Title

A GAL80 collection to inhibit GAL4 transgenes in *Drosophila* olfactory sensory neurons

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Running Title

GAL80 in *Drosophila* olfactory neurons

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ABSTRACT

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

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.

68 In *Drosophila*, most olfactory sensory neurons (OSNs) typically expresses a
69 single odorant receptor (OR) from a genomic repertoire of 60 genes (Vosshall et al.
70 1999; Robertson, Warr, and Carlson 2003; Vosshall, Wong, and Axel 2000; Clyne et al.
71 1999; Goldman et al. 2005). The promoter of an OR gene can be employed to label
72 specific subsets of OSNs with a particular transgene (Fishilevich and Vosshall 2005;
73 Couto, Alenius, and Dickson 2005). ORs, which vary in sensitivity and specificity to a
74 wide range of different odorants, determine the firing kinetics and odor-response
75 dynamics of each OSN (Hallem and Carlson 2006; Hallem, Ho, and Carlson 2004;
76 Couto, Alenius, and Dickson 2005; Fishilevich and Vosshall 2005; de Bruyne, Clyne,
77 and Carlson 1999; de Bruyne, Foster, and Carlson 2001; Dobritsa et al. 2003; Elmore et
78 al. 2003; Kreher et al. 2008).
79
80 Most OSNs express Odorant Receptor Co-Receptor (Orco), a highly conserved
81 member of the olfactory receptor family (Krieger et al. 2003; Vosshall and Hansson
82 2011), in addition to a single selected OR. Though Orco usually does not contribute to
83 the structure of the odorant binding site (Jung, borst, and Haag 2011; Nakagawa and
84 Vosshall 2009; Nichols and Luetje 2010; P. L. Jones, Pask, and Rinker 2011), it is
85 essential for odorant-invoked signaling in flies. Without Orco, the co-expressed OR
86 cannot localize to the dendritic membrane or relay an odor-evoked signal (Larsson et al.
87 2004; Benton et al. 2006). Orco null flies are largely anosmic, though some
88 chemosensation remains due to the presence of ionotropic receptors and gustatory
89 receptors, which do not require Orco to function (Silbering et al. 2011; Benton et al.

2009; Kwon et al. 2007; W. D. Jones et al. 2007). 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 (Vosshall, Wong, and Axel 2000; Fishilevich and Vosshall 2005; Couto, Alenius, and Dickson 2005; Stocker et al. 1990). 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

113 crossing and PCR screening. Classical genetic strategies thus limit the number and
114 complexity of combinatorial genotypes that one can achieve. More challenging
115 experimental questions demand more facile and versatile genetic tools.
116
117 The GAL4/UAS gene regulation system has become a *defacto* standard in
118 studies of *Drosophila*. GAL4 is a yeast transcription activator that binds to the Upstream
119 Activating Sequence (UAS) and induces expression of downstream genes (Giniger,
120 Varnum, and Ptashne 1985). By driving GAL4 expression from an OR promoter,
121 specific expression of a *UAS-transgene* can be obtained for any OSN subtype. An *OrX-*
122 *GAL4* line exists for almost every OR. This collection of GAL4 lines is a powerful toolbox
123 since different *UAS-transgenes* can be introduced into a line via conventional mating.
124 For example, human α-synuclein has been expressed in OSNs to model human
125 Parkinson's disease (A. Y. Chen et al. 2014). Alternatively, protein expression levels
126 can be knocked down using any specified *UAS-RNAi* transgene.
127
128 A variety of existing compatible effectors can be used study different aspects of
129 neuronal communication. The *UAS-Kir2.1* effector is used as an example in
130 experiments described below. This inward rectifier potassium channel electrically
131 inactivates the neurons that express it (Hodge 2009; Baines et al. 2001; Johns et al.
132 1999). Similarly, *shibire*^{ts} or tetanus toxin can be used to silence synaptic
133 communications (van der Bliek and Meyerowitz 1991; kitamoto 2002; Kitamoto 2001; M.
134 S. Chen et al. 1991; Sweeney et al. 1995; Baines et al. 1999), *reaper/grim/hid* genes
135 can be used to physically kill neurons by inducing their own apoptotic pathways (Song

