Genetic and Neural Mechanisms that Inhibit *Drosophila* from Mating with Other Species

Pu Fan,1,2,9 Devanand S. Manoli,2,3,9 Osama M. Ahmed,5 Yi Chen,7 Neha Agarwal,2 Sara Kwong,2 Allen G. Cai,2 Jeffrey Neitz,6 Adam Renslo,6 Bruce S. Baker,8 and Nirao M. Shah2,4,5,*

1State Key Laboratory of Biomembrane and Membrane Biology, School of Life Sciences, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China
2Department of Anatomy
3Department of Psychiatry
4Center for Reproductive Sciences
5Neuroscience Program
6Small Molecule Discovery Center, Dept. of Pharmaceutical Chemistry
University of California, San Francisco, San Francisco, CA 94158, USA
7Department of Biological Chemistry, HHMI, University of California, Los Angeles, Los Angeles, CA 90095, USA
8Janelia Farm Research Campus, HHMI, Ashburn, VA 20147, USA
9These authors contributed equally to this work
*Correspondence: nms@ucsf.edu
http://dx.doi.org/10.1016/j.cell.2013.06.008

SUMMARY

Genetically hard-wired neural mechanisms must enforce behavioral reproductive isolation because interspecies courtship is rare even in sexually naïve animals of most species. We find that the chemoreceptor Gr32a inhibits male *D. melanogaster* from courting diverse fruit fly species. Gr32a recognizes nonvolatile aversive cues present on these reproductively dead-end targets, and activity of Gr32a neurons is necessary and sufficient to inhibit interspecies courtship. Male-specific Fruitless (FruM), a master regulator of courtship, also inhibits interspecies courtship. Gr32a and FruM are not coexpressed, but FruM neurons contact Gr32a neurons, suggesting that these genes influence a shared neural circuit that inhibits interspecies courtship. Gr32a and FruM are not coexpressed, but FruM neurons contact Gr32a neurons, suggesting that these genes influence a shared neural circuit that inhibits interspecies courtship. Gr32a and FruM also suppress within-species intermale courtship, but we show that distinct mechanisms preclude sexual displays toward conspecifics. Although this chemosensory pathway does not inhibit interspecies mating in *D. melanogaster* females, similar mechanisms appear to inhibit this behavior in many other male drosophilids.

INTRODUCTION

A species can be defined as a set of organisms that share a gene pool and breed with each other (Darwin, 1860; Dobzhansky, 1937; Mayr, 1988). The lack of interspecies breeding results from mechanisms that promote breeding with conspecifics and those that interpose a reproductive barrier between species. Reproductive barriers can occur prior to or after fertilization. If fertilization is successful, there exist genetic pathways that lead to sterile or inviable interspecies hybrids (Coyne and Orr, 1998; Orr et al., 2004; Wu and Ting, 2004). Anatomy, physiology, and geographical isolation impose prefertilization barriers to interspecies breeding. Mechanisms that inhibit sexual displays toward other species are also important prefertilization barriers because such courtship increases predation risk and is energetically and reproductively wasteful. Recognition of conspecifics prior to mating is critical in habitats where many species coexist. Indeed, closely related species of fish, amphibians, and birds do not interbreed despite sharing territory (Blair, 1964; Dobzhansky and Mayr, 1944; Konishi, 1985; Seehausen and van Alphen, 1998). Despite the prevalence of behavioral reproductive isolation and its importance to evolution, the neural pathways that suppress interspecies courtship are poorly understood.

*D. melanogaster* offers a powerful model to study behavioral reproductive isolation. Many drosophilids coexist in nature and the mechanisms that influence courtship in *D. melanogaster* are well studied (Billeter et al., 2006; Dahanukar and Ray, 2011; Siwicki and Kravitz, 2009; Spieth, 1952). Behavioral reproductive isolation appears to operate in *D. melanogaster* because interspecies hybrids are rarely found in nature (Barbash, 2010; Spieth, 1974). The absence of such hybrids does not simply reflect their inability to mature or survive in nature, and previous work suggests that neural pathways that inhibit interspecies courtship in *D. melanogaster* are important for reproductive isolation (Dukas, 2004; Sturtevant, 1920).

We employed behavioral and genetic screens to identify mechanisms that inhibit courtship of *D. melanogaster* males toward other species. We find that Gr32a is required to detect aversive cues on such atypical mating targets and that Gr32a sensory neurons are necessary and sufficient to inhibit courtship of other drosophilids. FruM, a master regulator of male courtship...
Demir and Dickson, 2005; Manoli et al., 2005; Ryner et al., 1996; Stockinger et al., 2005), also suppresses interspecies courtship. Gr32a and FruM are not coexpressed, but Gr32a neurons appear to contact FruM neurons, suggesting that these genes function in the same neural circuit to inhibit courtship of other species. Gr32a and FruM also suppress conspecific intermale courtship (Manoli et al., 2006; Miyamoto and Amrein, 2008). However, we show that distinct mechanisms inhibit courtship of conspecific males and flies of other species. In addition, our observations suggest that other drosophilids employ similar pathways to enforce behavioral reproductive isolation.

