The *hector* G-Protein Coupled Receptor Is Required in a Subset of *fruitless* Neurons for Male Courtship Behavior

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**Abstract**

Male courtship behavior in *Drosophila melanogaster* is controlled by two main regulators, *fruitless* (*fru*) and *doublesex* (*dsx*). Their sex-specific expression in brain neurons has been characterized in detail, but little is known about the downstream targets of the sex-specific FRU and DSX proteins and how they specify the function of these neurons. While sexual dimorphism in the number and connections of *fru* and *dsx* expressing neurons has been observed, a majority of the neurons that express the two regulators are present in both sexes. This poses the question which molecules define the sex-specific function of these neurons. Signaling molecules are likely to play a significant role. We have identified a predicted G-protein coupled receptor (GPCR), CG4395, that is required for male courtship behavior. The courtship defect in the mutants can be rescued by expression of the wildtype protein in *fru* neurons of adult males. The GPCR is expressed in a subset of *fru*-positive antennal glomeruli that have previously been shown to be essential for male courtship. Expression of 4395-RNAi in GH146 projection neurons lowers courtship. This suggests that signaling through the CG4395 GPCR in this subset of *fru* neurons is critical for male courtship behavior.

**Introduction**

One critical aspect of understanding how complex behaviors are regulated is to understand the circuits and molecules that are required to generate and display the behavior. Sexual behavior offers an excellent model for the study of complex behaviors because it is both hard-wired and responsive to environmental cues. Courtship behavior in male fruit flies consists of well-defined courtship steps that can easily be studied to examine the effect of mutants.

Sex-specific behaviors in *Drosophila* are under the control of the same master upstream regulators that also regulate somatic sex [1,2,3,4,5]. They control the sex specific generation of two key male regulators, *fruitless* (*fruM*) and *doublesex* (*dsxM*). The male-specific *fruM* and *dsxM* proteins are both critically required for normal male courtship behavior [6,7,8,9]. Detailed insight has been gained into the expression pattern of these genes and their regulation by the sex-determination mechanisms, and details are starting to emerge on the sex-specific circuits these neurons may form. While an increasing number of differences are being observed between males and females in the number of *fru* and *dsx* neurons and the connections they make, it is also evident that many of the neurons that express *fruM* and *dsxM* form the male circuits are not absent in females [6,7,8,9,10,11,12,13,14,15,16,17,18,19,20]. Thus, it is likely that it is the expression of sex specific molecules in these neurons, or the sex-specific nature of their input and/or output that is critical for the observed sex specific behavior. Exploring which genes are expressed in these neurons and how this determines their function and their connections is therefore a critical next step in trying to understand how mating behavior is regulated. Both Fru and Dsx are transcription factors that in microarray studies have been shown to control a large number of genes [21,22]. However, little is known to date about the significance of these genes, in which cells they might be expressed and what biological role they might play. Given the crucial importance of communication among neurons in circuits, we hypothesized that signaling molecules play an important role in the function of male specific circuits. We describe here the identification of a novel putative G-protein coupled receptor (GPCR), encoded by the gene CG4395, that is required for male courtship. Mutants for the gene, which we named *hector* (*hec*), have courtship defects that can be rescued by expression of the wildtype protein in *fru* neurons. Co-localization studies of *fru* and *hec* suggest that the 4395 GPCR is required in a subset of glomeruli in the antennal lobes.

**Results**

CG4395 mutants have reduced male courtship

To examine whether CG4395 has a role in male courtship behavior, we tested two different pBac insertions, *PBac(00)06077* and *PBac(00)4274*. CG4395 is located at 11D4. The insertions are located in the last intron of the gene, 4 bp apart, but in different orientations. In order to control for genetic background, we outcrossed the mutants to a cantonized *w*1118 strain for ten generations. Since the gene is located on the X chromosome, we crossed the mutants to Canton-S wildtype in reciprocal crosses and tested male progeny with or without the mutant chromosome. We observed a significant drop in the courtship index (CI) for both
mutants (Figure 1A). The CI expresses the fraction of time a male spends performing any of the steps of the courtship ritual during the observation period. Although courtship of the mutant males is quantitatively reduced, it is not absent. To assess whether particular steps of courtship are affected in the mutants, we quantified latency (time to first orientation towards the female), fraction of wing extension during the overall courtship time, and the number of attempted copulations (Table 1). Mutant males are capable of all steps of courtship but perform them less frequently and seem to lack “motivation” to court. Control short-term activity assays did not show a difference among the genotypes tested (Figure 1B), indicating that the observed courtship phenotype was not caused by general sluggishness or sickness. To further assess the mutant phenotype, we performed additional courtship assays in red light (Figure 1C–E). Under these conditions, pheromonal input plays a major role in mate recognition. Without the visual input, courtship indices are generally lower, also in the control flies. The mutants show a similarly reduced defect in comparison to control males as in white light (Figure 1C). The same is true when they are paired with a matured virgin female (Figure 1D) (young virgin females were used in the other courtship assays). To further examine the effect of the mutation on male courtship behavior, we tested male-male courtship. Mutant males did not exhibit increased male-male courtship when compared to wildtype males and desat1573 mutant males whose male-male courtship phenotype has previously been described [23] (Figure 1E). Thus, mutant males appear to be able to distinguish between males and females.

