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RESEARCH ARTICLE

Potencies of effector genes in silencing odor-guided behavior in *Drosophila melanogaster*

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ABSTRACT

The genetic toolbox in *Drosophila melanogaster* offers a multitude of different effector constructs to silence neurons and neuron populations. In this study, we investigated the potencies of several effector genes – when expressed in olfactory sensory neurons (OSNs) – to abolish odor-guided behavior in three different bioassays. We found that two of the tested effectors (*tetanus toxin* and *Kir2.1*) are capable of mimicking the *Orco* mutant phenotype in all of our behavioral paradigms. In both cases, the effectiveness depended on effector expression levels, as full suppression of odor-guided behavior was observed only in flies homozygous for both Gal4-driver and UAS-effector constructs. Interestingly, the impact of the effector genes differed between chemotactic assays (i.e. the fly has to follow an odor gradient to localize the odor source) and anemotactic assays (i.e. the fly has to walk upwind after detecting an attractive odorant). In conclusion, our results underline the importance of performing appropriate control experiments when exploiting the *D. melanogaster* genetic toolbox, and demonstrate that some odor-guided behaviors are more resistant to genetic perturbations than others.

KEY WORDS: *Drosophila*, Behavior, Effector genes, Olfaction

INTRODUCTION

Much of the success of *Drosophila melanogaster* as a model organism in neuroscience is attributable to its genetic tractability. Binary expression systems such as the Gal4/UAS system can be used to drive expression of specific effector genes to genetically defined target neuron populations, allowing visualization of morphology and activity, and artificial activation and/or silencing (Venken et al., 2011). This way, the contribution of genetically identifiable neuronal subpopulations of sensory systems to the overall perception and evaluation of a given sensory stimulus can be studied in detail.

Drosophila melanogaster detects odors using an array of olfactory sensory neurons (OSNs) located in sensory hairs termed olfactory sensilla. Olfactory sensilla are located on two types of head appendages, the antennae and the maxillary palps (Stocker, 1994; Vosshall and Stocker, 2007). Most OSNs are activated by more than one odorant and most monomolecular odorants, and, more importantly, natural 'odors' consisting of several monomolecular odorants typically activate multiple OSN classes (de Bruyne et al., 1999, 2001; Hallem and Carlson, 2006; Hallem et al., 2004; Pelz et al., 2006; Silbering et al., 2011; Dweck et al., 2016). Therefore, the identity of most odors is encoded in the combinatorial activity of the OSN population as a whole (Malnic et al., 1999). Nevertheless, recent evidence suggests that the innate hedonic valence of odors can already be predicted on the basis of the identity of OSNs the odors activate (Ai et al., 2010; Dweck et al., 2013, 2015a,b; Grosjean et al., 2011; Knaden et al., 2012; Kurtovic et al., 2007; Min et al., 2013; Ronderos et al., 2014; Semmelhack and Wang, 2009; Stensmyr et al., 2012; Suh et al., 2004; Thoma et al., 2014). It is therefore tempting to remove specific OSN populations from the activity pattern and in this way to investigate their contribution to the overall valence of a given odor.

As a rule with few exceptions, each OSN expresses one type of ligand-binding chemoreceptor, which defines its ligand specificity (Couto et al., 2005; Fishilevich and Vosshall, 2005). With one exception, i.e. the CO_2 detection system comprising two gustatory receptors (GRs) (Jones et al., 2007; Kwon et al., 2007), all antennally expressed olfactory chemoreceptors in *D. melanogaster* belong to one of two gene families, the evolutionarily ancient ionotropic receptors (IRs) detecting mainly – but not exclusively – hydrophilic chemicals (Abuin et al., 2011; Ai et al., 2010; Benton et al., 2009; Grosjean et al., 2011; Min et al., 2013; Silbering et al., 2011) and the insect-specific odorant receptors (ORs) (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). All *D. melanogaster* ORs rely on the ubiquitously expressed co-receptor *Orco* for intracellular trafficking (Larsson et al., 2004) and signal transduction (Sato et al., 2008; Wicher et al., 2008), and OR-expressing OSNs lacking the *Orco* protein are generally unresponsive to odors. This genetic make-up of the OR-based olfactory system of *D. melanogaster* with variable ligand-binding ORs and a common co-receptor is ideally suited to investigate the effect of the removal of an OSN population expressing a particular OR on odor evaluation. Even in the absence of an observable effect of silencing a specific OSN population under control of the promotor of the odor-binding OR, efficiency of silencing can be controlled by targeting the silencing effector gene to the whole OR-expressing OSN population under control of the *Orco* promotor.