136 and Steller 1999; Abrams 1999), or ricin toxin can be expressed ectopically to kill
137 neurons. Conversely, neurons can be selectively activated with *trp1a* or a variety of
138 other channelrhodopsin transgenes (Boyden 2011; Pulver et al. 2009).
139
140 If GAL4 is a standard on-switch for nearly any desired transgene, GAL80 is the
141 logical off-switch. GAL80 binds the GAL4 transcriptional activation domain, thereby
142 preventing recruitment of RNA polymerase (Ma and Ptashne 1987). GAL80 crosses are
143 much more convenient than classical breeding approaches (**Figure 2**). In order to have
144 a single functional OSN in an otherwise silent olfactory system, the traditional method
145 uses an Orco null mutation (Larsson et al. 2004). In this genetic setup, Orco mutant flies
146 are mostly anosmic, but function is restored to one OSN subset with *Or-GAL4, UAS-*
147 *Orco* transgenes (Olsen, Bhandawat, and Wilson 2007; DasGupta and Waddell 2008;
148 Hoare, McCrohan, and Cobb 2008; Hoare et al. 2011; Benton et al. 2006; Fishilevich et
149 al. 2005) (**Figure 2a**). An *Orco-GAL4, UAS-effector, Or-GAL80* method can be used
150 instead (**Figure 2b**). Kir2.1 is used as an example of an effector (Hodge 2009; Baines et
151 al. 2001; Johns et al. 1999). Classical breeding strategies (**Figure 2a**) may look less
152 complicated on paper than GAL80 crosses (**Figure 2b**) but are actually more time-
153 consuming and limited. The Orco mutation must be homozygous. Since most
154 *Drosophila* transgenes are embedded into the same two chromosomes (2 or 3)
155 recombination and PCR screening may be required to achieve this homozygosity.
156
157 Neurons seldom operate autonomously, but rather groups of neurons coordinate
158 within a circuit to provide an organism with perception and behavior. An investigation of

159 the behavioral impact provided by a limited number of different functional neuronal
160 types would require additional genes. The elaboration of genotypes (**Figure 2**) to
161 restore pairs or groups of functional OSNs in a nonfunctional background normally
162 requires generations of crosses (followed by PCR screens for desired recombinants). A
163 GAL80 strategy can shorten this process by achieving similar results in only one or two
164 generations with no necessary recombinant creation. Furthermore, a GAL80 strategy
165 takes advantage of the interchangeable variety of existing *UAS-transgene* lines. Here
166 we describe a new collection of *OrX-GAL80* lines designed to complement existing *OrX-*
167 *GAL4* lines, and demonstrate their potential utility for neuroanatomical studies of the
168 *Drosophila* olfaction model.
169

170 **MATERIALS AND METHODS**

171 *Fly Stocks*

172 Flies were reared on standard cornmeal/molasses food and kept at 25C with a
173 16 hours on/8hours off light cycle. All lines were obtained from the Indiana University
174 Bloomington Stock Center and the Janelia Research Campus. Any recombinants made
175 were validated with PCR.

176 Stock List:

177 Or7a-GAL4 #23907

178 Or7a-GAL4 #23908

179 Or10a-GAL4 #9944

180 Or13a-GAL4 #9946

181 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

205 Gr21a-GAL4 #24147

206 Or22a-mcd8::GFP #52620

207 Gr21a-mcd8::GFP #52619

208 $Orco^2$ #23130

209 UAS-Orco #23145

210 UAS-mcd8::GFP #5130

211 UAS-mcd8::GFP #5137

212 UAS-Kir2.1 Janelia stock #3015545

213 UAS-Kir2.1 Janelia stock #3015298

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

215 pJFRC19-13xLexAop2-IVS-myr::GFP-p10 (attP8) Janelia stock #1171146

216 pJFRC59-13xLexAop2-IVS-myr::GFP-p10 (attP40) Janelia stock #3015445

217

218

219 *GAL80 Creation*

220 Primers were designed to capture the entire promoters described by (Couto,
221 Alenius, and Dickson 2005) (see **Table S1**). Promoters were amplified from genomic
222 DNA using Q5 High Fidelity PCR (NEB #M0491S) and added to entry vectors using the
223 pENTR/D-TOPO system (Invitrogen 2012b). Recombination with the pBP-GAL80Uw-6
224 (Addgene #26236) destination vector was done using the LR Clonase II system
225 (Invitrogen 2012a). To ensure no mutations, no gaps, and correct orientation, the
226 complete promoters were sequenced in the destination vector using the sequencing
227 primers shown in **Table S2**. PhiC31 site-directed transgenesis was performed by

228 Genetivision Inc. All GAL80 transgenes were inserted at the attP2 site. A single Or-
229 LexA line was also created using the Or22a-promoter entry vector and
230 pBPnlsLexA::p65Uw (Addgene #26230).
231
232 *Immunohistochemistry*
233 Female adult brains were dissected one day after eclosion in cold S2 Schneider's
234 Insect Medium (Sigma Aldrich #S0146) and fixed while nutating for 55 minutes at room
235 temperature in 2mL 2%PFA (Electron Microscopy Sciences #15713) in protein loBind
236 Tubes (Eppendorf #022431102). Brains were washed 4x, 15min per wash while
237 nutating with 2mL PBT buffer (1xPBS, Cellgro #21-040, with 0.5% TritonX-100, Sigma
238 Aldrich #X100). Brains were then blocked with 200µL 5% Goat serum (ThermoFischer.
239 #16210064) in PBT for 90 minutes while nutating, upright. Block was removed and 200
240 µL primary antibodies in PBT were added for 4 hours at room temperature and then
241 transferred to 4C for 36-48 hours while nutating, upright. Primary antibodies: mouse α-
242 bruchpilot (Developmental Studies Hybridoma Bank. #nc82-s) at 1:30, rabbit α-GFP at
243 1:1000 (Thermo Fischer #A11122), or rabbit α-Tom at 1:500 (clontech #632393).
244 Monoclonal antibody nc82 identifies Bruchpilot. Bruchpilot can serve as a general
245 neuropil marker because it is required in synaptic zones (Wagh et al. 2006). Larval
246 brains were collected from third instar larvae and fixed in 4% PFA. Primary antibodies:
247 mouse α-neuroglian (Developmental Studies Hybridoma Bank. #BP104) at 1:50 and
248 rabbit α-GFP at 1:500. Brains were washed 4x, 15min per wash while nutating with 2mL
249 PBT. 200µL secondary antibodies in PBT were then added for 4 hours at room
250 temperature and then 3 overnights at 4C while nutating upright. Secondary antibodies:

