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RESEARCH PAPER

Two sex pheromone receptors for sexual communication in the American cockroach

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Volatile sex pheromones are vital for sexual communication between males and females. Females of the American cockroach, *Periplaneta americana*, produce and emit two sex pheromone components, periplanone-A (PA) and periplanone-B (PB). Although PB is the major sex attractant and can attract males, how it interacts with PA in regulating sexual behaviors is still unknown. In this study, we found that in male cockroaches, PA counteracted PB attraction. We identified two odorant receptors (ORs), OR53 and OR100, as PB/PA and PA receptors, respectively. *OR53* and *OR100* were predominantly expressed in the antennae of sexually mature males, and their expression levels were regulated by the sex differentiation pathway and nutrition-responsive signals. Cellular localization of *OR53* and *OR100* in male antennae further revealed that two types of sensilla coordinate a complex two-pheromone-two-receptor pathway in regulating cockroach sexual behaviors. These findings indicate distinct functions of the two sex pheromone components, identify their receptors and possible regulatory mechanisms underlying the male-specific and age-dependent sexual behaviors, and can guide novel strategies for pest management.

sex pheromone | odorant receptor | sexual attraction | sex differentiation | cockroach

INTRODUCTION

Sexuality requires effective sexual communication between mature males and females. Volatile sex pheromones are widely used in animals, including insects, to attract potential mates, and are generally detected by dedicated odorant receptors (ORs) (Ebrahim et al., 2023; Kurtovic et al., 2007; Liberles, 2014; Linn Jr et al., 1987; Nakagawa et al., 2005). Transducing sex pheromone signals in insects requires sexually dimorphic elements, including antennal sensilla and sex pheromone receptors (i.e., peripheral circuitry), and the projection of sex pheromone-responsive neurons to dedicated glomeruli in the central nervous system (i.e., central circuitry). For example, in the silkworm, *Bombyx mori*, two ORs for sensing two female sex pheromone components with distinct functions, *BmOR1* and *BmOR3*, are expressed in a male-specific manner in specialized sensilla to ensure sexual attraction only by the opposite sex (Nakagawa et al., 2005; Xu et al., 2020). The male-specific expression of *BmOR1* and *BmOR3* is regulated by two terminal genes in the conserved sex differentiation pathway in insects, *doublesex* (*dsx*) and *fruitless* (*fru*), respectively (Burtis and Baker, 1989; Hopkins and Kopp, 2021; Peng et al., 2021; Ryner et al., 1996; Xu et al., 2020). In the fruit fly, *Drosophila melanogaster*, the male-specific volatile pheromone, 11-*cis*-vaccenyl acetate (cVA), activates the sexually monomorphic OR67d receptor in both sexes to mediate sexually dimorphic behaviors via different downstream processing pathways (Datta et al., 2008; Kurtovic et al., 2007; Ruta et al., 2010). Hence, it is particularly important to explore sex pheromone-receptor interaction and its regulatory mechanisms.

The American cockroach, *Periplaneta americana*, is one of the most widespread sanitary pests globally and is responsible for transmitting pathogens and disseminating potent allergens indoors (Pomés and Schal, 2020). Sexually mature *P. americana* females produce and emit two volatile sex pheromone components with closely related chemical structures, periplanone-A (PA) and periplanone-B (PB) (Okada et al., 1990; Roth and Willis, 1952). Interestingly, *P. americana* males are highly sensitive to the female sex pheromones (Okada et al., 1990; Sass, 1983). The males orient to the sex pheromone from a long distance, and close to the females, they display typical courtship behaviors, including waving of their antennae, raising and flapping their wings, and copulatory attempts (Okada et al., 1990; Roth and Willis, 1952) (for wild cockroach courtship, see

Video S1 in Supporting Information; for cockroach courtship in the lab, see Video S2 in Supporting Information). PB plays a major role in attracting males (Gemeno and Schal, 2004). The concentration of PA is lower than that of PB, and the function of PA has remained elusive for decades (Okada et al., 1990; Persoons et al., 1979; Seelinger and Gagel, 1985). Despite the identification of the sex pheromone of the American cockroach nearly half a century ago (Okada et al., 1990; Persoons et al., 1979; Seelinger and Gagel, 1985) and its total synthesis as a classic in organic chemistry (Still, 1979), the olfactory receptors that detect the two periplanones remain unknown (Gemeno and Schal, 2004). We previously sequenced the genome of *P. americana* and identified 154 candidate ORs (Li et al., 2018). RNAi knockdown of the odorant receptor co-receptor (*ORco*) impairs sex pheromone responses in male adults (Tateishi et al., 2020; Tateishi et al., 2022), further highlighting the importance of identifying specific ORs for the two sex pheromone components, PA and PB.

In this study, we first found that in *P. americana*, PB can attract adult males over a long distance, while PA counteracts PB attraction. Additionally, by employing various behavior assays, electrophysiology, sex- and age-specific transcriptomes, *Drosophila* genetics, and RNAi knockdown experiments, we identified OR53 as the receptor for PA/PB and OR100 as the receptor for PA. Fluorescence *in situ* hybridization (FISH) and single-cell sequencing localized OR53 and OR100 in two types of sensilla in the male antennae (MA). In summary, our findings revealed that two sex pheromone components coordinate cockroach sexual behaviors via two ORs located in two distinct types of sensilla. These findings can help us to develop novel ecologically sound strategies for controlling cockroach pests, which can transmit pathogens and disseminate allergens indoors.

RESULTS

PB attracts sexually mature males, while PA counteracts PB function

To investigate the onset of male sexual behaviors in the American cockroach, we first observed mating behavior in males from 1 to 9 d after eclosion (DAE) toward mature female adults. We found that male cockroaches began to mate on 4 DAE, with a mating rate of about 20%. The mating rate increased to nearly 50% at 5 DAE and more than 60% at 6 DAE and thereafter accumulated slowly and steadily (Figure 1A). Thus, as in most animal species, only sexually mature males display mating behavior in the American cockroach (Bilen et al., 2013; Roth and Willis, 1952; Södersten et al., 1977; Zhang et al., 2021).

We hypothesized that the levels of male sexual behaviors stimulated by the female sex pheromone should be consistent with their mating rates, as demonstrated above (Figure 1A). To test this hypothesis, we observed the responses of males to PB at 1, 3, 5, 7, and 9 DAE over the control solvent in a preference test using a Y-tube olfactometer. As expected, the preference for PB gradually increased during sexual maturation (Figure 1B; Figure S1A in Supporting Information), suggesting that only sexually mature males could effectively sense PB and engage in sexual behaviors. We performed the same preference test for PA to the control solvent but found no significant preference for PA in males from 1 to 9 DAE (Figure 1C; Figure S1B in Supporting Information). As it has been suggested that the ratio of PA:PB in mature females is about 1:10 (Persoons et al., 1979), we further tested whether the addition of low-concentration PA (10% PB) would affect PB attraction. We found that sexually mature males (i.e., 7 DAE, here and thereafter) preferred PB alone to the control solvent or a combination of PB and PA in the Y-tube olfactometer (Figure 1D). Notably, the combination of PB and PA was slightly, but not significantly, preferred over the control solvent by mature male cockroaches (Figure 1D). These results demonstrate that PB is the major sex attractant, while PA might counteract PB attraction.

As PA and PB might stimulate the same or distinct olfactory receptor neurons (ORNs) within basiconic sensilla on the surface of cockroach antennae (Nishino et al., 2012; Tateishi et al., 2020; Tateishi et al., 2022), we performed single-sensillum recording (SSR) in the antennae of sexually mature males stimulated by PB and PA. We found two types of sex pheromone-responsive basiconic sensilla. Type-1 sensilla (38 out of 50) robustly responded to the presence of both PB and PA, while type-2 sensilla (12 out of 50) responded strongly to PB but only weakly to PA (Figure 1E). We further performed cross-adaptation SSR with repeated stimulation with one sex pheromone component and a switch to the other component. We found that the application of PA induced strong responses even after the ORN became less responsive to repetitive PB stimulation (Figure 1F, top), suggesting that PA might activate a PB-unresponsive OR. In contrast, after repetitive PA stimulation, the application of PB failed to induce a robust response (Figure 1F, bottom), suggesting that PB can activate only a PA-responsive OR. In summary, these results suggest that PA and PB act on at least two distinct but shared ORs in at least two types of ORN-housing sensilla to mediate male sexual behaviors.

