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Investigating the impact of individual receptors on odor-guided behavior in *Drosophila* larvae by using the optogenetic tool Chrimson

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1. Abstract

The development of novel channelrhodopsins keeps improving the scope of optogenetics in *Drosophila*, allowing *in vivo* manipulation of neural activity with little interference and millisecond precision. Investigating the functional principles of the olfactory system of *Drosophila* larvae, the Gal4/UAS-System was used to express the chimeric channelrhodopsin CsChrimson in one olfactory receptor (OR) type at a time, to see if the stimulation of single olfactory receptor neurons (ORNs) can be sufficient for eliciting significant behavioral responses. OR42a and OR42b were the only receptors in which neural manipulation with CsChrimson evoked persistent positive responses, consistent with the specific properties of these two ORs, acting as detectors of food-source related odorants.

2. German Summary

Durch die Entwicklung von neuartigen Channelrhodopsinen werden die Anwendungsmöglichkeiten der Optogenetik kontinuierlich erweitert. Ektopische Expression von Channelrhodopsinen und deren Aktivierung durch bestimmte Lichtwellenlängen ermöglichen die Manipulation neuraler Aktivität, *in vivo* und mit nur minimalen Eingriffen. In der folgenden Arbeit wurde das olfaktorische System von *Drosophila melanogaster*-Larven manipuliert, um zu testen ob die Stimulation einzelner olfaktorischer Rezeptorneuronen (ORNs) für das Auslösen signifikanter Verhaltensreaktionen ausreicht. Das Gal4/UAS-System wurde dafür genutzt, um das chimäre Channelrhodopsin CsChrimson in einzelnen olfaktorischen Rezeptoren (ORs) zu exprimieren. Die transgenen Larven wurden in einer binären Phototaxis-Arena auf mögliche Verhaltensreaktionen getestet. Die Experimente lieferten signifikant positive Reaktionen für Larven mit CsChrimson-Expression in OR42a und OR42b, was mit der wichtigen Rolle dieser Rezeptoren bei der Erkennung von spezifischen, von Nahrungsquellen verbreiteten Geruchsmolekülen übereinstimmt.

3. Introduction

3.1 The olfactory system of *Drosophila melanogaster*

The reception of molecules is influencing behavior in every living organism, from single-celled bacteria and protozoa to all kinds of multicellular life forms. Olfactory systems provide animals with a remarkable skill that is crucial for the acquirement of food, mating and evading danger: the ability to discriminate between thousands of environmental odors.

Because of its simplicity and comparability to vertebrate systems, the olfactory system of the *Drosophila* larva is highly suitable for fundamental research on sensory reception and processing (Strausfeld and Hildebrand, 1999).

The paired dorsal organs are the principal olfactory organs in *Drosophila* larvae. Each dome sensillum is perforated by pore channels, enabling the passage of odorants to the dendrites of 21 olfactory receptor neurons (ORNs). The 21 ORNs are bundled in seven triplets and each ORN expresses a different set of olfactory receptors (ORs) that are seven-transmembrane-domain receptors, but seem to share only few characteristics with G-protein coupled receptors. *Drosophila* ORNs express the non-specific olfactory coreceptor 83b and usually one of about 25 specific endogenous ORs. In some instances, two specific endogenous ORs can be expressed in the same ORN. 14 of these specific OR genes are expressed both in larval and adult ORNs (OR2a, OR7a, OR10a, OR13a, OR19a, OR33a, OR33b, OR35a, OR42a, OR42b, OR43b, OR49a, OR67b, and OR88a), while the rest is expressed in larval ORNs only (OR1a, OR22c, OR24a, OR30a, OR45a, OR45b, OR59a, OR63a, OR74a, OR83a, OR85c, OR94a, OR94b) (Fishilevich *et al.*, 2005; Kreher *et al.*, 2005).

OR83b is a cation channel thought to form a heteromeric ligand-gated ion channel with the respective specific ORs. Binding of a ligand to the OR complex induces activation of an either solely ionotropic signaling cascade or in addition a metabotropic signaling cascade (Fishilevich *et al.*, 2005; Sato *et al.*, 2008; Wicher *et al.*, 2008).

After the transformation into electrical signals, the olfactory information is conveyed to the larval antennal lobe (LAL), where it is pre-processed. Each of the 21 ORNs connects with one of 21 glomeruli in the LAL. These glomeruli represent junctions with 21 second-order neurons, the olfactory projection neurons (PNs). The LAL glomeruli are also interlinked by inhibitory local interneurons (LNs), which are arranged in functional clusters (Masuda-Nakagawa *et al.*, 2010).

After pre-processing in the LAL, the olfactory information is delivered to two second-order olfactory processing centers: first to the mushroom body (MB) and then to the lateral horn. The 21 PNs connect each single LAL glomerulus with one of about 30 calycal glomeruli in the MB,

with some PNs targeting two different single calycal glomeruli (Ramaekers *et al.*, 2005). The MB has been shown to be involved in olfactory associative learning (Pauls *et al.*, 2010), while the role of the lateral horn has not been ascertained yet.

There is a rather straight input-output correlation up until this point: with one ORN connecting to one PN in the LAL and most PNs targeting only one calycal glomerulus, the activity pattern in the LAL is comparable to the activity pattern in the calyx. However, PNs can possibly connect with far more than one of a few hundred intrinsic mushroom body neurons (Matsuda-Nakagawa *et al.*, 2009).

3.2 Olfaction and Behavior

Odorants have been shown to induce a broad range of behavioral patterns in *Drosophila*, from oviposition and mating to avoidance reactions (Dweck *et al.*, 2013; 2015; Ebrahim *et al.*, 2015): It has recently been discovered that OR49a is sensitive to Iridomyrmecin, a pheromone produced by the parasitic wasp *Leptopilina*. It enables *Drosophila* larvae to avoid parasitic sites and adult flies to omit oviposition, using semiochemicals of their most abundant parasitoid for an extraordinary defense mechanism (Ebrahim *et al.*, 2015).

As *Drosophila* flies lay their eggs directly on the surface of food sources like rotting fruits, specific demands on the larval olfaction system are apparent: Larvae have to discriminate between appetitive and repellent sensory input against a highly concentrated background of odors.