251 AF568 goat α-mouse (Life Technologies #A11031) at 1:400 and AF488 goat α-rabbit
252 (ThermoFischer #A11034) at 1:800. Tubes were protected from light at all times after
253 secondary antibodies had been added. Brains were washed again 4x, 15min per wash
254 while nutating with 2mL PBT. Then washed with 1xPBS and mounted using Vectashield
255 mounting media (Vector Labs #H-1000). Confocal images were taken with Leica800
256 microscope.
257
258 *Single Sensillum Recordings*
259 SSRs were performed as described in Lin et al 2015 (Lin and Potter 2015). GFP
260 labeled ab1 and ab3 sensilla were identified using a Zeiss AxioExaminer D1 compound
261 microscope with eGFP filter cube (FL Filter Set 38 HE GFP shift free). A glass recording
262 electrode filled with ringers solution (7.5g of NaCl+0.35g of KCl+0.279g of $CaCl_2$-$2H_2O$
263 in 1L of H_2O) was inserted into the base of the sensillum. To test ab1 (Gr21a) response,
264 CO_2 was delivered through a tube ending with a Pasteur pipette that was inserted for 1
265 second into a hole in a plastic pipette directed at the antenna. This plastic pipette
266 (Denville Scientific Inc, 10ml pipette) carried a purified continuous air stream (8.3 ml/s)
267 that used a stimulus controller (Syntech) at the time of CO_2 delivery to correct for the
268 increased air flow. To test ab3 (Or22a) response, 20 µl of E2-Hexenal or Isoamyl
269 acetate (diluted to 1% in mineral oil) was pipetted on a piece of filter paper (1X2 cm) in
270 a Pasteur pipette. The Pasteur pipette was then inserted into the hole of the plastic
271 pipette that carried continuous air stream to the antenna. For odorant delivery, the
272 stimulus controller (Syntech) was used to divert a 1 s pulse of charcoal-filtered air (5
273 ml/s) into the Pasteur pipette containing the odorant.

274

275 Signals were acquired and analyzed using AUTOSPIKE software (USB-IDAC System;
276 Syntech). Spikes were counted in a 500 ms window from 500 ms after CO_2 delivery and
277 multiplied by 2 to calculate spikes/second. Then, the spikes in 1000ms before CO_2
278 delivery were subtracted to calculate the increase in spike rate in response to CO_2
279 (Δspikes/second). For each genotype, 6 flies (4-8 days old) were tested, with 1-3
280 sensilla tested in each fly.

281

282 **RESULTS**

283 *Design of GAL80 Constructs*

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

290

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

296 creating a limited number of OrX-GAL80s. This work is a logical extension and makes
297 many additional *OrX-GAL80s* available for general use.
298
299 *Testing GAL80 Efficacy and Specificity*
300 GAL80 lines were created for the following odorant receptor promoters: Or7a,
301 Or10a, Or13a, Or19a, Or22a, Or22b, Or33c, Or35a, Or42a, Or42b, Or43b, Or47a,
302 Or56a, Or59b, Or59c, Or67a, Or67d, Or71a, Or82a, Orco, Or85a, Or85b, Or85c, and
303 Gr21a. To examine GAL4 subtraction *in vivo*, *OrX-GAL80* flies were crossed to flies with
304 the genotype *OrX-GAL4, UAS-GFP*. OSNs expressing the same OR can be identified
305 from their specific glomerulus in the antennal lobe (**Figure 1**). *OrX-GAL4, UAS-GFP*
306 flies show robust expression of the GFP reporter gene in their respective glomeruli.
307 However, when *OrX-GAL80* is added to the genotype, GFP expression is entirely
308 absent, indicating a robust antagonism of GAL4 activity (**Figure 3**). The efficacy of
309 *Or22b-GAL80* could not be determined because the *OR22b-GAL4, UAS-GFP* control
310 did not show robust or reliable GFP signaling in the first place. The created *Or7a-*
311 *GAL80* line was not effective at subtracting GFP signal. Though these lines are not
312 included in Figure 3, they will still be available in the Bloomington Stock Center. Several
313 of the GAL80 lines also have expression in larvae. GAL4 subtraction was examined in
314 larval brains using the *UAS-GFP* reporter gene. In larvae, GAL80 reduced but did not
315 eliminate GAL4 activity (**Figure S1a**).
316
317 The *OrX-GAL80* lines were checked to ensure they would not have aberrant
318 expression in untargeted OSN subtypes. The pBP-GAL80uW-6 vector contains a