Figure 1. Mutations in the CG4395 GPCR reduce male courtship. Graphs show the courtship index CI (fraction of time males spend courting during the observation period) ± SEM of the indicated genotypes, or the performance of males in a control activity assay (# of line crossings ± SEM). N = 10. Data were analyzed by ANOVA followed by Tukey Kramer multiple comparisons. * p < 0.001). CG4395 mutants PBac(f06077) and PBac(f04274) have a reduced CI in comparison to the control (A), but no defects in activity (B). A similar reduction in courtship is also observed when the assays are performed in red light with either a young or aged virgin female (C, D). Mutant males do not court other males, in contrast to desat1573 males (E). Expression of 4395-RNAi using the promoter construct 4395-Gal4 reduces the CI to the levels observed in the PBac mutants in a wildtype as well as a 4395 mutant background (F); activity in the mutants is not different from the control (G). The PBac(f06077) mutant phenotype can be rescued by expressing wildtype 4395 using the 4395-Gal4 driver and two independent UAS-4395 responder lines (lines 1 and 2) (H), (I) Conditional rescue in adult males. UAS-4395 expression is restricted by the presence of tubP-Gal80ts at 18°C (induction –). Placement of 5 day old males at 32°C for 40 hours (induction +) releases the inhibition and leads to expression of 4395. Adult induction of 4395 leads to the rescue of the mutant phenotype.

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To ascertain that the mutant phenotype we observed is due to the disruption of the 4395 gene, we performed rescue experiments by expressing the wildtype gene in the mutants using the Gal4/UAS system. We created 4395-Gal4 transgenic flies by placing 4.7 kb of the 4395 promoter region upstream of Gal4 coding sequences. This fragment contains 1.2 kb upstream of exon 1, the non-coding exon 1, intron 1 and exon 1 right up to the translation start. UAS-4395 transgenic lines were produced by insertion of the 4395 ORF downstream of UAS. P{Bac(f06077)} mutant males carrying 4395-Gal4 and UAS-4395 were tested in courtship. The two independent UAS-4395 lines tested were capable of completely rescuing the mutant phenotype (p<0.001) (Figure 1H). When 4395-Gal4 was used to express a UAS-4395-RNAi transgene we observed a reduction in courtship that was indistinguishable from the P{Bac(f06077)} mutants, and which could not be lowered further when 4395-RNAi was expressed in a P{Bac(f06077)} mutant background (p<0.001). Locomotion activity was not affected in these mutants either (Figure 1 F, G). We conclude from these experiments that the CG4395 GPCR, which we named “hector” (hec), is required for male courtship behavior. We next asked whether hector is required during development or whether it has a physiological role in adult courting males. We used the Gal80° conditional expression system [24] to examine whether expression of hector only in adult mature males was capable of rescuing the mutant phenotype. At 18°C, Gal80° represses 4395-Gal4. Thus, UAS-4395 is not expressed. Upon transfer of the flies to 32°C, Gal80° is inactivated, 4395-Gal4 is active and leads to the expression of the UAS-4395 rescue transgene. Virgin males grown at 18°C were collected and aged individually at 18°C for seven days. They were then placed at 32°C for two days, allowed to adjust to room temperature for one hour and tested for courtship. Control animals were continuously kept at 18°C, allowed to adjust to room temperature for one hour, and tested in parallel to induced flies. We observed complete rescue in flies in which 4395 was induced in adult males (p<0.001), indicating that the gene plays a physiological, rather than a developmental, role in normal courtship (Figure 1 I). This rescue was dose-dependent, as induction for one day only led to partial rescue (data not shown).

