36A-C). GRASP signal is visible in dots and elongated tubes in the AL (Fig.36A’–C’). Co-localizations of nc82 and GRASP signal appear in white color. The merge of GRASP (green) and nc82 (magenta) shows that the tube like GRASP signal is not co-localized with nc82 (Fig.36A’’-C’’). This indicates that OA/TA cell axons do in part run along Orco positive OSNs without synaptic contact reflected by the continuous GRASP signal. The dot like GRASP signal is co-localized with nc82 in some regions (red arrows) while in other regions they are clearly not co-localized (Fig.36B’’ and C’’). This indicates that Orco positive OSNs have probably synaptic contact to OA/TA neurons, and cellular contact can be found all over the AL. The directionality of the synaptic transmission between the OA/TA neurons and OSNs cannot be determined using GRASP without pre- and postsynaptic markers. Still OA/TA neurons seem to connect synaptically with OSNs possibly providing a means of either modulation of the olfactory

information flow by OA/TA neurons or information transmission to OA/TA neurons.

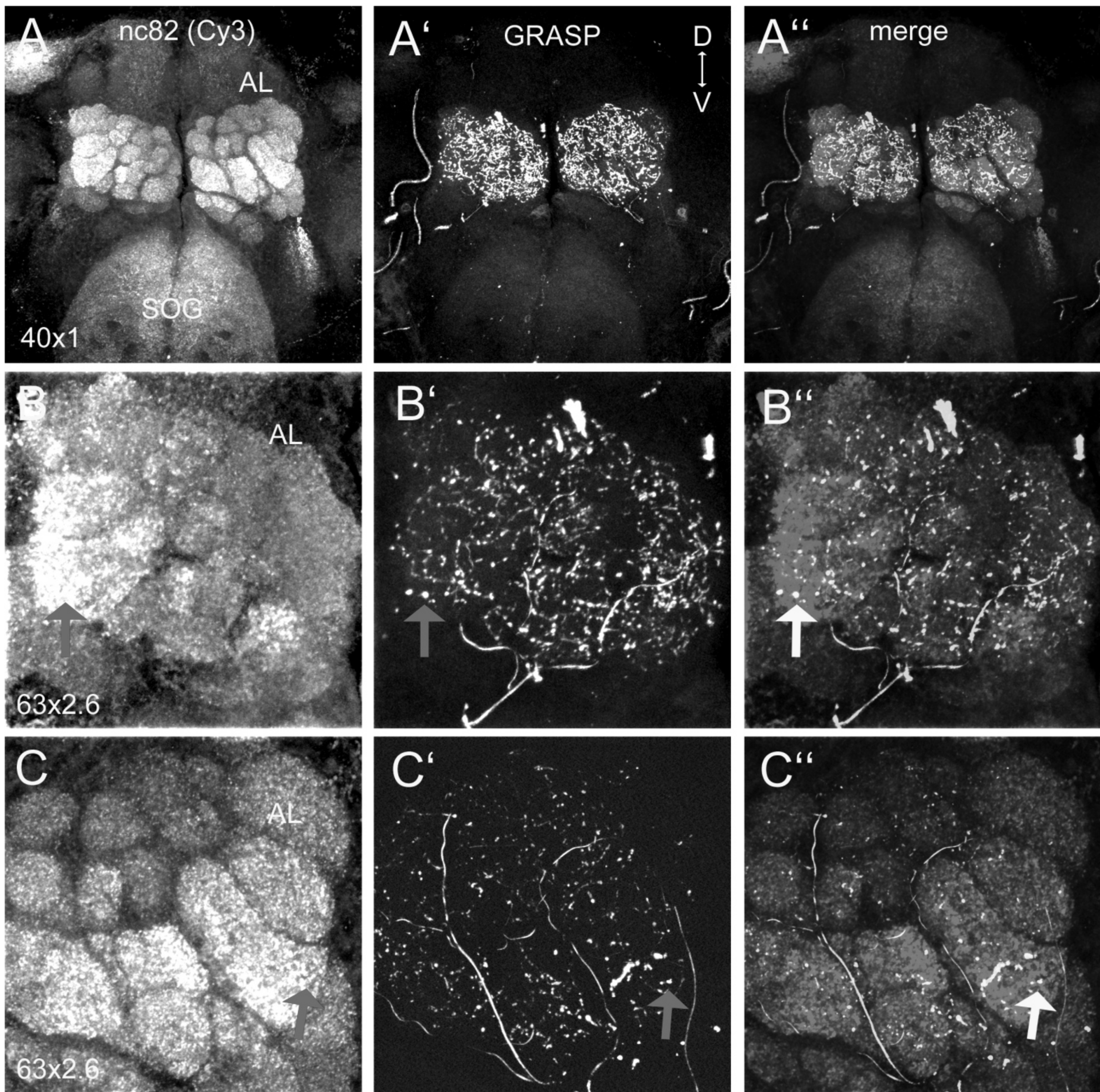


Figure 36. Contact between OSNs and OA/TA neurons in the AL. Adult male *w*^{1118}; *Tdc2*-GAL4/ *lexAop-CD4::spGFP1*; *Orco*-LexA/*UAS-CD4::spGFP1-10* CNS were stained against nc82. A-C Overview of adult CNS. nc82 (magenta) and GRASP (green) signal can be detected in the AL. B-C'' Magnification of the left (B-B'') and right AL regions (C-C''). Co-localization of nc82 and GRASP signal is visible in the AL and appears in white in the merge (indicated by red arrows). Magnifications as indicated. Scale bars represent 50µm (A-C) and 10µm (B-C'').

4 Discussion

4.1 Orco is not essential for odor perception

Previous studies indicate that in the absence of Orco in OSNs the perception of odors is abolished (Larsson *et al.*, 2004; Neuhaus *et al.*, 2005). Furthermore Orco has been proposed to act as a chaperone to link the OR-Orco complex to the axonal trafficking network to shuttle the complex to the sites of membrane insertion in the OSN dendrites (Larsson *et al.*, 2004; Benton *et al.*, 2006). This implicates that in *Orco*^{1} mutants the odor molecule specific receptor should be mis-localized due to the missing dendritic shuttle and therefore Orco dependent odor perception should be impaired. Here it is shown that *Orco*^{1} mutants can perceive and prefer odors but at higher than normal odor concentrations (Fig.13 and 14). This indicates that odors can be perceived via Orco independent mechanisms. One such mechanism could be that ORs are functional in the absence Orco. Orco and ORs form hetero- but also homodimers (Neuhaus *et al.*, 2005; Benton *et al.*, 2006; German *et al.*, 2013). It could be that in the absence of Orco the probability of OR-OR complex formation is increased and that the OR-OR complex can still be shuttled to the dendritic membrane but maybe to a lesser degree and there act in odor perception independently of Orco. One indication that this could work is that in previous studies in heterologous expression systems odor responses could be detected by ORs in the absence of Orco as long as the OR was inserted into the membrane (Neuhaus *et al.*, 2005; Wicher *et al.*, 2008; Smart *et al.*, 2008; Deng *et al.*, 2011). ORs acting as GPCRs are supposed to mediate OSN responses through the cAMP gated Orco cation channel. Loss-of the Orco would thus result in loss of the channel conducting the OSN response. One explanation how an olfactory response could still be elicited is that other cAMP gated ion channels (CNG) natural to the cell conduct the metabotropically mediated olfactory response (Smart *et al.*, 2008). One such channel could be the hyperpolarization-activated and cyclic-nucleotide-gated (HCN) I_h channel present in Drosophila (Marx *et al.*, 1999; Littleton and Ganetzky, 2000). It is involved in a variety of behaviors in Drosophila among which are circadian sleep/wake cycle, locomotor rhythm and the proboscis extension reflex (Chen and Wang, 2012; Gonazalo-gomez *et al.*, 2012). *in vitro* studies showed that with changes in cAMP levels the I_h channel kinetics changed (Gisselmann *et al.*, 2005). mRNA of this channel has been detected in the antennae and eyes of Drosophila (Marx *et al.*, 1999). Single sensillum recordings of OSNs expressing the I_h channel under the control of the *Orco*-GAL4 driver line showed increases in spontaneous

OSN activity and also prolonged activity after the odor exposure (Deng, 2009). This shows that CNGs are present in the antenna and can partly mediate and affect OSN odor responses. However, whether the I_h channel only contributes to olfactory stimulus conduction when it is overexpressed in the antennae is not known. Single sensillum recording or olfactory preference studies with expression of the I_h channel in Orco positive OSNs in the $Orco^1$ mutant background could answer this question. Still it could play a potential role in mediating cAMP dependent depolarizing cation currents and as such be a target of the metabotropic response to odor stimuli.

4.2 Impact of Orco on olfactory preference is odor dependent

Olfactory preference was either shifted or lost upon loss-of Orco (Fig.13 and 14). This indicates that Orco influences odor perception but not uniformly as could have been assumed if olfactory preference for all tested odors is abolished or always shifted. Since this is not the case there have to be Orco dependent and independent channels mediating Olfactory preference.

IRs but not ORs have been shown to be sensitive to AA providing a means to sense AA without Orco dependent olfactory reception (Silbering *et al.*, 2011). But AA is preferred at higher concentrations than in control flies (Fig.14C). The preference at higher AA concentrations could be explained by AA sensing IRs being less sensitive to AA. But the shift in preference due to loss-of Orco clearly indicates that Orco dependent olfactory sensing of AA increases sensitivity to AA. One explanation could be that IRs and ORs complement each other although no convergence of OR and IR projections in the AL on a common glomeruli have been observed (Silbering *et al.*, 2011). To investigate whether IRs are solely responsible for AA preference mutants without AA sensitive IRs could be tested in olfactory preference experiments. If the olfactory preference for AA is lost it means that IRs convey AA preference. A shift in preference would mean that IRs contribute to AA preference in combination with ORs since in Orco mutants the AA preference is also shifted. AA preference experiments with double mutants of both IRs and Orco could show whether IR and Orco independent mechanisms are involved. Still the shift in AA preference in *$Orco^1$* mutant flies indicates that Orco dependent olfaction is involved.

Olfactory preference for ethanol is lost upon loss-of Orco while preference for EtOAc was shifted to higher concentrations. This indicates that EtOH and EtOAc preference is mediated through different mechanisms. Only OR22a has been shown

to be responsive to ethanol and only weakly (Hallem and Carlson, 2006). This receptor is Orco dependent and thus loss-of Orco abolishes the response to low concentrations of ethanol (Larsson *et al*., 2004). Usually a higher odor concentration results in recruitment of additional more broadly tuned ORs resulting in additional odor signal channels (Hallem and Carlson, 2006; Kreher *et al*., 2008). Since no additional OR is known to be responsive to ethanol there might be no further recruitment of other ORs and therefore a diminished or abolished response. In case of EtOAc two receptors have been shown to be sensitive, OR42a and OR42b (Kreher *et al*., 2008). OR42b is sensitive to low EtOAc concentrations and OR42a to high concentrations effectively broadening the perceivable odor concentration range. One explanation could be that OR42b is Orco dependent and OR42a not. Therefore sensing of EtOAc at low concentrations is disrupted while higher concentrations can still be sensed and preferred. But a very recent study showed that *Orco*-GAL4 drives expression in neurons containing OR42a and b (Accepted Article, Grabe *et al*., 2014). Therefore it is likely but not definite that these neurons also express Orco. Still if both receptors are Orco dependent olfactory preference should be abolished and not shifted to higher concentrations. One reason for this phenotype could be the number of receptors that are sensitive to EtOAc. Including OR42a and b 7 ORs are stimulated by EtOAc (Hallem and Carlson, 2006). OR43b and OR59b show a strong response to EtOAc and OR22a, OR47a and OR85a a weak response. If the receptors are still present as homomeres in the OSNs it would mean that EtOAc is potentially able to excite the OSNs expressing the 7 ORs although higher concentrations are needed possible due to the loss of Orco as a CNG. This would possibly result in quantitatively more channels providing input to the AL for further processing as is the case for ethanol exciting one OR type and thus OSN type. This could lead to an increased glomerular activity profile and thus olfactory preference. Therefore Orco independent olfactory preference might depend on the number of excitable ORs. Silencing OSNs that express EtOAc sensitive receptors using an OR RNAi approach or establishing EtOAc sensitive OR mutants and testing them for EtOAc preference could answer whether the number of input channels are indeed responsible for the preference phenotype. Monitoring glomerular activity patterns via ca2+ imaging in Orco1 mutants could also show if more or stronger activity in certain glomeruli can be observed. Whether there are Orco and OR independent mechanisms to perceive EtOAc is not known so far.

