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A glial amino-acid transporter controls synapse strength and courtship in *Drosophila*

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Mate choice is an evolutionarily critical decision that requires the detection of multiple sex-specific signals followed by central integration of these signals to direct appropriate behavior. The mechanisms controlling mate choice remain poorly understood. Here, we show that the glial amino-acid transporter genderblind controls whether *Drosophila melanogaster* males will attempt to mate with other males. *Genderblind* (*gb*) mutant males showed no alteration in heterosexual courtship or copulation, but were attracted to normally unappealing male species-specific chemosensory cues. As a result, genderblind mutant males courted and attempted to copulate with other *Drosophila* males. This homosexual behavior could be induced within hours using inducible RNAi, suggesting that genderblind controls nervous system function rather than its development. Consistent with this, and indicating that glial genderblind regulates ambient extracellular glutamate to suppress glutamatergic synapse strength *in vivo*, homosexual behavior could be turned on and off by altering glutamatergic transmission pharmacologically and/or genetically.

Mate selection is an important decision that relies on proper detection and integration of multiple sensory cues. To aid the process, many animals perform elaborate courtship rituals that are designed to attract and differentiate between potential sexual partners. In the fruit fly *Drosophila melanogaster*, courtship typically begins when a male fly identifies and approaches a suspected conspecific female. To confirm his suspicions and to test whether she is sexually receptive, he will tap her with his foreleg (to evaluate nonvolatile pheromones via chemoreceptors on his leg), sing a species-specific courtship song (by extending and vibrating a wing) and lick her genitalia (to sample pheromones). If she is acceptable and does not reject him (by extending her ovipositor, striking him with her wings or legs, or simply running away), he will mount her, curl his abdomen and attempt copulation[1,2].

Much of the 'wiring' required for *Drosophila* courtship develops under the control of well-studied sex-specific transcription factors, including those encoded by the genes *transformer*, *fruitless*, *doublesex* and *dissatisfaction*, which also determine whether brains develop as 'male' or 'female'[3,4]. As expected, flies with genetically male brains carry out typical male behaviors and flies with genetically female brains show typical female behaviors.

Atypical behavior includes homosexual courtship. Homosexual (male-male or female-female) courtship, regardless of whether heterosexual (male-female) courtship is also altered, represents an inability to distinguish sex-specific cues or an inability to respond appropriately to these cues. In *Drosophila melanogaster*, the ability to discriminate between males and females depends on visual, acoustic and chemical cues, including 7-tricosene and *cis*-vaccenyl acetate (cVA), which are perceived by taste and olfaction, respectively[5,6]. Flies that do not produce 7-tricosene and/or cVA are courted by males, and male flies that cannot sense these pheromones inappropriately court other males.

But what controls whether cues such as 7-tricosene and cVA are attractive or repulsive? The central mechanisms controlling sexual behavior remain unknown. Here, we show that homosexual behavior in *Drosophila* is controlled by glutamatergic synapse strength, which in turn is regulated by a glial amino-acid transporter that we named genderblind on the basis of the mutant phenotype. Consistent with this conclusion, we found that we could turn homosexual behavior on and off in a period of hours by genetic alteration of genderblind abundance and/or by pharmaceutical manipulation of glutamatergic synapse strength. Genderblind represents a previously unknown form of neural circuit modulation and an unexpected means of regulating an evolutionarily critical behavior.

RESULTS

We observed that male flies carrying the *KG07905 P{SUPor-P}* transposon insertion in the *gb* (*CG6070*) gene showed frequent homosexual interactions, including singing to other males, genital licking and attempted copulation (**Fig. 1a** and **Supplementary Videos 1–5** online). In contrast, wild-type and control flies (including those carrying *P{SUPor-P}* transposon insertions in other genes) rarely showed these homosexual behaviors (**Fig. 1a** and data not shown).

The *P{SUPor-P}CG6070[KG07905]* insertion lies in the predicted 5′ UTR of the *gb* gene, and therefore might disrupt *gb* transcription, mRNA trafficking and/or mRNA stability. To determine whether *gb* mRNA was reduced in *gb[KG07905]* mutants, we carried out real-time RT-PCR. Quantitative real-time RT-PCR using mRNA extracted from adult male flies showed a significant reduction of *gb* mRNA in

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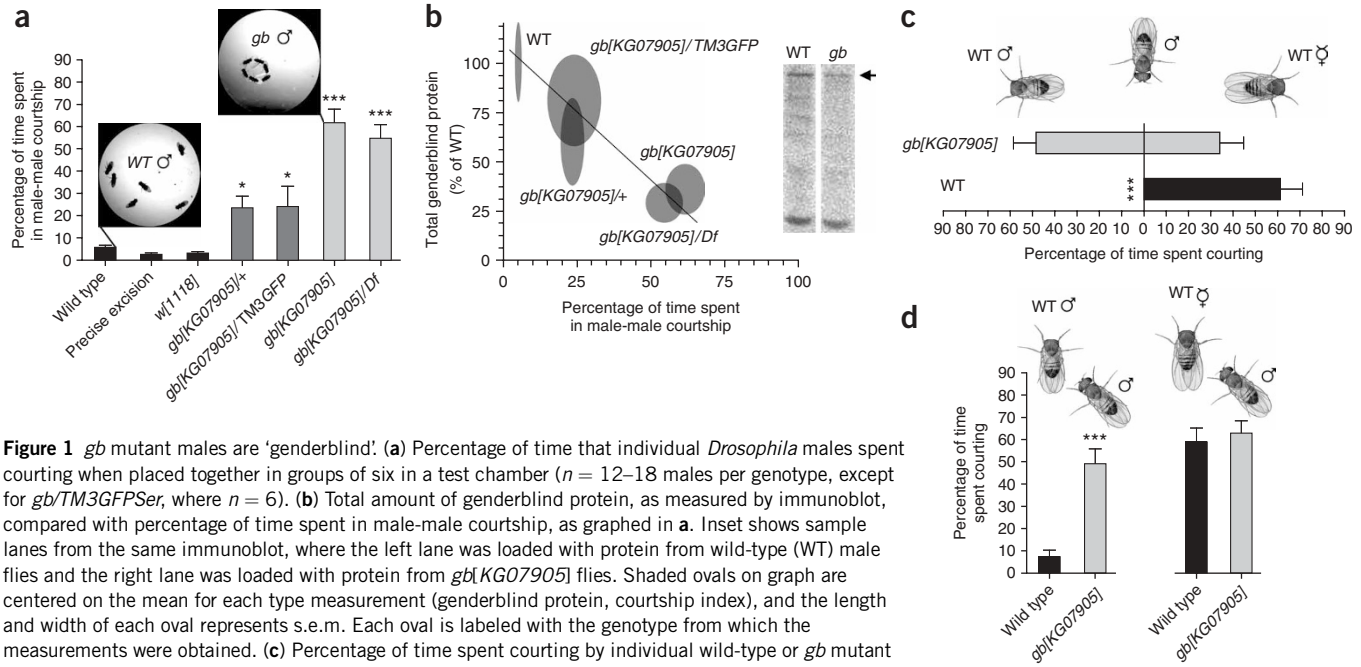