319 Drosophila Synthetic Core Promoter (DSCP). DSCP is an effective means of using
320 enhancer elements to drive strong expression (Pfeiffer et al. 2008), but it could also
321 cause the GAL80s to have nonspecific or leaky expression. Therefore, a version of
322 pBP-GAL80uW-6 was cloned with the DSCP removed. However, when the DSCP was
323 absent, GAL80 expression was insufficient to subtract GAL4 activity (**Figure S1b**). A
324 few lines were tested to see if DSCP causes nonspecific GAL80 expression. For these
325 lines, an *OrY-GAL80* did not impede GAL4 activity of an *OrX-GAL4* neuron (**Figure**
326 **S1c**). Due to the uneven expression in an *Orco-GAL4, UAS-GFP* line, it could not be
327 determined if each OrX-GAL80 subtracts GAL4 from only one glomerulus in an
328 otherwise fully-labeled brain, but results shown in Figure S1c give reasonable
329 confidence that the GAL80s do not have widespread nonspecific expression. It can also
330 be noted that the GAL80 subtraction does not interfere with reporter gene expression in
331 a genetic system that does not use GAL4. When *Or22a-GAL80* is used in conjunction
332 with *Or22a-GFP*, containing no GAL4/UAS intermediary, the GFP is still expressed
333 (**Figure S1d**). These images, showing subtraction of reporter gene expression, confirm
334 that GAL4 activity is suppressed anatomically by the GAL80 lines.
335
336 To confirm GAL4 was suppressed physiologically by the GAL80s, Single
337 Sensillum Recordings (SSRs) were used to assay electrical activity of OSNs. *Gr21a-*
338 *GFP* was used to identify sensilla of interest without interfering with the
339 GAL4/UAS/GAL80 system. Gr21a neurons are housed in ab1 sensilla. Carbon Dioxide
340 exposure causes a robust response in Gr21a ab1C neurons (Hallem and Carlson 2006;
341 Hallem, Ho, and Carlson 2004; Fishilevich and Vosshall 2005; deBruyne:2001bs W. D.

342 Jones et al. 2007; Kwon et al. 2007; de Bruyne, Foster, and Carlson 2001). When
343 *Gr21a-GFP* flies were exposed to CO_2, their ab1C sensillar neurons showed robust
344 responses (mean Δspikes/s=88, N=8 sensilla). Kir2.1-containing neurons are expected
345 to show little to no spontaneous firing (Olsen, Bhandawat, and Wilson 2007; Hoare,
346 McCrohan, and Cobb 2008). Adding Kir2.1 to Gr21a neurons (genotype *Gr21a-GFP,*
347 *Gr21a-GAL4, UAS-Kir2.1)* greatly reduced spiking responses to CO_2 (mean
348 Δspikes/s=14, N=12 sensilla, p=0.01). When Gr21a-GAL80 was added (genotype
349 *Gr21a-GFP, Gr21a-GAL4, UAS-Kir2.1, Gr21a-GAL80),* responses to CO_2 were restored
350 (mean Δspikes/s=94, N=6 sensilla, $p < 0.001$. No significant difference from genotype
351 *Gr21a-GFP,* p= .26) (**Figure 4a**).
352
353 To make sure the system also worked for neurons expressing an OR protein (in
354 additional to a GR), SSR was also done with ab3 sensilla. Ab3 houses Or22a-
355 expressing neurons. This receptor is known to be activated by a diverse set of odorants,
356 including Isoamyl acetate and E2-hexenal (Hallem and Carlson 2006; Hallem, Ho, and
357 Carlson 2004; Fishilevich and Vosshall 2005; deBruyne:2001bs W. D. Jones et al.
358 2007; Kwon et al. 2007; de Bruyne, Foster, and Carlson 2001). A transgene was
359 necessary to visualize the neurons without interfering with the GAL4/UAS/GAL80
360 system, but *Or22a-GFP* was insufficiently bright to identify sensilla for SSR. A new
361 *Or22a-LexA* transgenic animal was therefore created using the same promoter that was
362 used to create the *Or22a-GAL80* gene (this line is also available through Bloomington).
363 When crossed to a *LexAop2-GFP* line, the ab3 sensilla showed bright fluorescence.
364 When *Or22a-LexA, LexAop2-GFP* flies were exposed to Isoamyl acetate or to E2-

hexenal, their sensillar neurons showed robust responses (mean Δspikes/s=67.86 and 27.71, N=7 and 7 sensilla, respectively). Unlike the Gr21a neurons, Kir2.1 expression in the Or22a neurons effectively eliminated both spontaneous and odor-evoked activity. (mean Δspikes/s=0, N=7 sensilla, p<.001 for both odorants). Both activities could be restored with the addition of the *Or22a-GAL80* gene (Isoamyl acetate: mean Δspikes/s =74.29, N=7 sensilla, p<.001; E2-Hexenal: mean Δspikes/s =25, N=7 sensilla, p<.001). Neurons showed some low-level responses to mineral oil alone, the solvent used for the odorants (**Figure 4b**). Only genotype 3 *Or22a-LexA, LexAop2-GFP, Or22a-GAL4, UAS-Kir2.1, Or22a-GAL80* showed significantly higher responses to mineral oil than genotypes *Or22a-LexA, LexAop2-GFP* (p=.01) and *Or22a-LexA, LexAop2-GFP, Or22a-GAL4, UAS-Kir2.1* (p=.005), but the latter two genotypes showed no significant response to mineral oil alone. The results in **Figure 4** confirm that GAL80 functions effectively to prevent GAL4-induced activity in OSNs.