Table 1. Analysis of courtship elements in the mutants.

|                  | Latency [s]   | Wing Extension (% time of total courtship) | Attempted copulations |
|------------------|--------------|-------------------------------------------|-----------------------|
| y/Y (1)          | 3.8±0.75     | 0.69±0.06                                 | 7.5±2.0               |
| P{Bac4395f04274} | 4.2±0.82     | 0.36±0.06 *                               | 5.2±1.5               |
| y/Y (2)          | 5.2±0.75     | 0.65±0.04                                 | 5.6±0.9               |
| P{Bac4395f04277} | 5.3±1.3      | 0.40±0.06 *                               | 3.1±0.9               |

Individual courtship steps in a standard courtship assay were analyzed for males of the indicated genotypes paired with a wild-type virgin female. Values are mean ± SEM (N = 10). Latency: The time to first orientation toward the female is indicated. For wing extension, the relative time engaged in this behavior relative to the total time spent courting was calculated. For attempted copulation, the total number of events is given. Copulation was not scored since the females were only a few hours old and resisted copulation.

*Values that were significantly different from those of the control flies.

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CG4395 is expressed in several brain areas

To establish where hector is expressed, we crossed 4395-Gal4 flies to UAS-lacZ and UAS-GFP reporter lines in which the reporters are located to the nucleus, and examined expression by immunohistochemistry. We observed widespread expression in adult male brains. Prominent expression was observed in what appeared to be mushroom body (mb) cell bodies (Figure 2A). To further characterize this expression, we performed co-localization of 4395-Gal4 driven β-Gal with Dco, the catalytic subunit of PKA which is preferentially expressed in mushroom bodies [25]. As previously reported, Dco is present in mb cell bodies and processes. We observed co-staining in a subset of Kenyon cells. However, hector is only expressed in a subset of the Dco expressing cells, and not all 4395-Gal4 expressing cells in the dorsal brain area are mushroom body cells (Figure 2B, C).

To further visualize the mushroom body neurons that express 4395-Gal4, we crossed 4395-Gal4 to UAS-mCD8::GFP, thus expressing membrane bound GFP in hector neurons. GFP was visualized by anti-GFP antibody staining and co-localization with Dco was examined. We observed expression of 4395-Gal4 in the α, β and γ lobes of the mushroom bodies, but not the α’ and β’ lobes (Figure 2 D, G–I). Consequently, 4395-Gal4 expressing neurons form a subset of the neurons seen in the peduncle. 4395-Gal4 neurons were further found to project to the wheel of the mushroom bodies and make additional contacts with mushroom body neurons. In addition to expression in the mushroom bodies, prominent labeling of central complex neurons was observed (Figure 2 E). Expression was observed in the ellipsoid body (mainly in the interior layers R1 and R2, but not R4), the fan-shaped body, the noduli and the lateral triangles, the dendrites of ellipsoid body neurons, as well as the protocerebral bridge. Additional expression is visible in the median bundle and a number of isolated larger cells. 4395-Gal4 projections were also observed in a subset of glomeruli in the antennal lobes. Staining was present in the glomeruli DM3, DL3, DA1, VA2a, VA1v and V6 (Figure 2 F). Labeled cell bodies close by suggest that the 4395-Gal4 neurons might be projection neurons.

hector is required in a subset of fru cells

Which of the cells expressing 4395-Gal4 might be the relevant cells for the role of hector in male courtship? While 4395-Gal4 expression in the mushroom bodies is prominent, it has previously been shown that the mb are not required for courtship behavior per se [26]. We confirmed this by expressing 4395-RNAi in the mb using the well-characterized mb-driver P247-Gal4. Like 4395-Gal4, P247 is predominately expressed in the α, β and γ lobes [27]. As expected, these males did not show courtship defects (Figure 3C).

The central complex is implicated in the control of locomotion. Since the hec– and hec mutants are normal in an activity assay and can perform all steps of courtship, a reduction of hector in the central complex is probably not a major contributor to the courtship defects we observed.