Figure 1 *gb* mutant males are 'genderblind'. (**a**) Percentage of time that individual *Drosophila* males spent courting when placed together in groups of six in a test chamber ($n = 12$–18 males per genotype, except for *gb/TM3GFPSer*, where $n = 6$). (**b**) Total amount of genderblind protein, as measured by immunoblot, compared with percentage of time spent in male-male courtship, as graphed in **a**. Inset shows sample lanes from the same immunoblot, where the left lane was loaded with protein from wild-type (WT) male flies and the right lane was loaded with protein from *gb*[*KG07905*] flies. Shaded ovals on graph are centered on the mean for each type measurement (genderblind protein, courtship index), and the length and width of each oval represents s.e.m. Each oval is labeled with the genotype from which the measurements were obtained. (**c**) Percentage of time spent courting by individual wild-type or *gb* mutant males placed in a chamber with both a passive (decapitated) wild-type virgin female and a passive (decapitated) wild-type male ($n = 12$–13). (**d**) Percentage of time spent courting by a single wild-type or *gb* mutant male placed in a chamber with either a passive (decapitated) wild-type male or passive (decapitated) virgin female ($n = 25$–32). All error bars represent mean ± s.e.m.

gb[*KG07905*] mutants compared with wild type, demonstrating that the *KG07905* insertion does indeed cause a loss of *gb* mRNA and that *gb*[*KG07905*] is a mRNA hypomorph (wild type, 1.0; *gb*[*KG07905*], 0.53 ± 0.10; $P = 0.02$, $n = 4$ samples of wild-type mRNA and 4 samples of *gb*[*KG07905*] mRNA, where extract from 3–7 adult males was used for each sample).

Loss of *gb* mRNA should lead to loss of genderblind protein. To confirm this, and to also determine whether incidence of male-male courtship might be directly proportional to genderblind protein loss, we measured genderblind protein from five different genotypes using immunoblots probed with antibody to genderblind (**Fig. 1b**). The total amount of genderblind protein in *gb*[*KG07905*] mutants was 35 ± 12% of that found in wild type ($P = 0.03$, $n = 4$ blots with 8–12 flies of each genotype), consistent with the reductions in *gb* mRNA that we measured in the same genotypes by real-time RT-PCR. Furthermore, there was a strong inverse correlation between total genderblind protein quantity and homosexual courtship (**Fig. 1b**; $n = 4$ blots with 8–12 flies of each genotype).

Three other experiments confirmed that the homosexual behavior observed in *gb*[*KG07905*] mutant male flies was caused by loss of *gb* function. First, precise excision of the transposon inserted in *gb* (*P*{*SUPor-P*}*CG6070*[*KG07905*]) completely rescued the courtship phenotype (**Fig. 1a**). Second, *gb* mutant homosexual courtship was phenocopied by expression of *gb* RNAi (described below). Third, a chromosomal deletion of *gb*, *Df*(*3R*)*Exel6206*, was unable to complement the defect induced by the mutation; double heterozygote (*Df/gb*) males showed high levels of homosexual courtship behavior, equal to that observed in *gb* mutant homozygotes (**Fig. 1a**).

Although *gb*[*KG07905*] mutants showed prominent homosexual behavior, they also showed heterosexual behavior. Therefore, they were presumably bisexual. To confirm this, *gb*[*KG07905*] and wild-type male flies were presented simultaneously with a wild-type passive (decapitated) male and a wild-type passive (decapitated) virgin female, either of which could be chosen as a sexual partner. Wild-type males always chose to court the female (**Fig. 1c**). In contrast, *gb* mutant males courted wild-type males and females with equal intensity and probability (**Fig. 1c**). Detailed examination of *gb* mutant heterosexual courtship and copulation revealed no alterations in copulation frequency, latency or duration (**Supplementary Fig. 1** online). *gb* mutant males also showed normal locomotor activity (**Supplementary Fig. 2** online). Thus, the *gb* courtship phenotype appears to be specific to male-male interactions.

To rule out possible group effects that might have arisen in our assays, we also carried out single-pair courtship assays using passive (decapitated) partners (**Fig. 1d**). These assays confirmed that individual *gb*[*KG07905*] mutant males court both males and females with equal likelihood, unlike wild-type males (**Fig. 1d**). Notably, precise excision males courted decapitated wild-type males more often than did wild-type males (precise excision male-male courtship: 29.8% ± 5.6, $n = 26$). However, precise excision males are white-eyed, and thus are effectively blind. Wild-type males assayed under dim red light, where they are also blind, show similar levels of homosexual courtship (**Fig. 2a**). Therefore, the level of courtship in precise excision males is equivalent to that of wild type under similar sensory constraints. Precise excision males engaged in heterosexual courtship with decapitated wild-type females 49.7% ± 5.0 of the time ($n = 42$), which was also indistinguishable from wild type.