DISCUSSION

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, using a GAL80 transgene will be more flexible than mutant lines and less cumbersome than crafting the required recombinants as the complexity of

388 the genotype increases. Though in some special circumstances, olfactory sensory
389 neurons have been shown to produce behaviors autonomously, this is not a widely
390 applicable principle, and further investigation upon this principle requires better tools.
391 For example, Fishilevish et al (2005) used larvae in their study to restore aversion with a
392 single functional OSN subtype, but the larval olfactory system may be fundamentally
393 different from adults in this respect., (Bhandawat et al. 2010) also showed that single
394 glomerular activity is sufficient to invoke a behavioral response, but that study was done
395 using an intact and fully functional olfactory background, so some neuronal cooperation
396 may still have occurred. (DasGupta and Waddell 2008) provided evidence that a single
397 functional OSN subtype is sufficient to learn odor discrimination, and Gao et al (2015)
398 gave convincing evidence of aversive restoration in adults with only one functional OSN.
399

400 However, the extent to which the restoration of single-OSN behavior depends on
401 the odorant and receptor used is still unknown. Only a small subset of receptors have
402 been tested. The current models of odor coding by the olfactory system predict that a
403 coordinated effort of many OSNs is usually required to produce a behavioral output.
404 Paired neurons in sensilla can affect the firing dynamics of their neighbors in the
405 periphery (Dobritsa et al. 2003; Su et al. 2012; Kazama and Wilson 2009), and
406 downstream neurons such as interneurons and projection neurons may rely on
407 synchronized input from multiple OSN types (Chou et al. 2010; Wilson 2011; Yaksi and
408 Wilson 2010; Hong and Wilson 2015; Kazama, Yaksi, and Wilson 2011; Olsen,
409 Bhandawat, and Wilson 2007; Ng et al. 2002; Acebes et al. 2011). GAL80 tools open

more possibilities to combinatorially activate subsets of neurons. The hope is that additional researchers will use the reagents and validate them in their own assays.

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

433 examples. Neurons usually exist in pairs or groups in sensilla within the olfactory
434 organs—antenna or maxillary palp. Neurons expressing the same OR are distributed
435 throughout the periphery, but project their axons onto the same glomerulus in the
436 antennal lobe of the brain. For example, all Or22a-expressing neurons synapse onto the
437 DM2 glomerulus while all Or82a-expressing neurons synapse onto the VA6 glomerulus.
438
439 **Figure 2: Advantages of using a GAL80 approach over a null mutation**. a) **Current**
440 **method with available reagents**. In order to examine a single type of Olfactory
441 Sensory Neuron (OSN) without interference from other OSNs, one can use an Orco null
442 mutant. Without Orco, ORs cannot reach the cell membrane or function properly. Orco
443 mutants are mostly anosmic (unable to smell.) A single OR can then be restored using
444 two transgenes, *OrX-GAL4* and *UAS-Orco*. *Or22a-GAL4* is shown here as an example.
445 This fly may require the making and validating of one or more recombinant
446 chromosomes, since the Orco mutation must be homozygous. In more complicated
447 systems, e.g. restoring more than one OSN, multiple recombinants would need to be
448 made and validated at a cost of several months of crossing. b) **Using a GAL80**. GAL80
449 is a potent GAL4 inhibitor. All olfactory neurons could be silenced using any number of
450 transgenes in an *Orco-GAL4, UAS-effector* (such as *UAS-Kir2.1*) genotype. A single
451 OSN subtype can then be restored using an *OrX-GAL80* (such as *Or22a-GAL80*). This
452 system requires no recombinant creation, and is amenable to the use of various
453 effectors or additional transgenes without requiring recombinant construction. (Receptor
454 appearance, orientation, and heterodimerization is based on previous designs by

