BIOLOGY LETTERS

rsbl.royalsocietypublishing.org

Research

Cite this article: Lone SR, Venkataraman A, Srivastava M, Potdar S, Sharma VK. 2015 *Or47b*-neurons promote male-mating success in *Drosophila*. *Biol. Lett.* **11**: 20150292. http://dx.doi.org/10.1098/rsbl.2015.0292

Received: 10 April 2015
Accepted: 1 May 2015

Subject Areas:
behaviour, ecology, neuroscience

Keywords:
Drosophila, courtship, sensory, olfactory, *Or47b*

Author for correspondence:
Vijay Kumar Sharma
e-mail: vsharma@jncasr.ac.in

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2015.0292 or via http://rsbl.royalsocietypublishing.org.

THE ROYAL SOCIETY PUBLISHING

Animal behaviour

Or47b-neurons promote male-mating success in *Drosophila*

Shahnaz Rahman Lone, Archana Venkataraman, Manishi Srivastava, Sheetal Potdar and Vijay Kumar Sharma

Chronobiology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, PO Box 6436, Jakkur, Bangalore, Karnataka 560064, India

Drosophila performs elaborate well-defined rituals of courtship, which involve several types of sensory inputs. Here, we report that *Or47b*-neurons promote male-mating success. Males with *Or47b*-neurons silenced/ablated exhibit reduced copulation frequency and increased copulation latency. Copulation latency of *Or47b*-manipulated flies increased proportionately with size of the assay arena, whereas in controls it remained unchanged. While competing for mates, *Or47b*-ablated males are outperformed by intact controls. These results suggest the role of *Or47b*-neurons in promoting male-mating success.

1. Introduction

Innate behaviours ranging from highly conserved circadian rhythms to specialized ones such as waggle dance of honeybees and migration of monarch butterflies have an endogenous basis. Such behavioural programmes are governed by dedicated neuronal circuits that respond to specialized sensory cues [1]. *Drosophila* exhibits ritualized courtship, which comprises a series of easily observable stereotypic events [2]. The genes and neurocircuitry involved in the regulation of courtship behaviour have been extensively studied [2], and it is known that it involves a cascade of events genetically programmed by two zinc-finger transcription factors *fruitless* (*fru*) and *doublesex* (*dsx*) [3]. Male-specific fru^M is expressed in about 2000 neurons in the central and peripheral nervous system, and is deemed necessary and sufficient for the development of almost all aspects of male courtship behaviour [4,5].

Males recognize females using a wide array of sensory modalities that are visual, olfactory, gustatory, tactile, acoustic and mechanosensory in nature [2,6]. Four olfactory receptors (*Or47b*, *Or65a*, *Or67d* and *Or88a*) that respond to fly odours belong to the same family of olfactory receptors [7]. In addition, there is another family of receptors that respond to chemosensory cues [8], and it has been shown that one of its members, *IR84a*, is able to detect food odours and is important for male courtship behaviour [9].

GABA-signalling in *Or47b*-neurons regulates pheromone receptivity in males, improving their ability to detect females [10]. Two Fru-positive glomerular targets of *Or67d* (DA1) and *Or47b* (VA1v) neurons show developmental plasticity and are involved in courtship behaviour [11,12]. In a recent study, Wang *et al.* [13] have shown that the courtship efficiency of two *Or47b* loss-of-function mutants ($Or47b^2$ and $Or47b^3$) is comparable to controls, suggesting that *Or47b* receptors may not be involved in courtship behaviour. However, *Or47b* null males show defects in courting hydrocarbon-free targets, which are otherwise vigorously courted by wild-type males, suggesting the role of *Or47b* receptors in detecting chemical compounds involved in sexual behaviours. Taken together, these results suggest that *Or47b*-neurons and VA1v glomeruli to which they are connected are crucial for the regulation of sexual behaviours in the fruit fly *Drosophila melanogaster*.