Altered sexual discrimination in *gb* mutant males could be a result of a misinterpretation of sex-specific sensory cues. To test this hypothesis and to identify these cues, we first measured homosexual courtship under dim red light, in which *Drosophila* are virtually blind. In this condition, wild-type and precise-excision control males showed slightly higher than normal homosexual courtship (**Fig. 2a**), confirming the importance of visual cues for sexual discrimination. However, *gb* mutant males still showed much higher homosexual courtship (**Fig. 2a**), indicating that misinterpretation of nonvisual cues is the

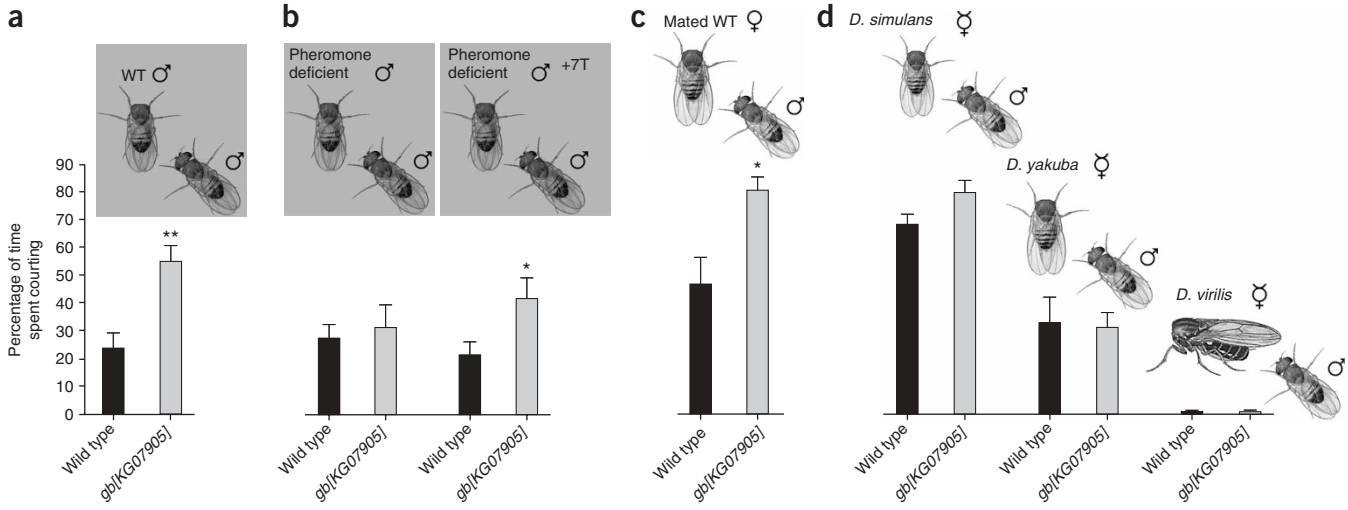

Figure 2 *gb* mutant males show altered responses to species-specific chemical sexual cues. (**a**) Percentage of time that single wild-type or *gb* mutant males spent courting when placed in a chamber with a passive (decapitated) wild-type male under dim red light ($n = 8$). (**b**) Percentage of time that single wild-type or *gb* mutant males spent courting when placed in a chamber under dim red light with a passive (decapitated) *desat1* mutant male that had (right) or had not (left) received topical application of pheromone 7-tricosene ($n = 18$–21). (**c**) Percentage of time that individual wild-type or *gb* mutant males spent courting a passive (decapitated) mated female ($n = 6$–11). (**d**) Percentage of time that passive (decapitated) virgin females of the listed species were courted by wild-type and *gb* mutant males ($n = 10$–13, except for *D. virilis*, $n = 5$). All error bars represent mean ± s.e.m.

primary cause of the *gb* mutant phenotype. To confirm this, we measured homosexual courtship directed toward *desat1* mutant males (**Fig. 2b**). *desat1* mutants are genetically deficient for the production of several pheromones, including 7-tricosene[7]. Homosexual courtship was reduced to wild-type levels when *gb* mutant males were partnered with *desat1* mutant males (**Fig. 2b**, left). However, homosexual courtship was restored to the high levels typical of *gb* mutants when synthetic 7-tricosene was topically applied to the cuticles of the *desat1* mutant male partners (**Fig. 2b**, right). Thus, *gb* mutant homosexual behavior represents an altered response to chemosensory cues, including 7-tricosene. Consistent with the idea that *gb* mutant males misinterpret chemical signals, *gb* mutant males also showed abnormally high courtship to mated wild-type females (**Fig. 2c**), which acquire inhibitory male chemical signals (including cVA) during copulation[8]. The chemical signals misinterpreted by *gb* mutant males appear to be species-specific, as *gb* mutant males reacted normally to potential partners from other *Drosophila* species (**Fig. 2d**).

To determine whether *gb* mutant males might overreact to other chemosensory stimuli, we carried out olfactory trap assays using standard *Drosophila* food as bait. Significantly more *gb* mutant males were trapped in these assays, compared with wild type or precise excision controls (wild type, 7.8 ± 4.6% trapped males after 12 h; precise excision, 11.0 ± 5.0%; *gb*[*KG07905*], 35.0 ± 9.6%; $P = 0.04$, $n = 9$–10 assays, 10 males per assay). This difference was confirmed in single-fly trap assays, where 60% of *gb* mutants were trapped after 34 h, compared with 33% of precise excision controls (precise excision, $n = 15$; *gb*, $n = 10$). These results support the idea that *gb* mutants have fundamental defects in chemosensory processing that cause them to overreact to certain chemical signals. We therefore turned our attention toward determining the mechanism by which genderblind might alter chemosensory processing.