455 Neuhaus et al. (2005), Benton et al. (2006), Smart et al. (2008), and (Smart et al. 2008;
456 Neuhaus et al. 2005; Benton et al. 2006; Benton 2009)
457
458 **Figure 3: OR-GAL80 reagents eliminate GAL4 activity**. All antennal lobes are stained
459 with anti-nc82 (a general neuropil marker, grey) and anti-GFP (green). The orientation
460 of each image is dorsal-up, ventral-down, lateral-right, medial-left. Scale bars indicate
461 20µm. Each of the brains shown has the genotype *OrX-GAL4, UAS-GFP*. The specific
462 receptor promoter is given above each column. The top row in each set shows GFP
463 expression in these lines without GAL80. Notice how each neuron's target in the
464 antennal lobe glomeruli is expressing GFP. Each bottom row shows the brains
465 containing an additional *Or-GAL80* gene. Note how GAL80 effectively inhibits GAL4
466 activity, as seen by the elimination of GFP expression. The images are representative of
467 the 5-20 brains examined per genotype. GAL4 inactivation was 100% penetrant in one
468 day old female flies.
469
470 **Figure 4: Olfactory neuron responses towards odors in Single Sensillum**
471 **Recordings (SSR).** In box plots on the left, each circle shows response in an individual
472 sensillum, and filled squares indicate the means. On the right of each plot, example
473 SSR traces are shown for each genotype. (* indicates .01>p>.005, ** indicates p<.001)
474 a) **Ab1C SSR responses.** Ab1C neurons are visualized using the *Gr21a-GFP* gene.
475 Top: Sensilla respond strongly to CO_2, and adding *Kir2.1* reduces response to CO_2.
476 Response is restored when *Gr21a-GAL80* is added. Bottom: Air was used as a control
477 for CO_2 experiments. Air does not cause an odor-evoked neuronal response, and

478 adding the *Kir2.1* or *GAL80* genes does not affect the spontaneous signaling
479 responses. b) **Ab3 SSR responses.** Ab3 neurons are visualized using the *Or22a-LexA*
480 *and LexAop2-GFP* genes. Sensilla respond to Isoamyl Acetate and to E2-Hexenal.
481 Adding *Kir2.1* eliminates both odor-evoked and spontaneous activity in these neurons.
482 Spontaneous and odor-evoked activity is restored when *Or22a-GAL80* is added.
483 Odorants were diluted in mineral oil and neurons from the GAL80 restorative genotype
484 did show low-level responses to mineral oil alone (bottom).
485

