Title

A GAL80 collection to nullify transgenes in *Drosophila* olfactory sensory neurons

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Summary

Fruit flies recognize hundreds of ecologically relevant odors and respond appropriately to them. The noise, redundancy and interconnectedness of the olfactory

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machinery complicate efforts to pinpoint the functional contributions of any component neuron or receptor to behavior. Some contributions can only be elucidated in flies that carry multiple mutations and transgenes, but the production of such flies is currently labor-intensive and time-consuming. Here, we describe a set of transgenic flies that express the *Saccharomyces cerevisiae* GAL80 in specific olfactory sensory neurons (OrX-GAL80s). The GAL80s effectively and specifically subtract the activities of GAL4-driven transgenes that impart anatomical, behavioral, and physiological phenotypes. OrX-GAL80s can allow researchers to efficiently activate only one or a few types of functional neurons in an otherwise nonfunctional olfactory background. Such experiments will improve our understanding of the mechanistic connections between odorant inputs and behavioral outputs at the resolution of only a few functional neurons.

Key Words

Olfaction; GAL80; Drosophila; Odorant Receptor; Sensory Neurons

Introduction

The olfactory system of *Drosophila melanogaster* is often the subject in studies of memory, evolution, gene choice, development and odorant-induced behavior. It is a good model system because of its relatively stereotyped neuronal circuitry, complex behaviors and convenient genetic tools.

In *Drosophila*, most olfactory sensory neuron (OSN) typically expresses a single odorant receptor (OR) from a genomic repertoire of 60 genes (Vosshall et al., 1999;

Robertson et al., 2003; Vosshall et al., 2000; Clyne et al., 1999; Goldman et al., 2005). The promoter of an OR gene can thus be employed to label specific subsets of OSNs with a particular transgene. ORs, which vary in sensitivity and specificity to a wide range of different odorants, determine the firing kinetics and odor-response dynamics of each OSN (de Bruyne et al., 1999; de Bruyne et al., 2001; Dobritsa et al., 2003; Elmore et al., 2003; Hallem et al., 2004; Couto et al., 2005; Fishilevich & Vosshall, 2005; Hallem & Carlson, 2006; Kreher et al., 2008).

Most OSNs express Odorant Receptor Co-Receptor (Orco), a highly conserved member of the olfactory receptor family (Krieger et al., 2003; Vosshall and Hansson, 2011), in addition to a single selected OR. Though Orco usually does not contribute to the structure of the odorant binding site (Nakagawa and Vosshall, 2009; Nichols and Luetje, 2010; Jones et al., 2011; Jung et al., 2011), it is essential for odorant-invoked signaling in flies. Without Orco, the co-expressed OR cannot localize to the dendritic membrane or relay an odor-evoked signal (Larsson et al., 2004; Benton et al., 2006;). Orco null flies are largely anosmic, though some chemosensation remains due to the presence of ionotropic receptors and gustatory receptors, which do not require Orco to function (Jones et al., 2007; Kwon et al., 2007; Benton et al., 2009; Silbering et al., 2011). The Orco promoter is consequently a convenient device for the expression of transgenes in most OSNs.

The olfactory organs, the antenna and maxillary palp, contain OSNs dendrites within structures called sensilla. ORs and Orco are embedded in the dendritic

membrane. OSN axons project to the antennal lobes in the brain of the animal. Each antennal lobe consists of ~50 globular synaptic sites called glomeruli. All OSNs on the periphery that expresses the same OR converge onto their own unique glomerulus. For example, all OSNs expressing Or22a will send axons to the DM2 glomerulus in the antennal lobe while all OSNs expressing Or82a will send axons to the VA6 glomerulus (**Figure 1**). The stereotyped organization of OSNs and their projections is known as the olfactory sensory map (Stocker et al., 1990; Vosshall et al., 2000; Couto et al., 2005; Fishilevich and Vosshall, 2005). The regularity of this map is a key feature that makes *Drosophila* olfaction such a useful model, as any aberration to the typical pattern will be apparent. The apparent simplicity of the map (**Figure 1**), however, obscures mechanistic complexities that are yet to be discovered, in part because necessary tools remain unavailable.

Drosophila geneticists have traditionally relied on genetic mutations or deletions to understand how complex biological systems normally work. Most alleles are recessive, so homozygotes must be bred over multiple generations. Achieving homozygosity of a mutation while also adding transgenes to the system often requires the creation of recombinant chromosomes produced after multiple generations of crossing and PCR screening. Classical genetic strategies thus limit the number and complexity of combinatorial genotypes that one can achieve. More challenging experimental questions demand more facile and versatile genetic tools.

The GAL4/UAS gene regulation system has become a *defacto* standard in studies of *Drosophila*. GAL4 is a yeast transcription activator that binds to the Upstream Activating Sequence (UAS) and induces expression of downstream genes (Giniger et al., 1985). By driving GAL4 expression from an OR promoter, specific expression of a *UAS-transgene* can be obtained for any OSN subtype. An *OrX-GAL4* line exists for almost every OR. This collection of GAL4 lines is a powerful toolbox since different *UAS-transgenes* can be introduced into a line via conventional mating. For example, human α-synuclein has been expressed in OSNs to model human Parkinson's disease (Chen et al., 2014). Alternatively, protein expression levels can be knocked down using any specified *UAS-RNAi* transgene.

A variety of existing compatible effectors can be used study different aspects of neuronal communication. *UAS-Kir2.1* effector is used as an example in experiments described below. This inward rectifier potassium channel electrically inactivates the neurons that express it (Johns et al., 1999; Baines et al., 2001; Hodge, 2009). Similarly, *shibire*^{ts} or tetanus toxin can be used to silence synaptic communications (van der Bliek and Meyerowitz, 1991; Chen et al., 1991; Sweeney et al., 1995; Baines et al., 1999; Kitamoto, 2001; Kitamoto, 2002), *reaper/grim/hid* genes can be used to physically kill neurons using their own apoptotic pathways (Abrams, 1999; Song and Steller, 1999), or ricin toxin can be expressed ectopically to kill neurons. Conversely, neurons can be selectively activated with *trp1a* or a variety of other channelrhodopsin transgenes (Pulver et al., 2009; Boyden, 2011).