OR53 and *OR100* are predominantly expressed in the antennae of sexually mature males

As only sexually mature males displayed a preference for the sex pheromone, we speculated that there was a male-biased and age-dependent expression of the sex pheromone receptors in the cockroach antennae. To screen for candidate ORs, we first performed RNA-seq analyses in various tissues, including antennae, forelegs, heads, mouthparts, testes, and wings, from sexually mature males, using the antennae from the sexually mature females as a control. Among the 109 OR genes revealed by the transcriptome of the male antennae, 43 ORs were identified as highly expressed genes in the male antennae ($|\log_2 FC|>1$, FDR<0.05). These ORs were sorted according to their fragments per kilobase of transcript per million mapped reads (FPKM), and the top 20 ORs were selected for further analysis (Figure 2A). Interestingly, the top three highly expressed OR genes, *OR53*, *OR100*, and *OR87*, as well as *ORco* that encodes the OR co-receptor, displayed both sex- and tissue-biased expression in the male antennae, while most of the other ORs showed tissue- but not sex-biased expression (Figure 2A; Figure S2 in Supporting Information). Quantitative real-time PCR (qPCR) further showed that *OR53* and *OR100* were predominantly expressed in the male antennae, while *OR87* and *ORco* were highly expressed in both male and female antennae (FA) (Figure S3 in Supporting Information).

We also collected antennae from males at 1, 3, 5, 7, and 9 DAE for RNA sequencing (RNA-seq) as males responded to females or their sex pheromone in an age-dependent manner (Figures 1A

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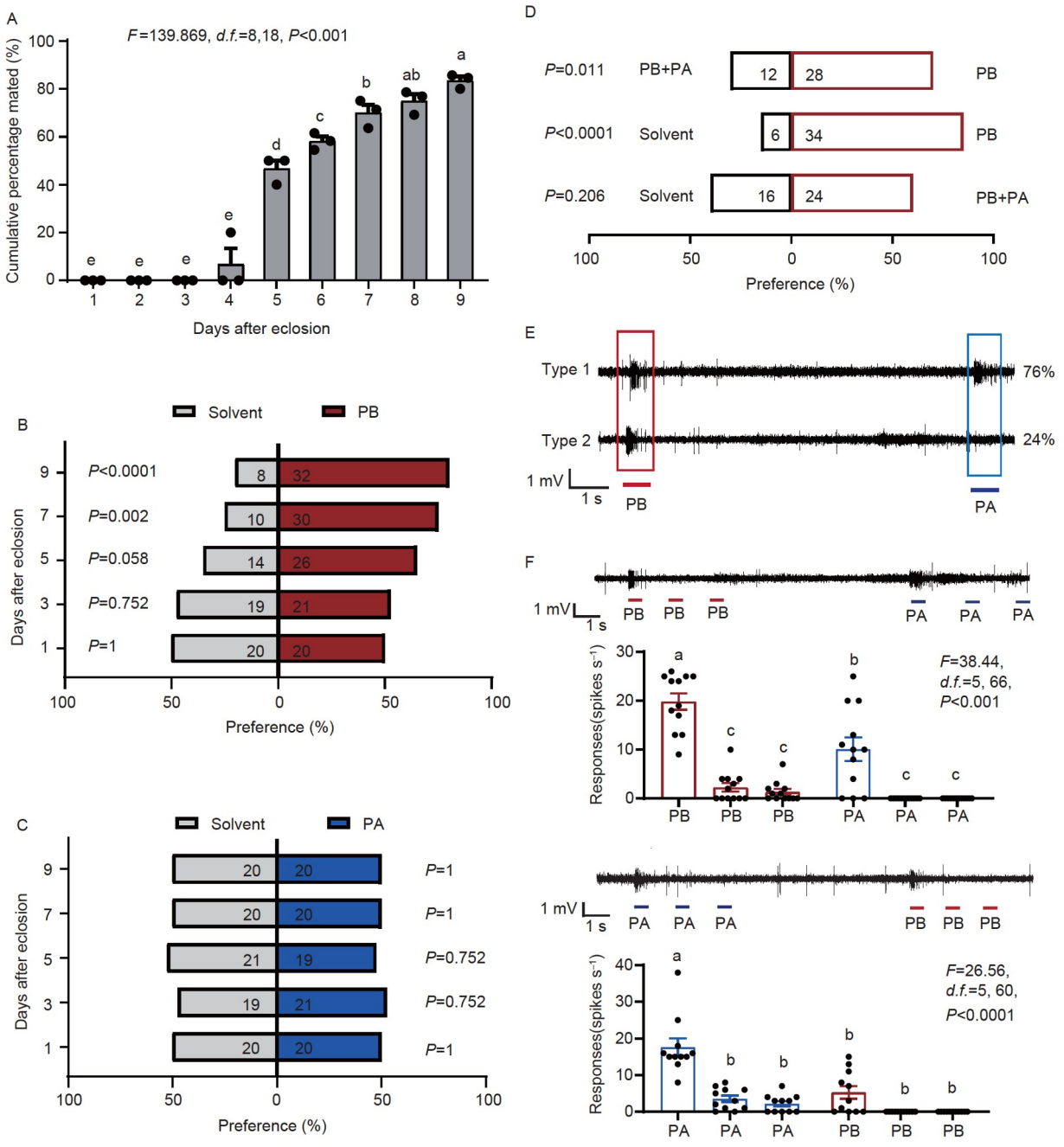

Figure 1. Sex pheromone components PA and PB elicit distinct sexual behavioral responses in adult male American cockroaches. A, The mating rate per day of male adults at 1 to 9 DAE. Females were sexually mature virgins. Three biological replicates were performed, and each used ten pairs of male and female adults. B and C, Preference of male adults at 1 to 9 DAE between PB (1 ng applied to a filter paper in 10 μL dichloromethane) and the control solvent dichloromethane (10 μL) (B) or between PA (1 ng) and dichloromethane (10 μL) (C) forty repeats were performed. D, Preference of male cockroaches at 7 DAE between PB (10 ng), PA (1 ng)+PB (10 ng) and the control solvent dichloromethane (10 μL). The number in each bar indicates the total number of cockroaches that chose the odorant. E, Two types of SSR responses from the antennae of male American cockroaches to PB and PA (20 μg for each). Top, for type 1 SSR (~76%), both PB and PA induced strong spikes; bottom, for type 2 (~24%), PB but not PA induced obvious spikes. F, SSR responses to repetitive stimulation of two sex pheromone components. Top, SSR responses to three repetitive PB stimulations and three PA stimulations (n=12); bottom, SSR responses to three repetitive PA stimulations and three PB stimulations (n=11). Data are presented as mean±s.e.m. For A and F, different letters indicate statistically significant differences between groups based on one-way ANOVA with Tukey's honestly significant difference (HSD) test at α=0.05. For B–D, the χ^2 test was used.

and B). We identified 107 ORs in the transcriptome of antennae during sexual maturation. Among them, 32 ORs exhibited significant differences in their level of expression. Interestingly, the expression levels of *OR53* and *OR100* increased as the males matured sexually (Figure 2B). As confirmed by qPCR, the expression levels of *OR53*, *OR100*, and *ORco*, but not *OR87*, gradually increased from 1 to 9 DAE (Figure S3 in Supporting Information). These results indicate that *OR53* and *OR100* are predominantly expressed in the antennae of sexually mature males but not in females or sexually immature males, implying that they are potential ORs for sensing the American cockroach female sex pheromone.

We hypothesized that the male-biased expression of *OR53* and *OR100* might also be determined by the terminal genes in the