There are several ways to genetically silence neurons in *D. melanogaster*. Neurons can be ablated by expressing bacterial toxins or pro-apoptotic genes, synaptically silenced using *tetanus toxin* (*TeTx*) or a dominant negative form of dynamin (*shibire^{ts}*), or electrically silenced by ectopic expression or RNAi-induced downregulation of ion channels (Venken et al., 2011). Here, by targeting all OR-expressing OSNs, we test the efficiency of the expression of *diphtheria toxin* (*DTA*; Han et al., 2000), the pro-apoptotic gene *reaper* (*rpr*; Zhou et al., 1997), *TeTx* (Sweeney et al., 1995) and the potassium channel *Kir2.1* (Baines et al., 2001; Paradis et al., 2001) in OSNs using the Gal4/UAS system

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(Brand and Perrimon, 1993) in suppressing odor-guided behavior in three different behavioral bioassays, a two-choice trap assay, the Flywalk (Steck et al., 2012) and an open-field arena. Similar to the observation by others who investigated the efficiency in the motor system and the mushroom body (MB) (Thum et al., 2006), we found that the effector genes differed in their potencies of abolishing odor-guided behavior. *DTA* and *rpr* did not abolish odor-guided behavior in any of the bioassays. *Kir2.1* and *TeTx* were partially effective, and their potency depended on the type of bioassay and expression level. Importantly, our results show that it is absolutely crucial to perform appropriate control experiments when using the *D. melanogaster* genetic toolbox to dissect the contribution of individual neuron populations to behavior.

MATERIALS AND METHODS

Flies

Flies were reared on standard cornmeal medium at 23°C, 70% relative humidity under a 12 h:12 h light:dark regime. All experimental flies were 6–8 days old and were starved, but not water-deprived, for 24 h before the experiments.

We used Orco-Gal4 to drive expression of the effector genes *rpr*, *DTA*, *TeTx* and *Kir2.1* specifically in Or-expressing OSNs (for details on original genotypes and sources, see Table 1). In addition, we performed experiments in Canton S wild-type and *Orco[2]* mutant flies. All Gal4 and UAS lines were backcrossed to w^{1118} flies to reduce variability conferred by the genetic background.

Chemicals

All monomolecular odorants were purchased from Sigma-Aldrich or FLUKA at the highest purity commercially available and diluted in mineral oil (also Sigma-Aldrich). In addition, we used commercially available balsamic vinegar in Flywalk and open-field arena experiments.

Trap assay

Trap assays were performed as previously described (Knaden et al., 2012) (Fig. 1A). The testing chamber consisted of a plastic box (length 10.5 cm, width 7.5 cm, height 9.5 cm) containing two traps constructed from smaller plastic vials (diameter 3.1 cm, height 4.3 cm). Flies could enter the traps through the cut end of a pipette tip, but once inside could not leave the traps. One of the cups contained a 0.2 ml PCR reaction tube containing a round piece of filter paper (diameter 1 cm) loaded with 100 µl of a 10^{-3} dilution of ethyl acetate (ETA) in mineral oil as an attractive odor source. The other trap served as a control, in which the filter paper was loaded with mineral oil only. Directly after preparation of the traps, cohorts of 40–80 flies of mixed sex were introduced into the testing box and allowed to choose between the traps for 24 h at 23°C and 70% relative humidity in complete darkness. Attraction was scored by calculating an attraction index (AI) as:

$$\mathrm{AI} = (n_{\mathrm{odor}} - n_{\mathrm{control}})/n_{\mathrm{total}}, \quad (1)$$

where n_{odor} is the number of flies in the odor trap, n_{control} is the number of flies in the control trap and n_{total} is the total number of flies tested in the experiment. Positive AI values indicate attraction, negative values indicate repulsion.

Table 1. List of the used transgenic fly lines

Fly line	BL no.	Genotype	Source
Orco$^{-/-}$	23130	yw; +; *Orco*^{2}	Bloomington *Drosophila* Stock Center
UAS-*Kir2.1*	6596	w; P{w[+mC]=UAS-Hsap\KCNJ2.EGFP}1/(CyO); +	Bloomington *Drosophila* Stock Center
UAS-*Diphtheria toxin*	25039	w; P{w[+mC]=UAS-Cbbeta\DT-A.I}18/CyO; +	Bloomington *Drosophila* Stock Center
UAS-*Tetanus toxin*	28837	w; P{w[+mC]=UAS-TeTxLC.tnt}E2; +	Bloomington *Drosophila* Stock Center
UAS-*reaper*	5824	w; P{w[+mC]=UAS-rpr.C}14; +	Bloomington *Drosophila* Stock Center
Orco-Gal4 driver line		yw; +; *Orco*->Gal4	Andre Fiala

Flywalk

Flywalk experiments were performed as previously described (Steck et al., 2012; Thoma et al., 2014). Briefly, 15 individual flies, starved for 24 h, were placed in parallel aligned glass tubes and their positions recorded under red-light conditions (λ=630 nm) over a period of ∼8 h. Flies were continuously exposed to a humidified airflow (∼20°C, ∼75% relative humidity) of 0.3 liters min^{-1} (20 cm s^{-1} in the glass tubes). Repeated odor pulses (inter-stimulus interval 90 s) were released from a multicomponent stimulus device (Olsson et al., 2011) loaded with 100 µl of odor dilutions in mineral oil. Responses were calculated as the mean distance flies covered within 4 s of encounter with the odor pulse.

Open-field arena

The open-field arenas consisted of rectangular polystyrene Petri dishes (125 mm to each side and 16 mm high) with a central hole (diameter 7 mm) in the lid. The hole was occluded with gauze from the inside and a round piece of filter paper (diameter 10 mm) from the outside. This way, flies could not physically contact the odor that was pipetted on the filter paper. The arena was illuminated by red LEDs (λ=630 nm) from above and monitored using a webcam (HD Pro Webcam C920, Logitech, Lausanne, Switzerland) from below.

