The odorant receptor Or47b promotes sexual receptivity in mated Drosophila melanogaster females

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Abstract

Female sexual receptivity, the likelihood to accept mating, is critical for reproduction and is determined by a combination of genetics and experience. The precise mechanisms underlying the effect of experience and genetics on female receptivity are, however, poorly understood. The mating behaviour of *Drosophila melanogaster* is a model to investigate this as females are sexually receptive when virgin but reduce receptivity once mated. After showing that the chromosomal basis for virgin and mated female receptivity is different, we show that the Odorant Receptor (OR) *Or47b*, which senses volatile cues emitted by flies of both sexes, is necessary for mated, but not virgin female receptivity. We then show that the Odorant Receptor Neurons (ORN) expressing *Or47b* form the main olfactory channel affecting mated female receptivity as females functionally lacking all classical ORs but *Or47b* have a wild-type receptivity level, while those lacking all ORs have an aberrantly high receptivity. These data indicate that *Or47b* ORN regulate mated female sexual receptivity through inhibition, a finding supported by the necessity of the inhibitory neurotransmitter GABA in the *Or47b* ORN. *Or47b* ORNs are part of a sexually dimorphic olfactory circuit and feminization of these neurons leads to faster mating in males and masculinization leads to decreased receptivity in mated females. We show that this circuit includes the *Or88a* ORN. Strikingly, *Or88a* mutant females have increased virgin sexual receptivity but normal mated female receptivity, while the ablation of the *Or88a* ORN only reduces mated female receptivity. We provide a hypothetical model explaining the roles of *Or88a* and *Or47b* in virgin and mated females. Here, we pinpoint a genetic basis to female post-mating sexual receptivity.

Keywords

Female sexual receptivity, virgin mating latency, remating latency, Mating rate, Olfaction, Odorant receptor neurons, *Or47b*, *Or88a*. 
**Introduction**

Sexual promiscuity, or mating with multiple partners, is observed in both males and females across taxa. The reproductive consequences of sexual promiscuity are, however, different for the two sexes: males dramatically increase their reproductive success each time they successfully fertilize a female, while females only marginally increase theirs because their fecundity is more constrained by egg production than by sperm availability (Bateman, 1948; Lefevre and Jonsson, 1962; Manning, 1967). In addition, mating reduces female immunity and lifespan suggesting that females should tightly regulate their level of sexual promiscuity (Arnqvist and Nilsson, 2000; Chapman et al., 1995; Jennions and Petrie, 2000; Kamimura, 2007; Kuijper et al., 2006; Priest et al., 2008; Rice et al., 2006; Schwenke and Lazzaro, 2017). Evolutionary modelling predicts that a female’s promiscuity depends on her level of sexual receptivity, likelihood to accept mating (Kokko and Mappes, 2012). The reproductive history of the female influences this level of receptivity as virgin females are very receptive in order to commence reproduction, while mated females show decreased receptivity possibly to increase choosiness (Jennions and Petrie, 2000; Kokko and Mappes, 2005). This predicts that the sexual receptivity of virgin and mated females is based on different rules and mechanisms.

The behavioural analysis of *Drosophila melanogaster* females coupled with the tractability of the genetics and neurobiology of this species offers a model for investigating the mechanisms (Laturney and Billeter, 2014; Linder and Rice, 2005; Sirot et al., 2015; Yamamoto and Kohatsu, 2017; Yamamoto et al., 2014). *D. melanogaster* female’s natural sexual promiscuity is demonstrated by the production of offspring from four to six different males by wild-caught females (Imhof et al., 1998; Ochando et al., 1996) and indirect estimates indicate females mate at least once per day (Giardina et al., 2017). Moreover, laboratory assays demonstrate that female sexual receptivity, as determined by the number of copulations per female per day, varies depending on the female’s genetic background, as well as her nutritional and social environments (Billeter et al., 2012; Gorter et al., 2016; Krupp et al., 2008; Kuijper and Morrow, 2009; Newport and Gromko, 1984). These features make *D. melanogaster* an ideal species to investigate sexual receptivity and promiscuity. However, our understanding of what determines female receptivity and how it differs between virgin and mated females is limited.

External factors that influence *D. melanogaster* female sexual receptivity are well described and include social context and female nutritional state (Billeter et al., 2012; Fricke et al., 2010; Gorter et al., 2016; Harshman et al., 1988; Laturney and Billeter, 2014), but the genetic factors influencing female receptivity are less clear. Most genetic mutants affecting female remating rate, an indication of mated female receptivity, belong to the pathway that responds to Sex peptide, a peptide transferred by males to females during mating that reduces female sexual receptivity (Mack et al., 2006; McGraw et al., 2004; Yapici et al., 2008). These genes, therefore, set the response to male factors, but may not be directly responsible for setting the level of female receptivity. This idea is backed-up by sexual selection and quantitative genetic experiments on female remating rate, which implicate genes involved in
female reproductive tract physiology, olfaction (Giardina et al., 2011) and immune response (Lawniczak and Begun, 2004) in variation in remating rate (Giardina et al., 2011; Gromko and Newport, 1988a; Lawniczak and Begun, 2004; Linder and Rice, 2005; Wigby and Chapman, 2004). However, causal relationships between these loci and female receptivity are not yet established. Additionally, most neurogenetic research has focused on virgin female sexual receptivity (Aranha et al., 2017; Bussell et al., 2014; Sakai et al., 2009; Zhou et al., 2014), which do not seem to be involved in mated female receptivity, suggesting different neuronal pathways for the two. This idea is supported by selection experiments which suggest that virgin female mating latency and mated female mating latency are two independent variables as the first does not correspondingly change after selection for fast or slow remating (Gromko and Newport, 1988b; Pyle and Gromko, 1981).