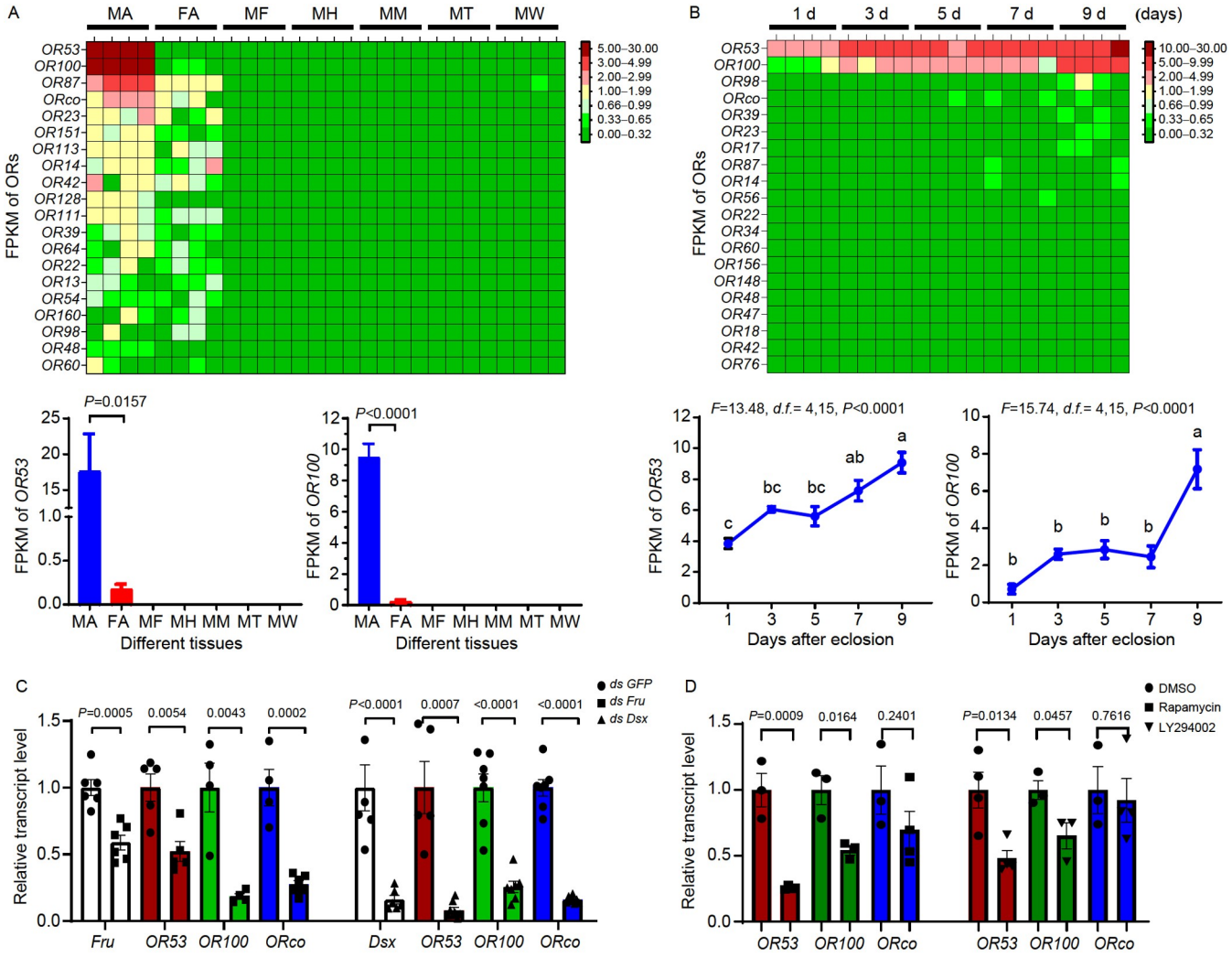

Figure 2. *OR53*and *OR100* are predominantly expressed in the antennae of sexually mature male American cockroaches. A, The heatmap indicating the ranking of the top 20 OR genes by FPKM expression level across various tissues. Statistical analysis was performed on the FPKM of *OR53* and *OR100* by extracting FPKM values from the corresponding section of the heatmap. MA, male antennae; FA, female antennae; MF, male forelegs; MH, male heads; MM, male mouthparts; MT, male testes; MW, male wings. n=4 biological replicates. B, The heatmap depicting the FPKM expression levels of the top 20 ORs from 1 to 9 DAE. The expression levels of *OR53* and *OR100* in the male antennae, captured at 1, 3, 5, 7, and 9 DAE, were analyzed by individually extracting FPKM values from the corresponding sections of the heatmap. n=4 biological replicates. C, qPCR showing the expression levels of *OR53*, *OR100*, and *ORco* in male antennae after RNAi knockdown of *fru* or *dsx*. *dsGFP* was used as the negative control. The mRNA levels were normalized by *rp49*. n=4–8 biological replicates. D, qPCR showing the expression levels of *OR53*, *OR100*, and *ORco* in male antennae after treatment with Rapamycin (a TORC1 inhibitor) and LY294002 (a PI3K inhibitor). DMSO was used as the negative control solvent. The mRNA levels were normalized to *rp49*. n=3–4 biological replicates. The qPCR and FPKM data are presented as mean±s.e.m. P values are based on unpaired two-tailed Student's t-test. Different letters in B indicate statistically significant differences between groups based on one-way ANOVA.

sex differentiation pathway, namely, *fru* and *dsx* (Hopkins and Kopp, 2021; Peng et al., 2021). We, thus, identified the male-specific *fru* (fru^M) and *dsx* (dsx^M) transcripts in the American cockroach through transcriptome sequencing of male antennae and sequence alignment with Fru and Dsx proteins in the German cockroach, *Blattella germanica* (Clynen et al., 2011; Wexler et al., 2019) (Figure S4 in Supporting Information). Using RNA interference (RNAi) to knock down fru^M or dsx^M expression, we found that the expression levels of *OR53*, *OR100*, and *ORco* were significantly reduced in the dsRNA-treated male antennae, suggesting that the male-biased expression of these ORs is positively regulated by fru^M and dsx^M (Figure 2C). The gradually increasing expression of *OR53* and *OR100* during sexual maturation suggests a close relationship between OR expression and nutrition/feeding after eclosion. In flies, cockroaches, and other insects, the nutrition-responsive insulin/insulin-like growth factor signaling (IIS) and target of rapamycin complex 1 (TORC1) play essential roles in mediating nutrition-promoted insect growth and reproduction (Li et al., 2018; Zhu et al., 2020). We found significant decreases in the expression levels of *OR53* and *OR100*, but not *ORco*, after injecting Rapamycin (inhibitor of TORC1) or LY294002 (inhibitor of an essential component of IIS, PI3K) (Zhu et al., 2020) (Figure 2D), indicating that the age-dependent expression of these ORs is regulated by IIS and TORC1. These results indicate that the sex differentiation pathway and nutrition-responsive signals determine the male-biased and age-dependent expression of sex pheromone receptors, respectively, ensuring the predominant expression of *OR53* and *OR100* in the antennae of sexually mature males.

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OR53 and OR100 are PB/PA and PA receptors, respectively

Transcriptome analyses suggested that OR53 and OR100 could be the receptors of PB and/or PA. To test whether the two ORs are the *bona fide* sex pheromone receptors, we ectopically expressed *OR53* and *OR100* in *Drosophila* cVA-sensing OR67d neurons (Datta et al., 2008) and recorded their electrophysiological responses to PA and PB. In the control male flies (genotype: *UAS-OR53*), *OR67d*-expressing ORNs responded specifically to cVA but not to PA or PB. However, in the transgenic *OR67d* mutant male flies, ectopic expression of the cockroach *OR53* (genotype: *UAS-OR53*; $OR67d^{GAL4}$, in which $OR67d^{GAL4}$ is an *OR67d* mutation) was sufficient to confer electrophysiological sensitivity to both PA and PB, with a stronger response to the latter. As the concentrations of PA and PB gradually increased, the neuronal responses also increased significantly (Figure 3A). Transgenic *OR67d* mutant male flies expressing cockroach *OR100* (genotype: *UAS-OR100*; $OR67d^{GAL4}$) lost the response to cVA and showed a robust response to PA even at low concentrations but not to PB even at high concentrations (Figure 3B). The results from the *Drosophila* genetic studies indicate that OR53 is sufficient to confer both PB and PA responses, while OR100 responds only to PA stimulation.

To further validate the results obtained from the *Drosophila* genetics studies, we knocked down *OR53* or *OR100* in cockroaches using RNAi and recorded electrophysiological responses of individual sensilla to PA and PB, respectively. The efficiency and specificity of RNAi knockdown were validated by qPCR (Figure 4A). We observed two types of responses from individual sensilla upon *OR53* RNAi. In most of the recorded sensilla (20 out of 25), knocking down *OR53* expression significantly weakened ORN responses to PB but had only a slight effect on the ORN responses to PA (Figure 4B). These sensilla most probably correspond to the type-1 sensilla in Figure 1E. In the rest of the recorded sensilla (5 out of 25), knocking down *OR53* expression diminished ORN response to both PA and PB (Figure 4B). This group of sensilla may correspond to the type-2 sensilla in Figure 1E. Likewise, we also observed two types of responses from individual sensilla when *OR100* is knocked down. In most of the recorded sensilla (16 out of 25), knocking down *OR100* expression specifically impaired ORN sensitivity to PA but not PB (Figure 4B). These sensilla may correspond to the type-1 sensilla. In the rest of the recorded sensilla (9 out of 25), knocking down *OR100* expression did not affect ORN response to either PA or PB (Figure 4B). These sensilla may correspond to type-2 sensilla. Statistical analyses showed that RNAi knockdown of *OR53* and *OR100* significantly impaired the response to PB and PA in the sensilla, respectively (Figure 4C). Accordingly, *OR53* RNAi eliminated the attraction of male adults to PB. However, sexually mature males with *OR100* knockdown did not prefer PB to the combination of PB and PA, but they still preferred PB to the control solvent (Figure 4D). Additionally, knocking down *OR53* reduced male mating rates with wild-type female cockroaches, while knocking down *OR100* did not significantly affect male mating rates (Figure 4E). These results unambiguously demonstrate that the sex pheromone component PB activates OR53 to induce sexual behaviors, while PA weakly activates OR53 and strongly activates OR100 and plays a relatively minor role in cockroach sexual behaviors.