**RESULTS**

**The Foreleg Is Essential to Inhibit Interspecies Courtship by Males**

We wished to identify male *D. melanogaster* sensory structures that inhibit courtship with other drosophilids. *D. melanogaster* males utilize vision, hearing, mechanosensation, smell, and taste during courtship (Figure 1A) (Acebes et al., 2003; Greenspan and Ferveur, 2000; Kowalski et al., 2004; Krstic et al., 2009; Robert-son, 1983; Spieth, 1974; Tompkins et al., 1980, 1982). Accordingly, we asked whether these modalities inhibited interspecies courtship by males.
courtship. We used conspecific or *D. virilis* females as mating partners of socially naive *D. melanogaster* males lacking specific sensory input (Figures 1B and 1G). *D. virilis* shared an ancestor with *D. melanogaster* ~40 million years ago (mya), and wild-type (WT) *D. melanogaster* males do not court *D. virilis* females (Figure 1H). Males lacking olfactory (antennae or maxillary palps) or auditory (antennae) structures as well as males tested in the dark courted conspecific but not *D. virilis* females (Figure 1). Gustatory cues are detected by neurons on mouthparts and on foreleg tarsi. Removal of all mouthparts led to dessication and a deterioration in general health and mating performance (data not shown). We therefore extirpated only the male labellum, the mouthpart that likely contacts the female. Such males courted conspecific, but not *D. virilis*, females (Figure 1). Males usually tap other flies with their foreleg tarsi prior to proceeding toward a conspecific, but not the mouthpart that likely contacts the female. Such males not shown). We therefore extirpated only the male labellum, the mouthpart that likely contacts the female. Such males courted conspecific, but not *D. virilis*, females (Figure 1). Males usually tap other flies with their foreleg tarsi prior to proceeding with courtship (Figure 1A) (Bastock and Manning, 1955). The foreleg is required to inhibit *D. melanogaster* males from courting *D. simulans* females, a species that diverged from *D. melanogaster* ~2 mya (Manning, 1959). Males lacking both foreleg tarsi courted conspecific and *D. virilis* females with a similar courtship index (CI), the fraction of time spent courting (Figures 1C and 1H). *D. virilis* females were not receptive to *D. melanogaster* males as evidenced by repeated kicking and walking away (data not shown). Nevertheless, tarsiless males reliably displayed sustained courtship, including courtship song and copulation attempts, toward *D. virilis* females (Figures 1H–1K). Thus, foreleg tarsi are required to inhibit *D. melanogaster* males from courting *D. virilis*, a distant drosophilid.

**Identification of Chemosensory Neurons that Inhibit Interspecies Courtship**

We sought to identify the foreleg neurons that inhibit interspecies courtship by males. The tarsi contain chemosensory neurons that detect contact-based chemical cues (Dethier and Chadwick, 1948; Dunipace et al., 2001; Frings and Frings, 1949; Scott et al., 2001). The fly genome encodes a gene family of gustatory receptors (Grs) that are expressed in chemosensory neurons (Clyne et al., 2000; Dunipace et al., 2001; Hallem et al., 2006; Scott, 2005; Scott et al., 2001). To identify Grs expressed in foreleg tarsal neurons, we used 20 published *Gr-GAL4* lines to express nuclear EGFP (*stingerGFP; UAS-stingerGFP*). We identified eight Grs expressed in male foreleg tarsi (Figures 2A–2H, S1, available online, and Table S1; see also Extended Experimental Procedures), some of whose expression patterns have been described (Bray and Amrein, 2003; Moon et al., 2009; Scott et al., 2001; Thorne and Amrein, 2008; Weiss et al., 2011).

We used these eight *Gr-GAL4* lines to ablate chemosensory neurons with *UAS-head involution defective (UAS-hid)* and assess their role in inhibiting interspecies courtship (Figure 2). Strikingly, ablation of Gr32a or Gr33a neurons, but not other Gr neurons, allowed *D. melanogaster* males to court *D. virilis* females (Figures 2I and 3). The extent and quality of courtship toward *D. virilis* females displayed by males lacking Gr32a or Gr33a neurons resembled that seen with conspecific females despite rejection by *D. virilis* females (Figure 3 and data not shown).

The specificity of the phenotype observed with Gr32a:hid and Gr33a:hid could reflect the possibility that only these GAL4 and HID pairings ablated the corresponding sensory neurons. We tested this directly by driving *stingerGFP* and *hid* in Gr neurons (*Gr:stingerGFP, hid* to visualize their loss. We find comparable reduction of sensory neurons with these eight Gr lines, with only an occasional escapee (Figures 2A–2H, S1, Table S1). Thus, the other Gr neurons we tested are not required to inhibit interspecies courtship. Although Gr32a and Gr33a are expressed in the foreleg and labellum, removal of the former but not the latter permits interspecies courtship. Thus, our findings indicate that Gr32a or Gr33a foreleg neurons inhibit courtship toward *D. virilis* females.

We tested whether Gr32a and Gr33a neurons also inhibited males from courting females of *D. simulans* and *D. yakuba*, species that diverged from *D. melanogaster* ~2 and ~10 mya, respectively. We find that Gr32a:hid and Gr33a:hid males avidly courted conspecific as well as *D. simulans*, *virilis*, and *yakuba* females (Figure 3). The vast majority of these assays had high levels of courtship, including attempted copulation by the experimental males (Figure 3). Males displayed attempted copulation most toward *D. virilis* females. In fact, *D. virilis* females move less and more slowly compared to the other females we tested, and this may allow males to attempt copulation more frequently. *D. virilis* females may also provide other cues (or lack chemorepellents) that elicit courtship in the absence of Gr32a or Gr33a neurons. In summary, Gr32a and Gr33a neurons inhibit courtship toward females of diverse species that last shared an ancestor with *D. melanogaster* 2–40 mya.

**Gr32a Inhibits Interspecies Courtship**

In the foreleg, most Gr32a neurons also express Gr33a (Moon et al., 2009). Thus, one or both of these Grs could be required to inhibit interspecies courtship. We tested *D. melanogaster* males null for Gr32a (Gr32a−/−) or Gr33a (Gr33a−/−) for courtship toward females of other species (Miyamoto and Amrein, 2008; Moon et al., 2009). Gr32a−/−, but not Gr33a−/−, males courted *D. simulans*, *virilis*, and *yakuba* females (Figure 4, Movies S1, S2, and S3). Gr32a−/− males displayed the entire range of courtship preceding copulation toward females of all species and copulated with conspecífics (Figure 4 and data not shown).

Two Grs, Gr5a and Gr66a, that detect sugars and bitter tastants, respectively, are broadly expressed in tarsal neurons (Chyb et al., 2003; Koganezawa et al., 2010; Thorne et al., 2004; Wang et al., 2004). Ablating Gr5a neurons (Gr5a:hid) did not permit courtship of other species (data not shown). Gr66a−/− males also do not court nonconspecific females (Figure S2). Thus, inhibition of interspecies courtship may not be a general function of chemoreceptors that detect aversive tastants. Rather, we have uncovered a role of Gr32a in restricting *D. melanogaster* males to courting conspecific females.