At the beginning of an experimental session, a single female fly was introduced into the arena and allowed to habituate to the new environment for 5 min. Afterwards, 10 µl of distilled water was carefully added to the filter paper under red-light conditions and without mechanical disturbances, and the fly was recorded at 30 frames s^{-1} for 10 min using Media Recorder 2 software (Noldus Information Technology, Wageningen, The Netherlands). Finally, 10 µl of balsamic vinegar was added to the filter paper and the fly was again recorded for another 10 min. Flies were then tracked offline by dynamic background subtraction using EthoVision XT software (Noldus Information Technology). Further analysis was performed using R (www.r-project.org).

For all experiments and corresponding sample sizes, the sample size numbers used in this study (Flywalk, N=15 flies; trap assay, N=8–15 replicates with each 40–80 flies; arena assay, N=20 flies) have been proven to yield significant results.

RESULTS

To investigate the potencies of different effector genes in silencing odor-guided behavior, we expressed the silencers *rpr*, *DTA*, *Kir2.1* and *TeTx* in OSNs under Orco-Gal4 control and examined odor-guided behavior in three different behavioral bioassays. Flies were heterozygous for both Orco-Gal4 and UAS-effector unless mentioned otherwise.

We first examined odor-guided behavior in a simple two-choice trap assay (Fig. 1A). Canton S wild-type flies as well as the parental

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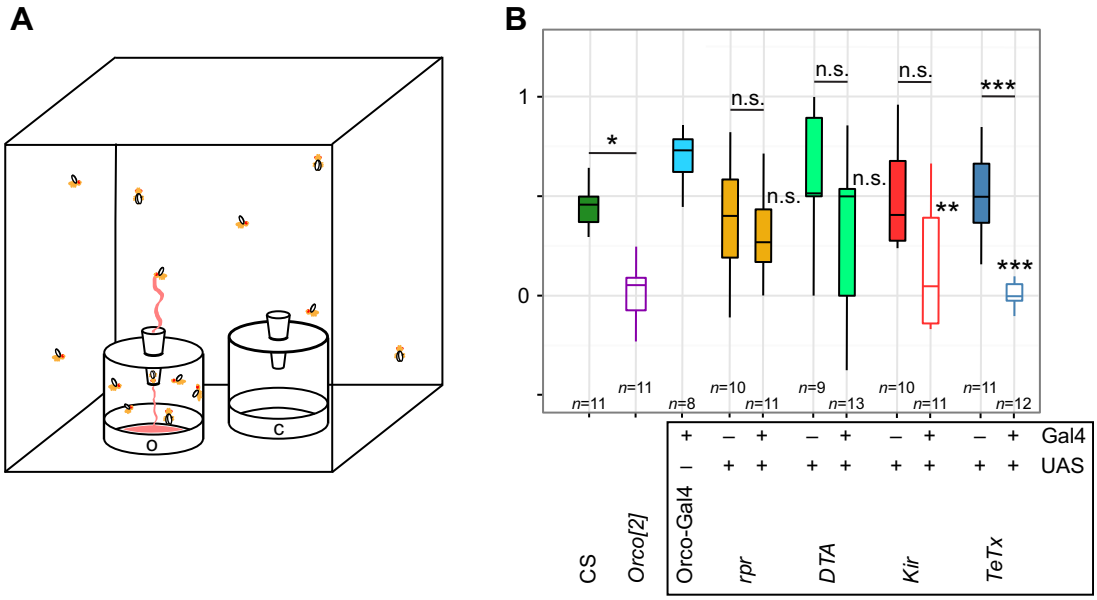

Fig. 1. Efficiency of effector genes in a two-choice trap assay. (A) Schematic representation of the trap assay. Forty to 80 flies are released in a plastic box containing two traps. One of the traps is loaded with odor in mineral oil, the other with the solvent mineral oil. Flies are allowed to choose between traps for 24 h and an attraction index (AI) is calculated. (B) Responses of control lines and flies expressing the effectors under Orco-Gal4 control to a 10^{-3} dilution of ethyl acetate (ETA). Filled boxes indicate statistically significant attraction (P<0.05, one-sample Wilcoxon rank sum test). Asterisks above boxplot show statistical significance between experimental flies and UAS controls, asterisks to the right of the boxes indicate statistical significance between experimental flies and Orco-Gal4 control (Kruskal–Wallis test with Dunn's *post hoc* test for multiple comparisons, P<0.05). CS, Canton S; *rpr*, *reaper*; *DTA*, *diphtheria toxin*; *TeTx*, *tetanus toxin*.