OR53 and *OR100* are localized in two types of ORN-housing sensilla

The electrophysiological results (Figures 1E and 4B) indicated that there were at least two types of ORN-housing sensilla expressing *OR53* and/or *OR100*. We hypothesized that most of the recorded sensilla (type-1) contain both OR53 and OR100 ORNs so they can respond to both PB and PA (Figures 1E and 3A), and knocking down one of the two receptors would not affect the function of the other (Figure 4B). We further hypothesized that some sensilla (type-2) might house only OR53 ORNs, so they should respond to PB robustly and to PA weakly (Figures 1E and 3A), and knockdown of OR53 should prevent response to both PB and PA (Figure 4B).

To test this hypothesis, we used FISH with mRNA probes to detect *OR53* and *OR100* expression in the antennae of male cockroaches. We first used mRNA probes against *OR53* and *ORco* and observed overlapped FISH signals in the dendritic regions of ORNs in basiconic sensilla in the male antennae (Figure S5A in Supporting Information). We also used mRNA probes against *OR100* and *ORco* and observed overlapped expression (Figure S5B in Supporting Information). These results indicate that mRNA probes are efficient and specific. When we used mRNA probes against *OR53*, *OR100*, and *ORco*, we observed that these three genes were often expressed in the same sensilla (Figure 5A), consistent with our hypothesis that the majority of basiconic sensilla contain both OR53 and OR100 ORNs.

To more systematically investigate the expression of OR genes, we performed single-cell sequencing using the antennae of sexually mature males. Based on the analysis of Uniform Manifold Approximation and Projection, (UMAP), effective cells were divided into 14 independent clusters according to their general expression profiles (Figure 5B). The gene expression level was measured for each cluster of cells, and cluster 2 was identified to be ORNs with high expression of *ORco* (Figure 5B). Further analysis of cluster 2 cells yielded 12 subtypes (Figure 5C). We found that 4 out of 12 subtypes expressed both *OR53* and *OR100* (in total, 31 cells expressing *OR53* and 39 cells expressing *OR100* in subtypes 2-3, 2-4, 2-5, and 2-8), which might correspond to ORNs of the major type-1 sensilla. We also found a subtype with predominant *OR53* expression (11 cells expressing *OR53* and 1 cell expressing *OR100* in the subtype 2-9), which might correspond to ORNs of the minor type-2 sensilla (Figure 5D). There could also be a small number of sensilla housing only OR100 ORNs (3 cells expressing *OR100* in the subtype 2-6) (Figure 5D). These results further support that there are at least two types of ORN-housing sensilla in the antennae of sexually mature males, with most of the sensilla housing both OR53 and OR100 ORNs, while a minority of the sensilla house only OR53 ORNs.

Based on these results, we propose a model of two-pheromone-two-receptor in two types of sensilla transducing sexual communication in the American cockroach (Figure 6).

DISCUSSION

The two components of the sex pheromone produced by female adults of the American cockroach, PA and PB, were identified more than 40 years ago (Persoons et al., 1979); however, to date, studies on their roles in male sexual behaviors have produced inconclusive results, and the olfactory receptors tuned

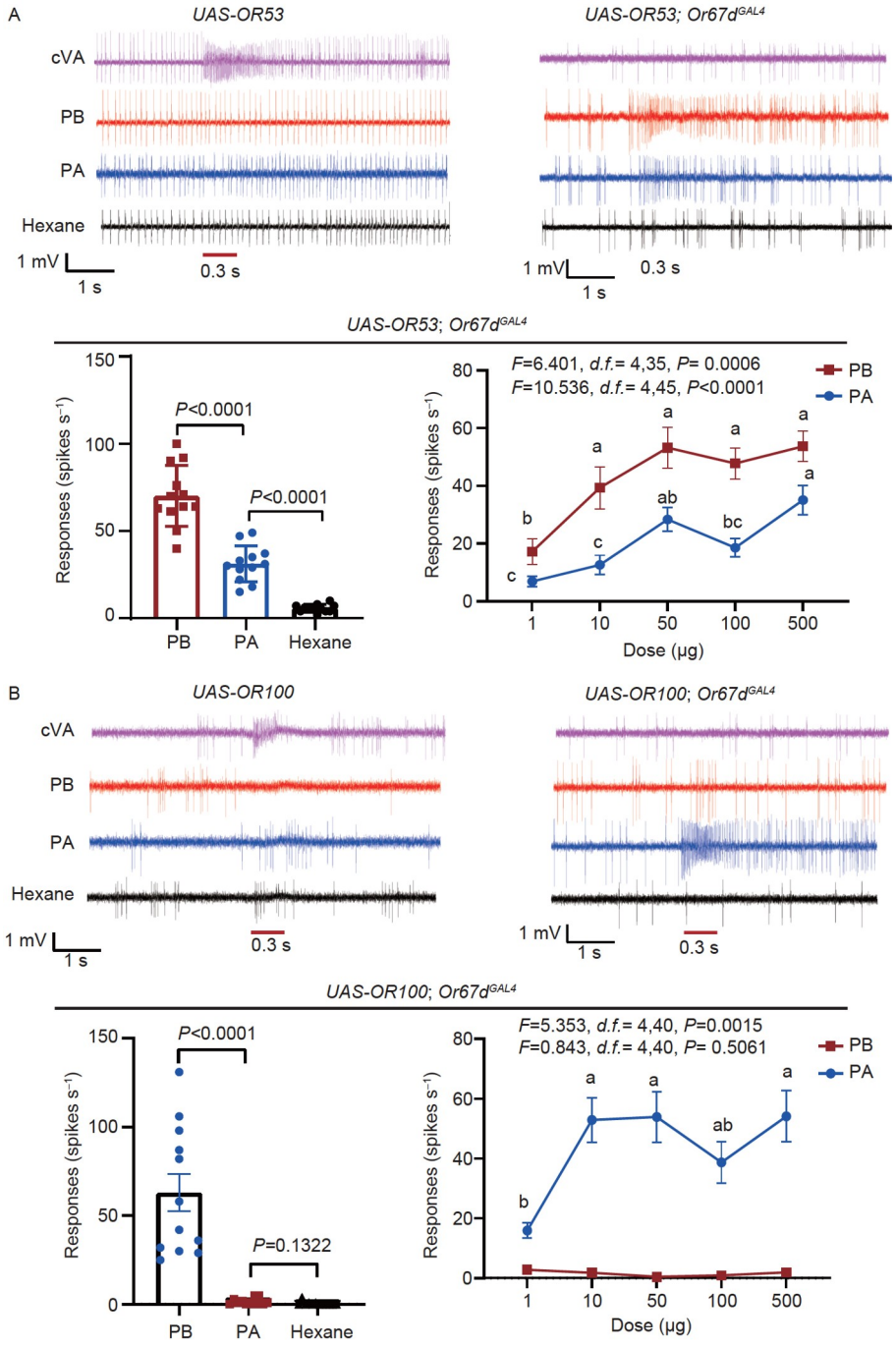

Figure 3. OR53 and OR100 are sufficient to confer PB/PA and PA sensitivity in transgenic *Drosophila melanogaster*. A, SSR responses of *OR53*-expressing neurons in the sensilla of *Drosophila*. *UAS-OR53* control flies showed no response to PA or PB. Ectopic expression of cockroach *OR53* in the *Drosophila OR67d* mutant male flies conferred sensitivity to PB and PA in *Drosophila* (*UAS-OR53*; *OR67d*^{GAL4}). SSR responses to PB were more pronounced than those to PA (100 μg each). PB and PA induced dose-dependent (1, 10, 50, 100, and 500 μg) SSR responses (n=8–10). Hexane was used as the control solvent. B, SSR responses of *OR100*-expressing neurons in the sensilla of *Drosophila*. Ectopic expression of cockroach *OR100* in the *Drosophila OR67d* mutant male flies conferred sensitivity specific to PA (100 μg), but not PB (100 μg), in *Drosophila* (*UAS-OR100*; *OR67d*^{GAL4}). PA induced dose-dependent (1, 10, 50, 100, and 500 μg) SSR responses (n=9). Hexane was used as the control solvent. Data are presented as mean±s.e.m. For panels on the left, P values are based on unpaired two-sided Student's t-test. For panels on the right, different letters indicate statistically significant differences between groups based on one-way ANOVA.