To look for internal factors determining female receptivity we turned to odour sensing mechanisms as the female olfactory system is important for setting sexual receptivity in both virgin and mated females. The spinster mutation dramatically reduces female virginal sexual receptivity (Sakurai et al., 2013). This effect is in part mediated by the functioning of this gene in the Or47b ORN as well as its secondary neurons (Sakurai et al., 2013). This odorant receptor has a documented impact on male sexual behaviour (Dweck et al., 2015b; Lin et al., 2016; Lone et al., 2015; Pitts et al., 2016; Wang et al., 2011; Zhuang et al., 2016) and responds to odours that are produced both by males and females (Dweck et al., 2015b; Lin et al., 2016; Pitts et al., 2016; van der Goes van Naters and Carlson, 2007). Additionally, Or47b is expressed in an ORN that innervates a sexually dimorphic glomerulus (VA1v) in the antennal lobe implying sex-specific roles (Stockinger et al., 2005). The olfactory system is also critical for mated females as we have previously demonstrated that classical odorant receptors are required for social-context dependent modulation of remating behaviour (Billeter et al., 2012).

Here we set out to understand the genetic and neuronal basis of female sexual receptivity and in particular the role of the odorant receptor Or47b in virgin and mated female sexual behaviour. We find that Or47b promotes sexual receptivity in females but this effect is restricted to mated females. We show that Or47b is necessary and sufficient for mated female sexual receptivity and both the receptor as well as its corresponding sensory neuron need to be present and able to release GABA for wild-type post-mating receptivity. These manipulations can lead to both a decrease and an increase in post-mating receptivity, indicating that Or47b ORN is part of a circuit that modulates sexual receptivity. In addition, we show that Or88a, an OR sharing common ligands with Or47b (Dweck et al., 2015b; Pitts et al., 2016; van der Goes van Naters and Carlson, 2007), is also implicated in female sexual receptivity, but in virgin state. We have drawn a hypothetical model to accommodate these corresponding and contradicting effects of the two ORN. These findings pinpoint a genetic basis to female post-mating sexual receptivity.
**Material and methods**

**Drosophila melanogaster stocks and genetics**

The wild-type strain used for all experiments was Canton-S (CS). Chromosomal introgression lines of Oregon-R (OR), another wild-type strain, and CS were generated by genetic crosses that introduced a single pair of the first, second or third chromosome from CS into the OR genome. This generated three introgression lines: CS;OR;OR, OR;CS;OR and OR;OR;CS. The semi-colon indicating the boundary between each of the four chromosomes of *D. melanogaster*. Due to its small size, the fourth chromosome is left out from manipulation and annotation, throughout this report.

Or47b rescue flies (+; Or47b; p{Or47b\textsuperscript{Canton-S}}) were generated by inserting a wild-type Or47b allele from CS back into the genome on the third chromosome. An Or47b mutation line with a genetic background similar to the rescue was used (+; Or47b\textsuperscript{3};ps31) for comparison. Additionally, an isogenised line of Or47b\textsuperscript{3} (+; Or47b\textsuperscript{3};+) was generated with CS as background control. For all Gal4-UAS combinations, control crosses were performed according to background in which the transgenes were inserted: we used CS flies for wild-type background and w\textsuperscript{1118};+;+ flies for w\textsuperscript{3} background.

Other flies used were Or47b-Gal4 (w\textsuperscript{3}; Or47b-Gal4;+, #9983, (Fishilevich and Vosshall, 2005)), Or88a-Gal4 (w\textsuperscript{3}; Or88a-Gal4, #23137 (Fishilevich and Vosshall, 2005)), Or67d-Gal4 (w\textsuperscript{3}; Or67d-Gal4;+, #9998, (Fishilevich and Vosshall, 2005)), +; UAS-hid/CyO (UAS-hid, #65403, (Zhou et al., 1997)), UAS-traF (w\textsuperscript{1118}; UAS-traF;+, #4590, (Ferveur et al., 1995)) and Orco\textsuperscript{1} (w\textsuperscript{3}; Orco\textsuperscript{1};+, #23129, (Larsson et al., 2004)) were obtained from Bloomington Stock Center. UAS-tra\textsubscript{2}RNAi (+;+; UAS-tra\textsubscript{2}RNAi, transflID 24499), UAS-dGAd1 (w\textsuperscript{1118};+; UAS-dGAd1-RNAi, transflID 32344) were obtained from the Vienna Drosophila RNAi Stock Center (Dietzl et al., 2007). Or47b-gal4 in Orco\textsuperscript{-} background (w\textsuperscript{3}; Or47b-gal4; Orco\textsuperscript{-}), Orco-gal4 in Orco\textsuperscript{-} background (w\textsuperscript{3}; Orco-gal4; Orco\textsuperscript{-}), UAS-Orco in Orco\textsuperscript{-} background (w\textsuperscript{3}; UAS-Orco; Orco\textsuperscript{-}) and a genomic rescue of Orco (w\textsuperscript{3}; +; Orco\textsuperscript{-}; p{Orco}, (Silbering et al., 2014)) were a gift from R. Benton and Or88a\textsuperscript{a} (w\textsuperscript{3}; Or88a\textsuperscript{a};+, (Pitts et al 2016)) was gifted by D. Smith. Or47b-gal4 and UAS-Or47b (Dweck et al., 2015b) in Or47b\textsuperscript{3} background (+; Or47b\textsuperscript{3}; Or47b-gal4 and +; Or47b\textsuperscript{3}; UAS-Or47b) were generated from lines kindly provided by M. Knaden. Or67d-gal4 (w\textsuperscript{3}; +; Or67d-Gal4, Orco\textsuperscript{1}), Or88a-gal4 (w\textsuperscript{3}; Or88a-Gal4, Orco\textsuperscript{1}) and double Gal4 of Or88a-gal4 and Or47b-gal4 (w\textsuperscript{3}; Or47b-Gal4, Or88a-Gal4, Orco\textsuperscript{1}) in Orco\textsuperscript{1} background were generated in multistep crosses of aforementioned lines.