If GAL4 is a standard on-switch for nearly any desired transgene, GAL80 is the logical off-switch. GAL80 binds GAL4 transcriptional activation domain, thereby preventing recruitment of RNA polymerase (Ma and Ptashne, 1987). GAL80 crosses are much more convenient than classical breeding approaches (**Figure 2**). In order to have a single functional OSN in an otherwise silent olfactory system, the traditional method uses an Orco null mutation (Larsson et al., 2004). In this genetic setup, Orco mutant flies are anosmic, but function is restored to one OSN subset with *Or-GAL4, UAS-Orco* transgenes (Fishilevich et al., 2005; Benton et al., 2006; Olsen et al., 2007; DasGupta and Waddell, 2008; Hoare et al., 2008; 2011) (**Figure 2a**). An *Orco-GAL4, UAS-effector, Or-GAL80* method can be used instead (**Figure 2b**). Kir2.1 is used as an example of an effector (Johns et al., 1999; Baines et al., 2001; Hodge, 2009;). Classical breeding strategies (**Figure 2a**) may look less complicated on paper than GAL80 crosses (**Figure 2b**) but are actually more time-consuming and limited. The Orco mutation must be homozygous. Since most *Drosophila* transgenes are embedded into the same two chromosomes (2 or 3) recombination and PCR screening may be required to achieve this homozygosity.

Neurons seldom operate autonomously, but rather groups of neurons coordinate within a circuit to provide an organism with perception and behavior. An investigation of the behavioral impact provided by a limited number of different functional neuronal types would require additional genes. The elaboration of genotypes (**Figure 2**) to restore pairs or groups of functional OSNs in a nonfunctional background normally requires generations of crosses (followed by PCR screens for desired recombinants). A

GAL80 strategy can shorten this process by achieving similar results in only one or two generations with no necessary recombinant creation. Furthermore, a GAL80 strategy takes advantage of the interchangeable variety of existing *UAS-transgene* lines. Here we describe a new collection of *OrX-GAL80* lines designed to complement existing *OrX-GAL4* lines, and demonstrate their potential utility for behavioral and neuroanatomical studies of the *Drosophila* olfaction model.

Results

Design of GAL80 Constructs

The following criteria were used to choose OR promoters for the collection. i) The ORs should be relevant to current research as shown by the number of studies that used it. ii) The ORs should represent a variety of expression patterns (larval or adult, antennae or maxillary palps, sensillary class etc.). iii) Finally, the ORs should reflect a variety of different odorant response profiles. The promoter regions were defined based largely on the work of Couto *et al*, 2005.

Equimolar expression of GAL4 and GAL80 is not always sufficient to effectively eliminate GAL4 activity so the pBP-GAL80uW-6 vector was used. This vector contains a modified GAL80 sequence, designed to increase the stability and expression of its gene product (Pfeiffer et al. 2010). A few *OrX-GAL80*s were already made with this vector and used effectively. Gao et al. (2015) pioneered the technique by creating a few OrX-

GAL80s. This work is a logical extension of their contributions and will make many GAL80s freely available for the continued development of the field.

Testing GAL80 Efficacy and Specificity

GAL80 lines were created for the following odorant receptor promoters: Or7a, Or9a, Or10a, Or13a, Or19a, Or22a, Or22b, Or33c, Or35a, Or42a, Or42b, Or43b, Or47a, Or56a, Or59b, Or59c, Or67a, Or67d, Or71a, Or82a, Orco, Or85a, Or85b, Or85c, and Gr21a. To examine GAL4 subtraction *in vivo*, *OrX-GAL80* flies were crossed to flies with the genotype *OrX-GAL4, UAS-GFP*. OSNs expressing the same OR can be identified from their specific glomerulus in the antennal lobe (**Figure 1**). *OrX-GAL4, UAS-GFP* flies show robust expression of the GFP reporter gene in their respective glomeruli. However, when *OrX-GAL80* is added to the genotype, GFP expression is entirely absent, indicating a robust antagonism of GAL4 activity (**Figure 3**). Several of these lines also have expression in larvae. GAL4 subtraction was examined in larval brains using the *UAS-GFP* reporter gene. In larvae, GAL80 reduced but did not eliminate GAL4 activity (**Figure S1a**).

The *OrX-GAL80* lines were checked to ensure they would not have aberrant expression in untargeted OSN subtypes. The pBP-GAL80uW-6 vector contains a Drosophila Synthetic Core Promoter (DSCP). DSCP is an effective means of using enhancer elements to drive strong expression (Pfeiffer et al., 2008), but it could also cause the GAL80s to have nonspecific or leaky expression. Therefore, a version of pBP-GAL80uW-6 was cloned with the DSCP removed. However, when the DSCP was

absent, GAL80 expression was insufficient to subtract GAL4 activity (**Figure S1b**). A few lines were tested to see if DSCP causes nonspecific GAL80 expression. For these lines, an *OrY-GAL80* did not impede GAL4 activity of an *OrX-GAL4* neuron (**Figure S1c**). Due to the uneven expression in an *Orco-GAL4, UAS-GFP* line, it could not be determined if each OrX-GAL80 subtracts GAL4 from only one glomerulus in an otherwise fully-labeled brain, but results shown in Figure S1c give reasonable confidence that the GAL80s do not have widespread nonspecific expression. It can also be noted that the GAL80 subtraction does not interfere with reporter gene expression in a genetic system that does not use GAL4. When *Or22a-GAL80* is used in conjunction with *Or22a-GFP*, containing no GAL4/UAS intermediary, the GFP is still expressed (**Figure S1d**). These images, showing subtraction of reporter gene expression, confirm that GAL4 activity is suppressed anatomically by the GAL80 lines.