We further confirmed the role of Gr32a in inhibiting interspecies courtship by using RNAi to knockdown Gr32a. We used the pan-neuronal C155-GAL4 to drive two separate RNAi constructs targeting Gr32a (Dietzl et al., 2007). Male flies expressing each of these transgenes courted conspecific females and females of other species (Figure S2). Thus, disruption of Gr32a function, either by a null mutation or by RNAi, permits *D. melanogaster* males to court females of many other drosophilids without disrupting courtship of conspecific females.
Gr32a Neurons Function Acutely to Inhibit Interspecies Courtship

Our findings so far suggest that activity of Gr32a neurons suppresses sexual displays toward nonconspecific females. We tested this possibility by expressing the temperature-sensitive dominant negative dynamin mutant, shibirets (UAS-shit), in Gr32a neurons (Kitamoto, 2001). At permissive temperatures, Gr32a:shit males courted conspecific, but not D. virilis, females (Figures 5A and 5C). However, at restrictive temperatures, when synaptic vesicle recycling is inhibited by Shi ts, these males courted D. virilis females as avidly as conspecific females (Figures 5A and 5C). Thus, functional silencing of Gr32a neurons permits interspecies courtship even though these neurons express WT Gr32a.

We tested whether heterologous excitation of Gr32a neurons inhibits interspecies courtship in Gr32a−/− males. We therefore generated males that expressed the heat-activatable cation channel, dTrpA1 (UAS-dTrpA1) (Pulver et al., 2009), in neurons that would normally express Gr32a (Gr32a−/−, Gr32a:dTrpA1). As expected, these flies courted D. virilis females at the permissive temperature (Figures 5B and 5D). By contrast, at an elevated temperature that activates dTrpA1 these males courted conspecific but not D. virilis females (Figures 5B and 5D). Thus, activity of Gr32a neurons abrogates interspecies courtship but does not

Figure 2. Identification of Gr Neurons in the Male Foreleg that Inhibit Interspecies Courtship

(A–H) Expression of different Grs (A–H) and ablation of Gr neurons (A’–H’) in foreleg tarsi. Whole-mount preparation of tarsal segments 4 and 5 (t4, t5) (A, A’, and C–H’) and t2 (B and B’) shown. More distal tarsal segments are on the left.

(I) Ablation of Gr32a or Gr33a neurons in D. melanogaster males permits courting of D. virilis females.

All statistical comparisons in this and subsequent figures were performed between experimental and the corresponding control genotypes. Error bars represent SEM; n = 5–10/genotype (A–H) and n = 8–12/genotype (I); ∗p < 0.001; scale bar, 50 μm.

Please see Figure S1 and Table S1.
appear to significantly inhibit courtship of conspecific females. In summary, functional activation of Gr32a neurons is necessary and sufficient to inhibit courtship specifically toward reproductively futile targets such as females of other species.

**Gr32a Is Required to Detect Aversive Ligands Secreted by Other Species**

We sought to determine the cues recognized by Gr32a that restrict courtship to conspecific females. Chemosensory cues encoded by cuticular hydrocarbons (CHs) profoundly influence social behavior in flies (Antony and Jallon, 1982; Billeter et al., 2009; Coyne et al., 1994; Ferveur, 2005; Grillet et al., 2012; Higgie et al., 2000; Jallon and David, 1987; Savarit et al., 1999). We asked whether cuticular extracts from *D. simulans*, *D. yakuba*, and *D. virilis* females inhibited courtship by *D. melanogaster* males. *WT* males courted oenocyteless (oe–) females (Billeter et al., 2009), including when oe– females were coated with conspecific cuticular extract, but they showed minimal courtship of oe– females coated with cuticular extracts from other species (Figure 5E). Strikingly, *Gr32a*–/– males courted oe– flies regardless of the source of the cuticular extract (Figure 5E). Thus, cuticular extracts from other drosophilids inhibit sexual displays by *WT melanogaster* males in a Gr32a-dependent manner.

We wished to identify the cuticular compounds that inhibit interspecies mating. The CH z-7-tricosene (7T; Figure S3) is secreted by *D. melanogaster* males and to ≥10-fold lesser extent by females (Jallon and David, 1987), and it inhibits intermale courtship (Ferveur, 2005; Lacaille et al., 2007). Moreover, Gr32a is required to detect 7T (Wang et al., 2011). Both sexes of *D. simulans* and *D. yakuba* secrete 7T in copious amounts (Jallon and David, 1987), and we asked whether 7T-coated oe– females would be courted by *D. melanogaster* males. We found...
that Gr32a−/−, but not WT, males courted oe− targets coated with physiological concentrations of 7T similar to control oe− or WT melanogaster females (Figure 5E). Although 7T is secreted by many drosophilids, it is essentially undetectable on the D. virilis cuticle. D. virilis, but not melanogaster, simulans, or yakuba, secrete the related CH z-9-tricosene (9T; Figure S3) (Ferveur, 2005; Liimatainen and Jallon, 2007). Gr32a−/−, but not WT, males courted 9T-coated oe− females vigorously (Figure 5E). Cuticular extracts from D. virilis appeared more effective than 9T alone in suppressing courtship of oe− females, suggesting the presence of other CHs on D. virilis that inhibit courtship. One such CH may be z-11-pentacosene (11P; Figure S3), which appears restricted to D. virilis (Ferveur, 2005). We synthesized 11P (Figure S3) and tested whether 11P-coated oe− females elicited courtship. We found that Gr32a−/−, but not WT, males courted such females vigorously (Figure 5E). Oe− females coated with both 9T and 11P did not elicit less courtship by WT males compared to 11P alone (Figure 5E), consistent with the notion that both cues are recognized by Gr32a. In summary, Gr32a is required to detect at least three CHs, 7T, 9T, and 11P, secreted by conspecific males or flies of other species but not by conspecific females, and this recognition inhibits courtship of such reproductively dead-end targets.