Orco-Gal4 flies were significantly attracted to ETA (Fig. 1B). Importantly, attraction was abolished in *Orco* mutant flies, suggesting that input from *Orco*-expressing OSNs is necessary to induce ETA attraction. Because we expressed the effector genes under Orco-Gal4 control and therefore in the expression pattern of *Orco*, effective silencing by the effector genes should recapitulate this loss of attraction. However, neither *rpr* nor *DTA* abolished attraction when expressed in OSNs (Fig. 1B). In both cases, the AI did not differ between experimental flies and the parental UAS controls. In contrast, attraction was abolished in flies expressing *Kir2.1* and *TeTx* in OSNs (Fig. 1B). However, we did not observe a significant difference between the AIs in *Kir2.1*-expressing flies and the corresponding parental UAS-control flies. This might be due to the low sample size and the generally higher behavioral variability of flies carrying effector constructs. From these experiments, we conclude that different effector genes are differentially effective in silencing *D. melanogaster* OSNs with the most effective one being *TeTx*.

Next, we investigated the effectors' potencies in suppressing odor-guided behavior in the Flywalk assay (Steck et al., 2012) (Fig. 2A). Importantly, in this bioassay, the localization of the odor source does not depend on chemotaxis along a chemical gradient, but, rather, on odor evaluation by the olfactory system and on wind direction as a directional cue for the localization of the odor source (anemotaxis). When presented with a 1-s pulse of the saturated headspace of an attractive 10^{-3} dilution of ethyl acetate in mineral oil, flies responded with instantaneous upwind trajectories, which were absent or only weak when flies were presented with the solvent mineral oil (MOL; Fig. 2B). Similar to the observation in the trap assay experiments, responses to ETA were abolished in *Orco* mutants (Fig. 2C). In addition to ETA, we examined fly behavior towards balsamic vinegar (BVI), methyl acetate (META; 10^{-3} dilution), 2,3-butanedione (BDN; 10^{-3} dilution), trans-2-hexenol (t2H; 10^{-1} dilution) and benzaldehyde (BEA; 10^{-1} dilution). Wild-type flies were significantly attracted by ETA, BVI, META and BDN, whereas t2H was behaviorally neutral and BEA responses were significantly lower than responses towards MOL. In contrast, the attraction induced by ETA, META and BDN as well as the repulsion induced by BEA were abolished in *Orco* mutant flies (Fig. 2D). *Orco* mutant flies retained a residual attraction towards BVI, which is probably conferred by the detection of acetic acid via IRs. In addition, the *Orco* mutant flies acquired attraction towards t2H, which, importantly, is not a false positive in this dataset, but highly reproducible in other datasets (data not shown). We included this odor, because we reasoned that efficient silencing of *Orco*-expressing OSNs should also recapitulate this gain of attraction toward t2H.

As already observed in trap assays, *DTA* and *rpr* also failed to abolish odor-guided behavior in most cases in the Flywalk paradigm (Fig. 2E,F). With the exception of the responses towards ETA, which were abolished in *Orco*-Gal4/UAS-*rpr* flies, all attraction responses were retained in flies expressing *DTA* and *rpr*. Also, in most cases in which flies expressing the two effectors differed in their responses from one of their parental control flies, responses were statistically indistinguishable from the other parental line (Fig. S1A,B). Similar to *DTA* and *rpr*, expressing *Kir2.1* in *Orco*-expressing OSNs failed to abolish any of the attractive responses and the aversion of BEA, whereas the gain of attractiveness of t2H observed in *Orco* mutant flies could be recapitulated by this manipulation, but not in parental controls (Fig. 2G, Fig. S1C). Importantly, *Kir2.1* was partly efficient in modifying attractant responses, as it significantly decreased responses compared with both parental control strains for BVI and BDN. Expressing *TeTx*, in contrast, abolished attraction towards META and reduced, but not abolished, responses towards BVI and BDN compared with parental controls, and failed to recapitulate the gain of attraction towards t2H (Fig. 2H, Fig. S1D). Also, the aversion induced by BEA was suppressed, which is