We have recently shown that genderblind is a highly conserved glial amino-acid transporter subunit and a critical regulator of ambient

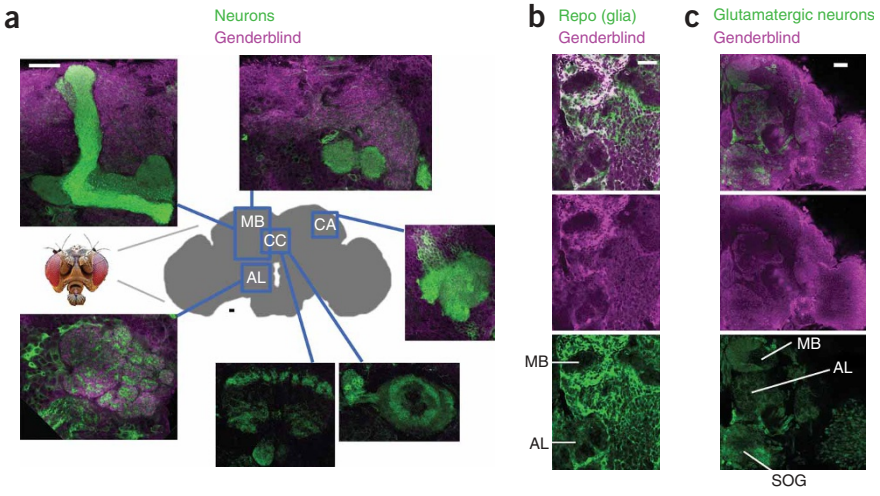

Figure 3 Genderblind (genderblind) protein is expressed in central glia surrounding glutamatergic neurons. (**a**–**c**) Single fluorescence confocal microscopy sections from male adult brains, stained with antibodies to genderblind (magenta) and CD8 (green). Colocalization is represented by white color. For each image, the transgenic transmembrane protein CD8::GFP was expressed in a specific tissue-type using the Gal4/UAS system. CD8 expression was driven with the neuronal driver *Elav-Gal4* (**a**), *Repo-Gal4* (which is expressed in a subset of glia, **b**) or the weak glutamatergic neuron driver *OK371-Gal4* (**c**). Selected brain structures are indicated in each panel. AL, antennal lobe; CA, calyx; CC, central complex; MB, mushroom body; SOG, subesophageal ganglion (see also **Supplementary Fig. 3**). Scale bars represent 25 μm.

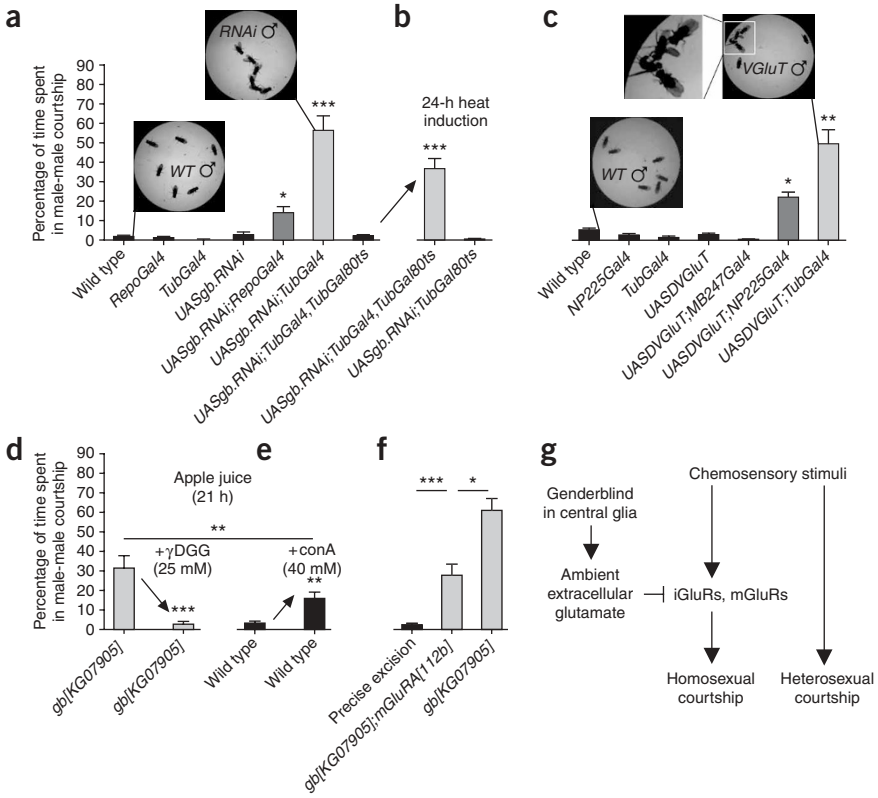

Figure 4 *Drosophila* male homosexual courtship is controlled by the strength of glutamatergic neurotransmission. (**a**–**f**) Percentage of time that individual males spent courting when groups of six males were placed together in a test chamber ($n = 12$–18, except for *RepoGal4*, *NP225Gal4*, *UASDVGluT*, *UASDVGluT;MB247Gal4* and *UASDVGluT;NP225Gal4*, where $n = 6$). All error bars represent mean ± s.e.m. (**g**) A model for how genderblind might regulate *Drosophila* sexual behavior.

extracellular glutamate[9]. In *gb*[*KG07905*] mutants, ambient extracellular glutamate is reduced to approximately 50% of normal[9]. Ambient extracellular glutamate bathes the nervous system and generally suppresses glutamatergic synapse strength via constitutive desensitization of glutamate receptors[9,10]. To test whether the homosexual behavior of *gb* mutant males might be attributable to increased glutamatergic synapse strength in chemosensory circuits, we carried out the following series of experiments. First, we used a genderblind-specific antibody to examine genderblind expression in the adult male brain. In particular, we examined whether genderblind protein might be expressed in the adult male nervous system near brain centers that are known to be involved in chemical sensation and integration (**Fig. 3**). As expected, genderblind was distributed throughout adult male *Drosophila* brain, including areas associated with olfactory and gustatory sensation and integration (**Fig. 3** and **Supplementary Fig. 3** online). More precisely, genderblind was detected in the subesophagial ganglia that receive inputs from gustatory neurons (some of which process 7-tricosene[6]), in the antennal lobe and in the calyces that are involved in the higher integration of pheromonal inputs (including olfactory inputs for cVA sensation[11,12]). In contrast, no expression was detected in the central complex region or in the different lobes of the mushroom bodies, which are involved in locomotion and olfactory learning, respectively[13,14]. Genderblind immunoreactivity was reduced to background levels after expression of *gb* RNAi, indicating that the antibody is specific (**Supplementary Fig. 4** online). We also looked to see whether genderblind is present in glia. In larvae, genderblind is exclusively expressed in glia[9]. Consistent with this, genderblind immunoreactivity in adult brains was excluded from neurons and was partially associated with cells expressing the glial transcription factor Repo (**Fig. 3a**,**b**). Genderblind was also abundant in areas of the brain containing glutamatergic neurons (**Fig. 3c**). Thus, immunohistochemical data support the possibility that genderblind could modulate glutamatergic neurotransmission in pathways that control processing and/or integration of chemical stimuli.