Olfactory stimulation induces an odor-guided crawling behavior, indicating a direct input-output relationship between single ORNs and ultimate behavior, with the individual ORNs already determining appetitive or aversive responses (Bellmann *et al.*, 2010; Störtkuhl and Fiala, 2011). However, olfaction is known to make use of the so-called combinatorial code: single odorants activate several ORNs and individual ORNs become activated by several odorants. Simulating larval olfaction with small numbers of receptors and odorants, Kreher *et al.* developed a linear model that could predict 74% of behavioral variation by evaluating the responses of only five receptors (Kreher *et al.*, 2008). This supports the idea that larval odor perception works in a combinatorial fashion similar to adult flies, while the contribution of individual ORNs remains up for discussion (Bellmann *et al.* 2010; Ebrahim *et al.*, 2015; Kreher *et al.*, 2008).

3.3 The Gal4/UAS-System

Established by Andrea Brand and Norbert Perrimon in 1993, the Gal4/UAS-System has become a valued tool for directing gene expression in specific cells or tissue in *Drosophila* and other model organisms. The system contains of two parts, the yeast transcriptional activator gene Gal4 and the upstream activation site (UAS), together enabling ectopic expression of a specific target or reporter gene fused to the UAS. As the transcriptional activator gene and its target gene are separated in different transgenic lines, the target gene is only activated in the progeny after crossing the transgenic parental lines. This allows the convenient generation of ready-made, balanced Gal4 and UAS driver lines (Brand and Perrimon, 1993).

3.4 The Channelrhodopsins Chrimson and CsChrimson

Chrimson (CnChR1) is a channelrhodopsin obtained from *Chlamydomonas noctigama*, assembled after *de novo* sequencing of algal transcriptomes. It has a spectral peak of 590 nm, making it the most red-shifted channelrhodopsin up to date. With more novel channelrhodopsins getting developed, the optogenetic field has gotten more impact over the last few years, providing specified tools for minimally invasive *in vivo* manipulation of cells and tissues, using only specific wavelengths of light as an activator (Klapoetke *et al.*, 2014).

Used in this work was not the original Chrimson channelrhodopsin, but a modified one named CsChrimson. CsChrimson is a chimeric channelrhodopsin also developed by Klapoetke *et al.*, replacing the N-Terminus of Chrimson with that of the CsChr N-Terminus to elevate membrane expression levels (Klapoetke *et al.*, 2014; <http://patents.justia.com/patent/20160039902>). The spectral peak is the same as Chrimson with 590 nm.

Channelrhodopsins are a subfamily of retinylidene proteins involved in phototaxis control of green algae. They are photoreceptor proteins with seven transmembrane domains that are not coupled to G-Proteins. Channelrhodopsins are able to mediate passive cation conductance similar to rhodopsin or other opsins, with the difference that they are not triggering channel gating indirectly via messengers. Instead the channelrhodopsins go through rapid photoisomerization reaction cycles, ultimately acting as cation channels themselves. The conformation changes are induced by the photoisomerizable chromophore retinal, starting to switch conformation from all-trans-retinal to 13-cis-retinal when it absorbs a photon. The begin of this photoisomerization induces further confirmation changes in the transmembrane protein complex, opening the pore to about 6 Å and thereby enabling passive cation conductance for several milliseconds, before the transmembrane protein complex is supposed to go into a closed, desensitized state and then into a closed ground state again (Nagel *et al.*, 2003; Klapoetke *et al.*, 2014).

3.5 Purpose of this Study

Described below is an experiment where the Gal4/UAS-System was used to express the channelrhodopsin CsChrimson in one of eleven larval ORs at a time. Experiments with light-activated channelrhodopsins like CsChrimson on *Drosophila* larvae are especially convenient because the larvae are almost transparent. Moreover, some channelrhodopsins can operate at wavelengths that are outside of the larval visible spectrum, allowing minimally invasive *in vivo* manipulation of neural activity or other tissues (Klapoetke et al., 2014; Xiang et al., 2011).

The experiments were conducted in a customized binary phototaxis assay, designed to capture either positive or negative odor-guided crawling behavior. By exposing the transgenic larvae to a specific light source on one side of the arena and observing whether the larvae would approach or avoid the light source, we tested if the stimulation of individual larval ORs with CsChrimson can be sufficient for eliciting significant behavioral responses. If the olfactory code is not strictly combinatorial, stimulation with CsChrimson could elicit significant positive or negative odor-guided crawling behavior, depending on the response profiles of the different ORNs.

Investigating the principles of invertebrate olfactory networks is promising in many ways, not only for the sake of basic research: Improving fundamental knowledge about functional principles of olfactory networks could accelerate innovations in different scientific fields, for example the finding of novel and sustainable agricultural control methods or the development of next generation biosensor systems (Son *et al.*, 2016).

4. Methods

4.1 Fly Stocks and Crossings

Fly stocks were grown at 25 °C on conventional agarose medium with a daily light/dark cycle of 12 hours. Crossings of female virgins carrying the CsChrimson reporter line with males of the different GAL4-OR driver lines were raised in the dark on food infused with all-trans retinal (Sigma, Germany). Food vials containing 5 ml food were infused with 50 µl from a stock solution of all-trans retinal in EtOH (40 mM), to reach a concentration of 0.4 mM in the food. Food vials were filled with 15% Sucrose in Ringer solution to collect randomly selected third instar larvae, which were transported and tested under exclusion of light. As Chrimson functionality depends on the sufficient intake of all-trans retinal during development, controls were obtained by the same crossing procedure, without adding any all-trans retinal as only variation.

4.2 Behavioral Testing

A plexiglass pane was strapped on top of the phototaxis assay. To simplify the placing of the larvae, small holes were drilled in the glass right over the middle of the trenches. The trenches are about 1,5 cm wide and 16 cm long and were marked to digitalize larval location. Trenches were about halfway filled with 1 % Agarose to ensure maximum crawling ability (Fig. 1).