To confirm GAL4 was suppressed physiologically by the GAL80s, Single Sensillum Recordings (SSRs) were used to assay electrical activity of OSNs. *Gr21a-GFP* was used to identify sensilla of interest without interfering with the GAL4/UAS/GAL80 system. Gr21a neurons are housed in ab1 sensilla. Carbon Dioxide exposure causes a robust response in Gr21a ab1C neurons (Jones et al., 2007; Kwon et al., 2007). When *Gr21a-GFP* flies were exposed to CO_2, their ab1C sensillar neurons showed robust responses (mean Δspikes/s=88, N=8 sensilla). Adding Kir2.1 to Gr21a neurons (genotype *Gr21a-GFP, Gr21a-GAL4, UAS-Kir2.1)* greatly reduced spiking responses to CO_2 (mean Δspikes/s=14, N=12 sensilla). When Gr21a-GAL80 was added (genotype *Gr21a-GFP, Gr21a-GAL4, UAS-Kir2.1, Gr21a-GAL80),* responses to CO_2

were restored (mean Δspikes/s=94, N=6 sensilla) (**Figure 4**). These results confirm that GAL80 is able to prevent GAL4-induced activity in a physiological experimental paradigm.

Behavior

To demonstrate how experiments can be simplified using GAL80 reagents, the GAL80s were used alongside a genetic strategy to create flies with a single functional OSN (**Figure 2**). The transgenic genotypes are easier to construct than those created by classical genetics, but the two systems are expected to be quite similar in behavior. Insect olfactory neurons do not require odor-evoked activity to develop with complete fidelity (Elmore and Smith, 2001; Wong et al., 2002; Dobritsa et al., 2003; Larsson et al., 2004; Berdnik et al., 2006;), and therefore both Orco null and *Orco-GAL4, UAS-Kir2.1* brains will have normal circuitry, despite their nonfunctional neurons. Orco null mutants also have greatly diminished spontaneous activity, and Kir2.1-containing neurons are expected to show little to no spontaneous firing (Hoare et al., 2008; Olsen et al., 2007).

A simple odor preference test was used to assay both types of adult flies. Flies were allowed to walk freely in a circular arena with four air quadrants separated by air flow but not physical barriers. After one minute, two quadrants along the diagonal received an odorant (Aso and Rubin, 2016). The diagonal quadrants receiving odorant were randomized. Flies were recorded and their motions tracked using Ctrax software (Branson et al., 2009) (**Figure 5a**). Response index was measured in each frame. Response Index= $Flies_{odor}$- $Flies_{air}$/Total Flies, so 0 indicates no response, +1 would be

229 total attraction, and -1 would be total aversion. Each N represented an experiment
230 involving 20-30 flies. Therefore, if an N had an RI of -0.5, about 23 of 30 flies were in
231 the air quadrant.
232
233 Or22a and Or82a were tested since they represent opposite ends of the odorant
234 tuning spectrum. Or22a is broadly tuned and its OSN responds to multiple odorants;
235 Isoamyl acetate (IAA) is one odorant that evokes a strong response. Or82a is narrowly
236 tuned, responding to only one known odorant, geranyl acetate (GAc) (de Bruyne et al.,
237 2001; Hallem et al., 2004; Fishilevich and Vosshall, 2005; Hallem and Carlson, 2006).
238 Or22a genotypes (**Figure 5b**) received 1:1000 IAA in mineral oil and Or82a genotypes
239 (**Figure 5c**) received 1:100 GAc in mineral oil. The mean response index for the middle
240 part of the odorant exposure (time = 75-105 seconds) was calculated. The positive
241 control was *Orco-GAL4* with no effector added; this line and the OrX-GAL80 lines were
242 made in a w^{1118} background. These flies find both odorants aversive, as shown by a
243 negative and significantly lower response index to all other genotypes ($p \leq .0001$ for IAA
244 and $p \leq .006$ for GAc). *Orco* null and *Orco-GAL4, UAS-Kir2.1* flies served as negative
245 controls and had response indices around 0, showing they are anosmic as expected.
246 *Or22a-GAL4, UAS-Kir2.1* flies did not find IAA aversive, and *Or82a-GAL4, UAS-Kir2.1*
247 flies did not find GAc aversive (**Figure 5b and c**), suggesting that Or22a and Or82a are
248 each necessary for behavioral responses to their respective odorants.
249
250 As noted previously, two genotypes for each OR had only a single functional
251 OSN subset in an otherwise nonfunctioning olfactory system (**Figure 2**). One group

represents the classic strategy (*OrX-GAL4, UAS-Orco* in an Orco null background) and the other represents the GAL80 strategy (*Orco-GAL4, UAS-Kir2.1, OrX-GAL80*). When O22a or Or82a OSN function is restored, neither of the single-functional OSN genotypes is sufficient alone to restore normal aversive behavior. However, for both Or22a genotypes with IAA and Or82a genotypes with GAc, the behavioral variance for the classical method vs the GAL80 method is comparable (F-test for the 75-105sec window is p=.44 for the Or22a/IAA group and p=.45 for the Or82a/GAc group). Since both groups have similar variance, this result supports the idea that the GAL80 strategy can be adopted as a substitute for the more traditional and time-consuming breeding strategy.

Discussion

The lack of aversive behavior in flies with a single functional OSN subtype is not consistent with some previous studies that showed one functional OSN was sufficient to restore aversive behavior. Fishilevish et al (2005) used larvae in their study to restore aversion with a single functional OSN subtype, but the larval olfactory system may be fundamentally different in this respect. Gao et al (2015) gave convincing evidence of aversive restoration in adults, though they didn't use Or82a and they tested Or22a with a different odorant (E2-hexenal) of a higher concentration. It could be that the principle of single-OSN aversive behavior is highly dependent on the odorant and receptor used. (Bhandawat et al., 2010) also showed that single glomerular activity is sufficient to invoke a behavioral response, but that study was done using an intact and fully functional olfactory background, so some neuronal cooperation may still have occurred.

DasGupta and Waddell (2008) provided evidence that a single functional OSN subtype is sufficient to learn odor discrimination, but the experiments here do not extend to assess learning or memory.