A Distinct Cellular Pathway Inhibits Interspecies Courtship

FruM isoforms are necessary and sufficient for most components of male courtship (Demir and Dickson, 2005; Gill, 1963; Hall, 1978; Ito et al., 1996; Manoli et al., 2005; Ryner et al., 1996; Stockinger et al., 2005). We tested whether FruM also restricts courtship to conspecifics. Males null for FruM (fru4−/−frusuT15) did not court any targets, including conspecific females, and this recognition inhibits courtship of such reproductively dead-end targets.

A Distinct Cellular Pathway Inhibits Interspecies Courtship

FruM isoforms are necessary and sufficient for most components of male courtship (Demir and Dickson, 2005; Gill, 1963; Hall, 1978; Ito et al., 1996; Manoli et al., 2005; Ryner et al., 1996; Stockinger et al., 2005). We tested whether FruM also restricts courtship to conspecifics. Males null for FruM (fru4−/−frusuT15) did not court any targets, including conspecific females, and this recognition inhibits courtship of such reproductively dead-end targets.

Figure 4. Gr32a Inhibits Interspecies Courtship

(A–P) Gr32a and Gr33a mutant and control D. melanogaster males were tested for courtship with females. (A–D) No difference in courting conspecific females between control and Gr32a or Gr33a mutants. (E–P) Gr32a, but not Gr33a, mutants court D. simulans (E–H), D. yakuba (I–L), and D. virilis (M–P) females. Error bars represent SEM; n = 10–24/genotype; *p < 0.05, **p < 0.001; NS = not significant. Please see Figure S2 and Movies S1, S2, and S3.
We therefore tested whether fru-1Δ/Δ or Gr32a+/− males courted males of other species. We find that FruM or Gr32a mutant males court conspecific, D. simulans and yakuba males, but not virilis males (Figures S4F and S4I), thereby revealing a broad, but not comprehensive, deficit in sex and species recognition. It is unlikely that a loss of sex recognition in FruM or Gr32a mutant males would permit them to court same-sex conspecifics as well as other drosophilids (Grosjean et al., 2008). Indeed, Gr32a+/− males also court conspecific males (Figure S4G) (Moon et al., 2009), but they do not court other drosophilids (Figures 4 and S4G). Moreover, males mutant for Ppk23, a Degenerin/Epithelial sodium channel expressed in FruM neurons in foreleg tarsi, court conspecifics of both sexes (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012), but these mutants did not court individuals of other species (Figures S2 and S4H). Thus, a loss of sex recognition is not sufficient to permit courtship of other species, and different molecular and cellular pathways regulate courtship of conspecific males and other drosophilids.

We wondered whether FruM functioned in Gr32a neurons to inhibit interspecies courtship. Gr32a neurons in adult foreleg tarsi and labellum do not express FruM (Figures 6B–6D, S4K–S4M, and data not shown). To preclude transient or weak, undetectable, FruM expression in Gr32a neurons, we utilized a validated RNAi strain (UAS-fruMIR) (Manoli and Baker, 2004) to knockdown FruM in Gr32a cells. However, Gr32a:fruMIR flies also did not court D. virilis females (Figure S4A). We cannot exclude the possibility that FruM regulates differentiation of Gr32a neurons prior to Gr32a expression to regulate interspecies courtship. Nevertheless, our findings indicate that FruM is not required in Gr32a neurons to inhibit interspecies courtship.

We tested whether Gr32a neurons might contact FruM neurons. We employed an enhanced variant of GFP reconstitution across synaptic partners (GRASP) (Feinberg et al., 2008) in which one component of GRASP is targeted to synapses, thereby restricting GFP reconstitution to synapses. Briefly, spGFP1-10 was targeted to synapses by fusing it to Neurexin (UAS-spGFP1-10::Nrx), a transmembrane protein involved in synapse formation and maturation (Knight et al., 2011), and spGFP11 was fused to CD4 (LexO-spGFP11::CD4) (Gordon and Scott, 2009) to permit cell-surface expression. Our strategy labeled a known...
synapse but not neighboring pre- or postsynaptic processes. L3 and Tm9 neurons have processes outside the M3 medullary layer, but only synapse within M3 (Gao et al., 2008; Yamaguchi and Heisenberg, 2011); correspondingly, we observed native GFP fluorescence only in M3 but not in L3 or Tm9 processes (Figures S4N–S4Q). In our experimental flies, we observed native GFP fluorescence in the ventral nerve cord (VNC) and the subesophageal ganglion (SOG) (Figures 6E–6G, see also Figures S4R–S4T), locations at which tarsal sensory neurons synapse with central neurons (Dunipace et al., 2001; Scott et al., 2001; Stocker, 1994). Such GRASP signal suggests synaptic contact between Gr32a and FruM neurons that will have to be verified with electron microscopy or electrophysiology. Removal of foreleg tarsi eliminated native GFP fluorescence in the VNC and the vertical limb of innervation in the SOG (Figures 6H and 6I), demonstrating that these contacts with FruM neurons emanated from foreleg Gr32a neurons (Wang et al., 2004). The residual GRASP fluorescence in the SOG is consistent with projections of proboscis Gr32a neurons. Our results are consistent with the notion that Gr32a and FruM function within a shared neural circuit to inhibit interspecies courtship.