To further explore the mechanism by which genderblind regulates homosexual behavior, we used RNAi (**Fig. 4a**,**b**). As expected, *gb* mutant homosexual behavior could be phenocopied by constitutive expression of *gb* RNAi using the Gal4/UAS system (*UASgb.RNAi;TubGal4*; **Fig. 4a**). To confirm that the *gb* RNAi homosexual phenotype was specific for knockdown of *gb*, we constitutively expressed RNAi against five different genes near *gb* using validated RNAi lines from the Vienna *Drosophila* RNAi Center[15] and the same *TubGal4* driver was used to drive *gb* RNAi. RNAi against *CG6074* (~2 kb immediately downstream of *gb*), *CG6066* (~5 kb upstream of *gb*) and *CG5880* (~4.5 kb upstream of *gb*) all caused lethality. RNAi against *CG5815* (<1 kb upstream of *gb*) and *CG5882* (~6.5 kb upstream of *gb*) caused neither lethality nor significant homosexual courtship (*TubGal4;UASRNAiCG5815*, 3.2% ± 2.4% of time spent in homosexual courtship, $n = 12$; *TubGal4;UASRNAiCG5882*, 2.4% ± 1.0% of time spent in homosexual courtship, $n = 12$). Thus, we attribute the *gb* RNAi homosexual phenotype to specific knockdown of *gb*.

Genderblind appeared to be expressed exclusively in glia. To confirm that the *gb* mutant homosexual phenotype was a result of the loss of glial genderblind, we carried out cell type–specific knockdown of *gb*. Duplication of the *gb* mutant phenotype by RNAi was maximal when *gb* RNAi was expressed in all brain tissues (**Fig. 4a**), but was only partial when *gb* RNAi was expressed under control of *RepoGal4* (**Fig. 4a**), consistent with the fact that some genderblind protein is expressed in glia that do not express *Repo* (**Fig. 3b** and ref. 9). There is no available Gal4 driver that is specific for genderblind glia.

To test whether the *gb* mutant courtship phenotype could be a result of a developmental alteration rather than acute modulation of neural circuit function, we used inducible RNAi. The *TubGal80ts* transgene is a ubiquitously expressed conditional repressor of Gal4 that is active at low temperatures (25 °C), but not at high temperatures (30 °C). In *UASgb.RNAi;TubGal4,TubGal80ts* males at 25 °C, all genetic components for *gb* RNAi expression are present, but RNAi expression is actively repressed by *TubGal80ts*. *UASgb.RNAi;TubGal4,TubGal80ts* males showed low levels of homosexuality (similar to wild type) when reared continuously at 25 °C (**Fig. 4a**). However, when *UASgb.RNAi;TubGal4,TubGal80ts* adult males reared at 25 °C were moved to 30 °C 24 h before testing, the *gb* mutant homosexual phenotype was completely restored (**Fig. 4b**). Homosexual behavior in these conditions could not have been an artifact of high temperature or the presence of *TubGal80ts*, as *UASgb.RNAi;TubGal80ts* males at 30 °C did

not show homosexual behavior (**Fig. 4b**). This ability to switch on homosexual behavior in adult males suggests that genderblind regulates brain function rather than development, which is consistent with our hypothesis that genderblind indirectly regulates glutamatergic synapse strength.

If homosexual courtship in *gb* mutants was a result of increased glutamatergic synapse strength in the CNS, then increasing CNS glutamatergic synapse strength independent of genderblind should also cause high levels of homosexual behavior. To test this, we increased the strength of glutamatergic synapses in adult male brains by overexpressing the *Drosophila* vesicular glutamate transporter (DVGluT). Overexpression of DVGluT has previously been shown to overload synaptic vesicles with glutamate and lead to increased glutamate secretion at synapses[16]. As predicted, overexpression of DVGluT (*UASDVGluT;TubGal4*) caused high levels of homosexual courtship (**Fig. 4c**). *UASDVGluT;TubGal4*-induced homosexual courtship, as in *gb* mutants, included all aspects of sexual behavior, including singing, genital licking and attempted copulation. Occasionally, *UASDVGluT;TubGal4* males even attempted copulation with inappropriate body regions (for example, the head) (**Fig. 4c**), suggesting that increased glutamatergic synapse strength was a strong proximate cause of homosexual courtship and that homosexual courtship might represent a restricted example of general ectopic courtship. Overexpression of DVGluT in mushroom body neurons (*UASDVGluT;MB247Gal4*) had no effect (**Fig. 4c**), which is consistent with the lack of genderblind expression in mushroom bodies. But the *gb* mutant homosexual phenotype was partially duplicated by DVGluT overexpression specifically in adult brain chemosensory centers (*UASDVGluT;NP225Gal4*) (**Fig. 4c**), consistent with the idea that *gb* mutant homosexuality is a result of increased glutamatergic synapse strength in circuits associated with processing of chemical stimuli.