Despite some specific circumstances, the lack of aversive rescue is generally consistent with the current models of odor coding by the olfactory system. I.e. a coordinated effort of many OSNs is usually required to produce a behavioral output. Paired neurons in sensilla can affect the firing dynamics of their neighbors in the periphery (Dobritsa et al., 2003; Kazama and Wilson, 2009; Su et al., 2012), and downstream neurons such as interneurons and projection neurons may rely on synchronized input from multiple OSN types (Ng et al., 2002; Olsen et al., 2007; Chou et al., 2010; Yaksi and Wilson, 2010; Acebes et al., 2011; Kazama et al., 2011; Wilson, 2011; Hong and Wilson, 2015). The behavioral conditions here give no effect and are included as a demonstration of GAL80 possibilities. The hope is that additional researchers will use the reagents and validate them in their own assays and use them to study effects of interest.

The collection of GAL80 lines subtracts GAL4 activity efficiently and specifically in OSNs. In anatomical studies, reporter gene expression from the GAL4/UAS system is suppressed. Neurons silenced with Kir2.1 expression have normal firing capacity restored when GAL4 is antagonized using the GAL80 lines. In behavioral assays, the GAL80s have comparable variations to traditional methods that use mutant backgrounds. However, using a GAL80 transgene will be more flexible than mutant

lines and less cumbersome than crafting the required recombinants as the complexity of the genotype increases.

Though in some special circumstances, olfactory sensory neurons can produce behaviors autonomously, this is not a widely applicable principle. Researchers encounter a significant technical obstacle to the understanding of olfactory function if they need to create genotypes with small groups of interacting neurons in isolation. The tools presented here facilitate the activation or deactivation of combinations of particular neurons, thereby overcoming this obstacle. The lines are available to order through Bloomington Stock Center.

Author Contributions

Conceptualization, JE and IM; Methodology, JE; Formal Analysis, JE and AA; Investigation, JE and AA; Writing—Original Draft, JE; Writing—Review and Editing, AA, CP, IM; Visualization, JE and AA; Supervision, IM; Funding Acquisition, IM

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Figure Legends

Figure 1: Olfactory Sensory Map. Each neuron in the olfactory system expresses one type of odorant receptor (OR). Or22a (teal) and Or82a (gold) are used here as examples. Neurons usually exist in pairs or groups in sensilla within the olfactory organs—antenna or maxillary palp. Neurons expressing the same OR are distributed throughout the periphery, but project their axons onto the same glomerulus in the antennal lobe of the brain. For example, all Or22a-expressing neurons synapse onto the DM2 glomerulus while all Or82a-expressing neurons synapse onto the VA6 glomerulus.

Figure 2: Advantages of using a GAL80 approach over a null mutation. a) **Current method with available reagents**. In order to examine a single type of Olfactory Sensory Neuron (OSN) without interference from other OSNs, one can use an Orco null mutant. Without Orco, ORs cannot reach the cell membrane or function properly. Orco mutants are mostly anosmic (unable to smell.) A single OR can then be restored using two transgenes, *OrX-GAL4* and *UAS-Orco*. *Or22a-GAL4* is shown here as an example. This fly may require the making and validating of one or more recombinant chromosomes, since the Orco mutation must be homozygous. In more complicated

systems, e.g. restoring more than one OSN, multiple recombinants would need to be made and validated at a cost of several months of crossing. b) **Using a GAL80**. GAL80 is a potent GAL4 inhibitor. All olfactory neurons could be silenced using any number of transgenes in an *Orco-GAL4, UAS-effector* (such as *UAS-Kir2.1*) genotype. A single OSN subtype can then be restored using an *OrX-GAL80* (such as *Or22a-GAL80*). This system requires no recombinant creation, and is amenable to the use of various effectors or additional transgenes without requiring recombinant construction. (Receptor appearance, orientation, and heterodimerization is based on previous designs by Neuhaus et al. (2005), Benton et al. (2006), Smart et al. (2008), and Benton (2009).

Figure 3: OR-GAL80 reagents eliminate GAL4 activity. All antennal lobes are stained with anti-nc82 (a general neuropil marker, grey) and anti-GFP (green). The orientation of each image is dorsal-up, ventral-down, lateral-right, medial-left. Scale bars indicate 20μm. Each of the brains shown has the genotype *OrX-GAL4, UAS-GFP*. The specific receptor promoter is given above each column. The top row in each set shows GFP expression in these lines without GAL80. Notice how each neuron's target in the antennal lobe glomeruli is expressing GFP. Each bottom row shows the brains containing an additional *Or-GAL80* gene. Note how GAL80 effectively inhibits GAL4 activity, as seen by the elimination of GFP expression. The images are representative of the 5-20 brains examined per genotype. GAL4 inactivation was 100% penetrant in one day old female flies. Not shown: Or7a, Or9a, Or19a, Or22b, Or33c, Or56a, Or85b, Or85c, and Orco.

Figure 4: Olfactory neuron responses towards CO_2 in Single Sensillum Recordings (SSR). a) **Box plot of SSR responses.** Ab1C neurons in *Gr21a-GFP* flies respond strongly to CO_2, adding *Kir2.1* reduces response to CO_2, and response is restored when *Gr21a-GAL80* is added. Each circle shows response in an individual sensillum, and filled squares indicate the means. b-d) **Examples of SSR traces** for: b) *Gr21a-GFP*, c) *Gr21a-GFP, Gr21a-GAL4, UAS-Kir2.1*, and d) *Gr21a-GFP, Gr21a-GAL4, UAS-Kir2.1, Gr21a-GAL80*.