to each component remain unknown. Our findings confirmed that PB is the major sex attractant and provided evidence supporting the potential role of PA in counteracting PB attraction. Additionally, we identified OR53 and OR100 as the receptors for PB/PA and PA, respectively, and demonstrated their roles in coordinating cockroach sexual behaviors. Furthermore, we showed that OR53 and OR100 were expressed in two types of ORN-housing sensilla in the antennae of male cockroaches. Moreover, we found that the male-biased and age-dependent expression of *OR53* and *OR100* were at least partially regulated by the sex differentiation pathway and nutrition-responsive signals, respectively, to fine-tune sexual behaviors only in

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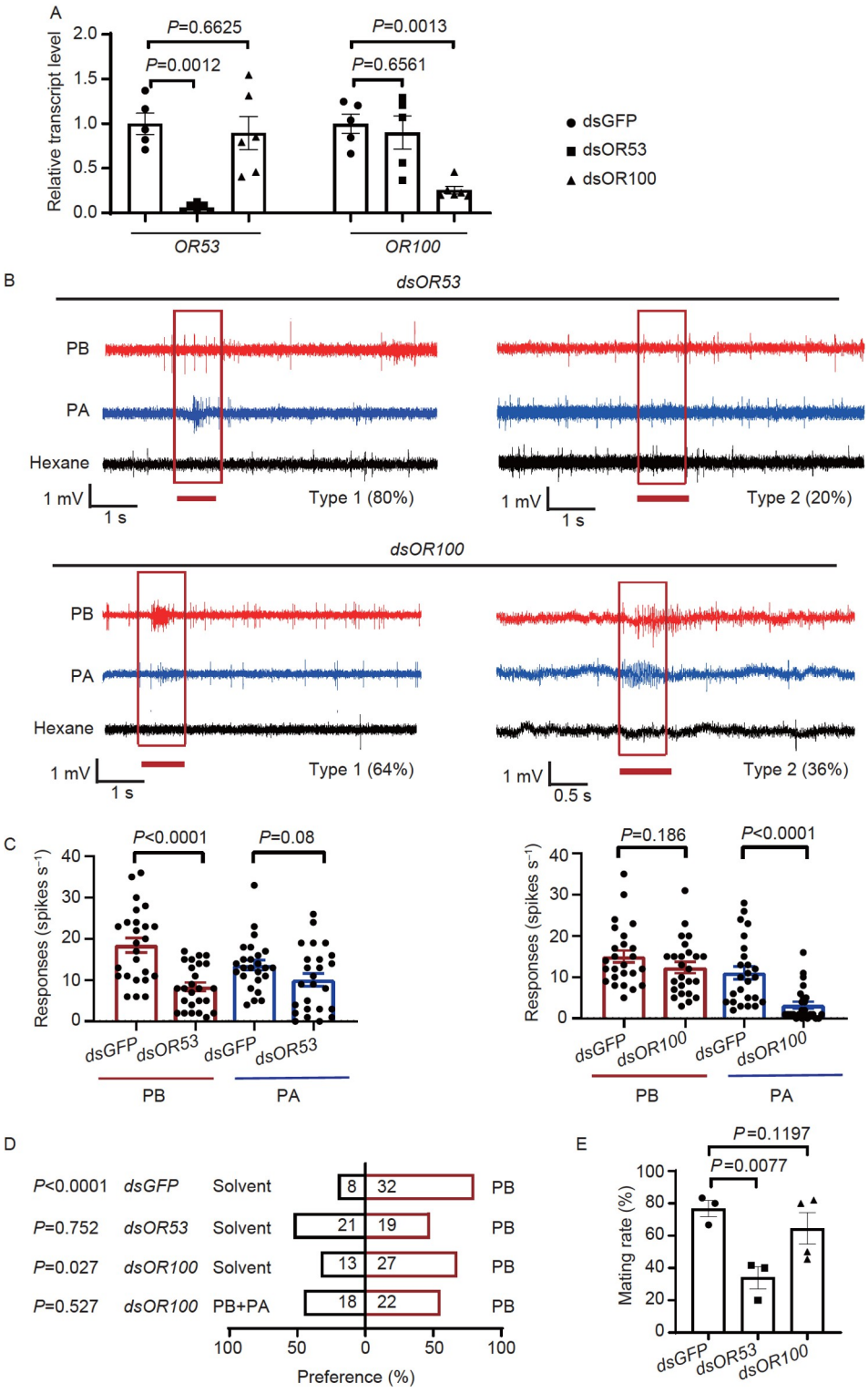

Figure 4. OR53 and OR100 are responsible for sensing sex pheromones in adult male American cockroaches. A, qPCR showing the RNAi efficiency and specificity of *OR53* and *OR100*. n=6 for *dsOR53*; n=5–6 for *dsOR100*; n=5 for *dsGFP*. B, Two types of SSR spike traces from basiconic sensilla on male antennae to PA and PB after knocking down *OR53* or *OR100*, respectively. Knockdown of *OR53* eliminated SSR response to PB, but not PA, in most samples (type-1, 20 out of 25) but abolished SSR response to both odors in a few samples (type-2, 5 out of 25). Knockdown of *OR100* impaired SSR response to PA, but not PB, in most samples (type-1, 16 out of 25) but did not affect SSR response to either odor in a few samples (type-2, 9 out of 25). n=25 for PB; n=25 for PA; n=25 for hexane. C, Statistical analysis of SSR response from male basiconic sensilla to PB and PA after knocking down *OR53* (left) or *OR100* (right). SSR responses (spikes s^{-1}) to PB and PA at a concentration of 1 μg μL^{-1}. n=25 for each. D, Behavioral responses of male cockroaches to various odorants in a Y-tube olfactometer after knocking down *OR53* or *OR100*. The number in each bar indicates the total number of males choosing the odorant. E, Cumulative mating rates of RNAi-mediated males with wild-type female cockroaches. Three or four biological replicates were performed, and n=34, 32 and 42 total males from left to right. Data are presented as mean±s.e.m. Statistical analysis for qPCR data in A, C and E were performed by unpaired two-tailed Student's t-test. For comparison between odorant preferences (D), the χ^2 test was used.