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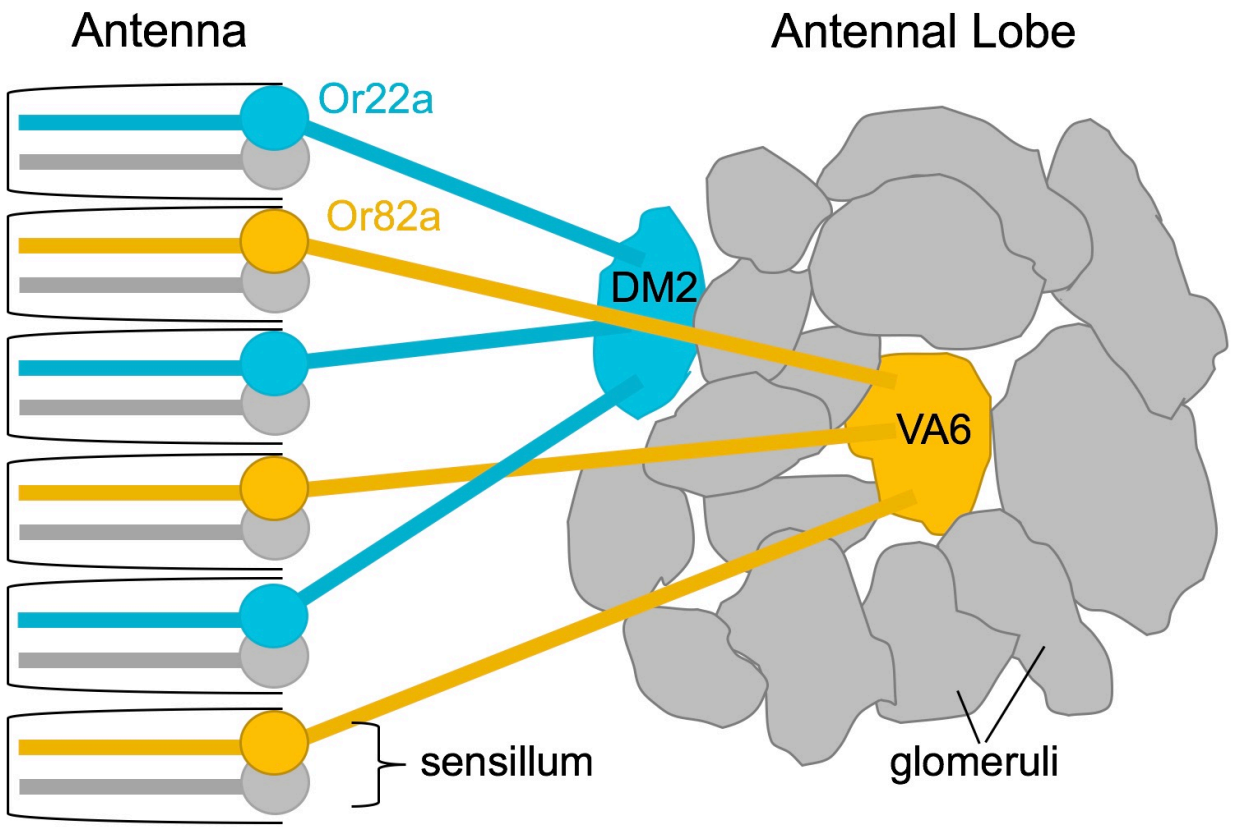
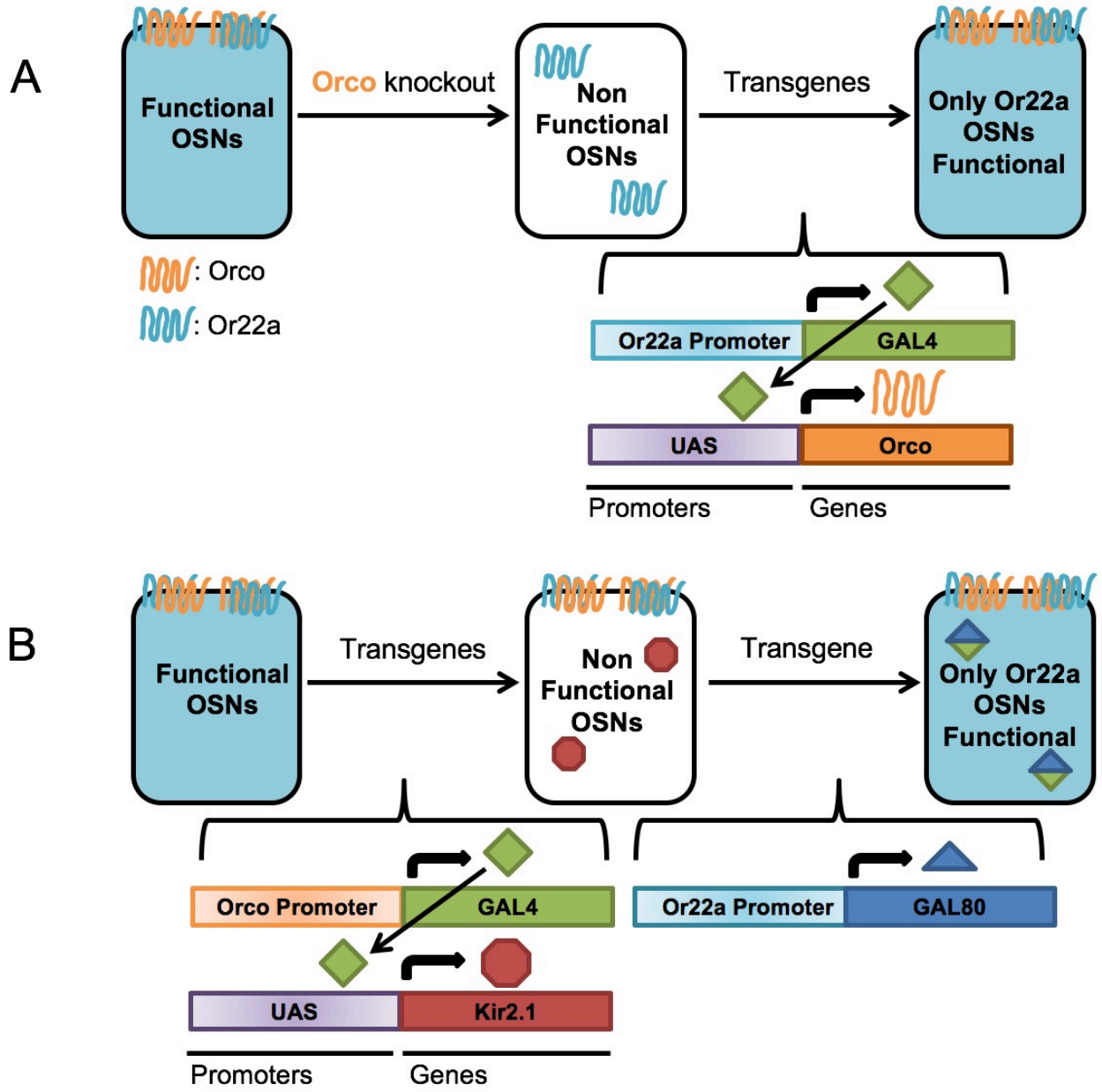