2. Material and methods

(a) Fly strains

Fly strains used are *Or47bGAL4* (NCBS, Bangalore), *iso31* (isogenic strain), *UASdti* (*dti* = *diphtheria toxin*), *UASdORKC1* (Todd Holmes, UC-Irvine), $Or47b^2$ and $Or47b^3$ (Bloomington). Expression of diptheria toxin (DTI) and *dORKC1* causes ablation and electrical silencing of neurons [14]. Fly line *iso31* is the genetic background strain in the DrosoDel project [15], and was used to backcross (for at least five generations) fly strains in our experiments. The driver and effector lines were confirmed [16].

(b) Copulation frequency assay

To estimate copulation frequency, freshly emerged virgin males and females were dispensed into small glass vials (25 × 90 mm; diameter × length), and 10 such vials per sex per assay were maintained for five days under 12 light : 12 dark cycles, after which lights were turned-off to create constant darkness (DD). All of our assays were carried out under DD using far-red light ($\lambda > 650$ nm) to eliminate confounding effects of visual cues on courtship and mating. After 9 h, 10 males and 10 females from a pair of vials were introduced into a mating arena ($n = 10$) comprising long glass vials (25 × 195 mm). Similarly, in a separate set of experiments, single male–female pairs ($n = 16$) were aspirated into fresh glass tubes (25 × 95 mm). Flies were monitored for the formation of mating pairs, and the number of such pairs formed in 3/5-min bins over a period of 15/30 min was used to estimate copulation frequency. All our results were verified in two independent trials. To assess the roles of males and females in successful mating, either males or females with ablated *Or47b*-neurons were used along with intact controls. Additionally, the copulation frequency of *Or47b* loss-of-function mutants ($Or47b^{2/2}$ and $Or47b^{3/3}$) and heterozygous controls ($Or47b^{2/+}$ and $Or47b^{3/+}$) was assayed either in groups or in pairs. Data were analysed by Kruskal–Wallis analysis of variance (ANOVA) followed by *post hoc* multiple comparisons using Dunn–Sidak's test.

(c) Copulation latency assay

To estimate copulation latency (time taken to initiate copulation), 9 h after the onset of DD, a single virgin male from either of the two genotypes (*Or47bGAL4/UASdti* or *Or47bGAL4/iso31*) was aspirated into a fresh glass vial approximately 10 min prior to the introduction of a virgin female ($n = 16$ pairs for each genotype and each experimental condition). Copulation latency was assayed in glass arenas of three sizes (5 × 65 mm = small tubes, 7 × 80 mm = large tubes and 25 × 90 mm = vials).

(d) Mating competition assay

Virgin flies were collected and maintained in a manner similar to that described above. One male each from the two genotypes (*Or47bGAL4/UASdti*, *Or47bGAL4/+* and *Or47bGAL4/UASdti*, *UASdti/+*) was introduced into a fresh glass tube (7 × 80 mm) approximately 10 min before a Canton S (*CS*) female was introduced. Mating success was estimated as the proportion of males of either genotype that were able to secure mating with *CS* females ($n = 16$ vials per replicate). Eye-colour (*Or47b*-ablated flies, dark-red compared with faint-red controls) was used to identify genotype of the successful male. Eye-colour of the successful male was observed first under red light, and subsequently confirmed under normal light.

3. Results

(a) Ablation/silencing of *Or47b*-neurons affects copulation frequency

We performed mating assays in groups of *Or47b*-ablated/silenced flies. ANOVA followed by Dunn–Sidak's multiple comparisons revealed that the copulation frequency of *Or47b*-ablated/silenced flies was significantly lower than that of intact controls (ablated: 10 min: $p < 0.05$; 15 min: $p < 0.05$ for *Or47bGAL4*, $p < 0.0001$ for *UASdti/+*; 20/30 min: $p < 0.0001$ for both controls, figure 1*a*,*b*; silenced: 10 min: $p < 0.05$, 15 min: $p < 0.01$, 20/25/30 min: $p < 0.0001$ for both controls, figure 1*c*,*d*). As results on ablated and silenced flies were similar, we chose *Or47b*-ablated flies for the next set of experiments. Ablation of *Or47b*-neurons using *UASdti* was confirmed by immunohistochemistry (electronic supplementary material, S1, $n = 20$, figure 1*e*,*f*). To avoid confounding effects of a large group of individuals on courtship/mating behaviours, we also carried out mating assays with male–female pairs and found that *Or47b*-ablated males show reduced courtship frequency compared with heterozygous controls (electronic supplementary material, figure S1).