Before conducting the actual experiments with transgenic larvae, the phototaxis assay was tested and optimized with wild-type larvae of the Canton-S strain, until continuously significant and positive behavioral responses were established. As reliable attractant for the larvae, 10 μ l of a 1:10 Balsamico vinegar with water dilution were placed on little blotting papers in holes in the plexiglass top at one end of the trenches. This was essential for creating an environment allowing unimpaired detection of odorants or the illusion of them. While one half of the assay remained relatively dark, the other half was illuminated with a gradient of red light (619 nm and 0.145 mW/mm²) from the bottom end in order to activate photoisomerization of CsChrimson in transgenic larvae, potentially creating the illusion of the presence of an odorant at the illuminated side of the trenches. Larvae were placed individually through a central opening in each of the trenches. Their positions within the trenches were recorded for 5 minutes and digitalized after each minute. A Logitech C-615 webcam with removed infrared cut-off filter was used to record the trials.

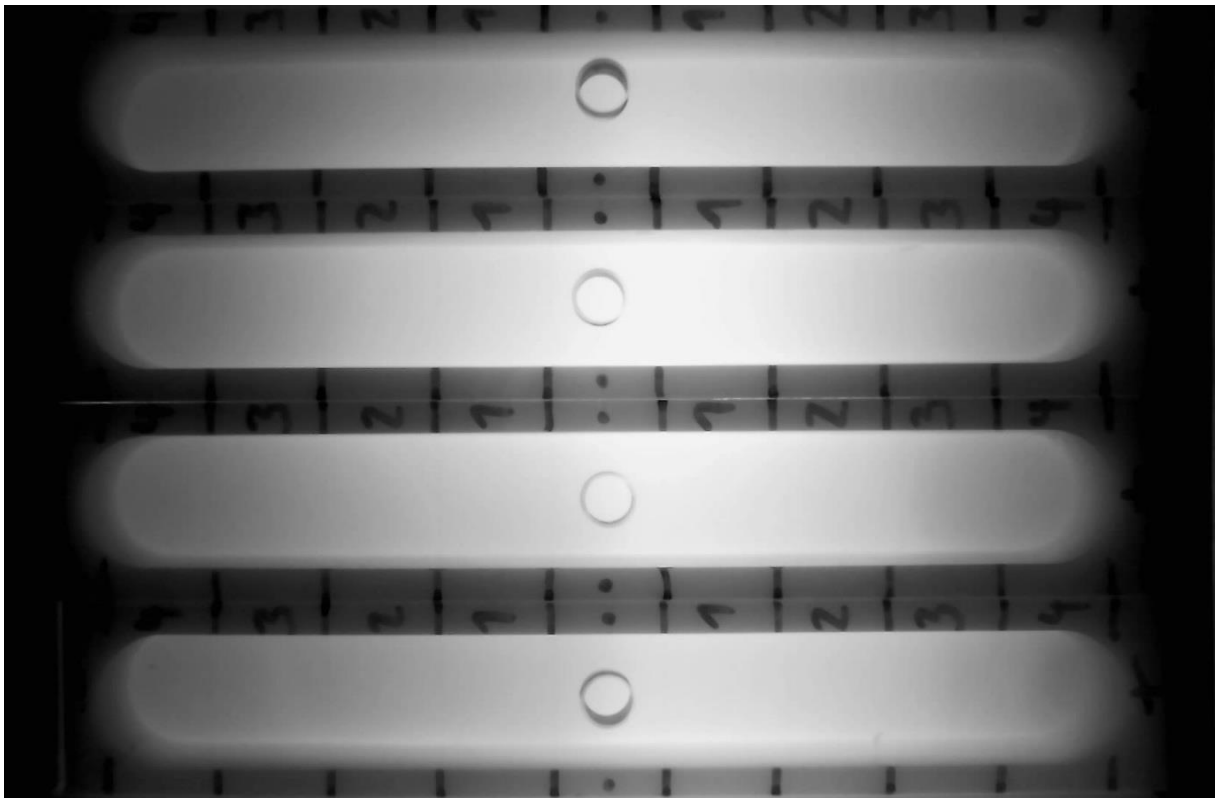


Fig. 1: The customized Phototaxis assay with four trays, as seen with the modified Logitech C-615 Webcam.

5. Results

Crossings were performed for each OR like described above. Transgenic larvae were raised on retinal infused food, after about 10 days they were collected and tested in the customized two-choice phototaxis assay. Trials were digitalized by hand, then the samples were tested for significance with Wilcoxon rank sum tests against zero as hypothetical median, performing individual tests for each minute.

Activating individual OSN populations by coexpressing Chrimson with single ORs in most cases did not provoke any significant behavior (Figs. 1-12). Only the artificial activation of OSNs expressing OR42a and OR42b resulted in significant attraction behavior (Figs. 6 and 7). These outcomes were further validated by raising control samples of OR42a and OR42b, performing the exact same crossing procedure without adding any retinal to the food vials. After testing the control samples in the customized phototaxis assay, Mann-Whitney-Tests were performed between transgenic samples and control samples for both ORs, again performing individual tests for each minute. The tests delivered significant correlations between transgenic samples and control samples of OR42a over the total of five minutes ($p_1 = 0.0050$; $p_2 = 0.0409$; $p_3 = 0.0271$; $p_4 = 0.0049$; $p_5 = 0.0033$). Tests also delivered significant correlations over the total of five minutes between transgenic and control samples of OR42b ($p_1 = 0.0037$; $p_2 = 0.0005$; $p_3 = 0.0015$; $p_4 = 0.01$; $p_5 = 0.01$).

Another significant attraction was found for the first minute when testing the OR49a samples ($p = 0.0267$). However, the attraction was restricted to one time point, so no further validation with control samples was conducted for this sample.