**Rearing conditions**

Flies were reared in a 12:12 h light/dark cycle (LD 12:12), lights on at 09:00 am local time (Zeitgeber time (ZT) 0), in 25°C. Rearing was done on medium containing agar (10g/L), glucose (167mM), sucrose (44mM), yeast (35g/L), cornmeal (15g/L), wheat germ (10g/L), soya flour (10g/L), molasses (30g/L), propionic acid (5mL/L of 1M) and Tegosept (2g/L) as described previously (Gorter and Billeter, 2017). This medium is called “Fly food” in this report. Virgin adults were collected using CO\textsubscript{2} anaesthesia and aged in same-sex groups of 20
in food vials for 5-8 days. Flies were raised in the same light and temperature conditions as rearing.

**Behavioural assays**

All assays were conducted in the presence of fly food and in the same light and temperature conditions as raising and rearing. Red light was utilized to monitor behaviour during the dark phase. All assays were conducted in small Petri dishes of 35x10 mm with 3 ml of food poured on the bottom as described previously (Gorter and Billeter, 2017). In short, one virgin male (wild-type CS) and one virgin female (of the indicated genotype) were aspirated into the Petri dish. All experiments began in the active phase in the afternoon between ZT 7 and 9. Webcam cameras (Logitech B910 webcam using the SecurityMonitor Pro software [Deskshare]) took pictures of the dishes at 2-min intervals for 24h unless stated otherwise to score the virgin mating latency, number of matings and latency to 1st remating (Billeter et al., 2012; Krupp et al., 2008). The number of matings was scored as start time of each mating. The virgin mating latency was calculated as time of first mating minus start time of the experiment (time male was inserted and dish placed under the camera). Outliers [based on average±(standard deviations*3)] or individuals that took longer than 1 hour to achieve the virgin mating were excluded from the datasets. This was not more than 10% of the data. The remating latency was defined as start time of second mating minus start time of first mating. If no second mating occurred remating latency was defined as end time of experiment minus start time of first mating. Flies that did not mate were excluded from all abovementioned analyses.

For male sexual receptivity and mutant female latency, one virgin (female or male) of the indicated genotype and one wild-type virgin counterpart (CS) were aspirated into the Petri dish. All experiments began in the active phase in the morning between ZT 0 and 1. Webcam cameras (Logitech B910 webcam using the SecurityMonitor Pro software [Deskshare]) took video recordings for one hour to score the virginal mating latency. Virginal mating latency was defined as the time between placement of the dish under the camera (right after the flies entered) until the start of the first successful copulation. Either the actual time was used or all matings were grouped in minutes over time in which mating started, creating a percentage per minute of couples that achieved mating.

**Glomerulus visualisation**

Adult whole brains were dissected 7 days after eclosion (emergence) in PBS and stained as follows. The whole brains were fixed after dissection for 60 minutes in 4% formaldehyde in PBS (on ice), rinsed three times with PBST (0.3% Triton X-100), blocked with 5% normal goat serum (NGS) in PBST for two hours (on ice), incubated in primary antibody in PBST and 5% NGS overnight (on ice), rinsed six times with PBST, incubated in secondary antibody in PBST and 5% NGS for four hours (room temperature), rinsed three times with PBST, rinsed three times with PBS and mounted in vectashield. Immunohistochemical staining were carried out using antibodies at the following dilutions: mouse monoclonal nc82 (1:50;
Hybridoma bank 431047) and Alexa-568 Goat Anti-Mouse IgG (1:600; Life Technologies A-11004). Stacks of 1 µm optical sections were obtained with a Zeiss LSM510 confocal microscope using Zen 2009 software and processed with Imaris version 7.6.5 software using the contour surface option. The volumes in µm³ obtained were averaged for two independent and blindly scored technical replicates of the same samples. Each glomerulus represents one datapoint.

Statistical analysis
Differences between groups were statistically analysed with mixed effect models with the lme4 package in R (Team, 2015). Genotype and time (for percentage mated over time) were included as fixed effects and tested for interaction, date and individual (for glomerulus data where a value for each glomerulus was obtained) were included as random factors whenever applicable. The best fitting model was selected by backwards elimination of non-significant fixed effects using log-likelihood ratio tests, these results are recorded in supplementary table 1. Normal distribution of residuals was inspected visually and data was tested on homogeneity of variances with Levene’s test. Whenever the data did not pass either one of the assumption, a log transformation was performed, if necessary followed by a Z transformation. When genotype is included in the model, all genotypes were post-hoc tested with a mixed effects model including two genotypes at the time, details are recorded in supplementary table 1 and significance is displayed in the figures either with stars or letters. When genotype is not a determining factor in the model, its p-value as main effect is reported in the figures.

Results
Different Chromosomal bases for virginal and mated female sexual receptivity
Laboratory wild-type strains mate several times in a continuous 24h assay (Billeter et al., 2012; Gorter et al., 2016; Krupp et al., 2008; Kuijper and Morrow, 2009). Mating frequency, however, differs dramatically between strains, a variation which is mainly explained by differences in female genotype (Billeter et al., 2012; Gromko and Newport, 1988b). The commonly used laboratory strains Canton-S (CS) and Oregon-R (OR) exhibit dramatic differences in female virgin mating latency, mating frequency and remating latency in response to CS males ((Billeter et al., 2012); figure 1). We generated and tested female chromosomal introgression lines between CS and OR to determine a chromosomal basis to these quantitative differences in female virgin and post-mating sexual receptivity.