(b) *Or47b*-neurons in males are necessary for maintaining normal copulation frequency

Copulation assay was done on: (i) *Or47b*-ablated (*Ordti*) males or intact (*Or47b/+*) males with *CS* females, (ii) *CS* males with ablated (*Ordti*) females or intact (*Or47b/+*) females. In (i), copulation frequency of the group with ablated males was significantly lower than the group with intact males (10 min: $p < 0.05$, 15 min: $p < 0.01$, 20 min: $p < 0.005$, 25/30 min: $p < 0.0005$; figure 2*a*,*b*). In (ii), we found no difference in copulation frequencies between the two groups ($p > 0.05$; figure 2*c*,*d*). These results suggest that *Or47b*-neurons in males rather than females are necessary for ensuring normal copulation frequency.

(c) *Or47b*-neurons enable males to efficiently secure mating

Regardless of size of the arena, *Or47b*-ablated flies exhibited significantly higher copulation latency compared with intact controls ($p < 0.05$; figure 2*e*). With increasing size of the arena, which would be expected to lower odourant concentrations, *Or47b*-ablated males took progressively longer to secure matings, while copulation latency of controls remained unchanged.

We also assayed the ability of *Or47b*-ablated males to secure matings with *CS* females by allowing them to compete against intact controls. Ablated males secured only approximately 25% of the matings, significantly lower than both the controls (approx. 75%; $p < 0.0001$; figure 2*f*), suggesting that *Or47b*-ablated males are less competitive in securing mates than controls.

(d) Flies with loss-of-function mutation in *Or47b* receptors do not show defects in mating behaviour

The copulation frequency of flies carrying loss-of-function mutation in the *Or47b* receptors ($Or47b^2$ and $Or47b^3$) did not differ from that of heterozygous controls ($Or47b^2/+$ and $Or47b^3/+$), when assayed in groups or pairs ($p > 0.05$; electronic supplementary material, figure S2). This suggests that *Or47b* receptors may not be involved in the regulation of mating behaviour.

4. Discussion

Or47b-silenced/ablated flies display reduced copulation efficiency, which suggests the role of *Or47b*-neurons in mating