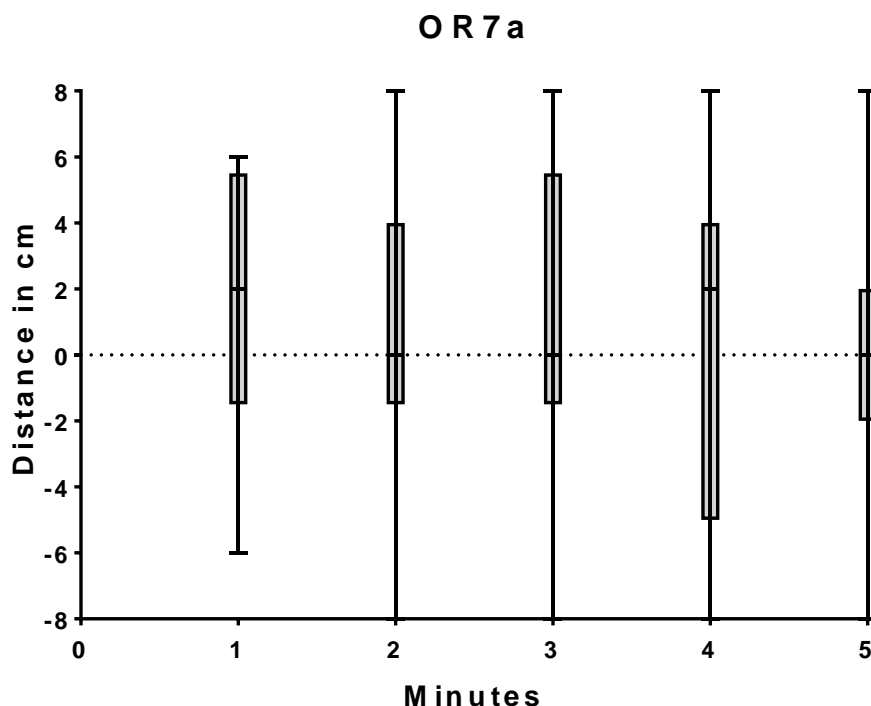


Fig. 2: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR7a promotor (n=20).

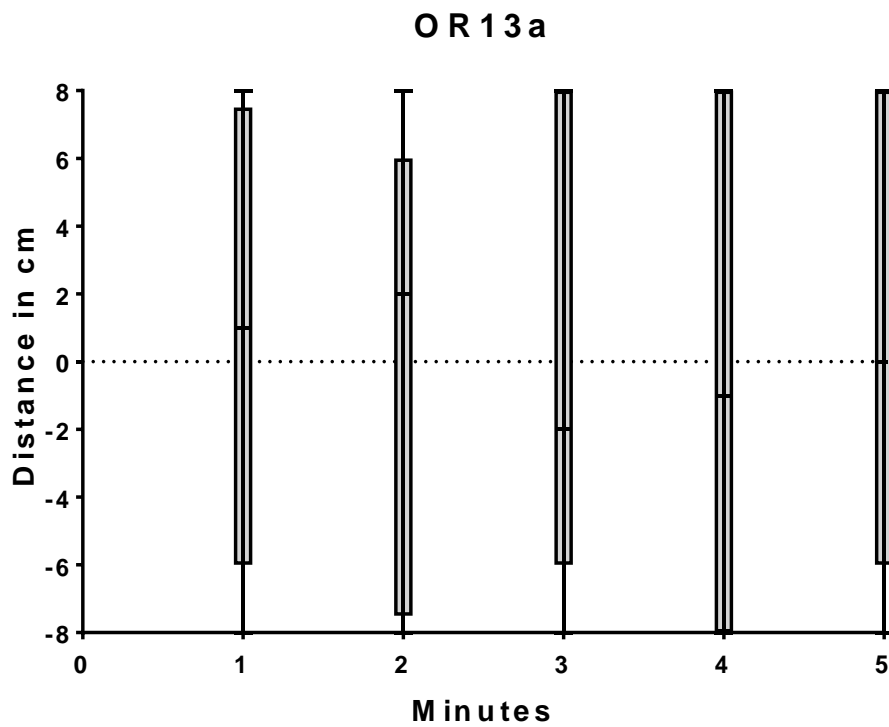


Fig. 3: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR13a promotor (n=20).

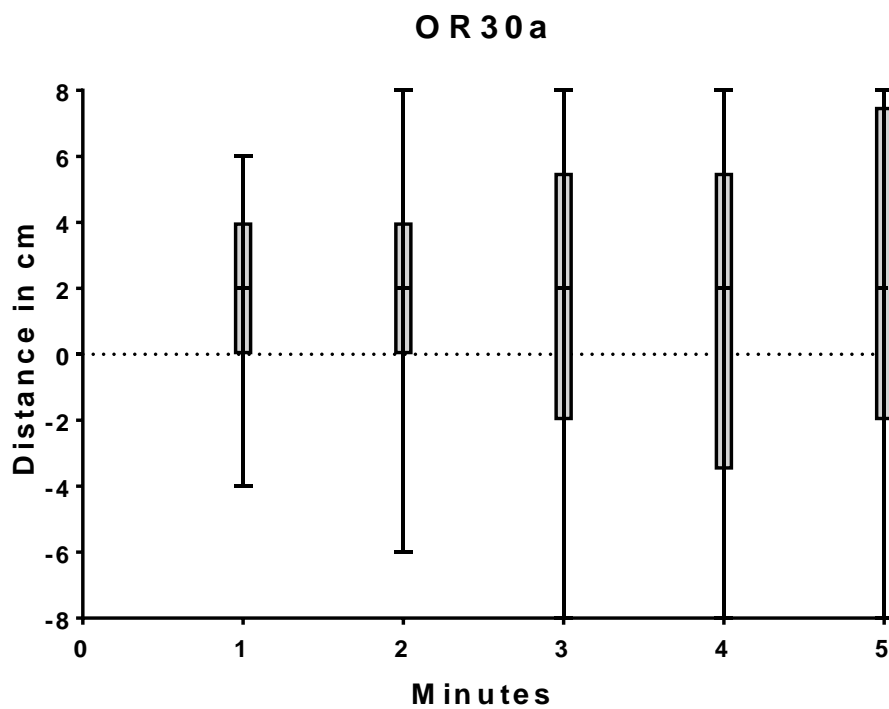


Fig. 4: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR30a promotor (n=20).