As a further test of the hypothesis that the *gb* mutant phenotype is a result of increased glutamatergic synapse strength, we pharmacologically and genetically altered glutamate receptor function (**Fig. 4d–f**). Gamma-D-glutamylglycine (γ-DGG) is a competitive glutamate-receptor antagonist. If *gb* mutant homosexuality is a result of increased glutamatergic neurotransmission, then γ-DGG should eliminate *gb* mutant homosexuality. As predicted, adult *gb* mutant male flies reverted to low (wild type) levels of homosexual courtship when fed apple juice containing 25 mM γ-DGG for 21 h (**Fig. 4d**). This dose of γ-DGG did not seem to disrupt coordination, and had no significant effect on locomotory activity (locomotor index: *gb*[*KG07905*], 52.2 ± 4.7, $n = 20$; *gb*[*KG07905*] + γ-DGG, 62.4 ± 4.4, $n = 21$; $P = 0.11$).

Glutamatergic neurotransmission is mediated by two different types of receptors: ionotropic (pore-forming) glutamate receptors and metabotropic (G protein–coupled) glutamate receptors. The increased glutamatergic neurotransmission underlying the *gb* mutant homosexual phenotype could occur via either receptor type or even both. Concanavalin A (ConA) is a glutamate-receptor agonist that inhibits ionotropic receptor desensitization. If *Drosophila* homosexual behavior is caused by increased glutamatergic neurotransmission via ionotropic glutamate receptors, then ingestion of ConA should induce homosexual behavior. Consistent with this, adult wild-type flies that were fed apple juice containing 40 mM ConA for 21 h before testing showed increased homosexual courtship (**Fig. 4e**). As with γ-DGG, the dose of ConA that we used did not seem to disrupt coordination and had no significant effect on locomotory activity (locomotor index: wild type, 67.4 ± 4.1, $n = 20$; wild type + ConA, 69.0 ± 3.9, $n = 20$; $P = 0.77$).

However, ConA (which disrupts transmission via ionotropic glutamate receptors) did not induce as high a level of homosexual courtship as was measured in either *gb* mutants or after ingestion of γ-DGG (which disrupts transmission via both ionotropic and metabotropic glutamate receptors). This suggests that the enhanced glutamatergic transmission causing *gb* mutant homosexual behavior is only partially attributable to overactivation of ionotropic glutamate receptors. To test whether some of the *gb* mutant homosexual behavior might also be a result of overactivation of metabotropic glutamate receptors, we measured homosexual courtship in *gb; mGluRA[112b]* double mutant males, in which loss of *gb* function was combined with a small deletion that specifically removes mGluRA, the only functional metabotropic glutamate receptor encoded by the *Drosophila* genome[17]. Deletion of mGluRA partially rescued the *gb* mutant homosexual phenotype (**Fig. 4f**), which is consistent with the idea that *gb* mutant homosexual courtship is a result of increased neurotransmission via both ionotropic and metabotropic glutamate receptors.

Taken together, our data suggest that *Drosophila* homosexual behavior is controlled by glutamatergic synapse strength and that genderblind normally suppresses homosexual behavior by suppressing glutamatergic synapse strength.

DISCUSSION

Our study was prompted by the observation that *gb*[*KG07905*] mutant males showed strong homosexual courtship. Similar homosexual courtship has been observed in flies with other transposon insertions[18,19]. In those cases, homosexual courtship was attributed to misexpression of *white*, an eye color gene that is commonly engineered into *Drosophila* transposons as a transgenic marker. Because *gb*[*KG07905*] mutants also contain a transgenic *white* gene, we were careful to consider the possibility that homosexual courtship in our experiments might simply be caused by misexpression of *white*. However, we saw no evidence that homosexual courtship can be triggered by the presence of *white*-expressing transposons that did not otherwise disrupt specific genes. For example, each of the mutant genotypes depicted in **Figure 4a–c**, plus the five additional RNAi-expressing genotypes described in the text, contain at least one *white*-misexpressing transposon insertion. Yet homosexual courtship in these genotypes was only increased after specific disruption of *gb* or glutamatergic transmission. In one genotype (*UASgb.RNAi;TubGal4,TubGal80ts*), there were at least three *white*-misexpressing transposon insertions in the fly genome, but no unusual homosexual courtship was observed until *gb* expression was disrupted by heat-shock induction of *gb* RNAi. Other studies have also cast doubt on the conclusion that *white* misexpression invariably causes male-male courtship[20,21].

The fraction of time spent in homosexual courtship by *gb*[*KG07905*] and *gb*[*KG07905*]*/Df* mutants was statistically identical (**Fig. 1a**), implying that *gb*[*KG07905*] is a null allele by traditional genetic criteria. However, our real-time PCR and genderblind immunoblot data clearly demonstrate that *gb*[*KG07905*] is not a null, and readers are cautioned not to over-interpret courtship index values. The maximum obtainable courtship is never 100%, even between wild-type male and female flies (**Fig. 1d**). Male flies spend substantial amounts of time in search and grooming behaviors (**Supplementary Videos 1–5**). Neither searching nor grooming counts as courtship behavior, and the maximal obtainable courtship values are therefore limited to 60–80%. Indeed, qualitatively far more vigorous courtship was measured after overexpression of DVGluT (**Fig. 4c** and **Supplementary Videos 1–5**), but this did not lead to a higher courtship index when compared with *gb*[*KG07905*] or *gb*[*KG07905*]*/Df* (compare **Figs. 1a** and **4c**), as noncourtship behavior was not substantially altered.

The fact that homosexual behavior in *Drosophila* seems to be controlled by glutamatergic circuits is notable, as the *Drosophila* CNS is generally thought to rely primarily on acetylcholine for

neurotransmission. However, there are increasing indications that glutamatergic transmission is also important, despite being overlooked, in the fly CNS, including evidence that (i) large portions of the *Drosophila* CNS are glutamatergic (**Fig. 3c** and ref. 22), (ii) *in situ* data show that many different ionotropic glutamate receptor subunits are expressed in the CNS[23,24], (iii) the ionotropic glutamate receptor subunit GluRIID has been shown to be important in central pattern generation[25] and (iv) both NMDA receptor homologs in the *Drosophila* genome are expressed in CNS memory centers and are required for proper olfactory memory formation[26].