Figure 5: Use of OR-GAL80 reagents in a behavioral experiment a) **Olfactory Arena Setup** (Aso and Rubin, 2016)**.** Flies are freely walking in a circular enclosure. The vertical and horizontal lines are added to indicate quadrant barriers, but no such physical barriers exist in the arena. All four quadrants receive air for one minute, and then two quadrants along a diagonal receive odorant for one minute. Diagonal quadrant pairing is randomized. In the example shown, flies find the odorant aversive and segregate into air quadrants. The colored outline around each fly was added during tracking using Ctrax software (Branson et al., 2009). The response index in each frame is measured. Response Index= $Flies_{odor}$- $Flies_{air}$/Total Flies. Each N represents a trial of 20-30 flies. E.g. A response index of -0.5 indicates ~23 of 30 flies are in the air quadrants and the odorant is aversive. b) **Comparison of reagents in olfactory behavior using Isoamyl acetate (IAA)**. Flies are added to the arena as described in A. Odorant is 1:1000 Isoamyl acetate (IAA) in mineral oil. Neurons expressing Odorant Receptor 22a (Or22a) respond to IAA. The top panel shows the mean response index of each genotype over the entire course of the trial. Dotted lines indicate the time

interval of the analysis shown in the bottom panel, time= 75-105 seconds. The bottom panel shows the mean response index of flies over the middle part of the odorant exposure. c) **Comparison of reagents in olfactory behavior using Geranyl acetate (GAc)**. Data are represented as described in B. Odorant is 1:100 Geranyl Acetate (GAc). Neurons expressing Odorant Receptor 82a (Or82a) respond to the odorant GAc. **For both B and C**: Positive control flies (red) respond to odorant with significantly more aversion than every other genotype ($p \leq .0001$ for IAA and $p \leq .006$ for GAc). Negative control flies (black and grey) cannot detect the odorant and have RIs around 0. The Or22a and Or82a receptors are necessary for normal aversive behaviors to their respective odorants (gold). Blue and Teal represent the genotypes as described in Figure 2. In each, when only a single OSN subtype is functional, it is insufficient to restore normal aversive behavior. But the two groups, i.e. using the GAL80 reagent (blue) or using the traditional Orco mutant method (teal), have similar variance ranges (F-test insignificant).

Materials and Methods

Fly Stocks

Flies were reared on standard cornmeal/molasses food and kept at 25C with a 16 hours on/8hours off light cycle. All lines were obtained from the Indiana University Bloomington Stock Center and the Janelia Farm Research Campus. Any recombinants made were validated with PCR.

Stock List:

Or7a-GAL4 #23907

Or7a-GAL4 #23908

Or9a-GAL4 #23918

Or10a-GAL4 #9944

Or13a-GAL4 #9946

Or13a-GAL4 #23886

Or19a-Gal4 #24617

Or22a-GAL4 #9951

Or22a-GAL4 #9952

Or22b-GAL4 #23891

Or33c-GAL4 #23893

Or35a-GAL4 #9967

Or42a-GAL4 #9970

Or42b-GAL4 #9971

Or43b-Gal4 #23894

Or46a-GAL4 #23291

Or47a-GAL4 #9981

Or56a-GAL4 #9988

Or59b-GAL4 #23897

Or59c-GAL4 #23899

Or67a-GAL4 #23904

Or67d-GAL4 #9998

Or71a-GAL4 #23121

Or82a-GAL4 #23125

Orco-GAL4 #23292

Orco-GAL4 #26818

Or85a-GAL4 #23133

Or85b-GAL4 #23911

Or85c-GAL4 #23913

Gr21a-GAL4 #24147

Or22a-mcd8::GFP #52620

Gr21a-mcd8::GFP #52619

$Orco^2$ #23130

UAS-Orco #23145

UAS-mcd8::GFP #5130

UAS-mcd8::GFP #5137

UAS-Kir2.1 Janelia stock #3015545

UAS-Kir2.1 Janelia stock #3015298

UAS-Kir2.1::eGFP Janelia stock #BS00312

GAL80 Creation

Primers were designed to capture the entire promoters described by Couto et al. (2005) (see **Table S1**). Promoters were amplified from genomic DNA using Q5 High Fidelity PCR (NEB #M0491S) and added to entry vectors using the pENTR/D-TOPO system (Invitrogen, 2012a). Recombination with the pBP-GAL80Uw-6 (Addgene #26236) destination vector was done using the LR Clonase II system (Invitrogen,

2012b). To ensure no mutations, no gaps, and correct orientation, the complete promoters were sequenced in the destination vector using the sequencing primers shown in **Table S2**. PhiC31 site-directed transgenesis was performed by Genetivision Inc. All GAL80 transgenes were inserted at the attP2 site.

Immunohistochemistry

Female adult brains were dissected one day after eclosion in cold S2 Schneider's Insect Medium (Sigma Aldrich #S0146) and fixed while nutating for 55 minutes at room temperature in 2mL 2%PFA (Electron Microscopy Sciences #15713) in protein loBind Tubes (Eppendorf #022431102). Brains were washed 4x, 15min per wash while nutating with 2mL PBT buffer (1xPBS, Cellgro #21-040, with 0.5% TritonX-100, Sigma Aldrich #X100). Brains were then blocked with 200µL 5% Goat serum (ThermoFischer. #16210064) in PBT for 90 minutes while nutating, upright. Block was removed and 200 µL primary antibodies in PBT were added for 4 hours at room temperature and then transferred to 4C for 36-48 hours while nutating, upright. Primary antibodies: mouse α-bruchpilot (Developmental Studies Hybridoma Bank. #nc82-s) at 1:30, rabbit α-GFP at 1:1000 (Thermo Fischer #A11122), or rabbit α-Tom at 1:500 (clontech #632393). Monoclonal antibody nc82 identifies Bruchpilot. Bruchpilot can serve as a general neuropil marker because it is required in synaptic zones (Wagh et al., 2006). Larval brains were collected from third instar larvae and fixed in 4% PFA. Primary antibodies: mouse α-neuroglian (Developmental Studies Hybridoma Bank. #BP104) at 1:50 and rabbit α-GFP at 1:500. Brains were washed 4x, 15min per wash while nutating with 2mL PBT. 200µL secondary antibodies in PBT were then added for 4 hours at room