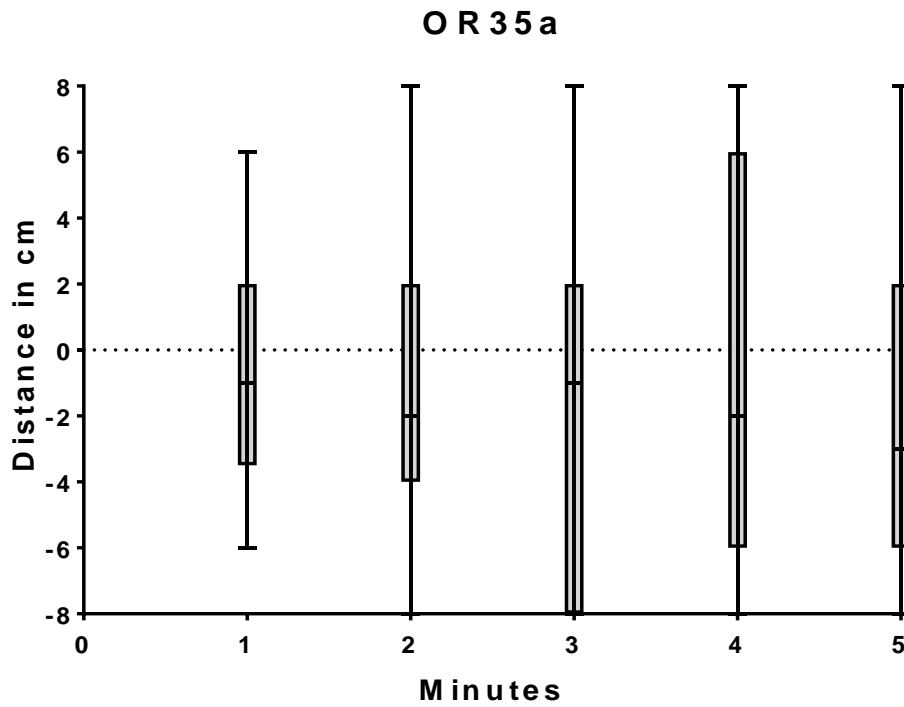


Fig. 5: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR35a promotor (n=20).

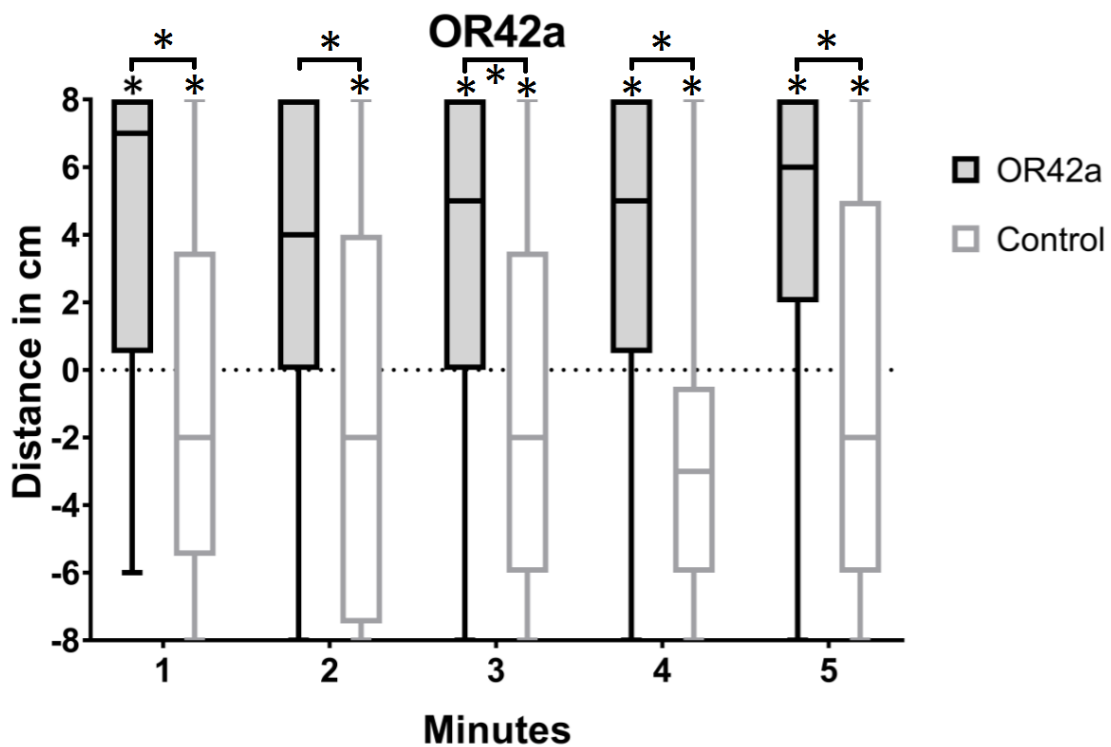


Fig. 6: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR42a promotor(n=20), with retinal free controls (n=20). Asterisks indicate significance for individual samples when tested against zero, and between the transgenic sample and the control sample for each minute.