4.3 Orco effect on olfactory aversion depends on the odor identity

Olfactory aversion was not affected in response to higher ethanol and EtOAc concentrations while in case of AA and AP it was shifted to higher concentrations in Orco mutants (Fig.13B, 14A and B). Around 70 to 80% of the OSNs express Orco (Larsson *et al.*, 2004). This could mean that Orco independent odor perception is possible through the remaining 20 to 30% of the OSNs. Hypothetically these OSNs could contain broadly tuned ORs that are responsive to high concentrations of various odors alone. One function could be to present a means of warning the fly of dangerous odor concentrations, which would explain why loss-of Orco does not affect aversion to EtOAc or EtOH. Still aversion to AP is reduced in Orco mutants and cannot be explained this way (Fig.14E and F). Nevertheless it could be possible but has not yet been investigated. The shift in aversion for AA that can be observed in the absence of Orco could be due to IRs sensing AA at higher concentrations (Silbering *et al.*, 2011). Assuming Orco independent broadly tuned ORs also respond to high AA concentrations in addition to the IRs the shift in aversion could be explained by both sensing modalities influencing the AA perception and processing. It would also mean that Orco dependent ORs either dominate or inhibit IR sensing and thus increase the sensitivity to AA and therefore shift AA aversion to lower concentrations. To test this hypothesis silencing of AA sensing IR OSNs or IR mutants could be used in AA preference experiments. If the aversion to AA is not changed compared to wild type flies it would mean that IRs are overruled or silenced by Orco dependent mechanisms. A shift in aversion to higher AA concentrations would indicate that Orco dependent and IR mechanisms are mediating AA aversion in concert. Double mutants for both Orco and IRs could show whether ORs and IRs are involved in AA aversion at all. However, how and if AA perception via IRs and Orco dependent ORs impact on AA aversion is unclear.

Apart from ORs and IRs that sense volatile compounds, GRs could also potentially play a role in olfactory preference and aversion. GRs are Orco independent and could be sensing the used odor compounds and mixtures due to droplets forming upon condensation of the odor mixtures. This is possible, since the glass beakers containing the flies and odor traps are illuminated for 16h from below resulting in vaporization of the fluids and condensation outside of the traps. This is definitely possible but since cold-white light sources are used the heat production should be minimal. Still this aspect has not been investigated here.

4.4 Orco regulates odor sensitivity for olfactory preference

Loss-of Orco results in an olfactory preference shift to higher odor concentrations compared to the control flies (Fig.14A and C). This indicates that Orco regulates odor sensitivity for olfactory preference. Even in the absence of odors Orco shows spontaneous channel conductance without odor stimulation resulting in spontaneous background activity in Orco expressing OSNs in vivo (Su *et al.*, 2012). This spontaneous activity is abolished upon loss-of Orco (Benton *et al.*, 2007; Deng *et al.*, 2011). One proposed function of the Orco dependent spontaneous activity is to reduce the spiking threshold of the OSNs and thus raise the sensitivity to incoming odors (Stengl, 2010, 2013). This would effectively allow the fly to sense lower odor concentrations than without the spontaneous activity in the background and a subsequently raised spiking threshold. Thus the loss-of Orco could possible result in decreased odor sensitivity but not necessarily result in complete odor perception abolishment in Orco expressing OSNs.

Another indication is that expression of ORs without Orco in heterologous expression systems showed that olfactory responses can be detected but longer exposure times and higher odor concentrations were necessary to produce cellular responses (Neuhaus *et al.*, 2005; Smart *et al.*, 2008; Deng *et al.*, 2011). Hypothetically, upon odor exposure the effect of the absence of Orco in the OSN would be that the elicited ca^{2+} response is weaker than in the presence of Orco in cells expressing the OR alone. That is likely due to two reasons: First, the Orco mediated ionotropic and metabotropic responses should be lost; Second if cell natural CNGs affect the metabotropic response there should be overall less CNGs available because the Orco as a CNG is gone. This means that likely only the cell natural CNG mediated depolarizing current is present resulting in a weaker response. It was shown that higher odor concentrations elicit stronger OSN responses (Hallem and Carlson, 2006). Therefore in Orco mutants a higher odor concentration is probably needed to result in a response comparable to responses to lower odor concentrations in wild type flies. If this is true *in vivo* it would explain why higher odor concentrations affect the behavioral response and low concentration cannot be sensed by the fly.

4.5 Odor identity assignment at low concentrations depends on Orco

Orco mutant flies can distinguish between water and single odors diluted in water in a concentration dependent manner (Fig.15). The ability to do so might be due to the possible Orco independent mechanisms described above. Still why they can sense and prefer odors over water when they need Orco to prefer single odors in complex odor mixtures is unclear. One possibility is that Orco independent ORs are retained as homomeres and the threshold excitability is reduced upon loss-of Orco. Since a complex odor mixture contains various odor compounds activating a number of ORs leading to various olfactory stimuli being processed the single odors cannot be resolved in the complex odor background with the additional information flow (Touhara, 2002; Yao *et al.*, 2005; Hallem and Carlson, 2006). This would mean that reduction of the mixture complexity could lead to single odor preference due to better odor resolution. Another possibility is odor mixture interaction where activity in one type of OSN leads to inhibition of another type of OSN (Schuckel *et al.*, 2009; Hillier and Vickers, 2011; Su *et al.*, 2011, 2012; Deisig *et al.*, 2012; Pregitzer *et al.*, 2012; Münch *et al.*, 2013). Still that control flies can form preference for the food odor mixture with the additional single odor shows that inhibition of single odor sensing by another odor in the mixture is unlikely in this case. Taking these possibilities into account it is likely that single odors cannot be resolved in the mixture due to the reduced OSN sensitivity while as a single odor the resolution is high enough. This does not implicate that the ability to discriminate single odor identities is retained. One indicator that loss-of Orco results in the inability to give odors proper identities is that Orco mutants fail to distinguish between single odors (Fig.17). It could be that the flies can sense that there is an odor because it possibly leads to an increase in OSN activity but cannot assign an identity to the odor and therefore perceive both odors as similar preferable. Higher odor concentrations might allow the fly to assign proper odor identities again since higher concentrations should also increase the OSN activity and thus the input into the AL and higher brain centers for processing. This could be true if odor identity is encoded by the activated OSNs in a receptor code which in turn leads to encoding of the odor identity by a glomerular code. Less input into the system would reduce the number of recruited glomeruli since higher odor concentrations have been shown to increase the number of recruited glomeruli (Knaden *et al.*, 2012). One way to test this hypothesis could be to map the glomerular activity code to a defined odor and monitor whether this code can be seen in Orco1

mutants at the same concentration as has been used before. This would answer whether odor identity is lost due to the loss-of Orco. In a further step it could be tested if increasing the odor concentration can recapitulate the glomerular activity code in Orco1 mutant flies that was mapped at lower the lower odor concentration in wild type flies.

4.6 The OA-VUMa2 neuron might mediate site-preference through reward substitution

OA has been associated with reward processing (Schwaerzel *et al.*, 2003; Kim *et al.*, 2007, 2013). It is known that reward can be substituted by OA/TA neuron activation in appetitive learning in larva and adult flies (Schroll *et al.*, 2006; Burke *et al.*, 2012). Furthermore it was previously shown that Site-preference can be induced by activation of OA/TA neurons with the "Hammer Frequency" (Schneider *et al.*, 2012). However which neurons and through what mechanism site-preference is mediated is not known. Site-preference implicates a preference for a certain area or place but OA is not involved in place memory (Heat gradient chamber experiments; Sitaraman *et al.*, 2010). Still OA plays a role in olfactory memory indicating that there are likely distinct forms of memory depending on the form of stimuli. Therefore the observed site-preference phenotype is likely due to olfaction and not place-memory. Here the results show that activation of the same subset of OA/TA neurons with other frequencies than the "Hammer Frequency" or constant light does not induce site-preference (Fig.21 and 22). This indicates that to induce site-preference a specific activation frequency is needed. The "Hammer frequency" derived from experiments on the OA VUMmx1 neuron from the honey bee. The VUMmx1 neuron shows the "Hammer Frequency" spiking pattern when sugar reward is presented (Hammer, 1993). Furthermore, activation of the VUMmx1 neuron with the "Hammer Frequency" can substitute for the unconditioned stimulus in associative olfactory learning. The VUMmx1 neuron fulfills the criteria of convergence on the conditioned stimulus (CS) pathway (see description of criteria in Hammer, 1993). In Drosophila the VUMa2 neuron has a similar innervation pattern as the VUMmx1 neuron in the honeybee (Busch *et al.*, 2009). This implies that the VUMa2 neuron might also fulfill the criteria for reward substitution. Therefore it is possible that the VUMa2 neuron fulfills a similar role as the VUMmx1 neuron in the honey bee and therefore the same or a very similar activation frequency pattern is necessary and no other frequencies work

to induce site-preference. One way to test this hypothesis could be to activate a subset of OA/TA neurons with the “Hammer Frequency” that does not include the VUMa2 neuron using e.g. different GAL4-driver lines to express ChR-2 like the 0665-GAL4 or 0891-GAL4 (Burke *et al.*, 2012). If site-preference can still be observed it would mean that the VUMa2 neuron does not play a role in mediating it, but a different subset of neurons. One way to link the experiments performed in the honey bee with the site-preference assay might be provided by the optogenetic site-preference setup. Theoretically the setup pairs food odor as the CS (conditioned stimulus) and neuronal activation replacing the US (unconditioned stimulus). Pairing is likely forward because the blue light might illuminate a broader area than the odor plume from the small odor trap opening. Therefore the fly would be first illuminated and thus neuronally activated before sensing the odor. Whether the odor plume is indeed perceived first or the neurons are activated first has not been investigated for this setup. One probable pitfall could be that the experiments in the VUMmx1 neuron could substitute for sugar reward that is mediated by gustation and not olfaction. Still, if the mechanism of reward substitution or reward reinforcement is a general function of the neuron and also transfers to olfactory inputs it could explain the observed olfactory site-preference possibly mediated by the OA-VUMa2 neuron.

4.7 DA neurons likely mediate site-aversion through negative reinforcement

It is known that DA mediates punishment learning and is generally thought to be involved in aversion (Schwaerzel *et al.*, 2003; Yarali and Gerber, 2010). In confirmation of the role of DA in aversive behavior, it is shown here that optogenetic activation of DA neurons (*Th*-GAL4) leads to site-aversion (Fig.20). One possible explanation for this phenotype could be trough punishment learning or negative reinforcement. It was shown previously that activation of *Th*-GAL4 neurons could substitute for punishment in aversive learning in drosophila larva (Schroll *et al.*, 2006). Furthermore, optogenetic and thermogenetic activation of subsets of DA PAM or PPL1 neurons resulted in negative reinforcement when paired with an odor presentation (Claridge-Chang *et al.*, 2009; Aso *et al.*, 2010; Aso *et al.*, 2012). Therefore it could be that the activation of DA neurons substitutes for negative reinforcement in association with the odor stimuli. A form of associative punishment

learning is possible because the fly is free to enter the blue light illuminated area repeatedly for a time span of at least 16h and thus learn from experience. In support of this, it could be shown that DA neurons react to US but not CS presentation (Riemensperger *et al.*, 2005). Following this notion, it could be that activation of the DA neurons substitutes for the US and the odor would represent the CS in the site-preference experiments here leading to site-aversion. The previous experiments showing the negative reinforcement properties of DA neurons did not involve specific neuronal activation frequencies (Claridge-Chang *et al.*, 2009; Aso *et al.*, 2010; Aso *et al.*, 2012). Therefore it is likely that other neuronal activation frequencies apart from the “Hammer Frequency” can induce site-aversion. This could be tested by activation of DA neurons with varying frequencies in the site-preference assay.

Another possible explanation for site-aversion is through increases in locomotion. It has been shown that activation of DA neurons increases locomotion and modulates the pattern of movement which (Lima and Miesenböck, 2005; Zhang *et al.*, 2007). This could be mediated by a pair of DA PPM-3 (protocerebral posterior medial-3) neurons that innervate the EB of the CC known to be involved in locomotor control (Kong *et al.*, 2010). In addition, direct activation of these neurons has been shown to induce locomotion. It is conceivable that increases in locomotion upon entering the blue light and thus activating DA neurons might render the flies unable to enter the trap because of the increased movement. To test this hypothesis, the *c346*-GAL4 driver line driving expression in the locomotion promoting DA PPM-3 neurons could be used to express ChR-2 (Kong *et al.*, 2010). Activation of these neurons should lead to increased locomotion. If site-aversion is due to increases in locomotion these flies should also show site-aversion.

DA also promotes arousal which increases activity levels through the photopigment cryptochrome (Cry; Kumar *et al.*, 2012). Cry´s are blue light sensitive photopigments that are not altered by the *norpA*^{1} mutation that renders the flies optically impaired. Therefore increases in activity could also be due to promotion of arousal by activation of DA neurons with addition of the Cry photopigment excitation due to the blue light by itself. Still activation of OA/TA neurons leads to increases in locomotion but also induces site-preference and not site-aversion (Fig.24). Taking this observation into account it is unlikely that site-aversion is due to increases in locomotion. Site-preference and aversion are therefore most likely not due to

changes in locomotor activity but punishment and reward reinforcement by activation of OA/TA and DA neurons respectively.