661 temperature and then 3 overnights at 4C while nutating upright. Secondary antibodies:
662 AF568 goat α-mouse (Life Technologies #A11031) at 1:400 and AF488 goat α-rabbit
663 (ThermoFischer #A11034) at 1:800. Tubes were protected from light at all times after
664 secondary antibodies had been added. Brains were washed again 4x, 15min per wash
665 while nutating with 2mL PBT. Then washed with 1xPBS and mounted using Vectashield
666 mounting media (Vector Labs #H-1000). Confocal images were taken with Leica800
667 microscope.
668
669 *Olfactory Arena*
670 Female flies aged 3-6 days were cold-plate sorted 1-2 days before the assay.
671 The arena was setup and the assay performed as described in (Aso and Rubin, 2016)
672 sans the optogenetic components. Odorants were diluted in mineral oil (Sigma Aldrich,
673 Geranyl Acetate #173495, Isoamyl Acetate #306967). After trying starved vs unstarved
674 flies and multiple odorant concentrations (data not shown), it was determined unstarved
675 flies at concentrations of 10^{-3} Isoamyl acetate and 10^{-2} Geranyl acetate gave the most
676 consistent and robust behaviors. Flies in video recordings were tracked using Ctrax
677 software (Branson et al., 2009).
678
679 *Single Sensillum Recordings*
680 SSRs were performed as described in Lin et al 2015 (Lin and Potter, 2015). GFP
681 labeled ab1 sensilla were identified using a Zeiss AxioExaminer D1 compound
682 microscope with eGFP filter cube (FL Filter Set 38 HE GFP shift free). A glass recording
683 electrode filled with ringers solution (7.5g of NaCl+0.35g of KCl+0.279g of $CaCl_2$-$2H_2O$

in 1L of H_2O) was inserted into the base of an ab1 sensillum. CO_2 was delivered through a tube ending with a Pasteur pipette that was inserted for 1 second into a hole in a plastic pipette directed at the antenna. This plastic pipette (Denville Scientific Inc, 10ml pipette) carried a purified continuous air stream (8.3 ml/s) that used a stimulus controller (Syntech) at the time of CO_2 delivery to correct for the increased air flow. Signals were acquired and analyzed using AUTOSPIKE software (USB-IDAC System; Syntech). Spikes were counted in a 500 ms window from 500 ms after CO_2 delivery and multiplied by 2 to calculate spikes/second. Then, the spikes in 1000ms before CO_2 delivery were subtracted to calculate the increase in spike rate in response to CO_2 (Δspikes/second). For each genotype, 6 flies (4-8 days old) were tested, with 1-3 sensilla tested in each fly.