The glomeruli of the antennal lobe in which 4395-Gal4 projections are found include the glomeruli that have been shown to contain fru expressing projection neurons (DA1, VA1v, V2Aa and V6) and have been implicated in male courtship [28,29]. They may therefore represent hector expressing cells within the antennal lobe that are important for male mating behavior.
Is the sexual identity of 4395-Gal4 expressing cells important for male courtship behavior? We used 4395-Gal4 to express traF, the key female regulator protein, to selectively feminize these cells in an otherwise normal male [30]. Males with feminized 4395-Gal4 cells had a significant decrease in their courtship index (Figure 3A). Conditional feminization only in adult flies did not affect male courtship, indicating that the relevant cells are sexually determined prior to adulthood (Figure 3B).

Figure 3. The sexual identity of 4395 Neurons is important for courtship. (A, B) Feminization of 4395-Gal4 expressing cells by the expression of UAS-traF reduces male courtship (A), indicating that these cells are sexually dimorphic. In contrast, conditional feminization in adult 5-day old males does not affect male courtship (B). (C) Expression of 4395-RNAi in the mushroom bodies using the P247-Gal4 driver does not affect courtship. N = 10. doi:10.1371/journal.pone.0028269.g003
These data demonstrate that the 4395-Gal4 cells that are important for courtship are sexually determined. This finding prompted us to examine the genetic relationship between fruitless, a major courtship regulator, and hector. When the hector mutation was placed in a heterozygous fra mutant background, we observed a significantly lower courtship index than in the hector mutants alone (p<0.001; Figure 4A). Heterozygous fra males do not have courtship defects. This indicates that the two genes are playing a role in the same overall genetic pathway that regulates normal male courtship behavior. This could indicate that hector is expressed in fra neurons, or and that the two kinds of neurons interact. To examine the two possibilities, we used fra-Gal4 to express 4395-RNAi, thus lowering hector in fra cells. We found that this led to a reduction of courtship to the levels observed in the hector PBac mutants, indicating the importance of signaling through the hector GPCR in fra neurons (p<0.001), Figure 4B). Again, the additional presence of the PBac^{hector} hector mutation in fra-Gal4/4395-RNAi flies did not further lower courtship. The mutants showed normal activity in a short term activity assay (Figure 4C).

To further test the hypothesis that hector is a downstream effector in fra neurons, we examined whether hector mutants could be rescued by the expression of hector protein only in fra neurons. As shown in Figure 4D, this was indeed the case. Furthermore, like in the case of the rescue with the 4395-Gal4 driver, conditional rescue in adult males using fra-Gal4 completely rescued the mutant phenotype (Figure 4E). Taken together these findings demonstrate that the hector GPCR is required in fra neurons of adult males for normal male courtship behavior.

Based on these findings, we suggest that 4395-Gal4 expressing cells that also express fra are likely to be the cells that require hector for courtship. To identify these cells, we generated flies that express a UAS-reporter under the control of 4395-Gal4 at the same time with a lexAoperator-reporter driven by fraPHexA [37]. We chose two kinds of reporters: One set with a nuclear localization signal, and the other expressing membrane-bound reporters in order to visualize neuronal projections. The results are shown in Figure 5. In the mushroom body area, numerous cells express both 4395-Gal4 and fra (Figures 5 A–F). In contrast to 4395-Gal4, fra is also expressed in the a’ and b’ lobes (Figures 5 L, L’, M, N). In order to assess the nature of cells that express both 4395-Gal4 and fra in the mushroom body area, we performed triple staining with anti-Deco. A representative optical section is shown in Figures 5 G–K. The cells co-expressing 4395-Gal4 and fra were found to be mushroom body neurons. We did not observe cells that co-expressed fra and 4395-Gal4 in the central complex. Staining in the antennal lobes was much weaker for both fra and 4395-Gal4 than in the other brain cells. fra expression was seen in glomeruli DA1 and VA1v (Figure 5Q), but presumably staining was too weak to see expression in VL2a and VA6, where its expression has also been reported [29]. hector expression was observed in the same subset of glomeruli already described in

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**Figure 4. CG4395 is required in fra neurons for male courtship.** Graphs show the courtship index CI (fraction of time males spend courting during the observation period) ± SEM of the indicated genotypes, or the performance of males in a control activity assay (# of line crossings ± SEM). N = 10. Data were analyzed by ANOVA followed by Tukey Kramer multiple comparisons. * p<0.001. (A) A mutation in CG4395 and fra interact gene in the same overall GPCR pathway significantly lower than the CI of PBac^{hector} alone. fra^{+/+} males have a normal CI. (B) Expression of 4395-RNAI by fra-Gal4 reduces the CI to the level of the PBac^{hector} mutants. The mutants have activity levels that are not different from the controls (C). (D,E) The PBac^{hector} mutant phenotype can be rescued by expression of wildtype UAS-4395 by fra-Gal4 (D). (E) Conditional adult expression of UAS-4395 by fra-Gal4 rescues the mutant phenotype. UAS-4395 expression is restricted by the presence of tubP-Gal80 at 18°C (induction –). Placement of 5 day old males at 32°C for 16 hours (induction +) releases the inhibition and leads to expression of 4395.