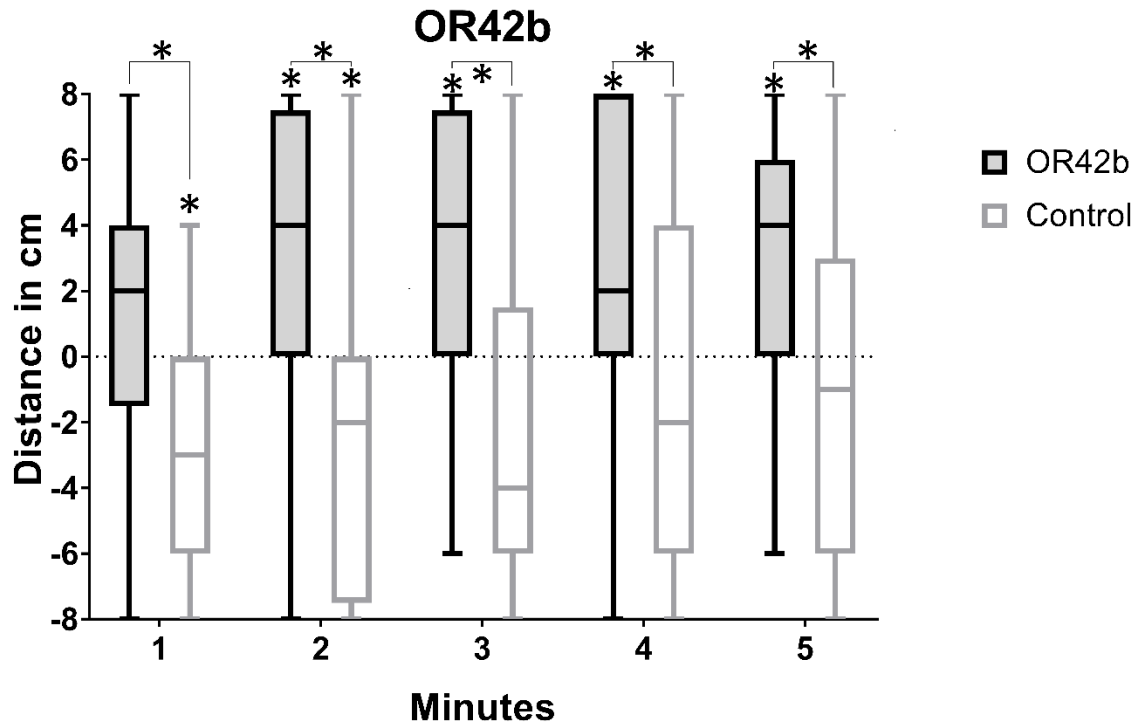


Fig. 7: Box plot for sample of transgenic larvae in which CsChrimson was expressed under the OR42b promoter (n=28), with retinal free controls (n=20). Asterisks indicate significance for individual samples when tested against zero, and between the transgenic sample and the control sample for each minute.

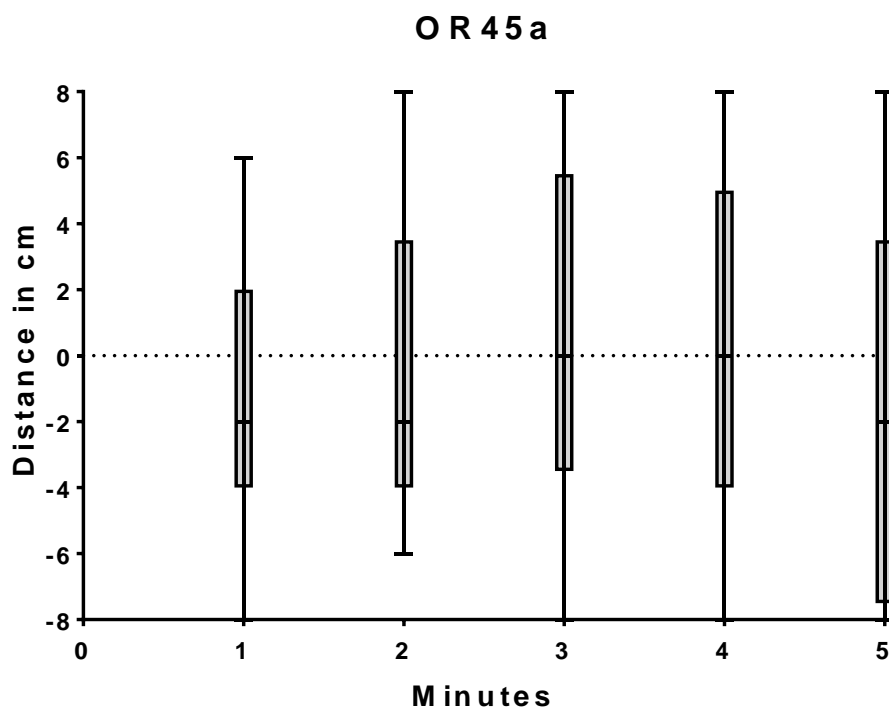


Fig. 8: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR45a promoter.

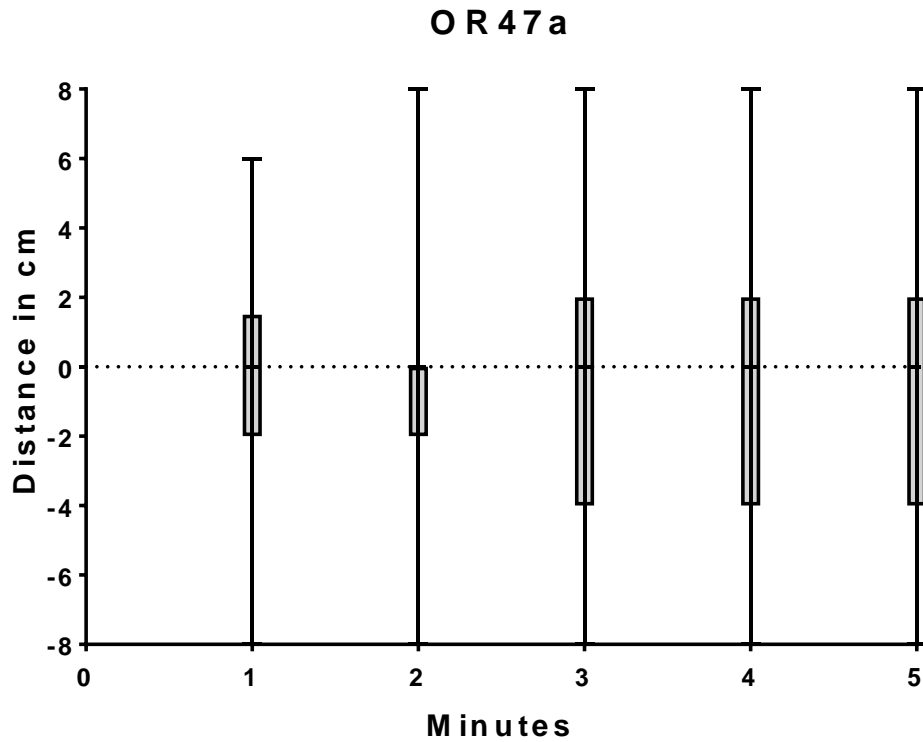


Fig. 9: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR47a promotor (n=20).