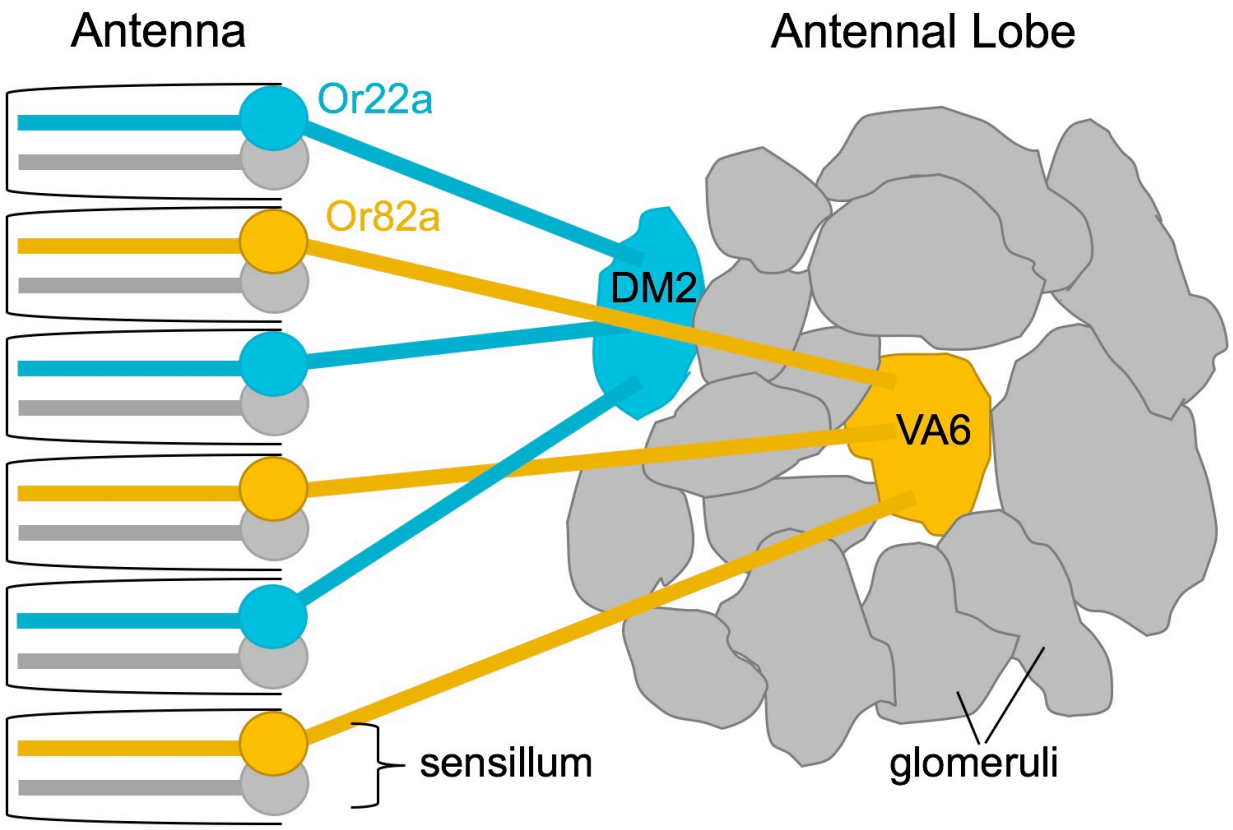

Figure 1

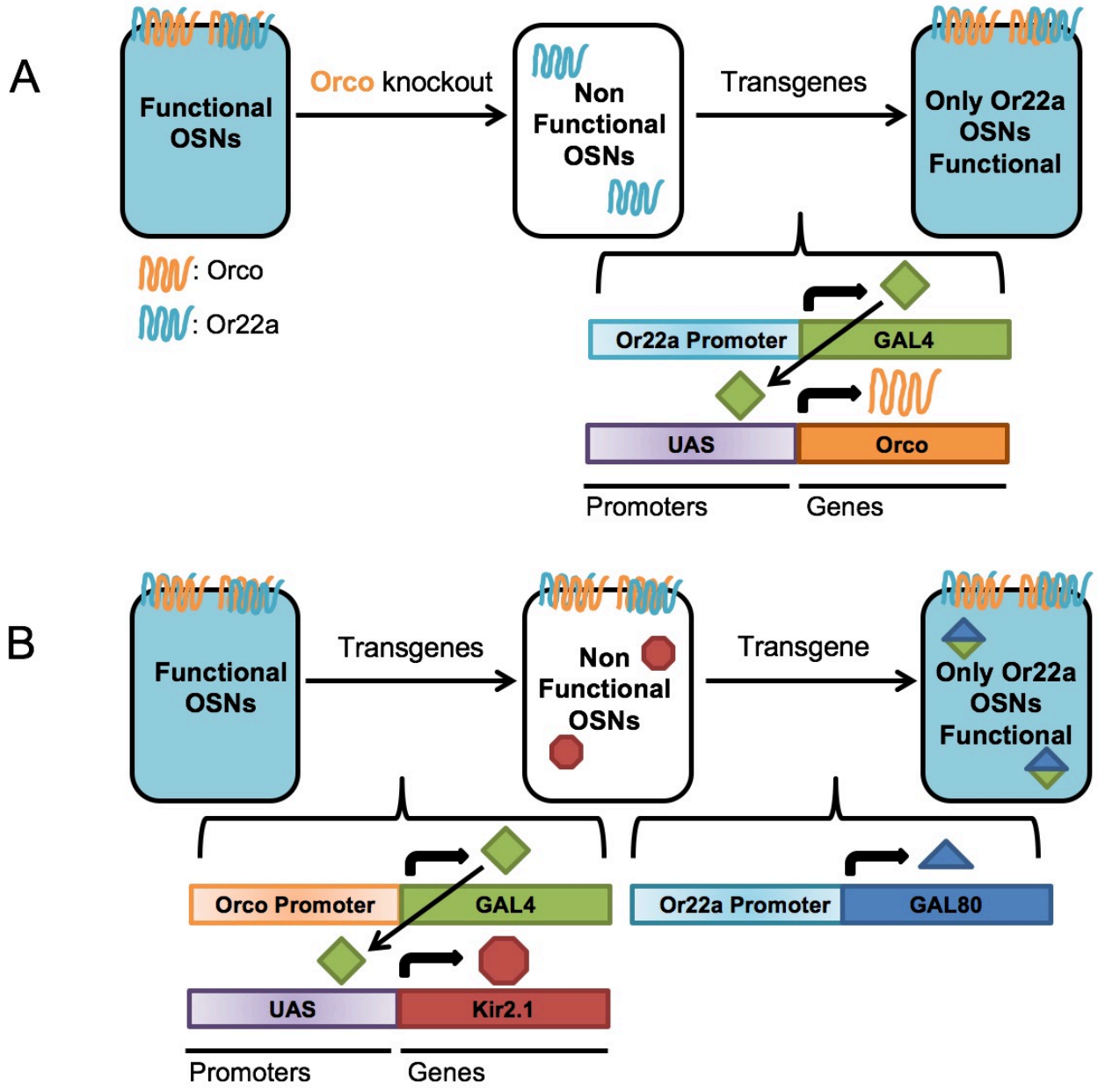

Figure 2

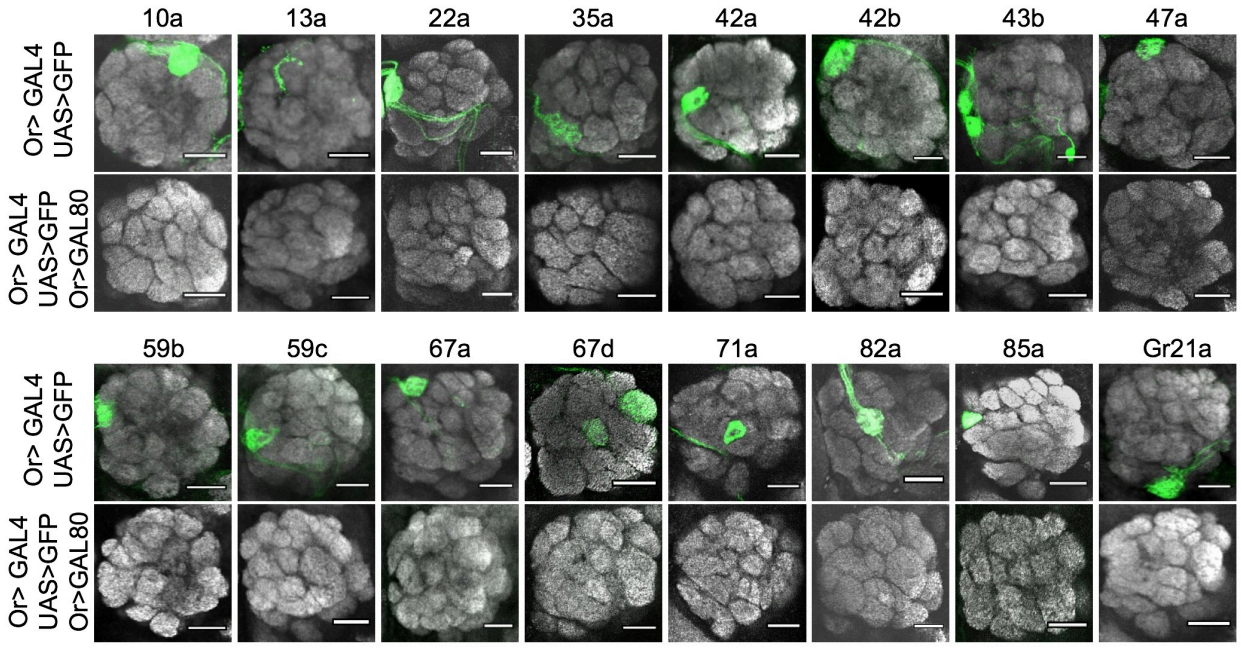