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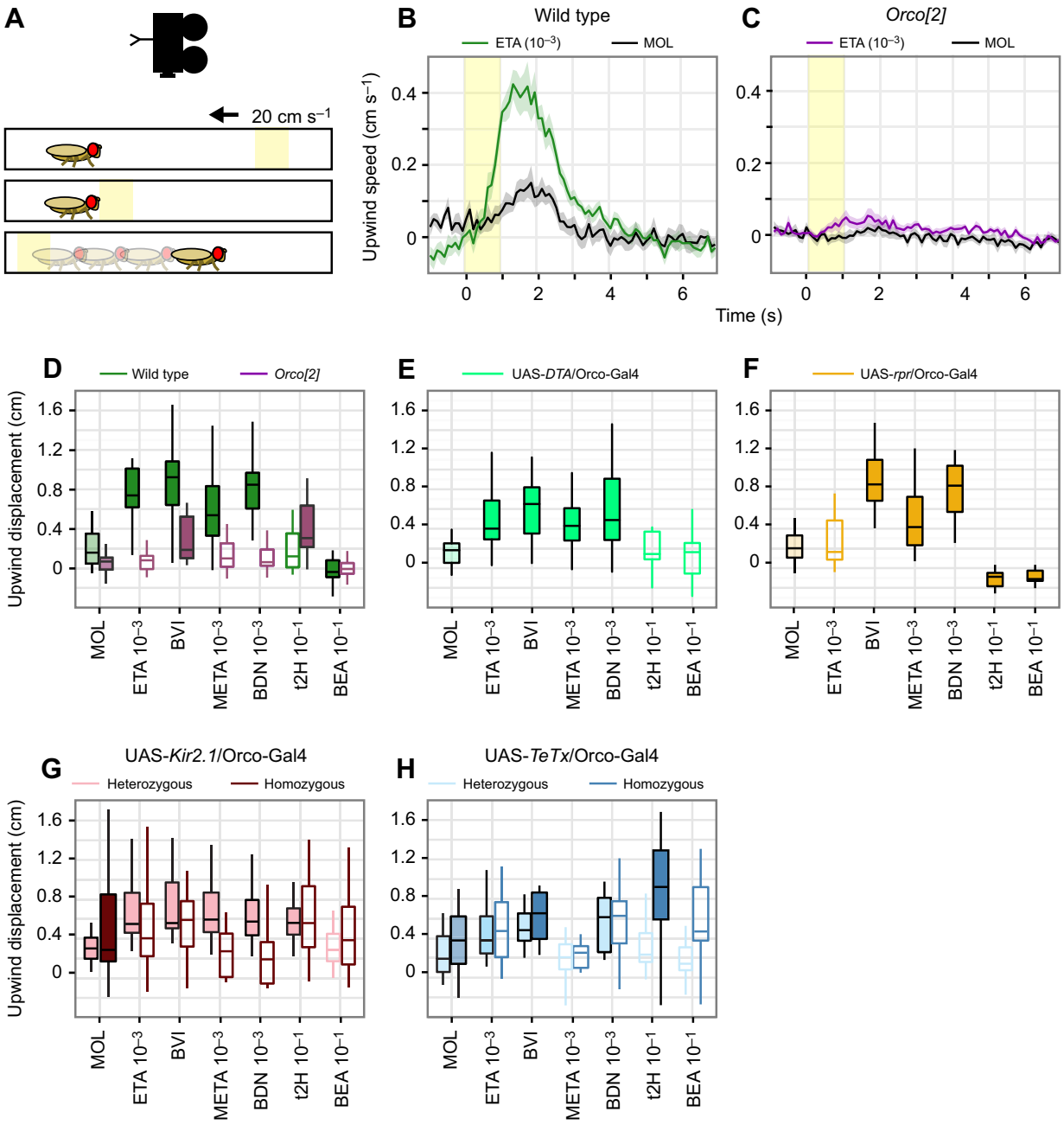

Fig. 2. Efficiency of effector genes in the Flywalk paradigm. (A) Schematic representation of the Flywalk paradigm. Individual flies are situated in small glass tubes and continuously monitored by an overhead camera. Odors are added to a constant airflow and fly movement after odor encounter is analyzed. (B) Speed trajectories after encounter with a 1 s pulse of a 10^{-3} dilution of ethyl acetate (ETA, green) and the negative control mineral oil (MOL; black; mean± s.e.m.; *n*=15 flies) in wild-type flies. (C) Speed trajectories after encounter with a 1 s pulse of a 10^{-3} dilution of ETA (violet) and the negative control MOL (black) in *Orco* mutant flies (mean±s.e.m.; *n*=15 flies). (D) Odor-induced upwind displacement in CS wild-type and *Orco* mutant flies to a set of odors. In D–H, filled boxes indicate statistically significant differences from the negative control MOL (transparent colors; Wilcoxon signed rank test, $P<0.05$, *n*=15 flies). (E,F) Odor-induced upwind displacement in flies heterozygously expressing *DTA* (E) and *rpr* (F) in the expression pattern of *Orco*. Note residual responses compared with *Orco* mutant flies shown in D. (G,H) Odor-induced upwind displacement in flies expressing *Kir2.1* (G) and *TeTx* (H) in the expression pattern of *Orco*. Lighter boxes show responses of flies expressing *Kir2.1* (G) or *TeTx* (H) heterozygously, darker boxes show responses of flies expressing *Kir2.1* (G) or *TeTx* (H) homozygously.

probably attributable to decreased responses towards the negative control compared with control genotypes, which can also be observed in *Kir2.1*-expressing flies (Fig. 2GH, Fig. S1CD). In summary, although *Kir2.1* and *TeTx* (and for one odor also *rpr*) reduced some of the odor-induced responses in the Flywalk paradigm, the neuronal silencing induced by the tested effectors was not absolute. None of the effectors succeeded in fully recapitulating the Orco mutant phenotype, at least not when heterozygously expressed. Because *TeTx* and potentially *Kir2.1* were effective in the trap assay and also displayed some effect in initial Flywalk experiments, we proceeded to investigate whether an increase in transgene expression levels by double-homozygous expression would increase the efficiency of these two effectors.

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Homozygously expressed *Kir2.1* was very efficient in abolishing odor-guided behavior in the Flywalk. *Kir2.1*-expressing flies did not show any statistically significant response to the presented odorants in the Flywalk paradigm (Fig. 2G). However, homozygous expression of *Kir2.1* could not fully recapitulate the *Orco* mutant phenotype, because responses towards BVI and t2H were also abolished. In contrast, homozygous expression of *TeTx* in *Orco*-expressing OSNs could fully recapitulate the *Orco* mutant phenotype (including residual responses to BVI and novel response to t2H; Fig. 2H).