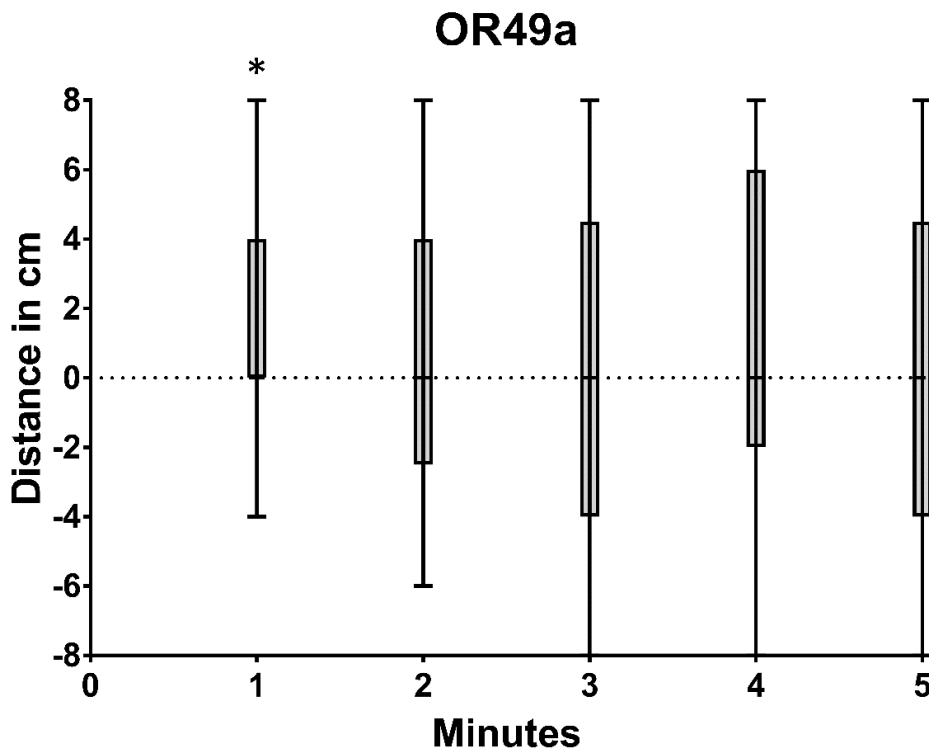


Fig. 10: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR49a promotor (n=30), Asterisk indicating significance for the first minute when tested against zero.

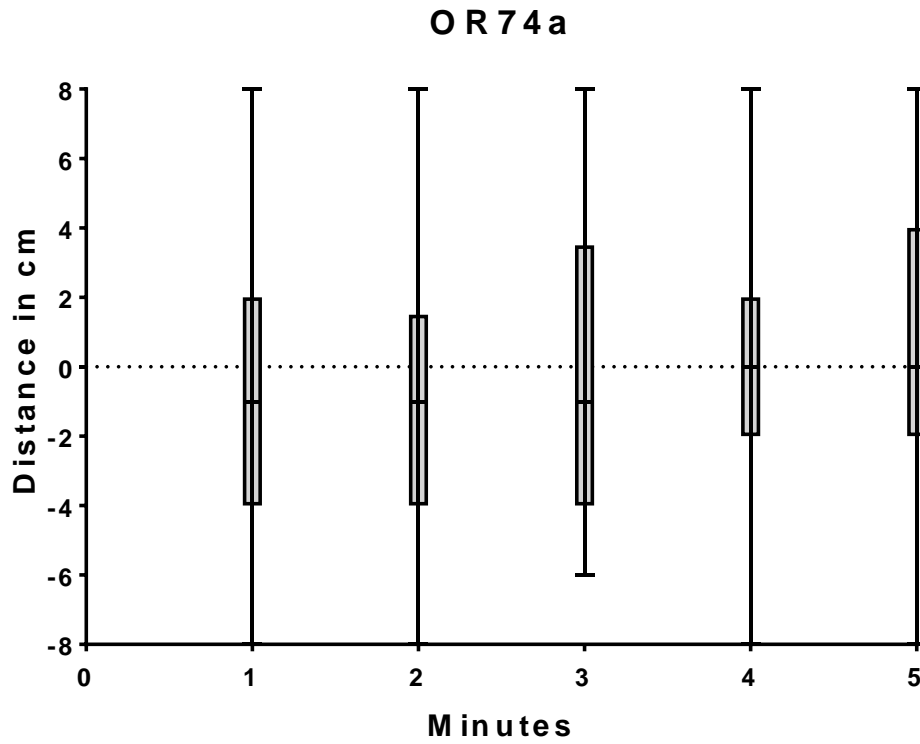


Fig. 11: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR74a promotor (n=16).