The enhancer trap P52A-GAL4 labels a bilateral set of ~60 FruM neurons (aDT6 neurons) within the SOG (Cachero et al., 2010; Manoli and Baker, 2004; Yu et al., 2010). Knockdown of FruM in aDT6 cells (P52A:fruMIR) permits males to sing and copulate without tapping a conspecific female (Manoli and Baker, 2004). Importantly, P52A:fruMIR males court conspecific females but not males, suggesting that sex recognition and mating can occur without tapping (Manoli and Baker, 2004). We wondered whether these males would court other species. Strikingly, P52A:fruMIR males courted D. simulans, virilis, and yakuba females and yakuba males (Figures 6J and S4J). In contrast to courtship of conspecific females, P52A:fruMIR males sang only after tapping nonconspecific flies (Table S2). Our findings suggest that males can recognize conspecific females as mating targets prior to tapping, which may be used to determine species membership before proceeding with courtship. In any event, aDT6 cells define a central neuronal
population that inhibits interspecies, but not conspecific intermale, courtship in a FruM-dependent manner. These findings provide further evidence showing that distinct cellular and molecular mechanisms inhibit intermale conspecific and interspecies courtship.

We tested whether aDT6 neurons are postsynaptic to Gr32a SOG projections using our enhanced GRASP variant. Despite the widespread expression of the P52A-GAL4 driver (Manoli and Baker, 2004), we did not observe native GFP fluorescence in the SOG (Figures S4U–S4V). The lack of GRASP signal does not reflect failure of expression of GRASP components because these could be visualized with immunolabeling (Figures S4W–S4W). We also did not observe apposition of Gr32a and aDT6 processes within the SOG using the fly brainbow system (Figure S4X and Movie S4; n = 11) (Hampel et al., 2011). Thus, if Gr32a and FruM aDT6 neurons inhibit interspecies courtship via a shared circuit, they are synaptically linked via one or more interposed neurons.

DISCUSSION

Mythological assertions notwithstanding, animals rarely pick mates from other species (Ovid, Metamorphoses). The
reproductive isolation imposed by inhibiting interspecies mating affords a powerful barrier to the admixing of gene pools. We have uncovered genes and neural pathways in *D. melanogaster* males that inhibit interspecies courtship. Although *D. melanogaster* females utilize unrelated mechanisms to reject males of other species, remarkably, many other drosophilids may employ a similar pathway to *D. melanogaster* males to reject nonconspecific females.

**Chemical Control of Interspecies Courtship**

Gr32a belongs to a family of contact-based chemoreceptors, whose putative ligands, tastants, and pheromones elicit robust spiking in sensory neurons (Hallem et al., 2006; Scott, 2005). Gr32a is required for the response to many aversive, bitter-tasting compounds, including alkaloids such as lobeline and the insect repellent N, N, diethyl-meta-toluamide (DEET). The Grs coexpressed with Gr32a, Gr33a, and Gr66a, also respond to these or other bitter, aversive tastants (Lee et al., 2010; Moon et al., 2006, 2009; Weiss et al., 2011). Here, we show that Gr32a is required for *D. melanogaster* males to detect diverse CHs found on other drosophilids and *D. melanogaster* males but not females. These CHs appear to serve as semiochemicals such that their presence on potential sexual partners permits *D. melanogaster* males to reject them as mates. These findings suggest a model wherein activation of Gr32a neurons by diverse cues may lead to avoidance of a potential food source or mate.

It is surprising that Gr32a is required for the recognition of diverse compounds such as alkaloids, the dialkylation DEET, and CHs. It is unknown whether Grs detect such ligands in the absence of additional coreceptors or cofactors. It is possible, therefore, that Gr32a partners with different coreceptors to detect these distinct cues (Figures S3E–S3I). Even though Gr32a, Gr33a, and Gr66a recognize alkaloids, only Gr32a is required to recognize CHs on flies. Although we have tested diverse drosophilids, Gr33a and Gr66a may recognize CHs that were not tested in this study. CH detection by these Grs may also be redundant to recognition by Gr32a. In any event, Gr32a is required for the detection of aversive CHs on nonconspecifics and for inhibiting interspecies courtship.

**A Molecular and Neural Pathway that Inhibits Interspecies Courtship**

Despite pioneering efforts (Coyne et al., 1994; Hollocher et al., 1997; Laturvey and Moehring, 2012; Manning, 1959; May and Dobzhansky, 1945; Moehring et al., 2006; Nanda and Singh, 2012; Ritchie et al., 1999; Shirangi et al., 2009; Smadja and Butlin, 2009; Spieth, 1949; Sturtevant, 1920), little is known about the neural pathways that inhibit interspecies mating. Gr32a appears to function in foreleg neurons to inhibit interspecies courtship, consistent with the observation that *D. melanogaster* males tap potential mates early during courtship. Labellar Gr32a neurons may be redundant to Gr32a foreleg neurons, they may lack a coreceptor essential for recognizing CHs, or their distinct central projections may not activate circuits that inhibit interspecies mating (Park and Kwon, 2011; Wang et al., 2004). Labellar Gr32a neurons are also likely activated during licking, a step by which males may be unable to disengage from mating. Indeed, courtship is thought to proceed via steps whose initiations depend on progressive sensory input (Manoli and Baker, 2004). Regardless, Gr32a foreleg neurons appear to inhibit interspecies courtship, and this foreleg inhibitory pathway is conserved across many drosophilids.

Heterologous activation of Gr32a neurons suppresses interspecies courtship by Gr32a−/− males. Such activation does not significantly inhibit courtship of conspecific females. In fact, distinct genes, chemosensory neurons, and pheromones are important for courting conspecific females (Bray and Amrein, 2003; Ejima and Griffith, 2008; Grosjean et al., 2011; Kurtovic et al., 2007; Lin et al., 2005; Lu et al., 2012; Thistle et al., 2012; Watanabe et al., 2011). Thus, neural pathways that elicit courting of conspecific females may override courtship-inhibiting signaling by Gr32a neurons. Our findings also suggest that, in addition to courtship-promoting neural circuits, evolutionary constraints can select for pathways such as Gr32a and FruM neurons that suppress courtship of reproductively futile targets.

Several observations show that Gr32a mutant males are not simply hypersexual. They court conspecific females in a WT manner (Miyamoto and Amrein, 2008) and spend less time court-
ing conspecific males than females. Gr32a mutants also court *D. virilis* females but not males, nor do they court ants and houseflies (data not shown), observations that suggest the existence of other pathways to inhibit such courtship. Thus, loss of Gr32a function does not lead to a release of sexual behavior toward all similarly-sized moving objects.