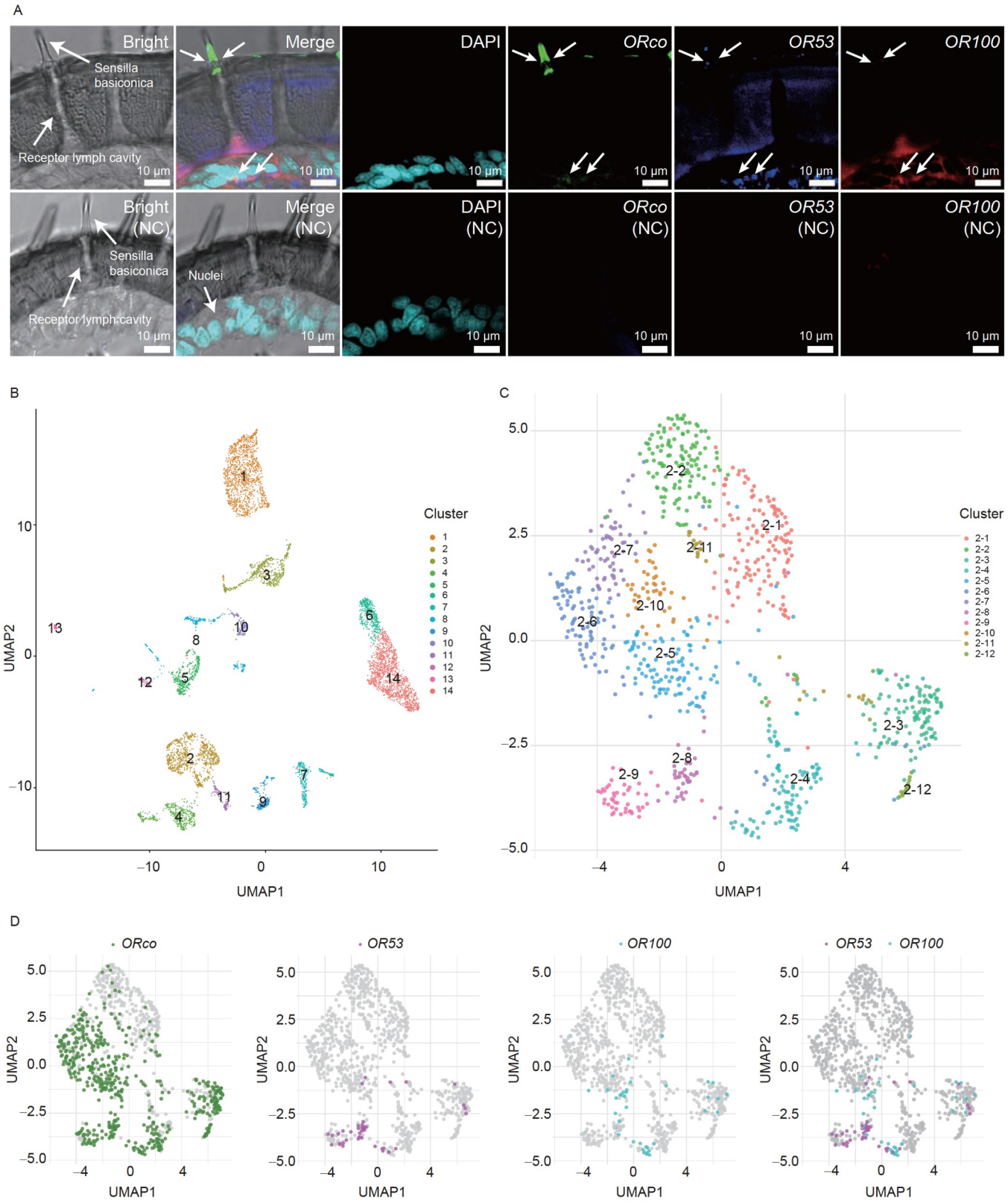

Figure 5. Cellular localization of *OR53* and *OR100* in the antennae of adult male American cockroaches. A, Co-localization of *ORco*, *OR53*, and *OR100* in basiconic sensilla on male antennae by FISH, which were labeled in green, blue, and red, respectively. The nuclei were labeled with DAPI. "Bright" indicates a bright-field microscopy image. "Merge" represents the overlay of signal channels for *OR53*, *OR100*, and *ORco*. NC, negative control. B, Clustering of antennal single-cell transcriptome. UMAP visualization of antennal cells identified 14 cell subpopulations. C, Further clustering of *ORco*-enriched cluster 2 cells into 12 subtypes. D, Distribution of *OR53* and *OR100* in 12 *ORco*-enriched subtypes of class 2 cells from single-cell transcriptome. Cells expressing both *OR53* and *OR100* were found in subtypes 2-3, 2-4, 2-5, and 2-8, while cells expressing predominantly *OR53* were detected in subtype 2-9.

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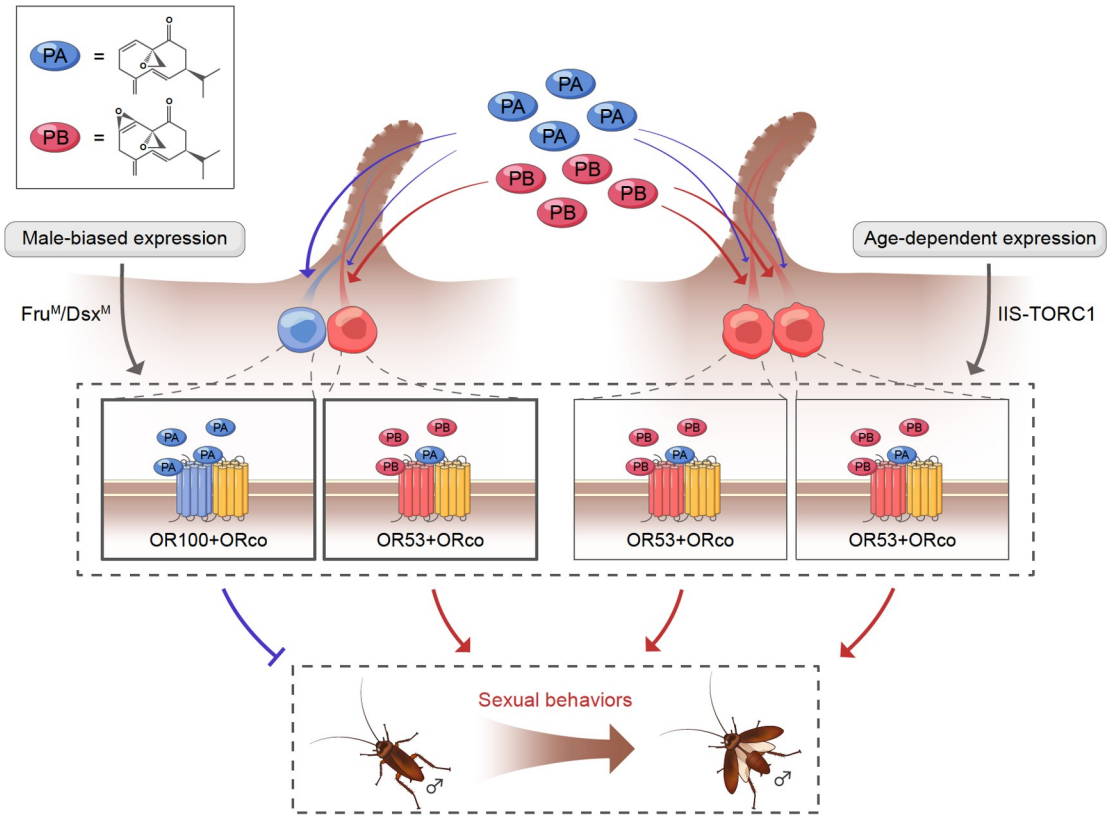

Figure 6. Two-pheromone-two-receptor pathway underlying sexual communication in the American cockroach. Sex pheromone components PB and PA emitted by females are, respectively, recognized by OR53 and OR53/OR100 in ORNs located in two types of basiconic sensilla in males. The basiconic sensilla possibly contain four ORNs (not shown), two of which are sensitive to sex pheromones. The major type of sensilla (left) houses two sex-pheromone sensing ORNs that express OR53 and OR100, respectively. For the minor type of sensilla (right), both sex-pheromone sensing ORNs express OR53. PB activates OR53 ORNs in both types of sensilla to promote male sexual behaviors, while PA mildly activates OR53 and strongly activates OR100 to counteract PB function. The male-biased and age-dependent expression of OR53 and OR100 are regulated by the sex differentiation pathway and nutrition-responsive signals, respectively, to ensure that sexual attraction to the female sex pheromone occurs only in sexually mature males.

sexually mature males. These results demonstrate a two-pheromone-two-receptor pathway in the regulation of cockroach sexual behaviors and have profound implications.

PB has been generally considered to be the major sex pheromone component that attracts males from a long distance, while PA functions at close range, but the exact role of PA is unclear. Our results confirm that PB, but not PA, acts as the major sex attractant. Adult males displayed a significant preference for PB to the control solvent, but they did not differentiate PA alone from the solvent control. Remarkably, although PA alone did not attract or repel adult males, its presence reduced attraction to PB, resulting in the combination of PA and PB (at a ratio of 1:10) being much less attractive than PB alone. Indeed, pheromone blends with counteracting components are widely used to modulate the sexual behaviors of insects (Chang et al., 2017; Johansson and Jones, 2007; Thomas, 2011). As the chemical structures of PA and PB are quite similar, with PA lacking an epoxy group (Okada et al., 1990; Persoons et al., 1979; Seelinger and Gagel, 1985), they might be synthesized through the same pathway with one compound as a precursor or breakdown product of the other. Based on these results, we hypothesized that the concentrations of PA and PB, as well as their relative levels, vary during sexual maturation in female adults so that a high concentration of PB can only be produced for the high sexual attractiveness in sexually mature females. This hypothesis will be tested in our future research.

Out of the over one hundred ORs, we identified OR53 and OR100 as sex pheromone receptors for PB/PA and PA, respectively, in cockroaches. First, these two OR genes were predominantly expressed in the antennae of sexually mature males but not females, suggesting their potential roles in sensing the female sex pheromone. Second, ectopic expression of *OR53* in *Drosophila* ORNs was sufficient to confer the response of these neurons to both PA and PB, with a stronger response to the latter, while OR100 sufficiently responded to PA but not PB. Third, knocking down *OR53* or *OR100* in cockroaches impaired their electrophysiological and behavioral responses to PB/PA and PA, respectively. Finally, knocking down *OR53* but not *OR100* significantly reduced male mating rates. Thus, at the molecular level, PB only activates OR53, while PA activates both OR100 and OR53 potentially with different thresholds. Our FISH experiments and single-cell transcriptome sequencing of antennae further revealed two distinct types of sensilla, the major type containing both OR53 and OR100 ORNs and the minor type containing only OR53 ORNs. We propose that PB activates OR53 ORNs in both types of sensilla to mediate sexual attraction to females, while PA might counteract PB attraction via two possible pathways: (i) PA competes with PB to activate the same receptor OR53 in both types of sensilla to weaken PB-induced sexual attraction, and (ii) PA activates its specific receptor OR100 in the major type of sensilla to prevent sexual behaviors. The dual

roles of PA through both OR53 and OR100 also explain why PA alone does not attract or repel adult males and why knocking down *OR100* does not significantly affect male mating rates. We further propose that such a two-pheromone-two-receptor pathway enables the interplay of sexual stimulation and prevention and fine-tunes proper levels of courtship and mating in males.