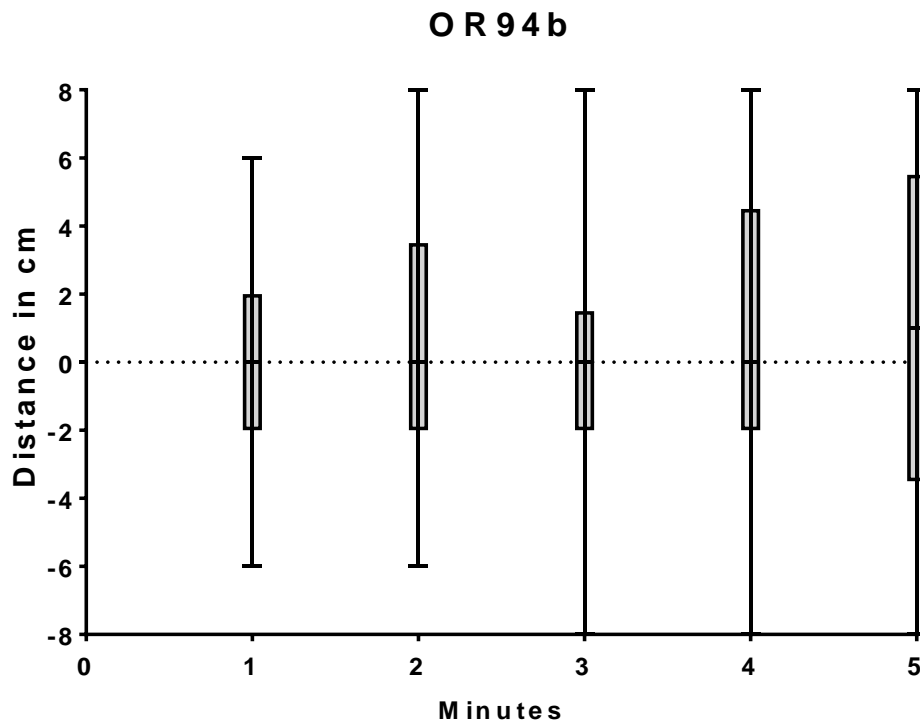


Fig. 12: Box plot for sample of transgenic larvae in which CsChrimson was expressed under control of the OR94b promotor (n=20).

6. Discussion

In vivo manipulation of tissue by using channelrhodopsins and optogenetics has become a valued method especially for *Drosophila melanogaster* larvae. Demonstrated here is an experiment where the Gal4/UAS-System was used to ectopically express the chimeric channelrhodopsin CsChrimson in one of eleven larval *Drosophila melanogaster* ORs at a time. The transgenic larvae were tested in a customized two-choice phototaxis assay, to see if the neural stimulation induced by the photo-activation of CsChrimson can be sufficient for evoking significant behavioral responses.

Before an actual discussion of the results, it must be remarked that the light intensity with 0.145 mW/mm² was powerful enough to activate CsChrimson. However, this experiment should be repeated with a new set-up allowing manual adjustment of light intensities. This would be suitable to address the fact that odors are naturally perceived at a myriad of different magnitudes, with the odorant concentration levels at which ORs give peak responses being highly variable (Kreher et al., 2008).

The results show a variance in the samples of OR7a, OR13a, OR30a, OR35a, OR45a, OR47a, OR74a and OR94b that is unfortunately allowing no solid conclusions about the functionality of CsChrimson in these ORs. However, as there was even no trend towards attraction or avoidance, the activation of ORNs expressing one of those receptors is obviously not sufficient to provoke any significant attraction or avoidance behavior.

The significantly positive behavioral response of the transgenic larvae from the OR49a sample during the first minute was quite surprising: OR49a is the receptor for detecting the *Leptopilina* pheromone Iridomyrmecin and associated to a network that serves *Drosophila* larvae and adults as a defense mechanism. Larvae displayed significant avoidance reactions towards the presence of Iridomyrmecin in a recent work of Ebrahim *et al.*, when tested with the actual odorant. They also displayed significant avoidance reactions when tested with channelrhodopsin-2 (ChR2) (Ebrahim *et al.*, 2015). Accordingly, CsChrimson-induced stimulation of OR49 was predicted to result in strong avoidance reactions. As this result cannot be brought in line with recent research, it will be carefully investigated in future experiments.

The samples of OR42a and OR42b displayed significant, positive behavioral responses against zero over the total of five minutes, with exception of the first minute for the sample of OR42b. Testing against controls with Wilcoxon rank sum tests revealed very solid significances between transgenic samples and control samples. This proves that the stimulation of individual ORs by photo-activation of CsChrimson can be sufficient for eliciting behavioral responses in *Drosophila melanogaster* larvae. As CsChrimson-evoked behavioral stimulation worked so remarkably well in these two ORs, the question comes up why it did not seem to work at all in the remaining ORs. It is likely that future experiments with a similar setup

will differ from this outcome, so that OR42a and OR42b will not be the only ORs strongly responding to the activation with CsChrimson. The high variance in other samples may be reduced by repeating the experiments with other light intensities. However, the fact that OR42a and OR42b are the only ORs responding so well to CsChrimson-induced behavioral stimulation under these conditions is of special interest, as they share some notable properties: OR42a and OR42b are highly sensitive to ethylacetate, propylacetate and ethylbutyrate. All three odorants are associated with the smell of rotting fruits, the natural food source of *Drosophila* larvae. OR42a is a low-affinity receptor and OR42b a high-affinity receptor for ethylacetate and propylacetate, and OR42a is a high-affinity receptor and OR42b a low-affinity receptor for ethylbutyrate (Grewal *et al.*, 2014). Appropriate stimulation results in highly appetitive behavioral responses. Additionally, other ORs can be recruited to respond to changes in odor concentration (Kreher *et al.*, 2008). While the ablation of OR1a or OR49a resulted in reduced chemotaxis to different single odors in an earlier study by Fishilevich *et al.*, ablation of OR42a resulted in chemotaxis defect towards 4 of 20 tested odorants. When OR1a and OR42a were rescued in otherwise anosmic larvae, there was a response recovery to more odors than the sum of rescuing 1a or 42a alone, hinting at a cooperative effect between ORNs (Fishilevich *et al.*, 2005). Again, the specific demands on *Drosophila* larval olfaction are remarked: Olfactory input from other odorants must be perceived and discriminated against the omnipresent, highly concentrated levels of background odorants detected primarily by OR42a and OR42b.

The result of this experiment is overall consistent with the specific conditions of OR42 and OR42b described above, further confirming their function as finely tuned detectors of food-related odors. My results also demonstrated that the olfactory code does not necessarily have to be strictly combinatorial, as single OR stimulation can be sufficient for eliciting behavioral responses. More research needs to be done to understand the underlying interactions and the true dimensions of this numerically simple olfactory system. This experiment should be adapted and repeated like described above. Using different lower light intensities might not only be important to avoid irritating the larvae. Trying out different low-level light intensities may also be a method of simulating different odor concentration levels. Thereby, more ORs could turn out to be responsive to CsChrimson-induced behavioral stimulation.

7. Acknowledgements

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Finally, a big thank you goes to my family. Thank you for giving me the opportunity to study and for your loving support throughout my whole life.

8. Declaration of Original Authorship

I hereby assure that I have written the present work independently with no other sources and no other help than indicated. All content taken from other sources has been marked. This has not been used as another exam work.

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Date

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Signature

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