4.8 OA/TA might mediate behavioral response selection for locomotion

OA and TA have been shown to be involved in locomotion mostly in studies on the larval body wall muscle NMJ (Yellmann *et al.*, 1997; Monastirioti *et al.*, 1996; Winther *et al.*, 2006). If there is a similar function for OA and TA neurons on NMJs in the adult fly is not known. Here it is shown that OA/TA neuron activation induces an increase in locomotion suggesting that OA/TA neurons modulate locomotion in adult flies (Fig.24). Six OA/TA neurons have descending projections to the ventral nerve cord (VNC; Busch *et al.*, 2009). These are the OA-VUMd1, -VUMd2, -VUMd3, -VL1, -VL2 and VPM1 neurons and have been suggested to potentially stimulate motor behaviors. The OA-VUMd3 neuron has been shown to be involved in courtship behavior (Certel *et al.*, 2007, 2010). Three OA and male *fruitless* positive neurons in the SOG are involved in pheromone induced promotion of aggression (Andrews *et al.*, 2014). Since aggression and courtship also involve locomotion it could be that these neurons are mediating the observed changes in locomotor activity. Use of Gal4-driver lines that drive expression in these neurons or in combination with GAL80 lines specifically inhibiting expression in selected cells like *Cha*-GAL80 (inhibiting GAL4 expression in cholinergic neurons) could illuminate whether they play a role in inducing locomotor activity.

OA has also been shown to regulate flight initiation and maintenance but the flies in the locomotion experiments performed here showed no flying or jumping behavior (Brembs *et al.*, 2007). It could still be that the flies would fly if the test arena would allow such movement.

Locomotion could also be modulated by OA neurons projecting into the CNS. Immunohistochemical studies with OA antibodies could show that immunoreactive processes can be detected at the EB a site associated with motor control although from which cells they originate has not been shown (Monastirioti, 1995). Furthermore, the EB shows high levels of OA receptors (Han *et al.*, 1998). This indicates that OA neurons could potentially influence locomotion at the EB, which would explain changes in locomotion upon activation of OA/TA neurons. But *Tdc2*-GAL4 OA/TA neurons do not innervate the EB (Busch *et al.*, 2009). Therefore other means have to provide the pathway to modulate locomotor activity in the CNS. DA neurons have

been proposed to be functionally downstream of OA neurons in appetitive short term memory and motivation through of OA receptors located on DA-PAM neurons (Burke *et al.*, 2012). Similarly it could be possible that the EB innervating PPM-3 neurons that are involved in locomotion receive input from OA/TA neurons to induce locomotor activity (Kong *et al.*, 2010). Still no evidence for synaptic connections forming such a pathway has been shown so far. Experiments to determine the presence of OA receptors on the PPM-3 neurons could elucidate whether there is a connection or possible influence of OA on the PPM-3 neurons. Still since OA is known to be involved in olfactory preference, site-preference, aggression and courtship behavior, all of which involve execution of movement, it is likely that the increase in locomotion is part of the motor program selection of one of these behaviors (Certel *et al.*, 2007, 2010; Schneider *et al.*, 2012; Andrews *et al.*, 2014).

4.9 OA/TA neuron activation frequency might mediate behavioral decision making

Most behaviors can likely be divided in groups of motor programs controlling for example feeding or locomotion. To interact with an environment the animal needs to make decisions on what and how to respond to incoming stimuli also depending on the internal state like e.g. hunger and thirst. A recent study showed that activation of neurons expressing the neuropeptide hugin induced the motor program for locomotion while suppressing the motor program for food intake in larvae (Schoofs *et al.*, 2014). Similarly three OA and Fruitless positive neurons in the SOG have been suggested to be involved in the regulation between aggression and courtship behavior in response to male pheromone sensing through the gustatory receptor Gr32a (Andrews *et al.*, 2014). Furthermore these neurons have been implicated in decision making (Certel *et al.*, 2007, 2010). These findings suggest a potential role of OA in decision making or response selection. Here we show that in addition to site-preference, locomotion can be influenced by stimulating the same set of OA/TA neurons (Fig.24). Inducing these behaviors required specific OA/TA neuron activation frequencies. One explanation for this could be that by activating all neurons that are labeled by the Tdc2-GAL4 line a vast number of responses and as such behaviors might be triggered through OA. At least two of these behavioral motor programs are aggression and courtship (Andrews *et al.*, 2014). Since locomotion is part of

preference behavior, or more precisely the execution of movement toward the preferred stimulus is, similar OA/TA neuron activation frequencies might influence both behaviors. Hypothetically, it could be that a subset of the activated OA/TA neurons are involved in the response selection of preference and as such locomotion behavior and the action of these neurons is mediated through the specific activation frequencies used here. This would effectively reduce the number of neurons that can trigger responses and therefore reduce conflicting decisions for other behavioral outputs that could overrule e.g. preference behavior. The finding that specific activation frequencies were needed to modulate preference and locomotion behavior supports this theory. Still constant neuronal activation of OA/TA neurons without specific frequency patterns have been shown to impact on for example appetitive memory formation and courtship (Schroll *et al.*, 2006; Andrews *et al.*, 2014). It remains elusive why promotion of certain behaviors does not require specific neuronal activation while promotion of other behaviors seemingly does. Without further experiments it can only be concluded that induction of site-preference and locomotion behaviors requires specific OA/TA neuron activation frequencies while other behaviors do not for unknown reasons.

One way to investigate whether OA/TA dependent response selection depends on the neuronal activation pattern would be to activate the same set of neurons with different frequencies in different behavioral paradigms. Since behavioral assays give readouts to specific selected behaviors only it could be that behavioral changes due to specific activation frequencies are simply overlooked. Therefore testing this would allow investigating if the used activation frequencies promote other behaviors as well. Another way could be to activate smaller subsets of OA/TA neurons using different GAL4-driver lines in combination with GAL80 to pinpoint the neurons mediating locomotion and site-preference

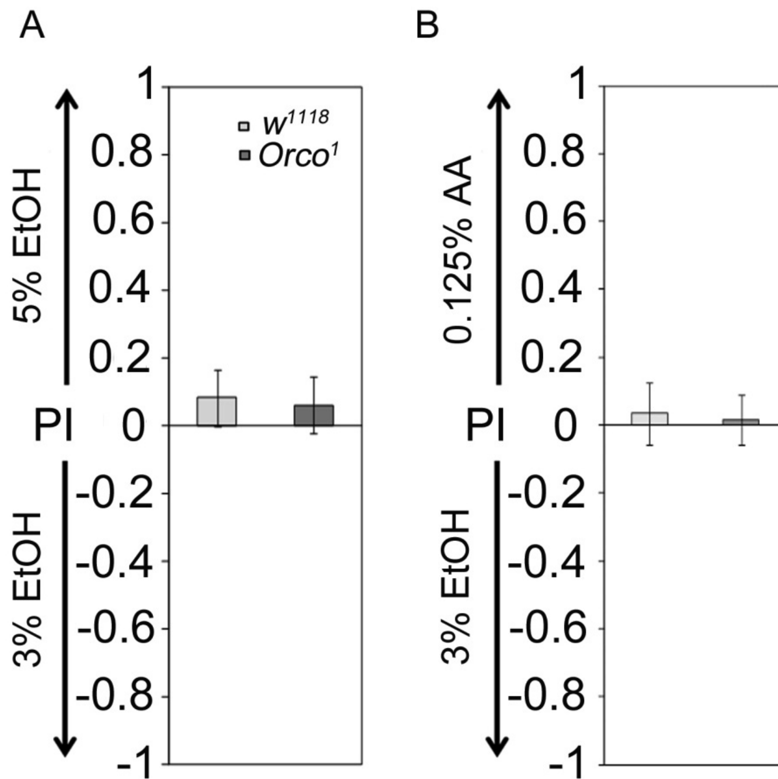
4.10 Concluding Remarks

Taken together, this study provides insights into the function of Orco and odor processing from a behavioral point of view. Furthermore, evidence is provided that OA/TA neurons regulate site-preference and locomotion behavior and could potentially play a role in response selection as has been suggested.

In the olfactory preference experiments it could be shown that Orco is not essential for olfactory preference but might rather aid in the odor processing by regulating odor sensitivity and odor identity assignment. The results further suggest that Orco independent mechanisms might act in concert with Orco dependent olfaction in shaping olfactory preference responses. To verify how and where Orco dependent and Orco independent mechanisms interact or if they interact at all further experiments are needed. Further studies using odors in complex odor mixture processing could help to understand how single odor identities might be conserved at natural concentrations.

It is shown here that behavioral output can differ and be regulated by activating the same population of OA/TA neurons with different frequencies. This indicates that response selection might be regulated by neuronal activity/spiking patterns and not by neurons simply being active or not. It seems like the activity pattern plays a major role in inducing behavioral output. If single behaviors and/or the switch between different behaviors is mediated via the activity of one neuron ore rather a group of neurons is unclear and needs to be further investigated. Future studies coupling neuroanatomical investigations and employing genetic tools to selectively activate or silence small populations of neurons could help understand how behaviors and behavioral switch is regulated.

Supplementary Figure 1. *w^{1118}/$Orco^{1}$*, PI ± SEM: (A) 0.08 ± 0.08/0.06 ± 0.08, N=24/22, P>0.05, (B) 0.03 ± 0.09/0.01 ± 0.07, N= 20/18, P>0.05. (student t-test, P<0.05).



Supplementary Table 1

	w^{1118}			$orco^{1}$		
EtOH [%]	PI	±SEM	N	PI	±SEM	N
0	-0.08	0.08	22	0.01	0.13	18
3	0.30	0.05	34	0.10	0.05	41
5	0.33	0.06	23	0.10	0.05	39
10	0.03	0.07	21	0.00	0.06	36
15	0.00	0.09	17	-0.14	0.06	41
23	-0.43	0.08	24	-0.46	0.07	18
EtOAc [%]	PI	±SEM	n	PI	±SEM	N
0	-0.01	0.06	26	-0.02	0.05	29
0.0025	0.18	0.04	48	-0.07	0.04	40
0.025	-0.18	0.05	27	0.00	0.06	28
0.25	-0.19	0.06	30	0.19	0.04	35
2.5	-0.84	0.03	19	-0.89	0.02	17
AA [%]	PI	±SEM	N	PI	±SEM	N
0	-0.04	0.08	11	0.07	0.10	11
0.0125	0.25	0.08	20	-0.02	0.07	21
0.125	0.19	0.08	19	0.10	0.07	13
1.25	0.25	0.08	20	0.15	0.04	20
5	-0.61	0.05	20	-0.22	0.06	19
12.5	-0.96	0.01	10	-0.51	0.06	9
AP [%]	PI	±SEM	N	PI	±SEM	N
0	0.06	0.07	22	-0.14	0.09	18
0.00005	-0.03	0.07	24	-0.08	0.09	23
0.0005	0.02	0.07	24	0.07	0.10	21
0.005	-0.07	0.10	25	-0.09	0.10	24
0.05	-0.19	0.10	27	-0.27	0.12	16
0.5	-1.00	0.00	13	-0.45	0.05	10

Supplementary Table 2

First Illumination cycle in 2s bins

	Control		Retinal		
bins [s]	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
0-2	0.002	0.002	0.002	0.002	0.197
2-4	0.003	0.004	0.003	0.004	0.094
4-6	0.004	0.004	0.004	0.004	0.156
6-8	0.004	0.005	0.004	0.005	0.006
8-10	0.005	0.004	0.005	0.004	0.017
10-12	0.004	0.004	0.004	0.004	0.007
12-14	0.005	0.004	0.005	0.004	0.019
14-16	0.006	0.005	0.006	0.005	0.040
16-18	0.007	0.005	0.007	0.005	0.949
18-20	0.007	0.004	0.007	0.004	1.000

First illumination cycle: Sum distances

	Control		Retinal		
	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
1st illu	0.041	0.031	0.074	0.039	0.006
1st pause	0.007	0.004	0.007	0.005	1.000
2nd illu	0.066	0.035	0.105	0.050	0.012
2nd pause	0.006	0.006	0.010	0.007	0.072
3rd illu	0.055	0.048	0.112	0.074	0.015
3rd pause	0.007	0.008	0.009	0.007	0.320