Thus, the tuning of cockroach sexual behaviors should depend on the precise control of both *OR53*/*OR100* expression in males and PA/PB levels in females. *OR53* and *OR100* are predominantly expressed in the antennae of sexually mature males, conferring efficient perception of sex pheromones. The male-biased expression of *OR53* and *OR100* is most likely determined by *fru* and *dsx* in the sex differentiation pathway (Chen et al., 2022; Hopkins and Kopp, 2021; Matson and Zarkower, 2012; Zarkower, 2001). Whether the transcription factors Fru^M and Dsx^M regulate *OR53* and *OR100* expression directly or indirectly is still unknown, although our preliminary bioinformatics analysis did not identify well-conserved binding sites of Fru and Dsx in the promoter regions of the two OR genes. Additionally, we provided preliminary evidence that the onset of sex pheromone reception is partially determined by the nutrition-responsive signals, IIS and TORC1, which are significantly up-regulated by feeding in adult cockroaches (Li et al., 2019; Zhu et al., 2020). Such a joint control by sex differentiation and nutritional signals may fine-tune sex pheromone reception in a sex-specific and age-dependent manner. As hypothesized above, the concentrations of PA and PB, as well as their relative levels, might determine the attractiveness of female cockroaches during sexual maturation and, in turn, stimulate male courtship robustness. Recently, the enzyme CYP4PC1 has been identified as the rate-limiting enzyme that controls the production of contact sex pheromones in adult females of the German cockroach (Chen et al., 2022). Interestingly, *CYP4PC1* expression is jointly up-regulated by sex differentiation genes and juvenile hormone (JH) signaling, which is also regulated by IIS and TORC1 (Zhu et al., 2020). Based on the findings in this study, it is plausible that the reception of contact sex pheromones in males of the German cockroach may also be sex-specific and age-dependent. The joint control of female attractiveness (sex pheromones) and male reception (sex pheromone receptors) by sex differentiation, JH, and nutrition-responsive signals may be casual, but mostly likely a potentially conserved mechanism evolved in both the American cockroach and the German cockroach to fulfill sex-specific roles and synchronize the timing for mating success.

The two-pheromone-two-receptor pathway and its identified regulatory mechanisms (Figure 6) provide insights into the control of sexual communication in general and also help develop novel strategies for cockroach management by interfering with cockroach sexual communication and reproduction.

MATERIALS AND METHODS

Animals

Periplaneta americana. The oothecae of the American cockroach, *Periplaneta americana*, were provided by the Jingxin Cockroach Farm, Yunnan Province, China, and maintained in our laboratory as described previously (Li et al., 2018). Briefly, the cockroaches were kept in a well-ventilated box measuring 45 cm×30 cm×30 cm, accommodating 400–500 insects. The cockroaches were provided with commercial rat food and clean water. All cockroaches were maintained at (28±1)°C and a relative humidity of 60%–70% under a 12/12 h light/dark cycle. To obtain pools of synchronized insects, newly emerged male adults were collected from the colony and placed in separate boxes.

Drosophila melanogaster. The ORFs encoding *P. americana OR53* and *OR100* genes were separately cloned into the pJFRC28-10XUAS-IVS-GFP-p10 vector. Primers used in the construction of the vector are listed in Table S1 in Supporting Information. Independent homozygous *UAS-OR53* and *UAS-OR100* transgenic flies were generated by Qidong Fungene Biotech (Qidong, China) and combined with $Or67d^{GAL4}$, which is also an *Or67d* mutant (Kurtovic et al., 2007), to establish homozygous w^+/w^+; *UAS-OR53*/*UAS-OR53*; $Or67d^{GAL4}/Or67d^{GAL4}$ and w^+/w^+; *UAS-OR100*/*UAS-OR100*; $Or67d^{GAL4}/Or67d^{GAL4}$ lines, respectively. *Drosophila* was fed on a cornmeal-agar-molasses medium and maintained at 25°C and relative humidity of 60% under a 12/12 h light/dark cycle (Liu et al., 2018).

Chemicals. The sex pheromones PB and PA (both with a purity of more than 95%) were produced by BioDuro-Sundia (Shanghai, China) and validated through HNMR and HPLC techniques. HPLC-grade hexane and dichloromethane were obtained from Sigma-Aldrich. Rapamycin and LY294002 were purchased from MedChemExpress (USA) (Zhu et al., 2020).

Behavioral assays.

Mating behavior assay. The mating test for cockroach adults was performed in plastic Petri dishes of 25 cm diameter and 4 cm height. For mating assays in wild-type cockroaches (Figure 1A), each male adult collected after eclosion was transferred into a Petri dish containing a sexually mature female at 7–9 DAE and videotaped continuously for nine days to estimate its cumulative mating rate. Ten pairs of male and female adults were used in each experiment, and this design was repeated three times. For the RNAi-mediated mating assay (Figure 4E), each interfered male adult was placed with three wild-type females at 7–9 DAE in a Petri dish and videotaped continuously for four days to evaluate the cumulative mating rate.

Y-tube olfactometer assay. The Y-tube olfactometer was used to assess olfactory responses in sexually mature males (7 DAE) toward PB and PA, which was conducted based on modified protocols (Guo et al., 2022; Wang et al., 2022). The olfactometer consisted of three arms: one 15 cm long arm and two 30 cm long choice arms with a 45° angle between them. The compounds PB and/or PA were applied onto a 3 cm×2 cm filter paper, serving as one odor source, while dichloromethane, the control solvent, was used as the other odor source. The amounts of PA and PB (0.1 ng in Figure S1 in Supporting Information and 1 ng in Figure 1B) were comparable to those used in previous studies (Okada et al., 1990; Seelinger and Schuderer, 1985). A humidified continuous air purified by activated granular carbon (100–150 mL min^{-1}, regulated by flow meters) was blown through each choice arm and carried the two odorants, which were on the filter paper. Individual wild-type males and dsRNA-injected males were placed at the downwind end of the common arm and allowed 5 min to acclimate to the experimental environment. The male was then released and allowed 10 min to make choices, in which only the first selection preference was analyzed. To minimize position bias, the positions of the two long arms were reversed. To

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prevent odor residues, a new Y-tube was used every five assays. Forty males were tested in each experiment.

SSR. SSR experiments were conducted to examine the response of individual sensilla to specific odorants. For SSR in *Drosophila*, transgenic flies at 3–5 DAE were placed in a plastic tube with a 1 cm diameter with their head and antennae fixed with dental wax before T1 sensilla on their antennae were used in recordings. For SSR in cockroaches, wild-type or RNAi-treated males at 7–9 DAE were immobilized on a glass slide, with their head and antennae further fixed with dental wax, and basiconica sensilla on their antennae were used in recordings. To prepare for SSR, Tungsten wire electrodes were electrolytically sharpened by a 40% KNO_2 solution. The recording electrode was inserted into the bottom of either the fly or cockroach sensilla using a micromanipulator (Narishige) connected to amplifiers (IDAC4, Syntech, Germany). The reference electrode was inserted into the eye. To prepare the chemical stimuli, a series of doses of volatile compounds were prepared in hexane using a stepwise dilution method. The working concentrations were prepared by a serial dilution from 100 to 0.1 μg μL^{-1} (100, 10, 1, 0.1 μg μL^{-1}). Hexane was used as a negative control. The solutions were prepared and stored at 4°C. Before every use, the solutions were vortexed at room temperature. To prepare the odorant cartridges, 5–10 μL of diluted pheromone compounds were applied onto a small piece of filter paper (1.5 cm in length), which was then inserted into a Pasteur pipette (ANPEL Lab Tech, Shanghai, China) and used to stimulate the antenna. Stimulus pulses were delivered for 300 ms. The average number of spikes generated by each odorant stimulation was recorded and calculated using Autospike v.3.9 (Syntech) (Wang et al., 2023).

RNA-seq. Various tissues of sexually mature American cockroaches were collected, including antennae, forelegs, heads, testes, mouthparts, and wings from males, as well as antennae from sexually mature females. The antennae of male cockroaches at various ages (from 1 to 9 DAE) were collected. Tissues were ground in liquid nitrogen for RNA extraction with TRIzol reagent (Invitrogen, USA). Four independent replicates were performed for each tissue group. The RNA concentration, purity, and integrity were measured to ensure high-quality RNA for sequencing. Paired-end reads were generated and sequenced on an Illumina platform (Illumina, USA) at Biomarker Technologies Corporation (Beijing, China). Raw sequences were processed into clean reads and mapped to the reference genome (Li et al., 2018). The gene expression levels were estimated using FPKM. Statistical analysis was performed to identify differentially expressed genes among the seven tissues and male antennae from 1 to 9 DAE, with a significance cutoff of FDR<0.05 and $|\log_2 FC|>1$. The corresponding FPKM values of ORs were extracted to compare the expression levels to screen for potential ORs.