Antenna
Antennal Lobe
Or22a
Or82a
DM2
VA6
sensillum
glomeruli

Figure 1

721

722



723

724 Figure 2

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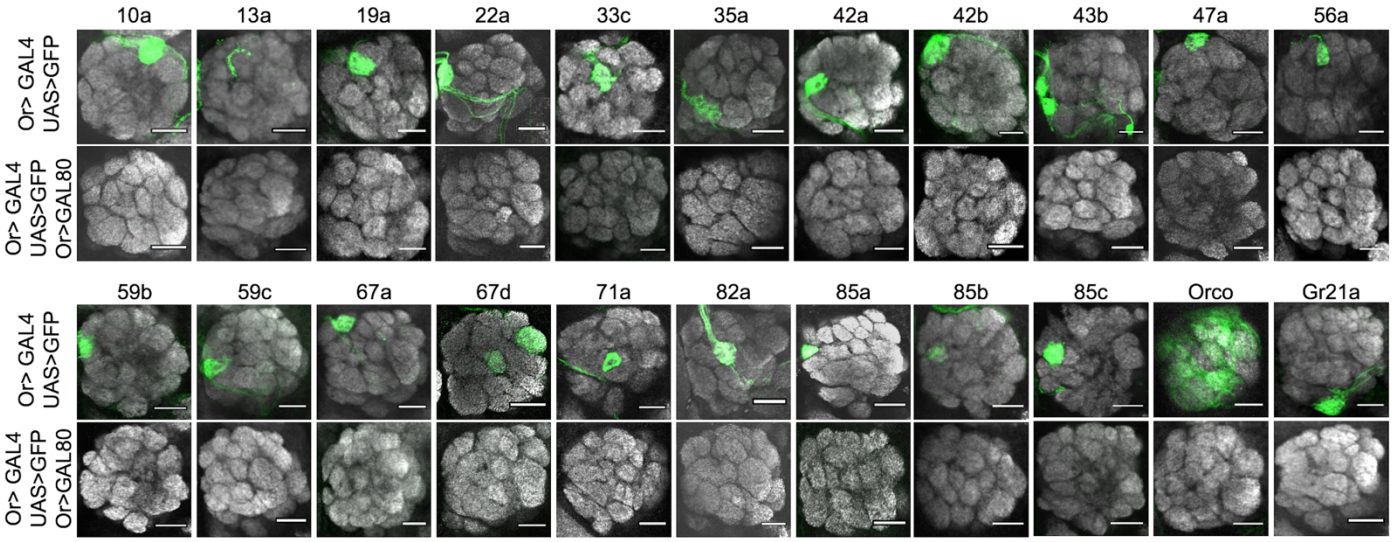
726



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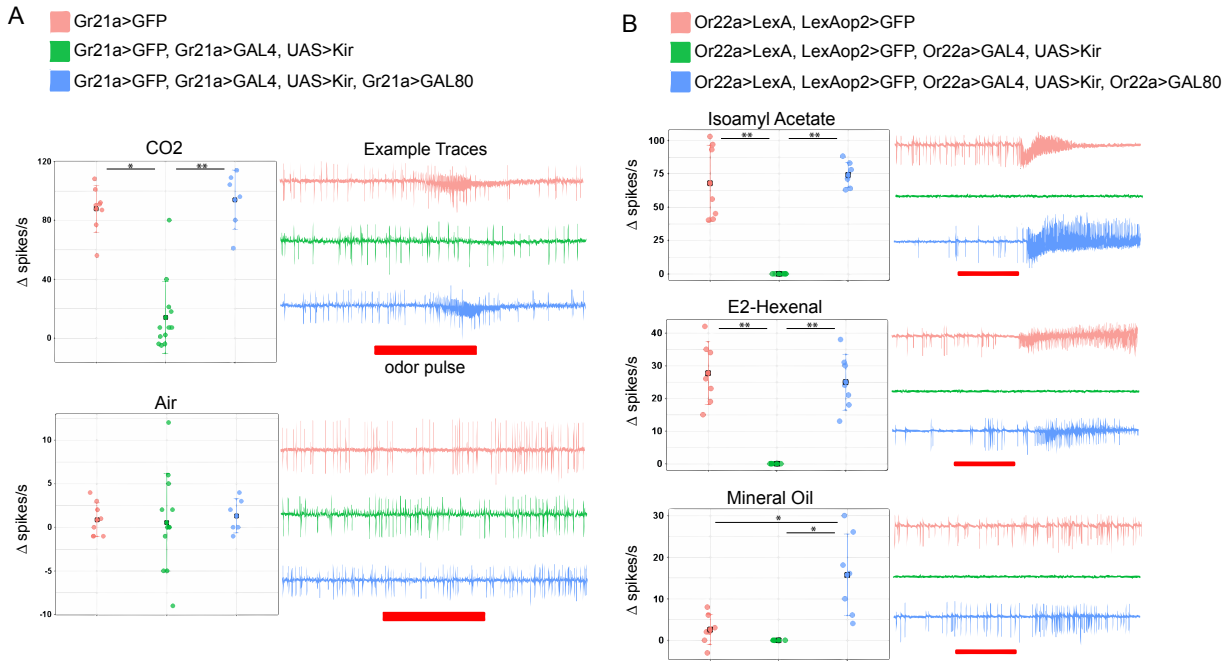
728 Figure 3

729 Figure 4


A
Gr21a>GFP
Gr21a>GFP, Gr21a>GAL4, UAS>Kir
Gr21a>GFP, Gr21a>GAL4, UAS>Kir, Gr21a>GAL80
CO2
Example Traces
Δ spikes/s
odor pulse
Air
B
Or22a>LexA, LexAop2>GFP
Or22a>LexA, LexAop2>GFP, Or22a>GAL4, UAS>Kir
Or22a>LexA, LexAop2>GFP, Or22a>GAL4, UAS>Kir, Or22a>GAL80
Isoamyl Acetate
E2-Hexenal
Mineral Oil