Supplementary Table 3

First Illumination cycle in 2s bins

	Control		Retinal		
bins [s]	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
0-2	0.003	0.007	0.002	0.005	0.467
2-4	0.007	0.008	0.009	0.010	0.604
4-6	0.010	0.008	0.009	0.008	0.560
6-8	0.006	0.005	0.006	0.007	0.658
8-10	0.002	0.003	0.004	0.006	0.912
10-12	0.001	0.003	0.002	0.003	0.329
12-14	0.001	0.002	0.000	0.002	0.575
14-16	0.000	0.000	0.002	0.006	1.000
16-18	0.001	0.002	0.001	0.002	0.865
18-20	0.002	0.005	0.001	0.002	0.558

First illumination cycle: Sum distances

	Control		Retinal		
	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
1st illu	0.002	0.005	0.003	0.007	0.467
1st pause	0.034	0.034	0.028	0.024	0.832
2nd illu	0.002	0.004	0.005	0.006	0.058
2nd pause	0.035	0.038	0.034	0.030	0.611
3rd illu	0.001	0.002	0.001	0.002	0.785
3rd pause	0.039	0.028	0.030	0.032	0.231

Supplementary Table 4

First Illumination cycle in 2s bins

	Control		Retinal		
bins [s]	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
0-2	0.002	0.003	0.006	0.008	0.017
2-4	0.010	0.007	0.014	0.010	0.141
4-6	0.011	0.005	0.014	0.008	0.189
6-8	0.011	0.006	0.012	0.007	0.669
8-10	0.011	0.006	0.016	0.008	0.031
10-12	0.012	0.005	0.015	0.006	0.457
12-14	0.015	0.006	0.018	0.007	0.133
14-16	0.015	0.007	0.017	0.009	0.716
16-18	0.016	0.007	0.017	0.008	0.887
18-20	0.008	0.006	0.019	0.010	0.001

First illumination cycle: Sum distances

	Control		Retinal		
	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
1st illu	0.086	0.034	0.112	0.046	0.125
1st pause	0.024	0.011	0.037	0.017	0.024
2nd illu	0.107	0.032	0.157	0.057	0.001
2nd pause	0.016	0.011	0.044	0.019	0.000
3rd illu	0.097	0.048	0.169	0.041	0.000
3rd pause	0.021	0.013	0.039	0.012	0.000

Supplementary Table 5

First Illumination cycle in 2s bins

	Control		Retinal		
bins [s]	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
0-2	0.002	0.005	0.002	0.003	0.514
2-4	0.010	0.010	0.011	0.011	0.982
4-6	0.012	0.006	0.014	0.007	0.369
6-8	0.014	0.009	0.013	0.007	0.983
8-10	0.012	0.008	0.014	0.008	0.346
10-12	0.015	0.008	0.014	0.009	0.645
12-14	0.013	0.007	0.014	0.006	1.000
14-16	0.017	0.007	0.017	0.006	0.983
16-18	0.018	0.008	0.018	0.008	0.878
18-20	0.011	0.009	0.014	0.008	0.283

First illumination cycle: Sum distances

	Control		Retinal		
	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
1st illu	0.095	0.042	0.099	0.041	0.677
1st pause	0.029	0.015	0.032	0.016	0.554
2nd illu	0.136	0.052	0.136	0.044	0.844
2nd pause	0.028	0.013	0.040	0.018	0.083
3rd illu	0.115	0.045	0.138	0.052	0.228
3rd pause	0.033	0.021	0.037	0.018	0.469

Supplementary Table 6

First Illumination cycle in 2s bins

	Control		Retinal		
bins [s]	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
0-2	0.005	0.005	0.006	0.008	0.783
2-4	0.014	0.008	0.014	0.010	0.891
4-6	0.015	0.007	0.013	0.008	0.659
6-8	0.015	0.005	0.012	0.007	0.197
8-10	0.016	0.006	0.015	0.008	0.867
10-12	0.017	0.005	0.014	0.007	0.186
12-14	0.017	0.005	0.017	0.008	0.553
14-16	0.016	0.006	0.016	0.010	0.843
16-18	0.018	0.005	0.016	0.009	0.421
18-20	0.017	0.005	0.018	0.010	0.750

First illumination cycle: Sum distances

	Control		Retinal		
	Mean [cm/min]	± STDV	Mean [cm/min]	± STDV	*P*-value
1st illu	0.133	0.039	0.130	0.051	0.891
1st pause	0.017	0.005	0.019	0.010	0.750
2nd illu	0.165	0.045	0.180	0.064	0.616
2nd pause	0.019	0.005	0.021	0.010	0.370
3rd illu	0.179	0.064	0.188	0.046	0.616
3rd pause	0.019	0.010	0.020	0.008	0.704

List of Abbreviations

AA	Acetic acid
AC	Antennal connective
AL	Antennal lobe
AN	Antennal nerve
AP	Acetophenone
ATR	*All-trans* Retinal
CC	Central complex
ChR-2	Channelrhodopsin-2
CNS	Central nervous system
DA	Dopamine
dH2O	Destilled water
EB	Ellipsoid body
eLN	Excitatory lateral neuron
EtOAc	Ethyl acetate
EtOH	Ethanol
FB	Fan shaped body
GR	Gustatory receptor
iLN	Inhibitory lateral neuron
IR	Ionotropic receptor
LH	Lateral neuron
LN	Mushroom body
MB	Neuromuscular junction
norpA	No receptor potential A
OA	Octopamine
on	Over night
OSN	Olfactory sensory neuron
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline + Triton x-1000
PI	Preference index
PN	Projection neuron
PPM-3	protocerebral posterior medial-3
RT	Room temperature
SEM	Standard error of the mean
SOG	Subesophageal ganglion
STDV	Standard deviation
TA	Tyramine
TDC	Tyrosine decarboxylase
TRP	Transient receptor potential
VM	Ventral median
VMlb	Ventral median labial
VMmd	Ventral median mandibular
VMmx	Ventral median maxillar
VPM	Ventral paired median
VUM	Ventral unpaired median
OR	Olfactory receptor
Orco	Olfactory receptor co-receptor
GPCR	G-protein coupled receptor
RNAi	Ribonucleic acid interference
PKC	Protein kinase C
cAMP	Cyclic adenosine monophosphate
Ach	Acetyl choline
VNC	Ventral nerve cord

References

Ai, M., Min, S., Grosjean, Y., Leblanc, C., Bell, R., Benton, R., Suh, G.S.B., 2010. Acid sensing by the Drosophila olfactory system. Nature 468, 691–695. doi:10.1038/nature09537

Andrews, J.C., Fernández, M.P., Yu, Q., Leary, G.P., Leung, A.K.W., Kavanaugh, M.P., Kravitz, E.A., Certel, S.J., 2014. Octopamine neuromodulation regulates Gr32a-linked aggression and courtship pathways in Drosophila males. PLoS Genet. 10, e1004356. doi:10.1371/journal.pgen.1004356

Asahina, K., Louis, M., Piccinotti, S., Vosshall, L.B., 2009. A circuit supporting concentration-invariant odor perception in Drosophila. J. Biol. 8, 9. doi:10.1186/jbiol108

Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A.B., Ito, K., Scholz, H., Tanimoto, H., 2012. Three Dopamine Pathways Induce Aversive Odor Memories with Different Stability. PLoS Genet 8, e1002768. doi:10.1371/journal.pgen.1002768

Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., Tanimoto, H., 2010. Specific dopaminergic neurons for the formation of labile aversive memory. Curr. Biol. 20, 1445–1451. doi:10.1016/j.cub.2010.06.048

Baier, A., Wittek, B., Brembs, B., 2002. Drosophila as a new model organism for the neurobiology of aggression? J. Exp. Biol. 205, 1233–1240.

Bamann, C., Kirsch, T., Nagel, G., Bamberg, E., 2008. Spectral characteristics of the photocycle of channelrhodopsin-2 and its implication for channel function. J. Mol. Biol. 375, 686–694. doi:10.1016/j.jmb.2007.10.072

Becher, P.G., Bengtsson, M., Hansson, B.S., Witzgall, P., 2010. Flying the fly: long-range flight behavior of Drosophila melanogaster to attractive odors. J. Chem. Ecol. 36, 599–607. doi:10.1007/s10886-010-9794-2

Bellmann, D., Richardt, A., Freyberger, R., Nuwal, N., Schwärzel, M., Fiala, A., Störtkuhl, K.F., 2010. Optogenetically Induced Olfactory Stimulation in Drosophila Larvae Reveals the Neuronal Basis of Odor-Aversion behavior. Front Behav Neurosci 4, 27. doi:10.3389/fnbeh.2010.00027

Benton, R., Sachse, S., Michnick, S.W., Vosshall, L.B., 2006. Atypical Membrane Topology and Heteromeric Function of Drosophila Odorant Receptors In Vivo. PLoS Biol 4, e20. doi:10.1371/journal.pbio.0040020

Benton, R., Vannice, K.S., Gomez-Diaz, C., Vosshall, L.B., 2009. Variant ionotropic glutamate receptors as chemosensory receptors in Drosophila. Cell 136, 149–162. doi:10.1016/j.cell.2008.12.001

Benton, R., Vannice, K.S., Vosshall, L.B., 2007. An essential role for a CD36-related receptor in pheromone detection in Drosophila. Nature 450, 289–293. doi:10.1038/nature06328

Bhandawat, V., Olsen, S.R., Gouwens, N.W., Schlief, M.L., Wilson, R.I., 2007. Sensory processing in the Drosophila antennal lobe increases reliability and separability of ensemble odor representations. Nat. Neurosci. 10, 1474–1482. doi:10.1038/nn1976

Brembs, B., Christiansen, F., Pflüger, H.J., Duch, C., 2007. Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. J. Neurosci. 27, 11122–11131. doi:10.1523/JNEUROSCI.2704-07.2007

Buchner, E., 1991. Genes expressed in the adult brain of Drosophila and effects of their mutations on behavior: a survey of transmitter- and second messenger-related genes. J. Neurogenet. 7, 153–192.

Buck, L., Axel, R., 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65, 175–187.

Bülthoff, H., 1982. Drosophila mutants disturbed in visual orientation. Biol. Cybern. 45, 63–70. doi:10.1007/BF00387215

Burke, C.J., Huetteroth, W., Owald, D., Perisse, E., Krashes, M.J., Das, G., Gohl, D., Silies, M., Certel, S., Waddell, S., 2012. Layered reward signalling through octopamine and dopamine in Drosophila. Nature 492, 433–437. doi:10.1038/nature11614

Busch, S., Selcho, M., Ito, K., Tanimoto, H., 2009. A map of octopaminergic neurons in the Drosophila brain. J. Comp. Neurol. 513, 643–667. doi:10.1002/cne.21966

Busch, S., Tanimoto, H., 2010. Cellular configuration of single octopamine neurons in Drosophila. J. Comp. Neurol. 518, 2355–2364. doi:10.1002/cne.22337

Carlson, J.R., 2001. Functional expression of a Drosophila odor receptor. Proc. Natl. Acad. Sci. U.S.A. 98, 8936–8937. doi:10.1073/pnas.171311198

Certel, S.J., Leung, A., Lin, C.-Y., Perez, P., Chiang, A.-S., Kravitz, E.A., 2010. Octopamine neuromodulatory effects on a social behavior decision-making network in Drosophila males. PLoS ONE 5, e13248. doi:10.1371/journal.pone.0013248

Certel, S.J., Savella, M.G., Schlegel, D.C.F., Kravitz, E.A., 2007. Modulation of Drosophila male behavioral choice. Proc. Natl. Acad. Sci. U.S.A. 104, 4706–4711. doi:10.1073/pnas.0700328104

Chen, S., Luetje, C.W., 2012. Identification of New Agonists and Antagonists of the Insect Odorant Receptor Co-Receptor Subunit. PLoS ONE 7, e36784. doi:10.1371/journal.pone.0036784

Chen, Z., Wang, Z., 2012. Functional study of hyperpolarization activated channel (Ih) in Drosophila behavior. Sci China Life Sci 55, 2–7. doi:10.1007/s11427-012-4270-6

Chentsova, N.A., Gruntenko, N.E., Bogomolova, E.V., Adonyeva, N.V., Karpova, E.K., Rauschenbach, I.Y., 2002. Stress response in Drosophila melanogaster strain inactive with decreased tyramine and octopamine contents. J. Comp. Physiol. B, Biochem. Syst. Environ. Physiol. 172, 643–650. doi:10.1007/s00360-002-0293-9

Chou, Y.-H., Spletter, M.L., Yaksi, E., Leong, J.C.S., Wilson, R.I., Luo, L., 2010. Diversity and wiring variability of olfactory local interneurons in the Drosophila antennal lobe. Nat. Neurosci. 13, 439–449. doi:10.1038/nn.2489

Claridge-Chang, A., Roorda, R.D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., Miesenböck, G., 2009. Writing memories with light-addressable reinforcement circuitry. Cell 139, 405–415. doi:10.1016/j.cell.2009.08.034

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., Carlson, J.R., 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in Drosophila. Neuron 22, 327–338.

Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J., Hirsh, J., 2005. Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J. Biol. Chem. 280, 14948–14955. doi:10.1074/jbc.M414197200

Crocker, A., Sehgal, A., 2008. Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J. Neurosci. 28, 9377–9385. doi:10.1523/JNEUROSCI.3072-08a.2008

Das, A., Sen, S., Lichtneckert, R., Okada, R., Ito, K., Rodrigues, V., Reichert, H., 2008. Drosophila olfactory local interneurons and projection neurons derive from a common neuroblast lineage specified by the empty spiracles gene. Neural Dev 3, 33. doi:10.1186/1749-8104-3-33

Dasari, S., Cooper, R.L., 2004. Modulation of sensory-CNS-motor circuits by serotonin, octopamine, and dopamine in semi-intact Drosophila larva. Neurosci. Res. 48, 221–227.

Davis, R.L., 1996. Physiology and biochemistry of Drosophila learning mutants. Physiol. Rev. 76, 299–317.

De Belle, J.S., Heisenberg, M., 1994. Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. Science 263, 692–695.

De Bruyne, M., Clyne, P.J., Carlson, J.R., 1999. Odor coding in a model olfactory organ: the Drosophila maxillary palp. J. Neurosci. 19, 4520–4532.

De Bruyne, M., Foster, K., Carlson, J.R., 2001. Odor coding in the Drosophila antenna. Neuron 30, 537–552.

Deisig, N., Giurfa, M., Lachnit, H., Sandoz, J.-C., 2006. Neural representation of olfactory mixtures in the honeybee antennal lobe. Eur. J. Neurosci. 24, 1161–1174. doi:10.1111/j.1460-9568.2006.04959.x

Deisig, N., Kropf, J., Vitecek, S., Pevergne, D., Rouyar, A., Sandoz, J.-C., Lucas, P., Gadenne, C., Anton, S., Barrozo, R., 2012. Differential interactions of sex pheromone and plant odour in the olfactory pathway of a male moth. PLoS ONE 7, e33159. doi:10.1371/journal.pone.0033159

Deng, Ying, 2009. Molecular mechanism of olfactory signal transduction in Drosophila Melanogaster. Doctoral Dissertation

Deng, Y., Zhang, W., Farhat, K., Oberland, S., Gisselmann, G., Neuhaus, E.M., 2011. The stimulatory Gα(s) protein is involved in olfactory signal transduction in Drosophila. PLoS ONE 6, e18605. doi:10.1371/journal.pone.0018605

Diegelmann, S., Bate, M., Landgraf, M. (2008). Gateway cloning vectors for the LexA-based binary expression system in Drosophila. Fly 2(4): 236--239.

Dobritsa, A.A., van der Goes van Naters, W., Warr, C.G., Steinbrecht, R.A., Carlson, J.R., 2003. Integrating the Molecular and Cellular Basis of Odor Coding in the Drosophila Antenna. Neuron 37, 827–841. doi:10.1016/S0896-6273(03)00094-1

Dweck, H.K.M., Ebrahim, S.A.M., Kromann, S., Bown, D., Hillbur, Y., Sachse, S., Hansson, B.S., Stensmyr, M.C., 2013. Olfactory preference for egg laying on citrus substrates in Drosophila. Curr. Biol. 23, 2472–2480. doi:10.1016/j.cub.2013.10.047

Elmore, T., Ignell, R., Carlson, J.R., Smith, D.P., 2003. Targeted mutation of a Drosophila odor receptor defines receptor requirement in a novel class of sensillum. J. Neurosci. 23, 9906–9912.

Emery, P., Stanewsky, R., Helfrich-Förster, C., Emery-Le, M., Hall, J.C., Rosbash, M., 2000. Drosophila CRY is a deep brain circadian photoreceptor. Neuron 26, 493–504.

Farooqui, T., Robinson, K., Vaessin, H., Smith, B.H., 2003. Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. J. Neurosci. 23, 5370–5380.

Feinberg, E.H., Vanhoven, M.K., Bendesky, A., Wang, G., Fetter, R.D., Shen, K., Bargmann, C.I., 2008. GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron 57, 353–363. doi:10.1016/j.neuron.2007.11.030

Feldbauer, K., Zimmermann, D., Pintschovius, V., Spitz, J., Bamann, C., Bamberg, E., 2009. Channelrhodopsin-2 is a leaky proton pump. PNAS 106, 12317–12322. doi:10.1073/pnas.0905852106

Fiala, A., Suska, A., Schlüter, O.M., 2010. Optogenetic Approaches in Neuroscience. Current Biology 20, R897–R903. doi:10.1016/j.cub.2010.08.053

Fox, L.E., Soll, D.R., Wu, C.-F., 2006. Coordination and modulation of locomotion pattern generators in Drosophila larvae: effects of altered biogenic amine levels by the tyramine beta hydroxlyase mutation. J. Neurosci. 26, 1486–1498. doi:10.1523/JNEUROSCI.4749-05.2006

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., Birman, S., 2003. Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. J. Neurobiol. 54, 618–627. doi:10.1002/neu.10185

Fuenzalida-Uribe, N., Meza, R.C., Hoffmann, H.A., Varas, R., Campusano, J.M., 2013. nAChR-induced octopamine release mediates the effect of nicotine on a startle response in Drosophila melanogaster. J. Neurochem. doi:10.1111/jnc.12161

Galizia, C.G., Sachse, S., Rappert, A., Menzel, R., 1999. The glomerular code for odor representation is species specific in the honeybee Apis mellifera. Nat. Neurosci. 2, 473–478. doi:10.1038/8144

Gao, Q., Chess, A., 1999. Identification of candidate Drosophila olfactory receptors from genomic DNA sequence. Genomics 60, 31–39. doi:10.1006/geno.1999.5894

Gao, Q., Yuan, B., Chess, A., 2000. Convergent projections of Drosophila olfactory neurons to specific glomeruli in the antennal lobe. Nat. Neurosci. 3, 780–785. doi:10.1038/77680

German, P.F., van der Poel, S., Carraher, C., Kralicek, A.V., Newcomb, R.D., 2013. Insights into subunit interactions within the insect olfactory receptor complex using FRET. Insect Biochem. Mol. Biol. 43, 138–145. doi:10.1016/j.ibmb.2012.11.002

Gervasi, N., Tchénio, P., Preat, T., 2010. PKA dynamics in a Drosophila learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase. Neuron 65, 516–529. doi:10.1016/j.neuron.2010.01.014

Getahun, M.N., Olsson, S.B., Lavista-Llanos, S., Hansson, B.S., Wicher, D., 2013. Insect Odorant Response Sensitivity Is Tuned by Metabotropically Autoregulated Olfactory Receptors. PLoS One 8. doi:10.1371/journal.pone.0058889

Gisselmann, G., Gamerschlag, B., Sonnenfeld, R., Marx, T., Neuhaus, E.M., Wetzel, C.H., Hatt, H., 2005. Variants of the Drosophila melanogaster I(h)-channel are generated by different splicing. Insect Biochemistry and Molecular Biology 35, 505–514.

Goldman, A.L., Van der Goes van Naters, W., Lessing, D., Warr, C.G., Carlson, J.R., 2005. Coexpression of Two Functional Odor Receptors in One Neuron. Neuron 45, 661–666. doi:10.1016/j.neuron.2005.01.025

Gonzalo-Gomez, A., Turiegano, E., León, Y., Molina, I., Torroja, L., Canal, I., 2012. Ih Current Is Necessary to Maintain Normal Dopamine Fluctuations and Sleep Consolidation in Drosophila. PLoS ONE 7, e36477. doi:10.1371/journal.pone.0036477

Gordon, M.D., Scott, K., 2009. Motor Control in a Drosophila Taste Circuit. Neuron 61, 373–384. doi:10.1016/j.neuron.2008.12.033

Grabe, V., Strutz, A., Baschwitz, A., Hansson, B.S., Sachse, S., 2014. Digital in vivo 3D atlas of the antennal lobe of Drosophila melanogaster. J. Comp. Neurol. doi:10.1002/cne.23697

Grosse-Wilde, E., Svatos, A., Krieger, J., 2006. A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor in vitro. Chem. Senses 31, 547–555. doi:10.1093/chemse/bjj059

Gruntenko, N., Chentsova, N.A., Bogomolova, E.V., Karpova, E.K., Glazko, G.V., Faddeeva, N.V., Monastirioti, M., Rauschenbach, I.Y., 2004. The effect of mutations altering biogenic amine metabolism in Drosophila on viability and the response to environmental stresses. Arch. Insect Biochem. Physiol. 55, 55–67. doi:10.1002/arch.10123

Gunaydin, L.A., Yizhar, O., Berndt, A., Sohal, V.S., Deisseroth, K., Hegemann, P., 2010. Ultrafast optogenetic control. Nat Neurosci 13, 387–392. doi:10.1038/nn.2495

Guo, S., Kim, J., 2010. Dissecting the molecular mechanism of drosophila odorant receptors through activity modeling and comparative analysis. Proteins 78, 381–399. doi:10.1002/prot.22556

Haddad, R., Weiss, T., Khan, R., Nadler, B., Mandairon, N., Bensafi, M., Schneidman, E., Sobel, N., 2010. Global features of neural activity in the olfactory system form a parallel code that predicts olfactory behavior and perception. J. Neurosci. 30, 9017–9026. doi:10.1523/JNEUROSCI.0398-10.2010

Hallem, E.A., Carlson, J.R., 2006. Coding of odors by a receptor repertoire. Cell 125, 143–160. doi:10.1016/j.cell.2006.01.050

Hallem, E.A., Ho, M.G., Carlson, J.R., 2004. The Molecular Basis of Odor Coding in the Drosophila Antenna. Cell 117, 965–979. doi:10.1016/j.cell.2004.05.012

Hallem, E.A., Nicole Fox, A., Zwiebel, L.J., Carlson, J.R., 2004b. Olfaction: mosquito receptor for human-sweat odorant. Nature 427, 212–213. doi:10.1038/427212a

Hammer, M., 1993. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature 366, 59–63. doi:10.1038/366059a0

Hammer, M., Menzel, R., 1998. Multiple Sites of Associative Odor Learning as Revealed by Local Brain Microinjections of Octopamine in Honeybees. Learning & Memory 5, 146 – 156. doi:10.1101/lm.5.1.146

Han, K.A., Millar, N.S., Davis, R.L., 1998. A novel octopamine receptor with preferential expression in Drosophila mushroom bodies. J. Neurosci. 18, 3650–3658.

Hardie, S.L., Zhang, J.X., Hirsh, J., 2007. Trace amines differentially regulate adult locomotor activity, cocaine sensitivity, and female fertility in Drosophila melanogaster. Dev Neurobiol 67, 1396–1405. doi:10.1002/dneu.20459

Heimbeck, G., Bugnon, V., Gendre, N., Keller, A., Stocker, R.F., 2001. A central neural circuit for experience-independent olfactory and courtship behavior in Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.A. 98, 15336–15341. doi:10.1073/pnas.011314898

Heisenberg, M., 2003. Mushroom body memoir: from maps to models. Nat Rev Neurosci 4, 266–275. doi:10.1038/nrn1074

Heisenberg, M., Borst, A., Wagner, S., Byers, D., 1985. Drosophila mushroom body mutants are deficient in olfactory learning. J. Neurogenet. 2, 1–30.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., Chrystal, M.A., Cravchik, A., Collins, F.H., Robertson, H.M., Zwiebel, L.J., 2002. G protein-coupled receptors in Anopheles gambiae. Science 298, 176–178. doi:10.1126/science.1076196

Hillier, N.K., Vickers, N.J., 2011. Mixture interactions in moth olfactory physiology: examining the effects of odorant mixture, concentration, distal stimulation, and antennal nerve transection on sensillar responses. Chem. Senses 36, 93–108. doi:10.1093/chemse/bjq102

Hoyer, S.C., Eckart, A., Herrel, A., Zars, T., Fischer, S.A., Hardie, S.L., Heisenberg, M., 2008. Octopamine in Male Aggression of Drosophila. Current Biology 18, 159–167. doi:10.1016/j.cub.2007.12.052

Huang, J., Zhang, W., Qiao, W., Hu, A., Wang, Z., 2010. Functional Connectivity and Selective Odor Responses of Excitatory Local Interneurons in Drosophila Antennal Lobe. Neuron 67, 1021–1033. doi:10.1016/j.neuron.2010.08.025

Ishizuka, T., Kakuda, M., Araki, R., Yawo, H., 2006. Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels. Neuroscience Research 54, 85–94. doi:10.1016/j.neures.2005.10.009

Jackson, F.R., Newby, L.M., Kulkarni, S.J., 1990. Drosophila GABAergic systems: sequence and expression of glutamic acid decarboxylase. J. Neurochem. 54, 1068–1078.