Genderblind has high homology to mammalian xCT proteins, which together with 4F2hc subunits, form heteromeric cystine/glutamate transporters that secrete glutamate in exchange for extracellular cystine[27]. Most of the focus on cystine/glutamate transporters to date has been on their ability to import cystine. However, cystine/glutamate transporters are also potentially important regulators of ambient extracellular glutamate bathing the nervous system. Pharmacological studies support the idea that cystine-glutamate transporters regulate ambient extracellular glutamate in rat brain[28], and we have recently shown that ambient extracellular glutamate in *gb* mutant flies is halved when compared with controls[9,29]. Ambient extracellular glutamate, in both mammals and flies, can regulate glutamatergic transmission via steady-state glutamate receptor desensitization[9,10]. Consistent with this idea, we were able to both phenocopy and rescue the *gb* mutant homosexual phenotype by pharmacological manipulation of glutamatergic transmission, including the use of the desensitization inhibitor ConA.

The findings stated here and in our recent description of *gb* mutant synaptic phenotypes[9] all support the idea that genderblind regulates ambient extracellular glutamate, and that this in turn regulates glutamatergic signaling in *Drosophila* chemosensory processing centers. Similar regulation, although perhaps not in chemosensory centers, may occur in mammals. In healthy mammalian brains, ambient extracellular glutamate concentration varies spatially and temporally[30–32], and these changes in ambient extracellular glutamate may contribute to behavioral states or mood[33]. For example, melatonin alters glial glutamate uptake and this triggers circadian changes in ambient extracellular glutamate[34]. Pharmacological manipulation of cystine/glutamate exchange in rats alters ambient extracellular glutamate, cocaine withdrawal and effects of phencyclidine[28,35,36]. However, the idea that genderblind-type transporters might volumetrically regulate glutamatergic signaling *in vivo* remains controversial. As a first step toward resolving this controversy, we cloned a *gb* cDNA using primers designed to amplify the *gb* cDNA that is predicted by Flybase. It was hoped that we could misexpress and overexpress *gb* to test whether specific glutamatergic circuits might be altered in a genderblind dose–dependent manner. However, pan-cellular expression of this cDNA failed to rescue the cellular phenotypes that we have recently described[9] or the behavioral changes that we describe here. Transgenic cDNA rescue in *Drosophila* does not always work, or can be misleading, and there are several reasons why our *gb* cDNA might have failed to rescue the mutant phenotypes. One possibility is that the *gb* locus encodes multiple protein isoforms and that these isoforms must be expressed in a specific spatiotemporal pattern to recapitulate normal synaptic circuit modulation. This conclusion is supported by quantitative RT-PCR data (see Methods). Genderblind-type transporters are also multi-subunit complexes, and expression of each subunit might need to be carefully coordinated for proper function[37–39].

In addition to demonstrating a behavioral role for genderblind, our results also suggest a physiological model for *Drosophila* sexual preference that parallels a model recently proposed for mice[40]. In this model (**Fig. 4g**), wild-type flies are 'pre-wired' for both heterosexual and homosexual behavior, but genderblind-based transporters suppress the glutamatergic circuits that promote homosexual behavior. In *gb* mutants, the repression of homosexual behavior does not occur and flies become bisexual. Heterosexual courtship is not altered in *gb* mutants, indicating that circuits driving heterosexual courtship are not regulated by genderblind. This could be because circuits promoting heterosexual courtship are not glutamatergic, or because they are perfused by a different ambient extracellular glutamate pool than the one that is regulated by genderblind-based transporters.

METHODS

Behavior. We measured and quantified grouped male homosexual courtship (**Figs. 1a** and **4a–f**) as follows. Males were collected at eclosion and kept individually in new vials with fresh food at 25 °C in a 12-h light/dark cycle for 5–8 d before testing, which always took place in the morning at approximately the same time. For testing, individual males were cold-anesthetized in a freezer (–20 °C) for 1.5 min for transferal to a single tube, and then immediately anesthetized for another 1.5 min for transfer to the observation chamber. Observation chambers (2.25 × 2.15 × 0.10 cm) were placed under a stereomicroscope equipped with a digital camera at ~22 °C. After placement in the observation chamber, the flies were allowed to recover for 2.5 min, followed by 10 min of digital video recording. The onset of ectopic courtship after RNAi-mediated knockdown of *gb* reached a peak approximately 40 min later than that of homozygous *gb*[*KG07905*] mutants. Therefore, RNAi-related phenotypes, including all associated controls, were quantified from 10 min of video starting 53 min after flies were placed in the observation chamber. After recording, videos were analyzed to measure the courtship index (percentage of time that a male is courting during a 10-min period) for each male (**Supplementary Videos 1–5**; all digital videos are available on request).

For some experiments (**Fig. 2b**), *desat*$^{1\text{-}1573}$ males that lacked male-specific cuticular hydrocarbons[41] were perfumed with either 1 ul of pentane (solvent) alone, or with 1,000 ng of 7-tricosene dissolved in 1 ul of pentane, as previously described[6]. Measurement and quantification of basal locomotion was carried out as previously described[42]. Single-pair courtship and copulation tests were performed and quantified as previously described[42,43]. Olfactory trap assays were carried out as previously described[44]. Flies were grown at 25 °C on standard cornmeal-malt medium with a 12-h light/dark cycle.

Pharmacology. For the pharmacological experiments (**Fig. 4d,e**), flies were grown and individually collected as described above, but male flies were individually transferred into an empty tube 21 h before testing that contained a drop (150 ul) of either apple juice alone ('Jewel' brand 100% apple juice from concentrate) or apple juice in which ConA (Sigma-Aldrich) or γDGG (Tocris-Cookson) were dissolved. After 5 h, another drop was added to replace the volume lost as a result of evaporation and/or ingestion.