Virgin CS females initiate mating twice as fast as OR virgins (figure 1A). When the genetic background of females is made-up of OR and the first chromosome is replaced with CS, virgin mating latency is intermediate between that of CS and OR. When the second or third chromosome are replaced for CS, virgin mating latency increases, but is statistically not significantly different from OR (figure 1A). Together this suggests that virginal mating
latency is under control of genes located on all 3 chromosomes and that the interaction between them is part of the determination of strain-specific virginal mating latency.

Within 24h, CS females have a shorter remating latency and a higher number of copulations compared to OR (figure 1B,C). When either the first or the third chromosome is replaced with CS, in OR background, the number of copulations and remating latency are not significantly different from that of wild-type OR females (figure 1B,C). However, when the second chromosome is substituted for that of CS, the latency to 1st remating is decreased and the number of copulations in 24 hours is significantly increased (figure 1B,C). These data suggest that genes located on the second chromosome influence remating latency and number of copulations. Genes on the second chromosome must also interact with genes on the other chromosomes as the difference between CS and OR is not completely bridged with only the second chromosome, which is particularly clear for number of copulations (figure 1C). The involvement of the second chromosome with remating behaviour is in line with previous evidence (Fukui and Gromko, 1991a), which showed that the second chromosome plays a major role in the response to selection on slow and fast remating.

Together these data show that female sexual receptivity has a chromosomal basis. However, the difference between virgin mating latency, mating frequency and remating latency suggests that virgin and post-mating receptivity are, at least partially, under the control of different genes and mechanisms.

**Figure 1: Female receptivity has a chromosomal basis** Virgin mating latency in minutes (A), latency to first remating in hours (B) and number of copulations in 24 hours (C) of two wildtype females, CS and OR, and three introgression females, with one chromosome of CS in OR background, versus CS wild type males (n=18-20). Dot plots with mean, SEM error bars and significant statistical output of post-hoc mixed effects models with genotype as dependent variable between all genotypes separately, significant difference between groups with different letters. For the full statistical output see supplementary table 1.

**Or47b influences mated but not virgin female receptivity**

Given that virgin and mated female sexual receptivity have a different chromosomal basis, we sought to uncover single genes that only affect mated female receptivity. Among the genes located on the second chromosome is Or47b, an odorant receptor gene expressed in the olfactory system implicated in male sexual behaviours (Dweck et al., 2015b; Lin et al., 2016; Lone et al., 2015; Wang et al., 2011; Zhuang et al., 2016). We first tested the hypothesis that Or47b is involved in female receptivity. Virgin Or47b− females paired with wild-type CS
males show a similar mating latency as their respective control lines (figure 2A). Additionally, rescuing expression of Or47b in an Or47b mutant female either by means of a genomic rescue construct (figure 2A) or by Gal4-UAS mediated expression of a wild-type Or47b cDNA (figure 2B) directly in Or47b neurons does not induce a change in virgin mating latency. Taken together, these data suggest little or no involvement of Or47b in virgin female sexual receptivity.

Or47b females have a significantly longer remating latency than their controls (figure 2C). This longer time to remating is accompanied with a decrease in number of copulations in 24h (figure 2E). These two mutant phenotypes are rescued by insertion of a wild-type Or47b allele (figure 2C,E) as well as by expression of an Or47b cDNA directly in Or47b ORNs (figure 2D,F). Together these data show that the Or47b gene promotes postmating receptivity both in frequency and in timing, but is not involved in virgin female receptivity.

Figure 2: The odorant receptor Or47b is necessary for female post-mating receptivity Virgin mating latency in minutes (n=14-18) (A), latency to first remating in hours (C) and number of copulations in 24h (E) (n=24-74) of Or47b females and their respective controls vs CS wildtype males. Virgin mating latency in minutes (n=26-34) (B), latency to first remating in hours (n=27-35) (D) and number of copulations in 24h (n=45-54) (F) of females with re-expression of Or47b in Or47b neurons (Or47b-Gal4-UAS-Or47b in Or47b background) and the two controls (Or47b-Gal4 in Or47b background and UAS-Or47b in Or47b background) vs CS wildtype males. Dot plots with mean, SEM error bars and significant statistical output of post-hoc mixed effects models with genotype as dependent variable between all genotypes separately, significant difference between groups with different letters. For the full statistical output see supplementary table 1.
The *Or47b* Odorant receptor neuron influences female sexual receptivity through inhibition

The *Or47b* gene clearly plays a stimulatory role in female post-mating sexual receptivity. To understand how this gene influences female receptivity, we investigated the function of the olfactory neuron in which it is expressed. *Or47b* is the only Odorant receptor expressed in the *Or47b* Odorant Receptor Neuron (ORN), which is embedded within a trichoid sensillum containing two other ORNs; *Or65a/b/c* and *Or88a* (Couto et al., 2005; Fishilevich and Vosshall, 2005). To determine the function of the *Or47b* ORN, we first ablated it by targeting expression of the pro-apoptic gene head involution defective (hid) specifically in the *Or47b* ORN. No consistent changes could be observed in both virginal mating latency and remating latency (figure 3A,B), but loss of these neurons leads to a significant decrease in number of mating (figure 3C). In terms of virgin mating latency and number of copulations, this result recapitulates the phenotype of *Or47b−*, however, is not consistent with 1st remating latency. As the ablation of *Or47b* ORNs does not precisely recapitulate the *Or47b* phenotype, we hypothesized that missing these neurons interferes with the neuronal circuitry underlying female sexual receptivity in a different way than merely missing the Odorant receptor that normally activates this neuron. To directly test the function of the *Or47b* ORNs within the olfactory circuitry regulating female sexual receptivity, we assayed the behaviour of females with only the *Or47b* ORNs active, while all other ORNs are silenced. All odorant receptors are co-expressed with the co-receptor Orco, and a null Orco mutation results in a absence of all ORs at the ORN membranes, making the flies unable to smell via the ORNs (Benton et al., 2006). As we previously reported, *Orco−* females have increased post-mating receptivity, both in mating frequency and remating latency ((Gorter et al., 2016); figure 3E,F). However, we observe no clear effect of *Orco−* on virgin mating latency (figure 3D). Rescuing expression of Orco in an Orco− background, either by Orco-Gal4 or a full genomic rescue, leads to a significant decrease in number of copulations, but a significant increase in latency to 1st remating is only observed for re-expression via Gal4-UAS compared to the respective control lines (figure 3E and 3F). Since ORNs normally only express one type of OR, combining Orco with the Gal4-UAS system allows for targeted expression of only one population of ORN by rescuing the expression of Orco in those cells alone (*Or47b*-Gal4>UAS-Orco). Strikingly, rescue of Orco expression only in *Or47b* ORN achieves a significant decrease in mating frequency and increase in latency to 1st remating that is the same behavioural result as expression in all ORNs (figure 3E,F). These data suggest that input from ORNs has an overall inhibitory effect on female post-mating receptivity and that the *Or47b* ORN is sufficient to cause this effect.