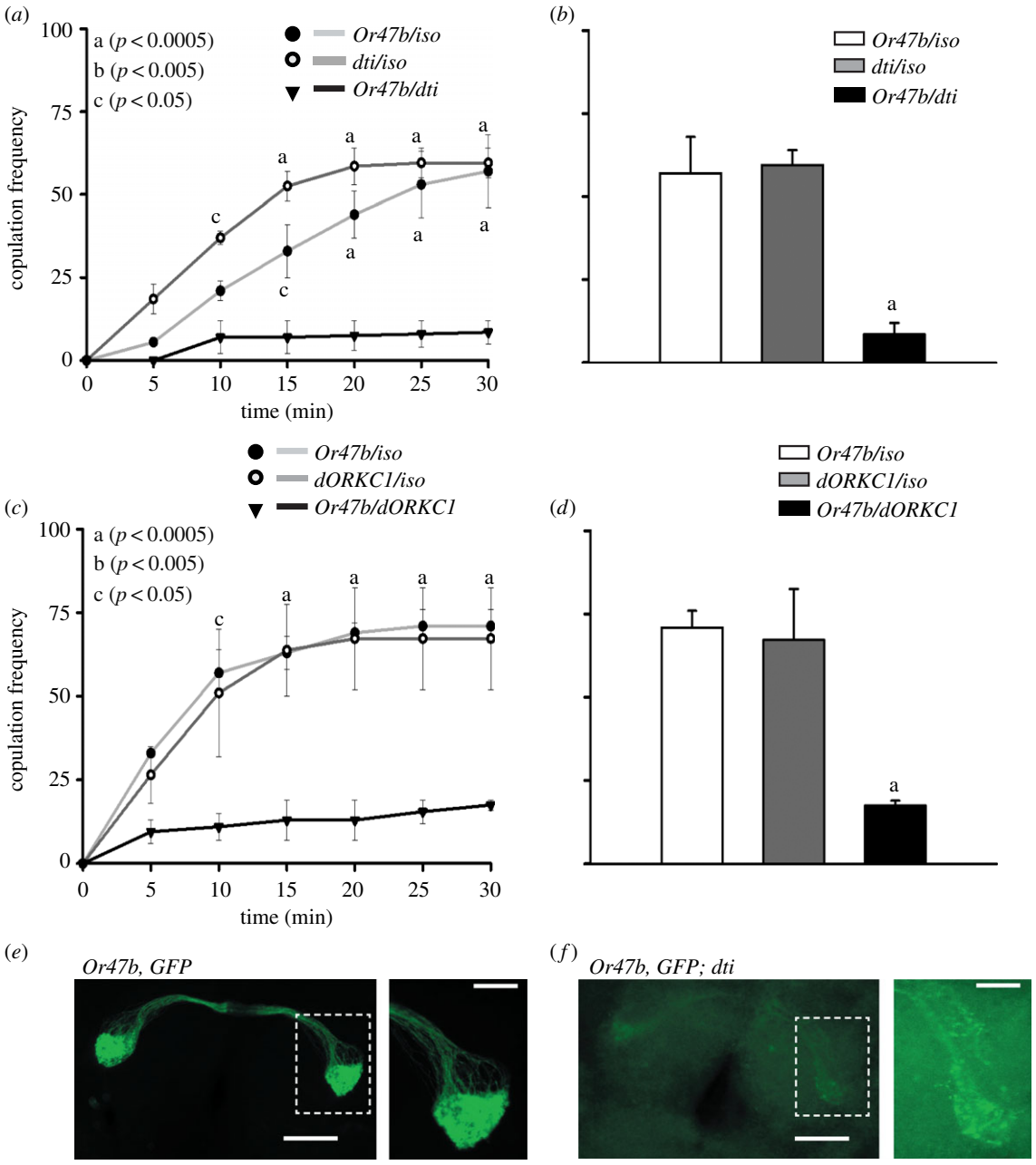

Figure 1. Ablation/silencing of *Or47b*-neurons affects copulation frequency. Copulation frequency profiles and copulation frequency at the end of 30 min of (*a*,*b*) ablated, (*c*,*d*) silenced (inverted triangle) and control flies [*UASdti*/+, *UASdORKC1*/+ (open circles), *Or47b*/+ (solid circles)]. Copulation frequency (percentage of flies copulating) is plotted along *y*-axis and time (min) is plotted along *x*-axis. Representative images for (*e*) *Or47b*, *GFP*, and (*f*) *Or47b*, *GFP; dti* brains along with magnified images of the highlighted region. Immunostaining against GFP shows projections of *Or47b*-neurons in the antennal lobes, which are markedly reduced in *Or47b*, *GFP; dti*. In controls, *iso* refers to *iso31*, used to backcross fly strains. Scale bars in the main figures (*e*,*f*) equal 50 µm and in the magnified images on the right equal 20 µm. Error bars in (*a*–*d*) represent standard error of the mean (SEM). (Online version in colour.)

behaviour. Copulation latency of *Or47b*-ablated flies increases proportionally with size of the assay arena whereas in controls it remains unchanged, which suggests that *Or47b*-neurons help males to track females in their vicinity efficiently. *Or47b*-ablated males were less efficient in securing mating when made to compete with intact controls, which further highlights the role of *Or47b*-neurons in mating behaviour.