So far, we showed that even effectors that successfully abolish odor-guided attraction in the trap assay may fail to do so in the Flywalk paradigm. What could be the reason for the differences in potencies observed in the different bioassays? Essentially, the two bioassays differ in two aspects: (1) in the trap assay, we tested cohorts of flies in contrast to individual flies in Flywalk, and (2) in the trap assay flies rely on chemotaxis along an odor gradient in contrast to the anemotactic odor source localization in Flywalk. To identify the reason for the conflicting results obtained so far, we next examined odor-guided behavior in a single-fly chemotactic assay similar to that described by others (Zaninovich et al., 2013). In this paradigm, we released individual flies in a square arena with a central odor source, recorded their positions and analyzed their distance from the central odor source (Fig. 3A). Because responses to single odorants are not very strong in this assay, we used balsamic vinegar as an attractant and distilled water as a negative control in these experiments.

When presented with water as a central odor source, CS flies typically spend most of the time at the edges of the arena and otherwise explore the whole arena without displaying spatial preferences (Fig. 3B). When presented with balsamic vinegar, in contrast, CS flies still spend a significant amount of time at the arena edges, but otherwise display intensive search behavior in the arena center (Fig. 3C). To analyze this observation quantitatively, we calculated the flies' mean distance from the arena center for both water and vinegar. Thus we observed that wild-type flies are attracted to the balsamic vinegar. Similar to wild-type flies, *Orco* mutant flies were also attracted to balsamic vinegar in this assay (Fig. 3D). In a more detailed analysis, we found that *Orco* mutants, compared with CS, spent significantly less time within 2 cm around the odor source when presented with vinegar (P=0.006, Wilcoxon rank sum test, n=20; Fig. S2B,C), suggesting that they are indeed impaired in their fine-scale search behavior, although they are still able to detect the odor source, probably detecting acetic acid using the IR-dependent olfactory subsystem.

We next tested flies heterologously expressing the different effector genes in this assay. According to the results obtained in the trap assay and Flywalk experiments, we did not observe any difference between *DTA*-expressing flies and their corresponding parental controls. All tested animals spent an equal amount of time within 2 cm of the odor source (Fig. 3E). The same was found for flies expressing *rpr* under *Orco*-Gal4 control. Although *Kir2.1* by trend abolished attraction in the trap assay experiment