The hypothesis that *Or47b* ORN is inhibitory in relation to female post-mating receptivity would suggest that this neuron expresses GABA, an inhibitory neurotransmitter. To directly test this hypothesis, we inhibited the conversion of glutamate to GABA in *Or47b* ORN by means of RNA interference against glutamic acid decarboxylase (dGAD1). No change in virgin mating latency could be observed (figure 3G). However, in support of our hypothesis, silencing GABA in *Or47b* ORNs resulted in decreased 1st remating latency and
increased mating frequency (figure 3H, I). We conclude that the Or47b ORN function as an inhibitory element in a neuronal circuit that influences female post-mating receptivity.
**Figure 3:** The inhibitory Or47b neuron influences female post-mating receptivity

Virgin mating latency in minutes (A), latency to first remating in hours (B) and number of copulations in 24h (C) of females with ablated Or47b neurons (Or47b-Gal4>UAS-hid) and the two controls (Or47b-Gal4 and UAS-hid) versus CS males (n=38-47). Virgin mating latency in minutes (D), remating latency (E) and number of copulations in 24h (F) of Orco- females with re-expression of Orco in Or47b neurons (Or47b-Gal4>UAS-Orco) and its controls, re-expression of Orco in all neurons (Orco-Gal4>UAS-Orco) and its controls, and Orco1 with genomic rescue (Orco1, {Orco}) and w1118 versus CS males (n=18-67). Statistical comparison between females with re-expression of Orco in specific neurons and their controls and w1118 with the mutant and genomic rescue. Virgin mating latency in minutes (G), latency to first remating in hours (H) and number of copulations in 24h (I) of females with inhibition of GABA production in Or47b neurons (Or47b-gal4>UAS-dGADi) and its controls versus CS males (n=27-30). Dot plots with mean, SEM error bars and significant statistical output of post-hoc mixed effects models with genotype as dependent variable between all genotypes separately, significant difference between groups with different letters. When genotype is not a determining factor in the model, its p-value as main effect is reported. For the full statistical output see supplementary table 1.

**Or47b neuron functions differently in males compared to females**

The Or47b mutation affects male mating latency and mating success (Dweck et al., 2015b; Lin et al., 2016; Zhuang et al., 2016). An observation that we reproduce here, in two genetic backgrounds (figure 4A,B). Or47b thus promotes both male and female sexual behaviours, but the influence is different between the sexes as Or47b only promotes sexual receptivity in mated females.

The Or47b neurons are part of a sexually dimorphic circuit given that the VA1v glomerulus it innervates has a different size in males and females (Kondoh et al., 2003; Stockinger et al., 2005). Glomerulus size difference is under the control of the sex determination gene fruitless, whose male specific isoform is sufficient for the development of a male-size VA1v glomerulus in an otherwise female animal (Stockinger et al., 2005). Given the different roles Or47b plays in male and female reproductive behaviour, we tested the hypothesis that the Or47b ORN functions differently because it is part of a sexually dimorphic neuronal circuit. We feminized the Or47b ORN by targeting traF in Or47b ORNs in an otherwise male individual. These feminized males show shorter mating latency (figure 4C,D), which means that these males achieve copulation faster. We also tested the effect of a masculinized Or47b neuron in an otherwise female individual by targeting RNAi-silencing of transformer-2 (tra-2) specifically in Or47b ORN. Females with these male-like Or47b ORN decreased number of copulations in 24 hours, but showed no change in virgin mating latency and 1st remating latency (figure 4E-G). This suggests a decrease in post-mating receptivity, but not fully as seen in the Or47b null mutants. Together this suggests that Or47b ORNs in female-state have a lower capability to inhibit sexual behaviour than that these same neurons have when they are in a male-state. We thus conclude that the sexual identity of the neuron in which Or47b is expressed confers different ability to modulate sexual receptivity.
The odorant receptor *Or47b* promotes receptivity

Figure 4: The *Or47b* neurons function differently in male and females

Male mating latency in minutes (A) and proportion of males mated over time (B) of *Or47b* males and their respective controls vs CS wild type females (*n*=15-19). Male mating latency in minutes (C) and proportion of males mated over time (D) of males with feminized development of *Or47b* neurons (*Or47b*-Gal4>UAS-traF) and the two controls vs CS wild type females (*n*=27-28). Virgin mating latency in minutes (E), latency to first remating in hours (F) and number of copulations in 24h (G) of females with masculinized development of *Or47b* neurons (*Or47b*-Gal4>UAS-tra;RNAi) and the two controls versus CS males (*n*=11-12). Dot plots with mean, SEM error bars and significant statistical output of post-hoc mixed effects models with genotype as dependent variable between all genotypes separately, significant difference between groups with different letters. Percentage plotted as single values with letters in the figure legends for the effect of genotype and letters on the line graph for the interaction effect of time*genotype*. When the difference between two groups is bordering significance, the p-value is reported in the figure. When genotype is not a determining factor in the model, its p-value as main effect is reported. For the full statistical output see supplementary table 1.
Feminization of *Or47b* ORN increases the volume of the olfactory glomeruli innervated by the *Or88a* ORN