Or47b-neurons express fru^{M}, which is activated by both male and female odours [7], and they therefore are believed to play a crucial role in male courtship behaviour [6]. Of the three olfactory neurons, *Or67d* projects to DA1, *Or47b* to VA1v and *IR84a* to VL2a [6]. These three glomeruli are larger in males than females, which could be the reason behind their greater role in males than in females [6]. Furthermore, *Or67d* and *Or47b* genes show higher expression in males than females, suggesting their male-specific roles [17]. Previous studies suggest that these two receptors are involved in promoting male reproductive fitness-related behaviours including male–male aggression and male–female courtship [12,13,18]. Furthermore, *Or47b* along with *Or88a* receptors promote mating by responding to both male and female odours [7,13]. Although the ligand recognized by *Or47b* receptors is yet unknown, there is enough evidence suggesting its role in male courtship.

Although ablation of *Or47b*-neurons reduces male-mating success, males with loss-of-function mutation in *Or47b* receptors ($Or47b^{2}$ and $Or47b^{3}$) do not show such defects [13]. This discrepancy could be owing to basic differences in the way sensory receptors and receptor neurons function and their impact on intra- and inter-glomerular interactions. One possible reason for this discrepancy could be that the function of *Or47b*-neurons in courtship and mating may be mediated by receptors other than olfactory receptors, such as IRs expressed in these neurons