Figure 3

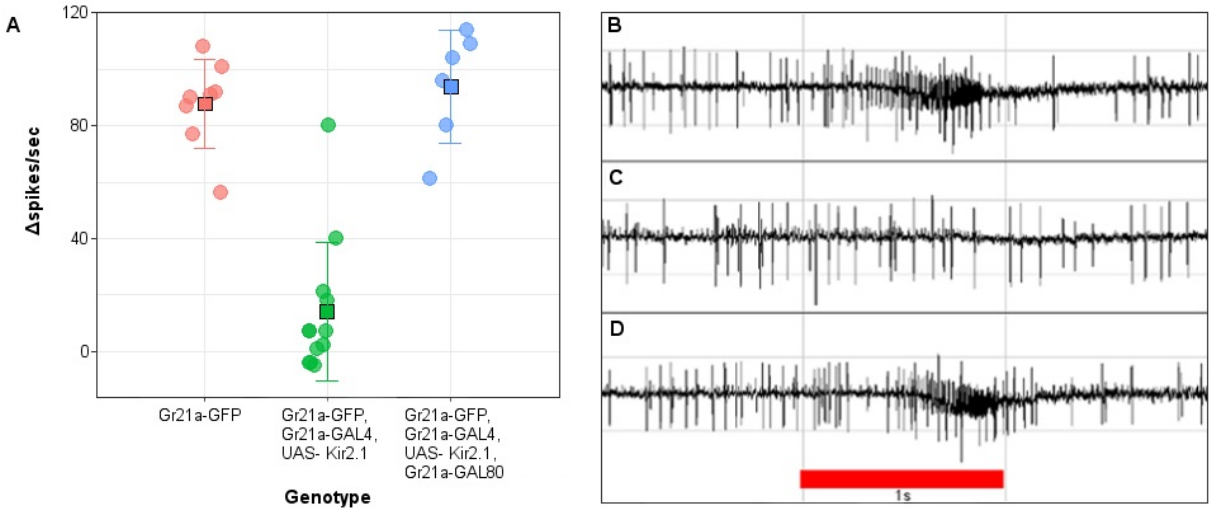

Figure 4

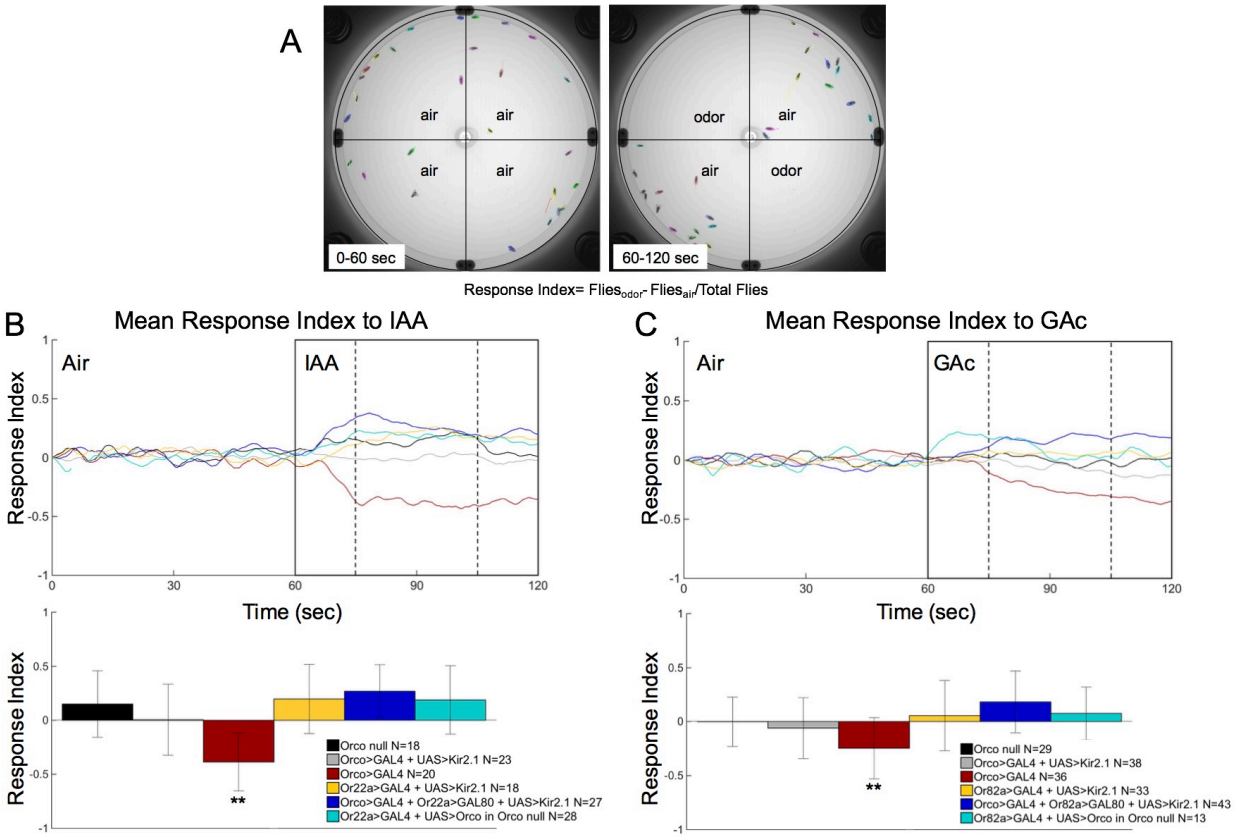

Figure 5