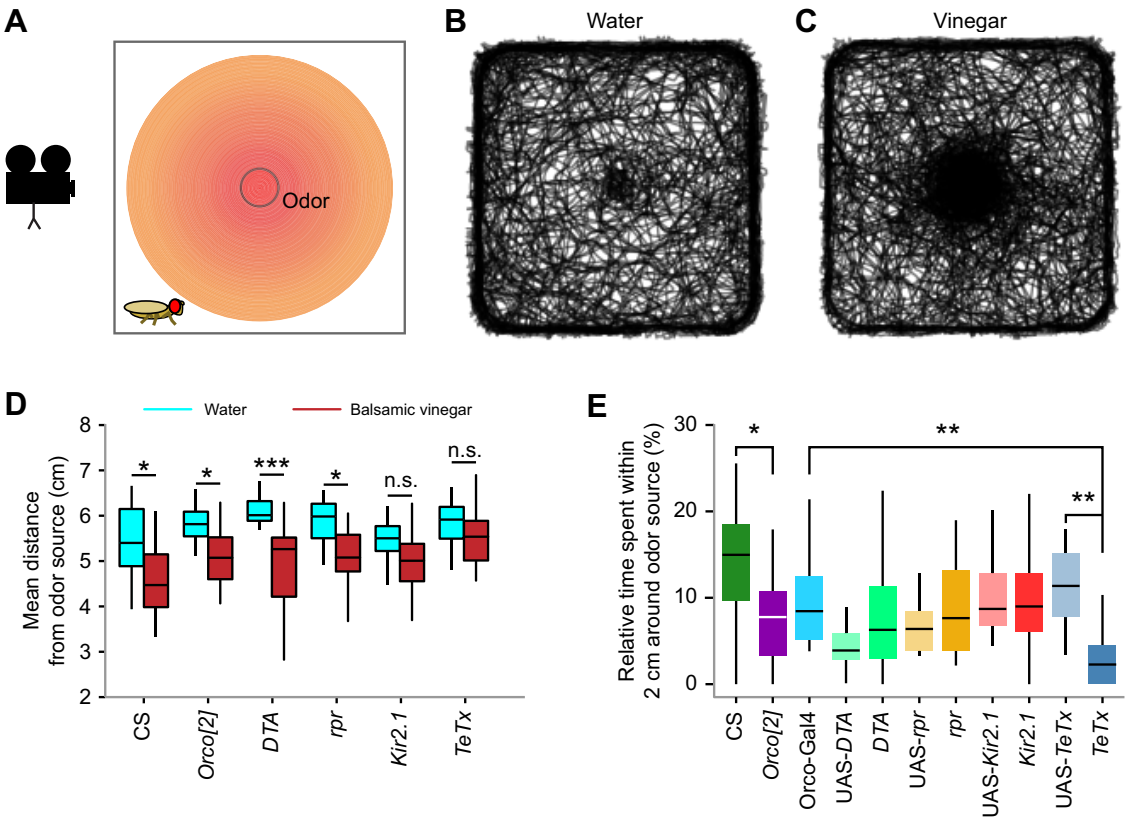

Fig. 3. Efficiency of effector genes in an open-field arena. (A) Schematic representation of the open-field arena. Individual flies are released in a square arena with a central odor source and their positions are recorded for 10 min. The odor source cannot be contacted physically by the fly. (B,C) Overlay of 20 fly trajectories when presented with water (B) and balsamic vinegar (C). Note increased search behavior in the central zone in C. (D) Mean distance from odor source of CS, *Orco[2]*, *DTA*-expressing, *rpr*-expressing, *Kir2.1*-expressing and *TeTx*-expressing flies. Water control is indicated by blue boxes, balsamic vinegar indicated by red boxes. Asterisks indicate significant differences (*P<0.05; **P<0.01; ***P<0.001; Friedman test; n=20 flies per genotype). (E) Relative time the tested flies spent within 2 cm around odor source (balsamic vinegar). Asterisks indicate significant differences (*P<0.05; **P<0.01; Wilcoxon rank sum test, Bonferroni-corrected; n=20 flies per genotype).

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and – if homozygously expressed – did so significantly in Flywalk experiments, we could not find any difference between experimental flies and parental controls in the open-field arena (Fig. 3E). This leads to the conclusion that *Kir2.1* expression is not able to reproduce the *Orco* mutant phenotype and therefore fails to completely abolish odor-guided behavior in this single-fly chemotactic bioassay. In contrast, *TeTx*-expressing flies showed a significantly lower attraction towards balsamic vinegar compared with parental controls (Fig. 3E). Experimental flies were not attracted to the water control or balsamic vinegar. Although they explored the arena and occasionally also passed the central odor source, they did not show any search behavior that is comparable to the behavior observed in the other genotypes (not shown).

We conclude that only two of the constructs we tested fully recapitulated the *Orco* mutant phenotype in all bioassays. However, when expressed heterozygously, both *rpr* and *DTA* failed to induce any expression-specific effect in any of the paradigms, while *Kir2.1* slightly reduced attraction in trap assays, but not in Flywalk or the open-field arena. *TeTx* appears to be the most efficient genetic tool for silencing OSNs, although it also failed to completely abolish odor-guided behavior in the *Flywalk* paradigm in the heterozygous expression regime. Only when expressed homozygously were both *TeTx* and *Kir2.1* able to abolish odor-guided behavior in Flywalk experiments, where *TeTx* fully recapitulated the *Orco* mutant phenotype. In addition, our results in combination suggest that the different potencies we observed in the three bioassays may at least be partially explained by the different demands on olfactory processing between chemotaxis and anemotaxis.

DISCUSSION

Our main objective in this study was to identify a genetic tool that is capable of reliably silencing single OSN populations in a large-scale approach to investigate the contribution of individual processing channels of the fly olfactory system to odor evaluation using the Flywalk paradigm. The contribution of OSN types to odor evaluation has so far been tested in several studies, each concerned with single OSN types using a wide variety of different behavioral paradigms (Ai et al., 2010; Dweck et al., 2013, 2015a,b; Grosjean et al., 2011; Kurtovic et al., 2007; Min et al., 2013; Ronderos et al., 2014; Semmelhack and Wang, 2009; Stensmyr et al., 2012; Suh et al., 2004). Also, a correlation between the activities of different projection neuron (PN) types and behavior to a large odor set has been established previously (Knaden et al., 2012). However, in order to establish causality rather than correlation, it is necessary to show that OSN output is necessary and sufficient to cause the observed behavioral effect. Therefore, the ability to silence OSN populations is essential to establish a causal relationship between OSN input and behavioral output. Because the behavioral effect of a loss of an individual OSN type can be rather cryptic and may not necessarily strongly affect responses towards the OSN's presumed cognate ligand (Keller and Vosshall, 2007), large-scale approaches will be needed to crack the olfactory valence code, and these depend on dependable genetic tools as it may not be possible to control for efficient silencing in every case.

Our data demonstrate that, at least when expressed heterozygously, none of the tested genetic silencing tools really silenced all OSNs in the literal sense of the word. That being said, the effectors clearly differed in their potencies, with *TeTx* being the most efficient, followed by *Kir2.1*, both of which significantly (*TeTx*) or at least by trend (*Kir2.1*) abolished odor-guided behavior in the trap assay and to some extent also in the open-field arena, and in the Flywalk paradigm when expressed homozygously. In contrast, expression of *DTA* and *rpr* did not affect odor-guided behavior in any of the bioassays. A similar ranking of the potencies of the tested silencers in the motor system has also been reported previously by others (Thum et al., 2006). Therefore, we conclude that these differences are at least in part intrinsic to the effectors, although target cell type and timing of expression may also contribute to the effectiveness.