Gr32a also regulates intrasexual aggression (Wang et al., 2011). Gr32a−/− males may court target flies of other species or conspecific males because they cannot fight with them. However, WT males did not attack *D. virilis* targets of either sex, and Gr32a−/− males courted *D. virilis* females vigorously. Rather than modulate aggression, functional activation or inactivation of Gr32a neurons regulated interspecies courtship with *D. virilis* females. It is possible that Gr32a first mediates species recognition, and if the fly is a male conspecific then Gr32a may activate aggression. Regardless, Gr32a inhibits interspecies courtship, and Gr32a neurons acutely inhibit courtship of reproductively futile targets such as members of other species.

Separable genetic and neural mechanisms in *D. melanogaster* males inhibit courtship of conspecific males and other species. Gr33a and Ppk23 inhibit courting of conspecific males but not other species. The few Gr33a foreleg neurons that do not express Gr32a may specifically preclude mating with conspecific males (Moon et al., 2009). FruM function in aDT6 neurons inhibits courtship of other species but not conspecific males. Thus, the mechanisms that inhibit interspecies and same-sex conspecific courtship are doubly dissociable.

**Molecular Mechanisms of Speciation**

One intuits that multiple sensory pathways recognize conspecifics as well as nonconspecifics. Strikingly, however, Gr32a sensory pathways alone are necessary and sufficient to inhibit courtship toward nonconspecifics of diverse drosophilids. Although sensory pathway evolution underlies many behavioral adaptations, Gr32a is, to the best of our knowledge, the first sensory receptor found to inhibit interspecies courtship behavior (Gracheva et al., 2010, 2011; Jiang et al., 2012; Jordt and Julius,
SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures four figures, two tables, and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.06.008.

ACKNOWLEDGMENTS

We are grateful to S.L. Zipursky for discussions and sharing GRASP reagents and data and to G. Meisner, A. Nern, B. Pfeiffer, and G. Rubin from JFRC for reagents and advice on GRASP studies. We thank S. Barondes, Y. Rao, and K. Yamamoto for support; C. Bargmann, E. Kandel, and Z. Knight for discussions; R. Axel, T. Clandinin, Y. Jan, D. Julius, S. Lomvardas, K. Scott, and Shah lab members for comments on the manuscript; the laboratories of J. Carlson, T. Clandinin, A. Dahanukar, G. Davis, U. Heberlein, L. Jan, and Y. Jan for sharing fly reagents or equipment; and the UCSF Nikon Imaging Center for providing critical imaging resources. This work was supported by China Scholarship Council (P.F.), NARSAD and Program for Breakthrough Biomedical Research (D.S.M. and N.M.S.), NSF graduate fellowship (O.M.A.), Career Awards in Biomedical Sciences from the Burroughs Wellcome Fund, Ellison Medical Foundation, McKnight Foundation for Neuroscience, and Sloan Foundation (N.M.S.).

Received: April 12, 2012
Revised: April 12, 2013
Accepted: June 10, 2013
Published: June 27, 2013

REFERENCES


Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

Drosophila Stocks, Culture, and Surgery
With the exception of the Gr32a-LexA and UAS-spGFP1-10::Nrx strains, the flies we have used in this study have been described before (Anand et al., 2001; Barolo et al., 2004; Goodwin et al., 2000; Gordon and Scott, 2009; Hampel et al., 2011; Kitamoto, 2001; Lu et al., 2000; Manoli and Baker, 2004; Melnattur et al., 2002; Scott et al., 2001; Wang et al., 2004). The D. pseudoobscura, simulans, virilis, and yakuba stocks were obtained from the Drosophila Species Stock Center at UC, San Diego. The UAS-mCD8GFP and UAS-hid strains were obtained from the Bloomington Drosophila Stock Center. All flies were in the Canton-S background except Gr32a (Oregon-R), and WT controls for the 3 mutant flies (Gr32a, Gr33a, Gr66a) were from the corresponding background strain.

The Gr32a-LexA DNA construct was generated by subcloning the 3.6 kb Gr32a promoter region into the pBPLexA::p65Uw vector lacking DSCP (Pfeiffer et al., 2010; Weiss et al., 2011). To generate the UAS-spGFP1-10::Nrx DNA construct, the N-terminal signal peptide and spGFP1-10 was PCRRed from constructs previously described (Feinberg et al., 2008) with S’ Eco RI site and 3’ Kpn I-TAA-Xba I sites, and cloned into pUAST resulting in pUAST::spGFP1-10. Beginning with residue 107, the Nrx coding sequence was amplified and cloned in-frame into the 3’ Kpn I site. Transgenic flies bearing these constructs were generated according to standard protocols. All flies and crosses were raised and maintained on standard cornmeal/molasses media at 25°C with 12:12 light:dark cycle and at 70% humidity. The only exceptions were for crosses using UAS-sh♯ (18°C), UAS-dTrpA1 (18°C), UAS-dBrainbow (18°C), UAS-hid (25°C or 29°C), and those involving RNAi (29°C). The Gr:hid behavioral screen to identify chemoreceptors that inhibit interspecies mating was done with flies raised at 25°C. We subsequently observed a more robust cellular ablation and behavioral phenotype when the flies were raised at 29°C, and we therefore present data for Gr32a:hid and Gr33a:hid with D. simulans and yakuba from flies raised at 29°C. Even under these optimal culture conditions, we observed essentially no ablation of Gr28a and Gr68a neurons (Gr:stingerGFP, hid), and these flies were not analyzed further.