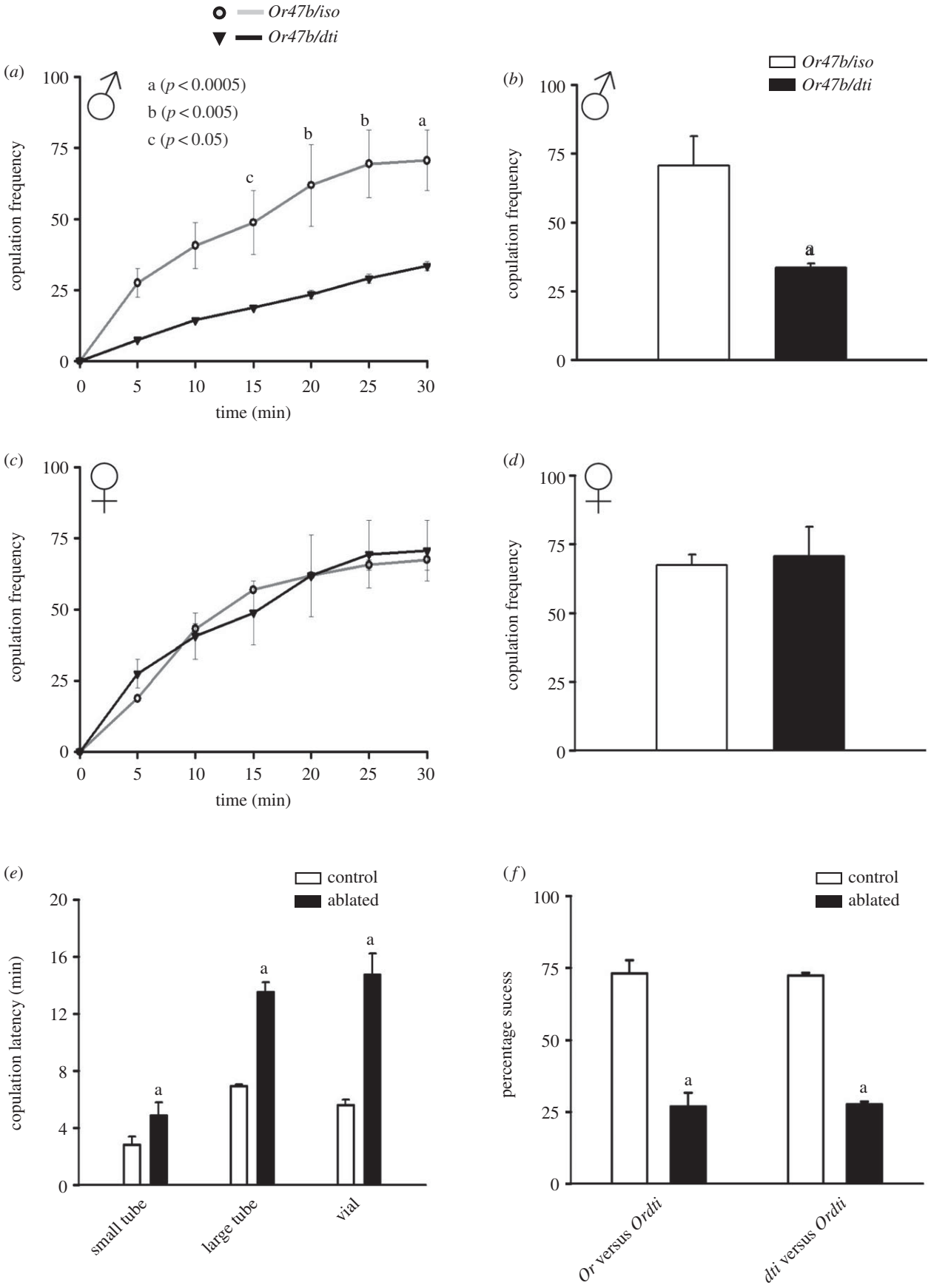

Figure 2. *Or47b*-neurons are necessary in males for normal copulation and male-mating success. Copulation frequency profiles and copulation frequency at the end of 30 min, in assays where (*a*,*b*) *Or47b*-ablated or control males are allowed to mate with *CS* females, or (*c*,*d*) *Or47b*-ablated or control females (inverted triangle for ablated and circles for intact) are allowed to mate with *CS* males. (*e*) Copulation latency of *Or47b*-ablated (dark bars) and intact control males (light bars) when assayed in three differently sized arenas. The *y*-axis represents time (min) taken by flies to initiate copulation (copulation latency). (*f*) Mating success of *Or47b*-ablated (dark bars) and intact control males (light bars) in competition to secure mating with *CS* females. *Or* and *dti* code for intact controls *Or47b*/+ and *dti*/+, whereas *Ordti* codes for *Or47b*-ablated flies (*Or47bGAL4*/*UASdti*). All other details same as in figure 1.

[8]. Another possible reason could be the interactions between glomeruli and the balance between lateral inhibition and lateral excitation [19]. This is probably skewed in such a way that it affects the ability of ablated/silenced males to sense females, which remains unaltered in null males. For example, there exist differences in the synaptic inhibition of GABA-signalling, which is known to modulate olfactory responses [19]. Pheromone-sensing olfactory neurons have high levels of GABA receptors, deemed important for tracking mates [10,20]. Expression of $GABA_BR2$-RNAi in *Or47b*-neurons is found to cause an increase in post-synaptic firing frequency, which suggests pre-synaptic inhibition by $GABA_BR2$ receptors. As the above two mutations in the *Or47b* receptor do not interfere with the development of neuronal projections, they have negligible impact on the $GABA_BR2$-signalling in VA1v glomeruli, causing no defect in male-mating efficiency. However, silenced/ablated *Or47b*-neurons would probably result in the release of pre-synaptic inhibition of $GABA_BR2$, causing increased post-synaptic firing and altered inter-glomerular signalling. Interestingly, change in the size of VA1v glomeruli also has a measurable effect on mating behaviour [11], indicating the role of pre-synaptic inhibition. Taken together, our studies suggest that *Or47b*-neurons promote male-mating success by enhancing mating efficiency.

Data Accessibility. All data are included in the main figures and electronic supplementary material.

Authors' Contributions. S.R.L. conceived the idea, designed experiments and analysed data. S.R.L. performed experiments along with A.V., M.S. and S.P., V.K.S. supervised the project and provided laboratory space. S.R.L. and V.K.S. drafted the manuscript, and all authors contributed to the writing and approved the final version of the manuscript.

Competing Interests. Authors declare no competing interests.

Funding. We received no funding for this study.

Acknowledgements. We thank Sheeba Vasu and two anonymous reviewers for suggesting improvements to the manuscript, Nisha, Pankaj, Antara, Rajanna and Muniraju for assistance during assays.