Jefferis, G.S.X.E., Potter, C.J., Chan, A.M., Marin, E.C., Rohlfing, T., Maurer, C.R., Luo, L., 2007. Comprehensive maps of Drosophila higher olfactory centers: spatially segregated fruit and pheromone representation. Cell 128, 1187–1203. doi:10.1016/j.cell.2007.01.040

Johnson, E., Ringo, J., Dowse, H., 1997. Modulation of Drosophila heartbeat by neurotransmitters. J. Comp. Physiol. B, Biochem. Syst. Environ. Physiol. 167, 89–97.

Jones, P.L., Pask, G.M., Rinker, D.C., Zwiebel, L.J., 2011. Functional agonism of insect odorant receptor ion channels. Proc. Natl. Acad. Sci. U.S.A. 108, 8821–8825. doi:10.1073/pnas.1102425108

Joseph, R.M., Devineni, A.V., King, I.F.G., Heberlein, U., 2009. Oviposition preference for and positional avoidance of acetic acid provide a model for competing behavioral drives in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 106, 11352–11357. doi:10.1073/pnas.0901419106

Kain, P., Chakraborty, T.S., Sundaram, S., Siddiqi, O., Rodrigues, V., Hasan, G., 2008. Reduced odor responses from antennal neurons of G(q)alpha, phospholipase Cbeta, and rdgA mutants in Drosophila support a role for a phospholipid intermediate in insect olfactory transduction. J. Neurosci. 28, 4745–4755. doi:10.1523/JNEUROSCI.5306-07.2008

Kazama, H., Wilson, R.I., 2008. Homeostatic matching and nonlinear amplification at identified central synapses. Neuron 58, 401–413. doi:10.1016/j.neuron.2008.02.030

Kazama, H., Wilson, R.I., 2009. Origins of correlated activity in an olfactory circuit. Nat. Neurosci. 12, 1136–1144. doi:10.1038/nn.2376

Kim, A.J., Lazar, A.A., Slutskiy, Y.B., 2011. System identification of Drosophila olfactory sensory neurons. J Comput Neurosci 30, 143–161. doi:10.1007/s10827-010-0265-0

Kim, Y.-C., Lee, H.-G., Han, K.-A., 2007. D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. J. Neurosci. 27, 7640–7647. doi:10.1523/JNEUROSCI.1167-07.2007

Kim, Y.-C., Lee, H.-G., Lim, J., Han, K.-A., 2013. Appetitive learning requires the alpha1-like octopamine receptor OAMB in the Drosophila mushroom body neurons. J. Neurosci. 33, 1672–1677. doi:10.1523/JNEUROSCI.3042-12.2013

Knaden, M., Strutz, A., Ahsan, J., Sachse, S., Hansson, B.S., 2012. Spatial Representation of Odorant Valence in an Insect Brain. Cell Reports 1, 392–399. doi:10.1016/j.celrep.2012.03.002

Kong, E.C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M.R., Heberlein, U., Bainton, R.J., Hirsh, J., Wolf, F.W., 2010. A Pair of Dopamine Neurons Target the D1-Like Dopamine Receptor DopR in the Central Complex to Promote Ethanol-Stimulated Locomotion in Drosophila. PLoS ONE 5, e9954. doi:10.1371/journal.pone.0009954

Kreher, S.A., Mathew, D., Kim, J., Carlson, J.R., 2008. Translation of sensory input into behavioral output via an olfactory system. Neuron 59, 110–124. doi:10.1016/j.neuron.2008.06.010

Krieger, J., Klink, O., Mohl, C., Raming, K., Breer, H., 2003. A candidate olfactory receptor subtype highly conserved across different insect orders. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 189, 519–526. doi:10.1007/s00359-003-0427-x

Kumar, S., Chen, D., Sehgal, A., 2012. Dopamine acts through Cryptochrome to promote acute arousal in Drosophila. Genes Dev. 26, 1224–1234. doi:10.1101/gad.186338.111

Lai, S.-L., Awasaki, T., Ito, K., Lee, T., 2008. Clonal analysis of Drosophila antennal lobe neurons: diverse neuronal architectures in the lateral neuroblast lineage. Development 135, 2883–2893. doi:10.1242/dev.024380

Laissue, P. p., Reiter, C., Hiesinger, P. r., Halter, S., Fischbach, K. f., Stocker, R. f., 1999. Three-dimensional reconstruction of the antennal lobe in Drosophila melanogaster. The Journal of Comparative Neurology 405, 543–552. doi:10.1002/(SICI)1096-9861(19990322)405:4<543::AID-CNE7>3.0.CO;2-A

Lange, A.B., 2009. Tyramine: from octopamine precursor to neuroactive chemical in insects. Gen. Comp. Endocrinol. 162, 18–26. doi:10.1016/j.ygcen.2008.05.021

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., Vosshall, L.B., 2004. Or83b Encodes a Broadly Expressed Odorant Receptor Essential for Drosophila Olfaction. Neuron 43, 703–714. doi:10.1016/j.neuron.2004.08.019

Lebreton, S., Becher, P.G., Hansson, B.S., Witzgall, P., 2012. Attraction of Drosophila melanogaster males to food-related and fly odours. J. Insect Physiol. 58, 125–129. doi:10.1016/j.jinsphys.2011.10.009

Lee, H.-G., Seong, C.-S., Kim, Y.-C., Davis, R.L., Han, K.-A., 2003. Octopamine receptor OAMB is required for ovulation in Drosophila melanogaster. Dev. Biol. 264, 179–190.

Lee, T., Lee, A. & Luo, L. (1999): Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development 126 (18): 4065-76

Lei, H., Vickers, N., 2008. Central processing of natural odor mixtures in insects. J. Chem. Ecol. 34, 915–927. doi:10.1007/s10886-008-9487-2

Lim, J., Sabandal, P.R., Fernandez, A., Sabandal, J.M., Lee, H.-G., Evans, P., Han, K.-A., 2014. The octopamine receptor Octβ2R regulates ovulation in Drosophila melanogaster. PLoS ONE 9, e104441. doi:10.1371/journal.pone.0104441

Lima, S.Q., Miesenböck, G., 2005. Remote Control of Behavior through Genetically Targeted Photostimulation of Neurons. Cell 121, 141–152. doi:10.1016/j.cell.2005.02.004

Lin, H.-H., Lin, C.-Y., Chiang, A.-S., 2007. Internal representations of smell in the Drosophila brain. J. Biomed. Sci. 14, 453–459. doi:10.1007/s11373-007-9168-0

Lin, J.Y., Lin, M.Z., Steinbach, P., Tsien, R.Y., 2009. Characterization of Engineered Channelrhodopsin Variants with Improved Properties and Kinetics. Biophysical Journal 96, 1803–1814. doi:10.1016/j.bpj.2008.11.034

Linster, C., Cleland, T.A., 2004. Configurational and elemental odor mixture perception can arise from local inhibition. J Comput Neurosci 16, 39–47.

Littleton, J.T., Ganetzky, B., 2000. Ion channels and synaptic organization: analysis of the Drosophila genome. Neuron 26, 35–43.

Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B.D., Aso, Y., Friedrich, A.B., Siwanowicz, I., Rubin, G.M., Preat, T., Tanimoto, H., 2012. A subset of dopamine neurons signals reward for odour memory in Drosophila. Nature 488, 512–516. doi:10.1038/nature11304

Liu, W.W., Wilson, R.I., 2013. Glutamate is an inhibitory neurotransmitter in the Drosophila olfactory system. Proc. Natl. Acad. Sci. U.S.A. doi:10.1073/pnas.1220560110

Lundin, C., Käll, L., Kreher, S.A., Kapp, K., Sonnhammer, E.L., Carlson, J.R., Heijne, G. von, Nilsson, I., 2007. Membrane topology of the Drosophila OR83b odorant receptor. FEBS Lett. 581, 5601–5604. doi:10.1016/j.febslet.2007.11.007

Luo, S.X., Axel, R., Abbott, L.F., 2010. Generating sparse and selective third-order responses in the olfactory system of the fly. Proc. Natl. Acad. Sci. U.S.A. 107, 10713–10718. doi:10.1073/pnas.1005635107

Marin, E.C., Jefferis, G.S.X.E., Komiyama, T., Zhu, H., Luo, L., 2002. Representation of the glomerular olfactory map in the Drosophila brain. Cell 109, 243–255.

Martin, F., Riveron, J., Alcorta, E., 2011. Environmental temperature modulates olfactory reception in Drosophila melanogaster. Journal of Insect Physiology 57, 1631–1642. doi:10.1016/j.jinsphys.2011.08.016

Marx, T., Gisselmann, G., Störtkuhl, K.F., Hovemann, B.T., Hatt, H., 1999. Molecular cloning of a putative voltage- and cyclic nucleotide-gated ion channel present in the antennae and eyes of Drosophila melanogaster. Invert. Neurosci. 4, 55–63. doi:10.1007/s101589900006

Masse, N.Y., Turner, G.C., Jefferis, G.S.X.E., 2009. Olfactory information processing in Drosophila. Curr. Biol. 19, R700–713. doi:10.1016/j.cub.2009.06.026

McClung, C., Hirsh, J., 1999. The trace amine tyramine is essential for sensitization to cocaine in Drosophila. Curr. Biol. 9, 853–860.

Min, S., Ai, M., Shin, S.A., Suh, G.S.B., 2013. Dedicated olfactory neurons mediating attraction behavior to ammonia and amines in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 110, E1321–1329. doi:10.1073/pnas.1215680110

Monastirioti, M., 2003. Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in Drosophila melanogaster. Dev. Biol. 264, 38–49.

Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., Budnik, V., 1995. Octopamine immunoreactivity in the fruit fly Drosophila melanogaster. J. Comp. Neurol. 356, 275–287. doi:10.1002/cne.903560210

Monastirioti, M., Linn, C.E., White, K., 1996. Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. J. Neurosci. 16, 3900–3911.

Münch, D., Schmeichel, B., Silbering, A.F., Galizia, C.G., 2013. Weaker ligands can dominate an odor blend due to syntopic interactions. Chem. Senses 38, 293–304. doi:10.1093/chemse/bjs138

Murlis, J., Elkinton, J.S., Cardé, R.T., 1992. Odor Plumes and How Insects Use Them. Annual Review of Entomology 37, 505–532. doi:10.1146/annurev.en.37.010192.002445

Nagaya, Y., Kutsukake, M., Chigusa, S.I., Komatsu, A., 2002. A trace amine, tyramine, functions as a neuromodulator in Drosophila melanogaster. Neuroscience Letters 329, 324–328. doi:10.1016/S0304-3940(02)00596-7

Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Musti, A.M., Bamberg, E., Hegemann, P., 2002. Channelrhodopsin-1: A Light-Gated Proton Channel in Green Algae. Science 296, 2395–2398. doi:10.1126/science.1072068

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., Bamberg, E., 2003. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. PNAS 100, 13940–13945. doi:10.1073/pnas.1936192100

Nagel, K.I., Wilson, R.I., 2011. Biophysical mechanisms underlying olfactory receptor neuron dynamics. Nat Neurosci 14, 208–216. doi:10.1038/nn.2725

Nakagawa, T., Sakurai, T., Nishioka, T., Touhara, K., 2005. Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. Science 307, 1638–1642. doi:10.1126/science.1106267

Neuhaus, E.M., Gisselmann, G., Zhang, W., Dooley, R., Störtkuhl, K., Hatt, H., 2005. Odorant receptor heterodimerization in the olfactory system of Drosophila melanogaster. Nat. Neurosci. 8, 15–17. doi:10.1038/nn1371

Ng, M., Roorda, R.D., Lima, S.Q., Zemelman, B.V., Morcillo, P., Miesenböck, G., 2002. Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. Neuron 36, 463–474.

Nichols, A.S., Chen, S., Luetje, C.W., 2011. Subunit contributions to insect olfactory receptor function: channel block and odorant recognition. Chem. Senses 36, 781–790. doi:10.1093/chemse/bjr053

Niewalda, T., Völler, T., Eschbach, C., Ehmer, J., Chou, W.-C., Timme, M., Fiala, A., Gerber, B., 2011. A combined perceptual, physico-chemical, and imaging approach to “odour-distances” suggests a categorizing function of the Drosophila antennal lobe. PLoS ONE 6, e24300. doi:10.1371/journal.pone.0024300

Nishikawa, K., Kidokoro, Y., 1999. Octopamine inhibits synaptic transmission at the larval neuromuscular junction in Drosophila melanogaster. Brain Res. 837, 67–74.