What may be the mechanistic reason for the observed differences? Both *DTA* and *rpr* act by ultimately killing their target cell. Whereas the action of *rpr* depends on the cellular apoptosis machinery and effectiveness of silencing may therefore vary depending on cell type, *DTA* is an inhibitor of protein synthesis and should therefore be ultimately lethal for all cell types. However, our results suggest incomplete ablation of the *Orco*-expressing OSN population for both *rpr* and *DTA*. Because we used rather high odor concentrations throughout the study, it is conceivable that a low number of surviving OSNs may be sufficient to evoke the behavior. The inefficiency of *DTA* is nevertheless surprising given its extreme toxicity. However, as a protein synthesis inhibitor, its action depends on cellular protein turnover rates and its effect may therefore be observable in flies older than those we tested.

In contrast to *DTA* and *rpr*, *TeTx* worked well in trap assays and both *TeTx* and *Kir2.1* in Flywalk if homozygously expressed. Furthermore, a heterozygous expression of *TeTx* was sufficient to abolish behavior in the open-field arena. Because expressing either *Kir2.1* or *TeTx* heterozygously did not phenocopy responses of *Orco* mutant flies in the Flywalk assay, silencing of OSN output is presumably also incomplete when using these constructs. Incomplete silencing was recently reported for the temperature-sensitive *dynamin* variant *shibire*^{ts}, which is also widely used in *D. melanogaster* behavioral studies and considered to be very effective. In that study, the authors showed that the expression of *shibire*^{ts} in OSNs reduced the responses in postsynaptic PNs by approximately 50% at the restrictive temperature (Liu and Wilson, 2013). A similar incomplete silencing of OSN activity may be a reason for the remaining responses in flies heterozygously expressing *Kir2.1* and *TeTx* in Flywalk, particularly because at least for some of the odors the concentrations we used were well above the behavioral threshold (Thoma et al., 2014).

But why do some constructs abolish behavior in some but not in other bioassays? We assume that the reason for the dependence on the type of bioassay lies in the navigational strategy employed to approach the odor source. In anemotactic assays such as Flywalk, the sole demand on the olfactory system is to identify and evaluate odors, whereas directional cues concerning the location of the odor source are provided by the wind direction. In chemotactic assays such as the trap assay or the open-field arena, odor source localization also depends on the olfactory system, in addition to odor identification and evaluation. *Drosophila melanogaster* larvae evaluate the direction of an odor gradient by an active sampling process, and respond behaviorally to small local concentration increments (Gomez-Marin et al., 2011; Louis et al., 2008). Adult vinegar flies have been demonstrated to be able to measure and respond to local concentration differences across their antennae in tethered paradigms (Borst and Heisenberg, 1982; Gaudry et al., 2013), although it is not entirely clear whether the slope of a natural odor gradient would be sufficiently steep to assess its direction by comparing the difference in inputs to the two antennae. Irrespective of whether adult flies assess the direction of odor gradients by comparing concentration across two spatially separated sensors, or by moving the sensors through the gradient and comparing concentration differences in time, both strategies probably depend on the full dynamic range and contrast of the olfactory system, both because local concentration increments may be tiny and because

they need to be measured under varying background conditions. Although *TeTx* and *Kir2.1* may not fully silence OSN output, they are likely to reduce dynamic range and contrast of the olfactory system already in the heterozygous state, and this reduction may be sufficient to disrupt chemotactic navigation, but insufficient to disrupt odor evaluation. This may be why the genetic manipulations had a stronger impact on fly behavior in trap assays and the open-field arena than in the Flywalk paradigm.

The observation that flies expressing *TeTx* in OSNs but not *Orco* mutant flies fail to locate the odor source in the open-field arena is rather puzzling and we can only speculate about possible reasons. *TeTx*-expressing flies clearly do not have motor deficits, which could have explained the results, because they also display odor responses in Flywalk (Fig. 2H). The effect of *TeTx* expression is unlikely to be an effect of the genetic background, because both parental strains were attracted by the odor source (Fig. S1D). Both *Orco* mutants and *TeTx*-expressing flies are likely to perceive balsamic vinegar, probably via IR-dependent detection of acetic acid, because both genotypes display attraction towards vinegar in Flywalk (Fig. 2D,F). Hence, our observations suggest that *Orco* mutants rely more on their IR nose during chemotactic close-range search behavior than do flies expressing *TeTx*. The *Orco* mutant strain we used has been published more than 10 years and – assuming an average generation time of 2 weeks – approximately 300 fly generations ago (Larsson et al., 2004). In contrast, the *TeTx*-expressing flies we tested were from the first generation with strongly reduced input from the OR nose. Considering that only 15 generations of experimental evolution are sufficient to induce a significant difference in learning abilities (Mery and Kawecki, 2002) and 30 generations of monogamy are sufficient to significantly reduce female fecundity (Innocenti et al., 2014), it is conceivable that 300 generations without an OR nose may have favored an altered usage and evaluation of the olfactory input from the IR-dependent olfactory system, although the selection pressure is probably low under standard laboratory culture conditions. This is of course highly speculative, but at the same time it appears to be the most parsimonious explanation for our observations and may provide an interesting future avenue of research in the evolution of odor-processing systems.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceived study and designed experiments: T.R., M.T., M.K. and B.S.H. Performed experiments: T.R. Analyzed data: T.R. and M.T. Wrote and revised manuscript: T.R., M.T., M.K. and B.S.H.

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