Every ORN projects to one particular part of the olfactory lobe where it connects with interneurons and projection neurons, whose synapses together form a neuropil structure called a glomerulus (Fishilevich and Vosshall, 2005). The glomerulus *Or47b* ORN project to, the VA1v, is 1.5 times bigger in males than females (Kondoh et al., 2003; Stockinger et al., 2005). We hypothesized that the feminization of *Or47b* ORNs in males results in a reduction in glomerular size. To test this hypothesis, we measured the volume of the VA1v (*Or47b* ORN), as well as two neighbouring glomerulus VA1d (*Or88a* ORN) and DA1 (*Or67d* ORN). Volumetric analysis did not reveal a volume reduction of the VA1v in feminized male brains as compared to their controls, but reveals an increase in the volume of the adjacent VA1d (figure 5B). That the VA1v glomerulus size is not affected by feminization of the *Or47b* ORN that innervates it is not completely surprising given that the glomerulus is made up of projections from the pre-synaptic *Or47b* ORN and post-synaptic projection neurons, who have themselves not been manipulated. Feminizing only pre-synaptic neurons is thus not sufficient to influence the volume of the glomerulus. However, a change in volume of the VA1d is unexpected because it is innervated by *Or88a* ORNs, which were not directly manipulated here and because this glomerulus is not naturally sexually dimorphic. This result suggests a connection between the development of neurons innervating the VA1v glomerulus and the size of VA1d.

![Image](image_url)

**Figure 5:** *Or47b* neuron manipulation changes the volume of the VA1d

Stacked picture of one olfactory bulb (A) with illustrative grey coloration of the glomeruli DA1 (top), VA1d (middle) and VA1v (bottom). Volume in μm³ for the glomeruli VA1v, VA1d and DA1 (B) of males with feminized development of *Or47b* neurons (*Or47b-Gal4-UAS-traF*) and the two controls (n=10-13). Dot plots with mean, SEM error bars and significant statistical output of post-hoc mixed effects models with genotype as dependent variable between all genotypes separately, significant difference between groups with different letters. When genotype is not a determining factor in the model, its p-value as main effect is reported. For the full statistical output see supplementary table 1.
The odorant receptor \textit{Or88a} influences both virgin and mated female sexual receptivity

The surprising change in the size of the VA1d glomerulus, innervated by the \textit{Or88a} ORN, following feminization of \textit{Or47b} is interesting because \textit{Or47b} and \textit{Or88a} share the same substrates (Dweck et al., 2015b; Pitts et al., 2016; van der Goes van Naters and Carlson, 2007). We hypothesized that \textit{Or88a} ORN is part of the same olfactory pathway as the \textit{Or47b} ORN regulating female sexual receptivity. We, therefore, tested the impact of \textit{Or88a} manipulation on female receptivity. For females with an \textit{Or88a} null mutation, virgin mating latency is significantly decreased compared to \textit{Or88a}/\textit{w}^{118} heterozygous females and \textit{w}^{118} wild-type females with an intermediate phenotype for the heterozygous females (figure 6A). Latency to 1$^{\text{st}}$ remating is increased for \textit{Or88a} compared to both controls, whereas the heterozygous controls show a reduced latency to 1$^{\text{st}}$ remating (figure 6B). The number of copulations, however, was not significantly affected by the mutation, but the heterozygous control shows an abnormally high number of copulations (figure 6C). This aberrant behaviour of heterozygous controls for post-mating receptivity might be explained by epistatic interactions between the genes from the two different sets of chromosomes leading to a different phenotype than either homozygous line. \textit{Or88a} mutation thus increases virgin receptivity and this phenotype can be partially rescued by one working copy of the gene, but \textit{Or88a} does not convincingly influence post-mating receptivity. Surprisingly, ablation of the \textit{Or88a} ORN does not significantly influence virgin mating latency (figure 6D) or 1$^{\text{st}}$ remating latency (figure 6E), but leads to a significant decrease in number of mating (figure 6F). Together this shows that \textit{Or88a} has an influence on sexual receptivity and that \textit{Or88a} is part of the circuit involved in post-mating receptivity as well as virgin receptivity.

Additionally, expression of odorant receptors only in \textit{Or88a} ORN or in both \textit{Or47b} and \textit{Or88a} ORN in \textit{Orco} $^-$ mutants leads to similar decrease in number of copulations and increases in latency to 1$^{\text{st}}$ mating as compared to \textit{Or47b} (figure 6H, I). This effect is specific to these two odorant receptors as expression in the \textit{Or67d} ORN alone is not sufficient to restore wild-type levels (figure 6H, I). The virginal mating latency is not significantly affected by any of the neuron groups, although a slight increase is visible for \textit{Or47b} ORN, \textit{Or88a} ORN and the combination of the two (figure 6G). Again, suggesting that the \textit{Or88a} neuron is an important player in the neuronal circuit that determines female post-mating receptivity and some indication that it might be involved in the virginal mating as well.
Figure 6: The Or88a Odorant receptor and neuron influences female receptivity
Virgin mating latency in minutes (A), latency to first remating in hours (B) and number of copulations in 24 h (C) of Or88a- mutant, w1118 control and heterozygous w1118;Or88a-/+ control females versus CS males (n=19). Virgin mating latency in minutes (D), latency to first remating in hours (E) and number of copulations in 24h (F) of females with ablated Or88a neurons (Or88a-Gal4>UAS-hid) and the two controls versus CS males (n=14-15). Dot plots with mean, SEM error bars and significant statistical output of post-hoc mixed effects models between all genotypes separately, significance between groups with different letters. When genotype is not a determining factor in the model, its p-value as main effect is reported. Virgin mating latency in minutes (G), latency to first remating in hours (H) and number of copulations in 24h (I) of Orco-females with re-expression of Orco in Or47b neurons (Or47b-Gal4>UAS-Orco), re-expression of Orco in Or67d neurons (Or67d-Gal4>UAS-Orco), re-expression of Orco in Or88a neurons (Or88a-Gal4>UAS-Orco), re-expression of Orco in both Or47b and Or88a neurons (Or47b-Gal4,Or88a-Gal4>UAS-Orco), one UAS-control and the Gal4-control for each set of neurons versus CS males (n=13-33). Dot plots with mean, SEM error bars and significant statistical output of post-hoc mixed effects models with all groups compared to the standard control UAS-Orco. The significance level is reported on top of the graph as: ns p>0.05 * p<0.05, ** p<0.001, *** p<0.0001. When genotype is not a determining factor in the model, its p-value as main effect is reported. For the full statistical output see supplementary table 1.
Discussion