Nolte, A., Funk, N.W., Mukunda, L., Gawalek, P., Werckenthin, A., Hansson, B.S., Wicher, D., Stengl, M., 2013. In situ tip-recordings found no evidence for an Orco-based ionotropic

mechanism of pheromone-transduction in Manduca sexta. PLoS ONE 8, e62648. doi:10.1371/journal.pone.0062648

Nuwal, N. (2010). Optogenetic investigation of nervous system functions using walking behavior and genome wide transcript analysis of Synapsin and Sap47 mutants of Drosophila. Dissertation Bayerische Julius-Maximilians Universität Würzburg

O'Dell, K.M., 1993. The effect of the inactive mutation on longevity, sex, rhythm and resistance to p-cresol in Drosophila melanogaster. Heredity (Edinb) 70 (Pt 4), 393–399.

Ogueta, M., Cibik, O., Eltrop, R., Schneider, A., Scholz, H., 2010. The influence of Adh function on ethanol preference and tolerance in adult Drosophila melanogaster. Chem. Senses 35, 813–822. doi:10.1093/chemse/bjq084

Okada, R., Awasaki, T., Ito, K., 2009. Gamma-aminobutyric acid (GABA)-mediated neural connections in the Drosophila antennal lobe. J. Comp. Neurol. 514, 74–91. doi:10.1002/cne.21971

Olsen, S.R., Bhandawat, V., Wilson, R.I., 2007. Excitatory interactions between olfactory processing channels in the Drosophila antennal lobe. Neuron 54, 89–103. doi:10.1016/j.neuron.2007.03.010

Olsen, S.R., Bhandawat, V., Wilson, R.I., 2010. Divisive normalization in olfactory population codes. Neuron 66, 287–299. doi:10.1016/j.neuron.2010.04.009

Olsen, S.R., Wilson, R.I., 2008. Lateral presynaptic inhibition mediates gain control in an olfactory circuit. Nature 452, 956–960. doi:10.1038/nature06864

Parnas, M., Lin, A.C., Huetteroth, W., Miesenböck, G., 2013. Odor discrimination in Drosophila: from neural population codes to behavior. Neuron 79, 932–944. doi:10.1016/j.neuron.2013.08.006

Pask, G.M., Jones, P.L., Rützler, M., Rinker, D.C., Zwiebel, L.J., 2011. Heteromeric Anopheline odorant receptors exhibit distinct channel properties. PLoS ONE 6, e28774. doi:10.1371/journal.pone.0028774

Pass, G., Agricola, H., Birkenbeil, H., Penzlin, H., 1988. Morphology of neurones associated with the antennal heart of Periplaneta americana (Blattodea, Insecta). Cell Tissue Res. 253, 319–326.

Patch, H.M., Velarde, R.A., Walden, K.K.O., Robertson, H.M., 2009. A candidate pheromone receptor and two odorant receptors of the hawkmoth Manduca sexta. Chem. Senses 34, 305–316. doi:10.1093/chemse/bjp002

Peter, M., 2004. Odorant receptor gene choice in olfactory sensory neurons: the one receptor–one neuron hypothesis revisited. Current Opinion in Neurobiology 14, 31–36. doi:10.1016/j.conb.2004.01.014

Pitts, R.J., Fox, A.N., Zwiebel, L.J., 2004. A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector Anopheles gambiae. Proc. Natl. Acad. Sci. U.S.A. 101, 5058–5063. doi:10.1073/pnas.0308146101

Pophof, B., 2002. Octopamine enhances moth olfactory responses to pheromones, but not those to general odorants. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 188, 659–662. doi:10.1007/s00359-002-0343-5

Potter, C.J., Luo, L., 2008. Octopamine fuels fighting flies. Nat. Neurosci. 11, 989–990. doi:10.1038/nn0908-989

Pregitzer, P., Schubert, M., Breer, H., Hansson, B.S., Sachse, S., Krieger, J., 2012. Plant odorants interfere with detection of sex pheromone signals by male Heliothis virescens. Front Cell Neurosci 6, 42. doi:10.3389/fncel.2012.00042

Pulver, S.R., Hornstein, N.J., Land, B.L., Johnson, B.R., 2011. Optogenetics in the teaching laboratory: using channelrhodopsin-2 to study the neural basis of behavior and synaptic physiology in Drosophila. Adv Physiol Educ 35, 82–91. doi:10.1152/advan.00125.2010

Pulver, S.R., Pashkovski, S.L., Hornstein, N.J., Garrity, P.A., Griffith, L.C., 2009. Temporal Dynamics of Neuronal Activation by Channelrhodopsin-2 and TRPA1 Determine Behavioral Output in Drosophila Larvae. Journal of Neurophysiology 101, 3075 –3088. doi:10.1152/jn.00071.2009

Rein, J., Mustard, J.A., Strauch, M., Smith, B.H., Galizia, C.G., 2013. Octopamine modulates activity of neural networks in the honey bee antennal lobe. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 199, 947–962. doi:10.1007/s00359-013-0805-y

Riemensperger, T., Völler, T., Stock, P., Buchner, E., Fiala, A., 2005. Punishment Prediction by Dopaminergic Neurons in Drosophila. Current Biology 15, 1953–1960. doi:10.1016/j.cub.2005.09.042

Riffell, J.A., Lei, H., Christensen, T.A., Hildebrand, J.G., 2009. Characterization and coding of behaviorally significant odor mixtures. Curr. Biol. 19, 335–340. doi:10.1016/j.cub.2009.01.041

Riveron, J., Boto, T., Alcorta, E., 2009. The effect of environmental temperature on olfactory perception in Drosophila melanogaster. Journal of Insect Physiology 55, 943–951. doi:10.1016/j.jinsphys.2009.06.009

Roeder, T., 1999. Octopamine in invertebrates. Prog. Neurobiol. 59, 533–561.

Roeder, T., 2005. Tyramine and octopamine: ruling behavior and metabolism. Annu. Rev. Entomol. 50, 447–477. doi:10.1146/annurev.ento.50.071803.130404

Roeder, T., Seifert, M., Kähler, C., Gewecke, M., 2003. Tyramine and octopamine: antagonistic modulators of behavior and metabolism. Arch. Insect Biochem. Physiol. 54, 1–13. doi:10.1002/arch.10102

Ronderos, D.S., Lin, C.-C., Potter, C.J., Smith, D.P., 2014. Farnesol-detecting olfactory neurons in Drosophila. J. Neurosci. 34, 3959–3968. doi:10.1523/JNEUROSCI.4582-13.2014

Root, C.M., Masuyama, K., Green, D.S., Enell, L.E., Nässel, D.R., Lee, C.-H., Wang, J.W., 2008. A presynaptic gain control mechanism fine-tunes olfactory behavior. Neuron 59, 311–321. doi:10.1016/j.neuron.2008.07.003

Root, C.M., Semmelhack, J.L., Wong, A.M., Flores, J., Wang, J.W., 2007. Propagation of olfactory information in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 104, 11826–11831. doi:10.1073/pnas.0704523104

Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., Yasukochi, Y., Touhara, K., Nishioka, T., 2004. Identification and functional characterization of a sex pheromone receptor in the silkmoth Bombyx mori. Proc. Natl. Acad. Sci. U.S.A. 101, 16653–16658. doi:10.1073/pnas.0407596101

Saraswati, S., Fox, L.E., Soll, D.R., Wu, C.-F., 2004. Tyramine and octopamine have opposite effects on the locomotion of Drosophila larvae. J. Neurobiol. 58, 425–441. doi:10.1002/neu.10298

Sargsyan, V., Getahun, M.N., Llanos, S.L., Olsson, S.B., Hansson, B.S., Wicher, D., 2011. Phosphorylation via PKC Regulates the Function of the Drosophila Odorant Co-Receptor. Front Cell Neurosci 5, 5. doi:10.3389/fncel.2011.00005

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., Touhara, K., 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452, 1002–1006. doi:10.1038/nature06850

Scheiner, R., Plückhahn, S., Öney, B., Blenau, W., Erber, J., 2002. Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. Behavioural Brain Research 136, 545–553. doi:10.1016/S0166-4328(02)00205-X

Scheiner, R., Steinbach, A., Claßen, G., Strudthoff, N., Scholz, H., 2014. Octopamine indirectly affects proboscis extension response habituation in Drosophila melanogaster by controlling sucrose responsiveness. J. Insect Physiol. doi:10.1016/j.jinsphys.2014.03.011

Schindelin, J. Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, Curtis Rueden, Stephan Saalfeld, Benjamin Schmid, Jean-Yves Tinevez, Daniel James White, Volker Hartenstein, Kevin Eliceiri, Pavel Tomancak and Albert Cardona (**2012**) Fiji: an open-source platform for biological-image analysis, *Nature Methods* 9(7): 676-682

Schlief, M.L., Wilson, R.I., 2007. Olfactory processing and behavior downstream from highly selective receptor neurons. Nat. Neurosci. 10, 623–630. doi:10.1038/nn1881

Schneider, Andrea. 2011. Analyse der mechanistischen Grundlagen von Ethanol Präferenz bei *Drosophila melanogaster.* Doctoral Dissertation

Schneider, A., Ruppert, M., Hendrich, O., Giang, T., Ogueta, M., Hampel, S., Vollbach, M., Büschges, A., Scholz, H., 2012. Neuronal basis of innate olfactory attraction to ethanol in Drosophila. PLoS ONE 7, e52007. doi:10.1371/journal.pone.0052007

Scholz, H., 2005b. Influence of the biogenic amine tyramine on ethanol-induced behaviors in Drosophila. J. Neurobiol. 63, 199–214. doi:10.1002/neu.20127

Scholz, H., Ramond, J., Singh, C.M., Heberlein, U., 2000. Functional ethanol tolerance in Drosophila. Neuron 28, 261–271.

Scholz, H., Ramond, J., Singh, C.M., Heberlein, U., 2000. Functional ethanol tolerance in Drosophila. Neuron 28, 261–271.

Schoofs, A., Hückesfeld, S., Schlegel, P., Miroschnikow, A., Peters, M., Zeymer, M., Spieß, R., Chiang, A.-S., Pankratz, M.J., 2014. Selection of Motor Programs for Suppressing Food Intake and Inducing Locomotion in the Drosophila Brain. PLoS Biol 12, e1001893. doi:10.1371/journal.pbio.1001893

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E., Fiala, A., 2006. Light-Induced Activation of Distinct Modulatory Neurons Triggers Appetitive or Aversive Learning in Drosophila Larvae. Current Biology 16, 1741–1747. doi:10.1016/j.cub.2006.07.023

Schuckel, J., Torkkeli, P.H., French, A.S., 2009. Two interacting olfactory transduction mechanisms have linked polarities and dynamics in Drosophila melanogaster antennal basiconic sensilla neurons. J. Neurophysiol. 102, 214–223. doi:10.1152/jn.00162.2009

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

Seki, Y., Rybak, J., Wicher, D., Sachse, S., Hansson, B.S., 2010. Physiological and morphological characterization of local interneurons in the Drosophila antennal lobe. J. Neurophysiol. 104, 1007–1019. doi:10.1152/jn.00249.2010

Selcho, M., Pauls, D., El Jundi, B., Stocker, R.F., Thum, A.S., 2012. The role of octopamine and tyramine in Drosophila larval locomotion. J. Comp. Neurol. 520, 3764–3785. doi:10.1002/cne.23152

Semmelhack, J.L., Wang, J.W., 2009. Select Drosophila glomeruli mediate innate olfactory attraction and aversion. Nature 459, 218–223. doi:10.1038/nature07983

Shakiryanova, D., Zettel, G.M., Gu, T., Hewes, R.S., Levitan, E.S., 2011. Synaptic neuropeptide release induced by octopamine without Ca2+ entry into the nerve terminal. Proc. Natl. Acad. Sci. U.S.A. 108, 4477–4481. doi:10.1073/pnas.1017837108

Shanbhag, S.R., Müller, B., Steinbrecht, R.A., 2000. Atlas of olfactory organs of Drosophila melanogaster 2. Internal organization and cellular architecture of olfactory sensilla. Arthropod Struct Dev 29, 211–229.