Experimental animals were collected within 12 hr of eclosion. The males were entrained at rearing temperature in isolation for 7–10 days. Virgin females were used 3–5 days after eclosion. The only exception to these ages was that we used male and female D. pseudoobscura and virilis at 10–14 days and 7–10 days after eclosion, respectively. In instances where behavioral assays were performed between males whose genotypes or species membership were not easily distinguishable, we trimmed the wings of target males bilaterally. The tarsi, antennae, and palps were surgically removed bilaterally under anesthesia 1–3 days prior to behavioral testing. The labellum was surgically removed under anesthesia 3–4 hr prior to behavioral testing. Oe– females and dBrainbow males were generated as described previously with the exception that dBrainbow males were raised at 18°C and not subjected to heat shock (Billeret et al., 2009; Hampel et al., 2011).

Histology
With the exception of GRASP samples, proboscis, forelegs, and CNS structures were dissected in ice-cold PBS, fixed in 4% paraformaldehyde at 22°C, washed, stained, and then mounted as described before (Manoli et al., 2005). Samples stained for GRASP components were processed as native GRASP fluorescence, with the exception that all staining and washes were conducted in PBT. Primary antibodies used were: 1) for visualization of GRASP components, mouse anti-GFP (1:1000; Invitrogen, #A11122; 1:100; Sigma, #G6539), rabbit anti-GFP (1:800; Abcam, ab290); 2) for dBrainbow imaging, rabbit anti-GFP (1:500; Invitrogen, #A11122), mouse anti-Myc (1:50; Developmental Studies Hybridoma Bank, clone 9E10), and rat-anti-HA (1:100; Roche, #11867423001). Secondary antibodies were AlexaFluor488 anti-rabbit (1:500, Molecular Probes, #A-11034), AlexaFluor488 anti-mouse (1:500, Molecular Probes, #A-11001) Cy3 anti-Rat (1:500, Jackson ImmunoResearch, #712-166-150), and Cy3 anti-mouse (1:500, Jackson ImmunoResearch, #715-166-150). Samples were imaged using a Zeiss LSM700 or Nikon Spectral C1si Confocal and processed in ImageJ. 3D-reconstruction of dBrainbow clones was performed using the Nikon Elements or Imaris software packages.

Preparation and Coating of oe– Females
Cuticular extracts were prepared by washing 25-30 D. melanogaster, simulans, or yakuba flies with 50 μL of hexane, briefly and gently vortexing, and aspirating liquid. Extracts from D. virilis were prepared using 15-20 flies in a similar volume. For transfer of extracts to oe– flies, 50 μL of extract were pipetted onto filter paper in a 1.5 ml tube and solvent allowed to evaporate for 30 min. 3-5 flies were placed into the tube, and gently vortexed 3x for 20s each with 20s pauses. For individual compounds, previous work has demonstrated that ~3% of the total amount of a given compound that is placed onto a filter paper as above is transferred to each fly when 7 flies are prepared at a time using this technique (Wang et al., 2011). We therefore transferred 30x of the desired dose of 7T, 9T, and 11P on to a filter paper and coated seven target flies at a time as above. Target amounts of individual compounds were as follows: 7T, 1 μg/fly (Wang et al., 2011); 9T, 0.1 μg/fly (Butler et al., 2009); 11P, 2 μg/fly (Oguma et al., 1992).

Drosophila Behavioral Assays
Courtship assays were performed at zeitgeber 6–10 hrs. Tarsectomized D. yakuba males were introduced via mouth pipette into the courtship chamber without anesthesia. All courtship assays were performed at 22°C except in studies using UAS-sh♯ and UAS-dTrpA1 in which case the flies were tested at 18°C or 31°C. For tests performed at 31°C, the flies were warmed at 31°C for
20 min (UAS-shR8 experiments) or 40 min (UAS-dTrpA1) prior to behavioral assays. The courtship assays were illuminated by a fluorescent ring lamp (22W) suspended ~4 cm above the behavioral chamber and recorded with a Sony camcorder (HDR-XR550V). To test the requirement of vision in inhibiting interspecies courting, the behavioral assays were performed in the absence of visible light, illuminated by infra-red LED, and recorded as above. Behavioral assays were scored blind to the relevant experimental variables such as genotype and temperature, using ScoreVideo, a software package developed in MATLAB (Wu et al., 2009). Individual steps in courtship, including tapping, unilateral wing extension (courtship song), abdominal bending (attempted copulation), and copulation were scored. In addition, we scored courtship as a separate parameter, defined as the period of time engaged in any of the above steps subsequent to the first wing extension. The CI was calculated as the time spent in courtship, as defined above, divided by the total observation time of 15 min.

11P Synthesis and Analysis

**Pentacose-11-yne**

A solution of n-BuLi (1.6 M in hexanes, 1.0 ml, 1.6 mmol, 1.1 eq.) was added dropwise with stirring to a cooled (~75 °C, bath temperature) solution of 1-dodecene (0.31 ml, 1.43 mmol) in dry THF (2 ml) containing HMPA (0.5 ml). The mixture was stirred for 2 hr then a solution of 1-bromotridecane (0.440 ml, 1.72 mmol, 1.2 eq.) in dry THF (0.5 ml) was added dropwise over 1–2 min. The mixture was allowed to warm to room temperature as the cooling bath melted. The reaction mixture was diluted with brine (10 ml) and extracted with hexane (2 × 10 ml). The combined extracts were dried (Na2SO4). The drying agent was removed by filtration. Silica gel (~1 g) was added and the filtrate was concentrated to dryness under reduced pressure. Flash column chromatography (RediSepRf SiO2 (12 g) 100% hexanes) gave the product (Rf = 0.40, 100% hexanes) as a clear, colorless liquid, that slowly crystallized upon standing at room temperature (0.574 g).

**(11Z)-pentacos-11-ene**

A suspension of the alkyne (0.5 g) and Lindlar’s catalyst (300 mg) in quinoline:EtOAc (13 ml [1:1]) was stirred overnight under a balloon temperature (0.574 g).

Statistical Analysis

Categorical data were analyzed with a Fisher’s exact test and a post hoc Holm-Bonferroni correction for multiple group comparisons. For other comparisons, we first tested whether data were normally distributed using Lilliefors’ goodness-of-fit test. Data not violating this assumption were analyzed with parametric tests (Student’s t test for two groups or one-way ANOVA); otherwise, data were analyzed using a nonparametric test (Kolmogorov-Smirnov test for two groups or Kruskal-Wallis test). A Tukey’s post hoc test following multiple group comparisons was used to determine which groups differed significantly.