References

1. Olsen SR, Wilson RI. 2008 Cracking neural circuits in a tiny brain: new approaches for understanding the neural circuitry of *Drosophila*. *Trends NeuroSci.* **31**, 512–520. (doi:10.1016/j.tins.2008.07.006)
2. Villella A, Hall JC. 2008 Neurogenetics of courtship and mating in *Drosophila*. *Adv. Gen.* **62**, 67–184. (doi:10.1016/S0065-2660(08)00603-2)
3. Manoli DS, Meissner GW, Baker BS. 2006 Blueprints for behavior: genetic specification of neural circuitry for innate behaviors. *Trends NeuroSci.* **29**, 444–451. (doi:10.1016/j.tins.2006.06.006)
4. Demir E, Dickson BJ. 2005 *fruitless* splicing specifies male courtship behavior in *Drosophila*. *Cell* **121**, 785–794. (doi:10.1016/j.cell.2005.04.027)
5. Manoli DS, Foss M, Villella A, Taylor BJ, Hall JC, Baker BS. 2005 Male-specific *fruitless* specifies the neural substrates of *Drosophila* courtship behavior. *Nature* **436**, 395–400. (doi:10.1038/nature03859)
6. Ziegler AB, Berthelot-Grosjean M, Grosjean Y. 2013 The smell of love in *Drosophila*. *Front. Physiol.* **4**, 72. (doi:10.3389/fphys.2013.00072)
7. van der Goes van Naters W, Carlson JR. 2007 Receptors and neurons for fly odors in *Drosophila*. *Curr. Biol.* **17**, 606–612. (doi:10.1016/j.cub.2007.02.043)
8. Benton R, Vannice KS, Gomez-Diaz C, Vosshall LB. 2009 Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* **136**, 149–162. (doi:10.1016/j.cell.2008.12.001)
9. Grosjean Y, Rytz R, Farine JP, Abuin L, Cortot J, Jefferis GS, Benton R. 2011 An olfactory receptor for food-derived odors promotes male courtship in *Drosophila*. *Nature* **478**, 236–240. (doi:10.1038/nature10428)
10. Root CM *et al*. 2008 A presynaptic gain control mechanism fine-tunes olfactory behavior. *Neuron* **59**, 311–321. (doi:10.1016/j.neuron.2008.07.003)
11. Kayser MS, Yue Z, Sehgal A. 2014 A critical period of sleep for development of courtship circuitry and behavior in *Drosophila*. *Science* **344**, 269–274. (doi:10.1126/science.1250553)
12. Kurtovic A, Widmer A, Dickson BJ. 2007 A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* **446**, 542–546. (10.1038/nature05672)
13. Wang L, Han X, Mehren J, Hiroi M, Billeter JC, Miyamoto T, Amrein H, Levine JD, Anderson DJ. 2011 Hierarchical chemosensory regulation of male–male social interactions in *Drosophila*. *Nat. Neurosci.* **14**, 757–762. (doi:10.1038/nn.2800)
14. Venken KJ, Simpson JH, Bellen HJ. 2011 Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron* **72**, 202–230. (doi:10.1016/j.neuron.2011.09.021)
15. Ryder E *et al*. 2011 The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster*. *Genetics* **167**, 797–813. (doi:10.1534/genetics.104.026658)
16. Lone SR, Sharma VK. 2012 *Or47b* receptor neurons mediate socio-sexual interactions in the fruit fly *Drosophila melanogaster*. *J. Biol. Rhythms* **27**, 107–116. (doi:10.1177/0748730411434384)
17. Shiao MS, Fan WL, Fang S, Lu MJ, Kondo R, Li WH. 2013 Transcriptional profiling of adult *Drosophila* antennae by high-throughput sequencing. *Zool. Stud.* **52**, 42. (doi:10.1186/1810-522X-52-42)
18. Wang L, Anderson DJ. 2010 Identification of an aggression-promoting pheromone and its receptor neurons in *Drosophila*. *Nature* **463**, 227–231. (doi:10.1038/nature08678)
19. Olsen SR, Wilson RI. 2008 Lateral presynaptic inhibition mediates gain control in an olfactory circuit. *Nature* **452**, 956–960. (doi:10.1038/nature06864)
20. Ng M, Roorda RD, Lima SQ, Zemelman BV, Morcillo P, Miesenbock G. 2002 Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. *Neuron* **36**, 463–474. (doi:10.1016/S0896-6273(02)00975-3)