Shang, Y., Claridge-Chang, A., Sjulson, L., Pypaert, M., Miesenböck, G., 2007. Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. Cell 128, 601–612. doi:10.1016/j.cell.2006.12.034

Silbering, A.F., Galizia, C.G., 2007. Processing of odor mixtures in the Drosophila antennal lobe reveals both global inhibition and glomerulus-specific interactions. J. Neurosci. 27, 11966–11977. doi:10.1523/JNEUROSCI.3099-07.2007

Silbering, A.F., Okada, R., Ito, K., Galizia, C.G., 2008. Olfactory information processing in the Drosophila antennal lobe: anything goes? J. Neurosci. 28, 13075–13087. doi:10.1523/JNEUROSCI.2973-08.2008

Silbering, A.F., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G.S.X.E., Benton, R., 2011. Complementary function and integrated wiring of the evolutionarily distinct Drosophila olfactory subsystems. J. Neurosci. 31, 13357–13375. doi:10.1523/JNEUROSCI.2360-11.2011

Sinakevitch, I., Strausfeld, N.J., 2006. Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. J. Comp. Neurol. 494, 460–475. doi:10.1002/cne.20799

Sinakevitch, I.T., Smith, A.N., Locatelli, F., Huerta, R., Bazhenov, M., Smith, B.H., 2013. Apis mellifera octopamine receptor 1 (AmOA1) expression in antennal lobe networks of the honey bee (Apis mellifera) and fruit fly (Drosophila melanogaster). Front Syst Neurosci 7, 70. doi:10.3389/fnsys.2013.00070

Sitaraman, D., Zars, M., Zars, T., 2010. Place memory formation in Drosophila is independent of proper octopamine signaling. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 196, 299–305. doi:10.1007/s00359-010-0517-5

Smart, R., Kiely, A., Beale, M., Vargas, E., Carraher, C., Kralicek, A.V., Christie, D.L., Chen, C., Newcomb, R.D., Warr, C.G., 2008. Drosophila odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. Insect Biochem. Mol. Biol. 38, 770–780. doi:10.1016/j.ibmb.2008.05.002

Stengl, M., 2010. Pheromone transduction in moths. Front Cell Neurosci 4, 133. doi:10.3389/fncel.2010.00133

Stengl, M., Funk, N.W., 2013. The role of the coreceptor Orco in insect olfactory transduction. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 199, 897–909. doi:10.1007/s00359-013-0837-3

Stocker, R.F., 1994. The organization of the chemosensory system in Drosophila melanogaster: a review. Cell Tissue Res. 275, 3–26.

Stocker, R.F., Lienhard, M.C., Borst, A., Fischbach, K.F., 1990. Neuronal architecture of the antennal lobe in Drosophila melanogaster. Cell Tissue Res. 262, 9–34.

Störtkuhl, K.F., Hovemann, B.T., Carlson, J.R., 1999. Olfactory adaptation depends on the Trp Ca2+ channel in Drosophila. J. Neurosci. 19, 4839–4846.

Strauss, R., Heisenberg, M., 1993. A higher control center of locomotor behavior in the Drosophila brain. J. Neurosci. 13, 1852–1861.

Su, C.-Y., Martelli, C., Emonet, T., Carlson, J.R., 2011. Temporal coding of odor mixtures in an olfactory receptor neuron. Proc. Natl. Acad. Sci. U.S.A. 108, 5075–5080. doi:10.1073/pnas.1100369108

Su, C.-Y., Menuz, K., Reisert, J., Carlson, J.R., 2012. Non-synaptic inhibition between grouped neurons in an olfactory circuit. Nature 492, 66–71. doi:10.1038/nature11712

Suh, G.S.B., Ben-Tabou de Leon, S., Tanimoto, H., Fiala, A., Benzer, S., Anderson, D.J., 2007. Light activation of an innate olfactory avoidance response in Drosophila. Curr. Biol. 17, 905–908. doi:10.1016/j.cub.2007.04.046

Suver, M.P., Mamiya, A., Dickinson, M.H., 2012. Octopamine neurons mediate flight-induced modulation of visual processing in Drosophila. Curr. Biol. 22, 2294–2302. doi:10.1016/j.cub.2012.10.034

Tanaka, N.K., Awasaki, T., Shimada, T., Ito, K., 2004. Integration of chemosensory pathways in the Drosophila second-order olfactory centers. Curr. Biol. 14, 449–457. doi:10.1016/j.cub.2004.03.006

Tanaka, N.K., Endo, K., Ito, K., 2012. Organization of antennal lobe-associated neurons in adult Drosophila melanogaster brain. J. Comp. Neurol. 520, 4067–4130. doi:10.1002/cne.23142

Thoma, M., Hansson, B.S., Knaden, M., 2014. Compound valence is conserved in binary odor mixtures in Drosophila melanogaster. J. Exp. Biol. 217, 3645–3655. doi:10.1242/jeb.106591

Touhara, K., 2002. Odor discrimination by G protein-coupled olfactory receptors. Microsc. Res. Tech. 58, 135–141. doi:10.1002/jemt.10131

Tsitoura, P., Andronopoulou, E., Tsikou, D., Agalou, A., Papakonstantinou, M.P., Kotzia, G.A., Labropoulou, V., Swevers, L., Georgoussi, Z., Iatrou, K., 2010. Expression and membrane topology of Anopheles gambiae odorant receptors in lepidopteran insect cells. PLoS ONE 5, e15428. doi:10.1371/journal.pone.0015428

Tsunoda, S.P., Hegemann, P., 2009. Glu 87 of channelrhodopsin-1 causes pH-dependent color tuning and fast photocurrent inactivation. Photochem. Photobiol. 85, 564–569. doi:10.1111/j.1751-1097.2008.00519.x

Von Nickisch-Rosenegk, E., Krieger, J., Kubick, S., Laage, R., Strobel, J., Strotmann, J., Breer, H., 1996. Cloning of biogenic amine receptors from moths (Bombyx mori and Heliothis virescens). Insect Biochem. Mol. Biol. 26, 817–827.

Vosshall, L. (2008.4.10). P{Or83b-GAL4.W}11.17.

Vosshall, L. (2009.10.27). Insertions at BDSC.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., Axel, R., 1999. A spatial map of olfactory receptor expression in the Drosophila antenna. Cell 96, 725–736.

Vosshall, L.B., Wong, A.M., Axel, R., 2000. An olfactory sensory map in the fly brain. Cell 102, 147–159.

Wang, J.W., Wong, A.M., Flores, J., Vosshall, L.B., Axel, R., 2003. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell 112, 271–282.

Wark, B., Lundstrom, B.N., Fairhall, A., 2007. Sensory adaptation. Curr. Opin. Neurobiol. 17, 423–429. doi:10.1016/j.conb.2007.07.001

Wetzel, C.H., Behrendt, H.J., Gisselmann, G., Störtkuhl, K.F., Hovemann, B., Hatt, H., 2001. Functional expression and characterization of a Drosophila odorant receptor in a heterologous cell system. Proc. Natl. Acad. Sci. U.S.A. 98, 9377–9380. doi:10.1073/pnas.151103998

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., Hansson, B.S., 2008. Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452, 1007–1011. doi:10.1038/nature06861

Wilson, R.I., 2013. Early olfactory processing in Drosophila: mechanisms and principles. Annu. Rev. Neurosci. 36, 217–241. doi:10.1146/annurev-neuro-062111-150533

Wilson, R.I., Laurent, G., 2005. Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the Drosophila antennal lobe. J. Neurosci. 25, 9069–9079. doi:10.1523/JNEUROSCI.2070-05.2005

Wilson, R.I., Turner, G.C., Laurent, G., 2004. Transformation of olfactory representations in the Drosophila antennal lobe. Science 303, 366–370. doi:10.1126/science.1090782

Winther, A.M.E., Acebes, A., Ferrús, A., 2006. Tachykinin-related peptides modulate odor perception and locomotor activity in Drosophila. Mol. Cell. Neurosci. 31, 399–406. doi:10.1016/j.mcn.2005.10.010

Wistrand, M., Käll, L., Sonnhammer, E.L.L., 2006. A general model of G protein-coupled receptor sequences and its application to detect remote homologs. Protein Sci. 15, 509–521. doi:10.1110/ps.051745906

Yaksi, E., Wilson, R.I., 2010. Electrical coupling between olfactory glomeruli. Neuron 67, 1034–1047. doi:10.1016/j.neuron.2010.08.041

Yao, C.A., Carlson, J.R., 2010. Role of G-proteins in odor-sensing and CO2-sensing neurons in Drosophila. J. Neurosci. 30, 4562–4572. doi:10.1523/JNEUROSCI.6357-09.2010

Yao, C.A., Ignell, R., Carlson, J.R., 2005. Chemosensory coding by neurons in the coeloconic sensilla of the Drosophila antenna. J. Neurosci. 25, 8359–8367. doi:10.1523/JNEUROSCI.2432-05.2005

Yarali, A., Gerber, B., 2010. A Neurogenetic Dissociation between Punishment-, Reward-, and Relief-Learning in Drosophila. Front Behav Neurosci 4, 189. doi:10.3389/fnbeh.2010.00189

Yellman, C., Tao, H., He, B., Hirsh, J., 1997. Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated Drosophila. Proc. Natl. Acad. Sci. U.S.A. 94, 4131–4136.

Zhang, T., Branch, A., Shen, P., 2013. Octopamine-mediated circuit mechanism underlying controlled appetite for palatable food in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 110, 15431–15436. doi:10.1073/pnas.1308816110

Zhang, W., Ge, W., Wang, Z., 2007. A toolbox for light control of Drosophila behaviors through Channelrhodopsin 2-mediated photoactivation of targeted neurons. European Journal of Neuroscience 26, 2405–2416. doi:10.1111/j.1460-9568.2007.05862.x

Zhou, C., Rao, Y., Rao, Y., 2008. A subset of octopaminergic neurons are important for Drosophila aggression. Nat. Neurosci. 11, 1059–1067. doi:10.1038/nn.2164

Zhu, J., Park, K.-C., Baker, T.C., 2003. Identification of odors from overripe mango that attract vinegar flies, Drosophila melanogaster. J. Chem. Ecol. 29, 899–909.

Zufall, F., Leinders-Zufall, T., 2000. The cellular and molecular basis of odor adaptation. Chem. Senses 25, 473–481.

Zumstein, N., Forman, O., Nongthomba, U., Sparrow, J.C., Elliott, C.J.H., 2004. Distance and force production during jumping in wild-type and mutant Drosophila melanogaster. J. Exp. Biol. 207, 3515–3522. doi:10.1242/jeb.01181

Publications

Research Articles:

Giang, T., Rauchfuss, S., Ogueta, M., Scholz, H., 2011. The Serotonin Transporter Expression in Drosophila melanogaster. Journal of Neurogenetics 25, 17–26. doi:10.3109/01677063.2011.553002

Schneider, A., Ruppert, M., Hendrich, O., **Giang, T.**, Ogueta, M., Hampel, S., Vollbach, M., Büschges, A., Scholz, H., 2012. Neuronal basis of innate olfactory attraction to ethanol in Drosophila. PLoS ONE 7, e52007. doi:10.1371/journal.pone.0052007

Ziegler, T., Orlov, N.L., **Giang, T.T.**, Truong, N.Q., Tao, N.T., Quyet, L.K., Khoi, N.V., Thanh, V.N., 2010. New records of cat snakes, Boiga Fitzinger, 1826 (Squamata, Serpentes, Colubridae), from Vietnam, inclusive of an extended diagnosis of Boiga bourreti Tillack, Le & Ziegler, 2004. Zool. Reihe 86, 263–274. doi:10.1002/zoos.201000009

Conference Abstracts:

Thomas Tam Giang, Andrea Schneider, Henrike Scholz. 2012. "The *Drosophila melanogaster* olfactory co-receptor Orco mediates odorant sensitivity in adult flies". 9th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany

Thomas Tam Giang, Andrea Schneider, Henrike Scholz. 2012. "The *Drosophila melanogaster* olfactory co-receptor Orco mediates odorant sensitivity in adult flies". 18th Regional Drosophila Meeting, Osnabrück, Germany

Thomas Tam Giang, Andrea Schneider, Henrike Scholz. 2013. "The *Drosophila melanogaster* olfactory co-receptor Orco mediates odorant sensitivity in adult flies". Nutrition Homeostasis Workshop, Limes Institute, Bonn

Thomas Tam Giang, Andreas Klein, Henrike Scholz. 2013. „Behavioral output depends on frequency specific neuronal activation". Neurobiology of Drosophila, Cold Spring harbor, USA

Declaration/Erklärung

Erklärung

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Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Henrike Scholz betreut worden.

Köln, 2014

Thomas Tam Giang

Acknowledgements

I would like to express my gratitude to

Prof. Dr. Henrike Scholz for providing me with the opportunity to do my PHD, excellent supervision, guidance and furthering my understanding of what it means to do research.

Prof. Dr. Siegfrid Roth for kindly joining my thesis committee as a proofreader and examiner.

Prof. Dr. G. Plickert for joining and agreeing to be the chair in my disputation.

My express thanks and gratitude goes to Manu and Gerbera for proofreading, patience, just being helpful by nature and if not for them I would have despaired a long time ago.

I also want to thank Helga for always helping out and showing me the way where no path was visible before.

From the bottom of my Heart I want to thank Lena for proofreading and being the unshakable pillar supporting me from more angles than seemed possible.

I also want to thank my family for always having faith and silently supporting me without complaint

Finally I want to express my thanks to all the members and ex members of the Scholz lab for making my life a brighter place: Li, Laura, Shreyas, Sravia, Sebastian, Jian Zheng and Jhamna.