Immunocytochemistry and confocal microscopy. Antibodies to genderblind[9] were used at a 1:600 ratio. Mouse monoclonal antibodies to CD8 (Caltag Laboratories) were used at a 1:100 ratio. FITC, TRITC-conjugated goat secondary antibodies to mouse or rabbit were obtained from Jackson Immunoresearch Laboratories and were used at a 1:400 ratio. Adult male brains were dissected in standard *Drosophila* saline (135 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM TES and 72 mM sucrose) and then fixed for 20 min in 4% paraformaldehyde fixative. Images were obtained using an Olympus Fluoview FV500 laser-scanning confocal microscope. Image analysis was carried out using ImageJ (US National Institutes of Health).

Quantification of genderblind mRNA and protein levels using real-time RT-PCR and immunoblots. *Drosophila* total RNA was isolated from wild-type and *gb*[*KG07905*] adult males using standard Trizol extraction[45]. mRNAs were reverse transcribed using oligo-dT primers and standard methods. PCR primers were used to amplify *gb* and *actin 5C* (*Act5C*) as a standard and control. We used two primers to amplify *gb*, 5′-CAC ATA GAT GGG CAC GAC AAC TAA G-3′ and 5′-CCT TTG GCG ATA AGA TTC TCG G-3′, which amplify predicted exons 3–5. Using these primers, there was no

detectable PCR product from animals that were homozygous for a deficiency, *Df(3R)Exel6206*, that completely removed the *gb* gene (homozygous *Df* animals were L1 lethal). Using a different primer pair that amplified predicted exons 1 and 2, we measured no decrease in *gb* mRNA in *gb*[*KG07905*] mutants by real-time RT-PCR, indicating that several products are synthesized from the predicted *gb* gene (data not shown). Real-time PCR was carried out using an MJResearch Opticon2 real-time thermocycler and quantitative fluorescent detection of SYBR green–labeled PCR product. Relative mRNA abundance was calculated using the 'ΔΔCT method', as previously described[46]. Briefly, $C(t)$ values for *gb* and an *Act5C* control were determined for each sample. *gb* mRNA abundance was normalized to the *Act5C* control in each genotype using $\Delta C(t)\text{sample} = C(t)gb - C(t)Act5C$. Normalized sample $C(t)$ values were then referenced to a wild-type control sample that was run in parallel (the 'calibrator'). The formula used was $\Delta\Delta C(t)\text{sample} = \Delta C(t)\text{sample} - \Delta C(t)$ calibrator. The amount of *gb mRNA* for each sample was reported relative to the calibrator (wild type) using $2^{-\Delta\Delta^{C(t)}}$.

Immunoblots were carried out using standard methods. Briefly, proteins from 8–12 adult male flies of each genotype were used for each blot. Blocking was carried out with 5% milk, and blots were incubated overnight with antibody to genderblind (1:2,500 or 1:5,000). Bands were detected using chemiluminescent detection (1 h in secondary), as per manufacturer's (Pierce) directions, and visualized using a BioRad Versadoc 4000 gel/blot imaging system. The antibody to genderblind recognized a single large band of approximately 120 kDa, revealing that genderblind, as previously reported for mammalian xCT proteins[37–39], runs in polyacrylamide gels as an apparent dimer. Conveniently, the antibody to genderblind also recognized a small (15 kDa) nonspecific band that served as an ideal loading control for accurate quantification of genderblind protein in each lane, using BioRad Quantity One gel/blot analysis software. Statistics for genderblind protein abundance in each genotype were derived from multiple independent protein isolations and several blots carried out over several days, and thus truly represent independent replicates.

Genetics and statistics. Wild-type *Drosophila simulans, yakuba* and *virilis* were provided by the Tucson stock center. Wild-type *Drosophila melanogaster* strains used in this study were *Oregon R* and *Dijon*. No statistically significant difference was observed between these two strains with regard to measurements performed for this study. Previously characterized *P{SUPor-P}CG6070* [*KG07905*] mutants[9] were generated by the *Drosophila* Gene Disruption Project[47] and are available from the Bloomington Stock Center. *UAS-DVGluT* flies[16] were generously provided by A. DiAntonio (Washington University), *OK371Gal4* flies[22] by H. Aberle (MPI-Tubingen), *mGluRA[112b]* flies[17] by K. Broadie (Vanderbilt University), *MB247Gal4* flies[48] by T. Zars (University of Missouri-Columbia) and *NP225Gal4* flies[49] by R. F. Stocker (University of Fribourg). *Df(3R)Exel6206* (*w1118; Df(3R)Exel6206, P{XP-U}Exel6206/TM3, Sb1 Ser1*), *RepoGal4* (*P{Gal4}Repo/TM3, Sb*) and *TubGal4* (*P{TubP-Gal4}LL7/TM3, Sb*) flies were obtained from the Bloomington Stock Center and re-balanced over TM3 GFP Ser for use in our experiments. The deficiency *Df(3R)Exel6206* completely deletes *gb/CG6070*, as well as six other genes 5′ to *gb* and 8 genes 3′ to *gb*.

Construction of the RNAi transgene against *gb* was previously described[9]. RNAi transgenes against *CG6074*, *CG6066*, *CG5880*, *CG5815* and *CG5882* were obtained from the Vienna *Drosophila* RNAi Center[15]. The transformant line numbers for these five RNAi lines are 31148, 35064, 1264, 22203 and 27532, respectively.

For comparison of multiple groups, statistical significance was determined using ANOVA (for Gaussian distributed data) or Kruskal-Wallis (nonparametric) tests followed by either a Bonferroni or a Dunn's post test to compare genotypes. For comparison between paired normally distributed data, a Student's paired *t*-test was used. When data distributions had unequal variance, *P* for two-group comparisons was computed using an unpaired *t*-test with Welch's correction. In figures, statistical significance is indicated by asterisks. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.G. made the original observation that *gb* mutant males courted each other and was responsible for all genetic and pharmacological manipulations, immunohistochemistry and most of the behavioral experiments and analysis. M.G. was responsible for some locomotory tests, the heterosexual copulation measurements and the *desat* mutant male experiments and contributed to decapitated partner courtship tests. H.A. was responsible for the *gb* real-time RT-PCR and GB immunoblot data. D.E.F, Y.G. and J-F.F. were responsible for experimental design and interpretation of results and writing the article.

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