Here we show that the sexual receptivity of mated and virgin females have different genetic bases. We first find that different chromosomes explain quantitative differences in virgin and mated female receptivity between two wild-type strains showing that these two mating statuses are influenced by different genetic architectures. We back-up this conclusion by uncovering one gene that is specifically involved in mated, but not virgin, female sexual receptivity. This gene, Or47b, encodes an odorant receptor whose deletion leads to decreased remating frequency. A rationale for the existence of different genetic bases is the necessity of high sexual receptivity, and decreased choosiness, for virgin females to diminishing the likelihood of remaining unmated (Kokko and Mappes, 2005; Markow, 2011). Once mated, choosiness should increase and sexual receptivity decrease as finding additional mates mainly serves to increase the genetic quality of her offspring (Jennions and Petrie, 2000). Our finding that a gene coding for an olfactory receptor that senses fly compounds (Dweck et al., 2015b; Lin et al., 2016; van der Goes van Naters and Carlson, 2007) influences the sexual receptivity of mated, but not virgin females is in line with this argument. Sensing of volatile compounds from flies allows the gathering of information about social context and maybe male quality. Deploying this sense once mated may thus allow more information to be gathered and thus higher choosiness. This same logic was already demonstrated for food in D. melanogaster, where females become more attracted to food-derived odours (Hussain et al., 2016; Ribeiro, 2013) and increase receptivity (Gorter et al., 2016) once mated. Distinct mechanisms for virginal and mated female sexual receptivity might therefore be the evolutionary outcome of different selective pressure on virgin and mated females mate choice: virgin females might maximize receptivity to avoid the costs of virginity (Kokko and Mappes, 2005; Markow, 2011), while mated females may be more choosy to avoid the costs of unnecessary mating (Kamimura, 2007; Kuijper et al., 2006; Wigby and Chapman, 2004).

A further consideration about the function of Or47b in determining female sexual receptivity is our discovery that this gene has sexually dimorphic effects. We confirm the involvement of Or47b in male sexual behaviour (Dweck et al., 2015b; Lin et al., 2016; Lone et al., 2015; Wang et al., 2011; Zhuang et al., 2016), and further show that the sex of the Or47b ORNs modulates their effect on male and female sexual behaviour. Indeed, feminizing the Or47b ORN in an otherwise male individual leads to increased sexual activity of those males. Interestingly, masculinization of the same ORN in an otherwise female individual leads to decreased receptivity showing that the effect of Or47b on sexual receptivity is dependent on the developmental state of the neuron that expresses it. Such sexual dimorphism might help to buffer the different needs of males and females in terms of the consequences of sexual promiscuity and resolve potential intra-locus sexual conflict. Indeed, our data suggest that Or47b ORN is more influential in the modulation of female receptivity than male courtship as the Or47b mutation has more striking effects in females than in males. In male mating latency there is, next to the effect of Or47b, a significant difference between CS wild-type males and both mutants as well as the rescue line (figure 3A,B). This difference cannot solely be explained by Or47b and suggests that other genetic factors play an important role in
determining male arousal. This lower influence of Or47b on male arousal may serve to prevent courtship initiation in the presence of males or mated females that send inhibitory signals like cis-Vaccenyl acetate (cVA) and 7-tricosene (7T) (Laturney and Billeter, 2016). When these inhibitory stimuli are absent, sexual activity is further increased in individuals with normal Or47b activity. This is illustrated by an increase in male-male courtship towards oenocyteless males, which cannot produce cuticular hydrocarbons like 7T (Billeter et al., 2009; Dweck et al., 2015b; Zhuang et al., 2016). The male-male interaction in this example is abolished by perfuming the target males with 7T alone (Billeter et al., 2009; Wang et al., 2011).

The different effects of Or47b in virgin and mated females and in females and males cannot be explained by sex-specific expression of this receptor. Instead, our experiments suggest that different effects of Or47b comes from the properties of the olfactory neuronal circuitry it is part of. Our data indicate that the Or47b ORNs function within a circuit that differs between males and females and between virgin and mated females. Based on our behavioural experiments and neuronal manipulations, we propose a hypothetical model of this circuitry in females (figure 7). This model illustrates how positive and negative stimuli can lead to both increases and decreases of female receptivity after Or47b manipulation.