SUPPLEMENTAL REFERENCES


Figure S1. Identification and Ablation of Gr Neurons in the Male Foreleg, Related to Figure 2
(A–H) Expression (A–H) of different Gs (Gr:stingerGFP) and ablation (A’–H’) of Gr neurons (Gr:stinger GFP, hid) in foreleg tarsi. Whole-mount preparation of all tarsi (t1–t5) shown, with more distal tarsi to the left. n= 5–10/ genotype; scale bar, 50 μm.
Figure S2. Gr32a, but Not Gr66a or Ppk23, Inhibits Interspecies Courtship, Related to Figure 4

Control and experimental males were tested for courtship with females of various species.

(A–D) No difference in courting conspecific females between control, Gr66a null, and flies with knockdown of Gr32a (C155:Gr32aIR-1 or C155:Gr32aIR-2). As expected, Ppk23 null males show reduced courtship of conspecific females (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012).

(E–P) Males with a knockdown of Gr32a, but not Gr66a or Ppk23 mutant males, court D. simulans (E–H), D. yakuba (I–L), and D. virilis (M–P) females.

(Q) Gr32a−/− males court D. pseudoobscura.

Error bars represent SEM; n = 10–24/genotype; *p < 0.05, †p < 0.01, |p < 0.001.
Figure S3. Analytical Data for 11P, Related to Figure 5

(A) $^{13}$C NMR spectrum collected at 75 MHz.
(B) $^1$H NMR spectrum collected at 300 MHz.
(C) GCMS of purified 11P, as detected by TIC (total ion current, mass spectrometric).
(D) m/z composition of the dominant peak in the chromatogram shown in (C).
(E) Chemical structure of $\alpha$-7-tricosene (7T).
(F) Chemical structure of $\alpha$-9-tricosene (9T).
(G) Chemical structure of $\alpha$-11-pentacosene (11P).
(H) Chemical structure of lobeline.
(I) Chemical structure of N, N, diethyl-meta-toluamide (DEET).
Figure S4. Distinct Mechanisms Inhibit Conspecific Intermale and Interspecies Courtship, Related to Figure 6

(A) Consistent with the lack of FruM expression in Gr32a neurons, driving expression of a FruM RNAi in male Gr32a neurons does not lead to courtship of D. virilis females.

(B–D) No overlap in FruM (frulex:stinger GFP) and P52A (P52A:tdTomato) cells in foreleg tarsi. P52A:tdTomato reveals previously unreported expression (Manoli and Baker, 2004) in the cuticle and other nonneural tissue in the foreleg, but these cells do not coexpress FruM in the tarsi.

(E) Enumeration of FruM (frulex:stinger GFP) and P52A (P52A:tdTomato) cells in foreleg tarsi and tibia.

(F) Gr32a null males court conspecific, D. simulans and yakuba, but not virilis, males.

(G) Gr33a null males court conspecific males, but not males of other species.

(H) Ppk23 null males court conspecific males, but not males of other species.

(I) fru1/fru4-40 males court conspecific, D. simulans and yakuba, but not virilis, males.

(J) Males with a knockdown of FruM in aDT6 neurons (P52A:fruMIR) court D. yakuba males. As reported previously, these flies do not court conspecific flies (Manoli and Baker, 2004). Taken together with the courtship phenotypes of Gr32a, Gr33a, and Ppk23 null males, our results suggest that it is possible to dissociate at a molecular and cellular level the atypical courtship of conspecific males from that of animals of other species.

(K–M) No coexpression of FruM and Gr32a in foreleg tarsi of D. melanogaster males (K–M). Enlarged version of the panels shown in main Figures 6B–6D. A cell that appears colabeled for FruM and Gr32a in a Z projected image (arrow in M) in fact represents two distinct cells in different optical slices expressing either FruM (K–M) or Gr32a (K–M) but not both.

(N–Q) Schematic of connectivity between L3 and Tm9 neurons in the M3 layer of the medulla (N); gray box represents the area of the histological images shown to the right (O–Q). Native GRASP fluorescence is visualized in the M3 layer (O, Q) but not in other regions of the medulla (visualized with nc82 immunolabeling in P, Q) although these regions do contain processes from L3 or Tm9 neurons.

(R–T) Switching the expression of the GRASP components also labels contacts between Gr32a (Gr32a:LexA) and FruM (fruM:GAL4) neurons in the SOG and T1 VNC of D. melanogaster males (Gr32a:spGFP11::CD4, fru:spGFP1-10::Nrx), as visualized by native GRASP fluorescence.

(U–V) No contacts between Gr32a and aDT6 neurons in the SOG, as visualized by native GRASP fluorescence, in D. melanogaster males (Gr32a:spGFP11::CD4, P52A:spGFP1-10::Nrx). The neuropil (magenta) is immunolabeled with nc82.

(W–W) Immunolabeling confirms expression of the individual GRASP components in the SOG of D. melanogaster males (Gr32a:spGFP11::CD4, P52A:spGFP1-10::Nrx).

(X) dBrainbow-labeled clones in D. melanogaster males (hs:Cre, Gr32a:GAL4::VP16, P52A:GAL4, UAS-dBrainbow) show no overlap between axonal arbors of Gr32a neurons (green and magenta clones) in the SOG and aDT6 (green clones) soma and local dendritic arbors (Miyamoto and Amrein, 2008). aDT6 axon projections can be visualized as two thick green vertical bands exiting the top of the figure. See also Movie S4 for 3D reconstruction of the optical stack.

Error bars represent SEM; n = 10–31/experimental cohort for all panels except (W–W*, n = 5); *p < 0.01, **p < 0.001; NS = not significant; scale bar, 50 μm (B–D), 10 μm (O–Q), and 20 μm (V–X).