PacBio sequencing and gene cloning. Total RNA was isolated from male antennae of sexually mature cockroaches using the TRIzol reagent and assessed as described previously (Li et al., 2022). The extracted RNA was sequenced using PacBio Sequel instruments at the Biomarker Technologies (Beijing, China). High-quality full-length transcripts were recovered and mapped to the reference genome for further structural analysis (Li et al., 2018). Alternative splicing events were identified using the AStalavista tool. The coding sequences of *OR53*, *OR100*, *OR87*, and *ORco* were amplified from cDNA using genomic information (Li et al., 2018) and RNA-seq data. Additionally, the coding sequence of male-specific *dsx* and *fru* derived from PacBio long-read sequencing were cloned from the antennal cDNA library. To obtain the full-length sequence of *OR100*, cDNA ends (RACE) were rapidly amplified with *OR100*-specific primers using a SMARTer RACE 5′/3′ Kit (Clontech, USA). Purified PCR products were sequenced directly using a 3730xl DNA Analyser (Applied Biosystems, USA). The molecular weights of the encoded proteins were predicted using ExPASy (http://web.expasy.org/compute_pi/), and the protein domain architectures were predicted using SMART (http://smart.embl-heidelberg.de/). Sequences of fru^M and dsx^M between American cockroaches and German cockroaches were aligned using Uniprot (https://www.uniprot.org/align) and visualized using the GeneDoc program. The genomic organization of fru^M and dsx^M was analyzed using GSDS 2.0 (http://gsds.gao-lab.org/). The male-specific *fru* and *dsx* isoforms were validated through reverse-transcription PCR (RT-PCR). All the primers used are listed in Table S1 in Supporting Information.

Double-stranded RNA (dsRNA) preparation and RNAi. The dsRNAs for *OR53* (400 bp), *OR100* (301 bp), *ORco* (401 bp), *fru* (216 bp), *dsx* (357 bp), and *GFP* (340 bp) were synthesized using the T7 RiboMAX Express RNAi System (Promega, USA) following the manufacturer's protocol. The fragments for *in vitro* synthesis were amplified from a cDNA library before they were sequenced. The primers for dsRNA synthesis of these genes are listed in Table S1 in Supporting Information. The synthesized dsRNA was diluted to 1 μg μL^{-1} and stored at −80°C before injection. For *in vivo* injection into cockroaches, a volume of 2 μL of dsRNA at 1 μg μL^{-1} for each gene was delivered into the insect's abdomen using a 10 μL volume injector twice, once at 3 DAE and again at 5 DAE. At 7 DAE, total RNA was isolated from the antennae and reverse-transcribed to investigate the relative transcript levels of OR genes by qPCR. All qPCR analyses were performed with four to six biological replicates.

qPCR. Tissues including the antennae, forelegs, heads, mouthparts, testes, and wings of adult males at 7–9 DAE, along with the antennae, forelegs, mouthparts and ovaries of adult females at 7–9 DAE, as well as the antennae in males at 1–9 DAE were dissected, and male antennae were also dissected after knocking down *fru* and *dsx* genes and treatment with rapamycin and LY294002. Total RNA was isolated from the dissected tissues using the TRIzol reagent. cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase and Oligo (dT) primers. qPCR was performed on a QuantStudio™ 6 Flex qPCR system (Thermo Fisher Scientific, USA) using Hieff qPCR SYBR Green Master Mix (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China). The transcription levels of ribosomal protein 49 (rp49) were used for normalization. Three to six replicates were performed for each test group.

Chemical inhibitor treatments. Newly emerged male adult cockroaches were transferred to a new container and sexually isolated. A volume of 2 μL of Rapamycin or LY294002 at a concentration of 2 μg μL^{-1} in dimethyl sulfoxide (DMSO) was injected into these males at 3 DAE and again at 5 DAE (Zhu et al., 2020). DMSO was used as a negative control. Total RNA was isolated from male antennae at 7 DAE. The relative transcript levels of OR genes were assessed by qPCR

FISH. The FISH experiment was conducted using modified protocols (Bantignies et al., 2011; Liu et al., 2018). Briefly, DIG-labeled probes for *OR100* (245 bp) were synthesized using the T7 Quick High Yield Transcription Kit (Beyotime, Shanghai, China) and the DIG RNA labeling mix (Roche, Switzerland). Fluores-

cence-labeled probes for *OR53* (237 bp, Alexa Fluor® 555) and *ORco* (410 bp, Alexa Fluor® 488) were prepared using the FISH Tag RNA Multicolor Kit (Invitrogen). Antisense probes were the positive signal, while the sense probes were the negative control. An intact antenna was dissected from an adult male cockroach at 7–9 DAE before antenna fragments were fixed in 4% paraformaldehyde for 48 h. This was followed by additional fixation in a 50% sucrose solution at room temperature for 24 h, other additional fixation steps, and a series of incubations. Antenna sections were then frozen in Tissue-Tek O.C.T. Compound (Sakura, The Netherlands) and prepared as 10-μm sections using a CM 1950 microtome (Leica, Germany) on slides. The slides were treated with proteinase K (20 μg mL^{-1}) for 10 min before incubation in 5% formaldehyde for 25 min. The slides were hybridized with either the DIG- or fluorescein-labeled probe at a final concentration of 1 μg mL^{-1}. The hybridization process involved denaturation of the probes and incubation at 56°C for 20 h. For post-hybridization, the sections were incubated with specific antibodies. For DIG-labeled probes, the sections were incubated with an anti-DIG-AP antibody (Roche, Switzerland) diluted at a ratio of 1:400. HNPP/Fast Red TR (Roche) was used as a fluorescent substrate for detection. After each incubation step, the sections were washed with ice-cold PBT (0.3% Tween 20). DAPI was used for nuclear staining. Finally, the sections were washed with PBT and fixed on slides. They were observed and photographed under a confocal laser scanning microscope (Olympus FV3000, Olympus Corporation, Japan) at 488 nm, 555 nm, or 647 nm, with the same parameters set for all images.

Single-cell sequencing. Fresh male cockroach antennae were dissected at 9 DAE and quickly processed to prepare a single-cell nuclei suspension for quality control according to the recommended method on the official platform of 10x Genomic (Pleasanton, USA) and Biomarker Technologies Corporation (Beijing, China). A microfluidic chip was used to generate gel beads in emulsion (GEMs) by encapsulating individual cell nuclei, reagents, and gel beads in oil droplets. Inside the GEMs, cells were lysed, and RNA was reverse-transcribed with primers containing a Cell Barcode and Unique Molecular Identifier (UMI). After breaking the GEMs, full-length cDNA was amplified through PCR before it was purified. The cDNA library was sequenced using the Illumina NovaSeq 6000 platform with a sequencing depth of 50,000 reads per cell. Sequencing data were processed using Illumina BaseSpace software version 1.0 for base calling and demultiplexing as described (Wang et al., 2023). The Cell Ranger Single-Cell Software Suite version 7.1.0 was used to align the sequenced reads to the *P. americana* reference genome. Low-quality cells were filtered out using Seurat (version 4.0). After quality control, a total of 7,600 cells were retained. Globally, the filtered cells exhibited a median of 1,384 unique molecular identifiers (UMIs) per cell, mapping to a total of 18,691 genes. UMAP was used for two-dimensional visualization of cell clusters

Statistical analysis. Significant differences in the mating rates of male cockroaches, the spike responses from SSR in wild-type cockroaches and transgenic flies, the FPKM and expression levels of ORs in various tissues and stages were tested for using one-way ANOVA with Tukey's multiple-comparisons test when necessary. The FPKM of *OR53* and *OR100* between male and female antennae, the expression levels of ORs after *fru*-RNAi, *dsx*-RNAi, rapamycin or LY294002 treatment, and the spike responses in *OR53*-RNAi and *OR100*-RNAi-treated cockroaches were compared using a two-tailed unpaired *t*-test. Preference in the Y-tube olfactometer was analyzed using the χ^2 test. *P* values were determined by nonparametric Wilcoxon signed-ranks test. All the error bars associated with the means indicated s.e.m. Differences were considered significant at $P<0.05$. All statistics were performed using IBM SPSS Statistics v.19.0 software. All other data were plotted with GraphPad Prism v.8.0.2.

Compliance and ethics

The author(s) declare that they have no conflict of interest.

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Supporting information

The supporting information is available online at https://doi.org/10.1007/s11427-023-2548-3. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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