**Figure 7: model of neuronal circuitry determining female sexual receptivity** Hypothetical neuronal circuitry model including the odorant receptor neurons (ORN) Or47b and Or88a as well as other previously identified determinants of female post-mating receptivity (A). The interaction between the two ORN, with in black the ORN we suggest activated in the female state mentioned on top and in grey the inhibited ORN (B). Connections ending with - represent inhibitory signals. Connections ending with -> represent excitatory signals. The dashed square box depicts the sensillar environment the neurons reside in.
Based on our finding of increased receptivity when ORN activity is compromised in *Orco* (figure 3E,F), ORN stimuli is assumed inhibitory for female receptivity. When all ORN stimuli are absent, positive stimuli perceived through other modalities than odorant receptors, like yeast availability sensed via ionotropic receptors (Gorter et al., 2016), courtship song (Laturney and Billeter, 2014) and sperm storage (Newport and Gromko, 1984), can more easily increase post-mating receptivity (illustrated in figure 7A). When the ORN of *Orco* are restored for *Or47b* or *Or88a*, but not *Or67d*, the mating frequency is significantly decreased. This suggests that *Or47b* and *Or88a* are both inhibitory factors that can impact post-mating receptivity. However, our data also suggests these odorant receptors have specialized and opposite functions as *Or47b* only impacts post-mating receptivity (figure 2A,B), while *Or88a* only showed changes in virgin receptivity (figure 6A). We therefore suggest that these neurons inhibit each other’s output (figure 7B), resulting in only one of the two being active at each moment. This inhibition could for example occur through presynaptic neurotransmitter signalling (Root et al., 2008), by decreasing the electrical current within the sensillum they are both housed in (Su et al., 2012; van der Goes van Naters, 2013) or at the site of the olfactory lobe. Indeed, these neurons could easily modulate each other as they share the same sensillum, project to glomeruli located directly next to each other (Couto et al., 2005; Fishilevich and Vosshall, 2005) and it was even suggested that *Or47b* neurons might innervate the glomerulus of *Or88a* (VA1d), but with fewer innervations (Bhalerao et al., 2003; Fishilevich and Vosshall, 2005).

To explain when each neuron is activated and inhibits the other (figure 7B), we need to consider that they are activated by the same substrates (Dweck et al., 2015b; Pitts et al., 2016; van der Goes van Naters and Carlson, 2007). Although, *Or88a* was suggested to respond to substrates at longer distance for aggregation, while *Or47b* used those substrates on short range to determine sexual behaviour in males (Dweck et al., 2015b). This suggests that *Or88a* is more sensitive to the same substrates and the initial state would be an activated *Or88a* ORN that inhibits *Or47b* signalling and sends inhibitory signals to the integration center for virgin mating (figure 7A). This explains why Or47b manipulation does not change virgin sexual receptivity (figure 2A,B and 3A,D,G). Upon mating, the physiological state of *Or47b* might get modulated to become active and it will start inhibiting *Or88a*, thereby also inhibiting its modulatory effect on *Or47b*. This change in neuron activity after mating is inspired by the finding that the sensory output of olfactory and gustatory neurons was changed due to upregulation of Sex Peptide Receptors in those cells (Hussain et al., 2016). Additionally, this explanation allows for *Or47b* to show less sensitivity to the same substrates supporting a more stringent decision making process in mated vs virgin females.

However, to understand the effects of eliminating *Or47b* or *Or88a* functioning via genetic mutation or neuron manipulation we also need to consider their respective valence. When *Or47b* is eliminated, through both genetic mutation and neuron ablation, this leads to decreased post-mating receptivity (figure 2C-E). Since *Or47b* is absent, the switch to *Or47b* activity cannot occur and *Or88a* will keep influencing receptivity in a mated state as well (figure 7). *Or88a* should, therefore, be more inhibitory than *Or47b* normally is to see a
decrease in sexual receptivity. GABA inhibition in Or47b ORN, on the other hand, leads to increased post-mating receptivity (figure 3H,I), because it cannot send its inhibitory signal to a putative integration center, but the ORN’s activity does block Or88a from being active (figure 7A,B). Interestingly, the effect of Or88a elimination differs per manipulation. Genetic mutation does not affect post-mating receptivity (figure 6B,C), because Or47b gets normally activated. However, removing the Or88a ORN from the circuit leads to no change in virgin receptivity (figure 6D) and a decrease in post-mating receptivity (figure 6E,F), which cannot be explained easily by our model (figure 7). Since Or47b is assumed to have a lower valence and the Or88a ORN is not there to inhibit, Or47b might take on the function of Or88a in influencing virgin mating, hence little change in sexual receptivity occurs. The decrease in post-mating receptivity might be due to increased sensitivity or activity of Or47b in the absence of the Or88a ORN. Although the interaction between Or47b and Or88a still need empirical data, our findings support a model in which these two neurons modulate each other to determine sexual receptivity for both virgin and mated females (figure 7B). Further analysis on these key players is, however, needed to understand in depth how they are connected, communicate and differentiate tasks, to accommodate the sexual needs of males, virgin and mated females.

**Authors and Contributors**

J.A.G, J.L. and J.-C.B designed and interpreted the study. J.A.G generated the data for all figures and performed the statistical analyses. J.A.G and J.-C.B wrote the paper. All authors read and commented on the paper.

**Acknowledgements**

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### Supplementary Information

**Figure 1:** Female receptivity has a chromosomal basis.

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**Figure 2:** The odorant receptor Or47b promotes receptivity.

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**Figure 3:** The inhibitory Or47b neuron influences female post-mating receptivity.

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Figure 4: The Or47f neurons function differently in male and females.

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Figure 5: Or47f neuron manipulation changes the volume of the V4L.

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Chapter 5

Chapter Title

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Log transformed, sometimes followed by normalization. When applicable a random effect for date was added to the output is shown here with t
that complied with the rules of normality and homogeneity were tested with mixed effects linear models and the Supplementary Table 1 Detailed statistics

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**Log likelihood ratio test**  **Test statistics (contingency table convert)**

Supplementary Table 1 Detailed statistics All models in this table were performed using R version 3.2.2. All data that complied with the rules of normality and homogeneity were tested with mixed effects linear models and the output is shown here with t- and p-values. When either normality or homogeneity could not be satisfied, the data were log transformed, sometimes followed by normalization. When applicable a random effect for date was added to the models.