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CI of the mutant flies was still significantly lower than in wildtype, (Figure 6D). Courtship in all flies was reduced, as expected. The mutant flies and examined their courtship in red light which contains the olfactory receptors, in control CG4395 males showed significantly reduced courtship in white light as well as red light (Figure 6A, B)). This indicates a role for CG4395 in a subset of GH146 projection neurons. However, the effect was not as pronounced as in fru-Gal4/4395-RNAi flies, suggesting an additional role for CG4395 outside of GH146, and a different strength of the two drivers. Using 4395-RNAi, a projection neurons, or a different strength of the two drivers. Using 4395-RNAi, suggesting an additional role for CG4395 outside of GH146 that binds cis vaccenyl acetate (cVA) [18,32,33]. The 4395-RNAi flies responds to cis vaccenyl acetate (cVA) [18,32,33]. The agreement with previous findings that these structures are dispensable for basic courtship.

Expression of hector is also observed in a number of structures of the central complex. The central complex plays a crucial role in the control of locomotion [40]. However, our short term activity assays which reflect motor behavior did not indicate locomotion defects in the hector mutants we examined. Furthermore, we did not observe co-expression of hector and fru in central complex neurons (see below).

Our initial genetic interaction studies indicated that hector and fru act in the same overall pathways that regulate male courtship behavior. Remarkably, when we expressed hector in fru neurons by using the fru-Gal4 driver, this expression was sufficient to completely rescue the hector mutant phenotype, indicating that hector is required in fru neurons. Furthermore, expression of 4395-RNAi in fru neurons using fru-Gal4 leads to a mutant phenotype that is indistinguishable from the ones observed in the P Bac mutants. This implies a crucial role for hector in the control of male courtship behavior in a subset of fru neurons that co-express fru-Gal4 and 4395-Gal4. When we examined the co-expression of fru-Gal4 and hector we observed extensive co-labeling in the lateral protocerebrum. Not all hector expressing cells also express fru, and only a subset of fru cells expresses hector. Triple labeling experiments with anti-Dos, a mushroom body marker [25], demonstrated that the cells in the lateral protocerebrum that express both fru and hector are mushroom body neurons. As described above, mushroom bodies are not required for male courtship. This implies that cells outside the mushroom bodies, where hector and fru are co-expressed, are the cells that are required for male courtship. While we observed a few additional isolated cells that express both, the only other neurons where we have observed hector expression where fru is also expressed are neurons in a subset of glomeruli in the antennal lobes. Although expression of our reporters in the antennal lobes was much weaker than in other parts of the brain, we have observed hector projections in glomeruli DM3, DL3, DA1, VA2a, VA1v and V6. This includes all of the glomeruli that have been shown to be innervated by both fru ORN and fru projection neurons (DA1, VA1v, VL2a and V6). [28,29]. The best characterized among them is the DA1 glomerulus. It is innervated by the olfactory receptor neuron Or67d and has been shown to be involved in the perception of 11-cis-vaccenyl acetate (cVA) in both males and females [41,42,43]. cVA acts as a repellant in males, and an attractant in females. Or67d mutant males show increased courtship towards other males, but maintain courtship towards females. In contrast to the findings with Or67d mutants, we have not observed male-male courtship in hector mutants. We have also not observed an increase in latency, i.e. the time to first orientation towards the female, which is thought to be an indicator of impairment in perceiving pheromonal cues. Significantly, Stockinger et al. (2005) have shown that silencing of all of the fru projection neurons in the antennal glomeruli resulted in male-female courtship defects that were as strong as when they silenced all fru-expressing neurons [29]. This result implies an important role for these glomeruli in male-female courtship. Given the presence of hector projections in these glomeruli, and the results we obtained using fru-Gal4 driven rescue as well as hector knockdown by RNAi, we propose that the phenotypes we observe are in large parts caused by the absence of hector signaling in these glomerular fru neurons. Hence, signaling through the hector GPCR is likely a crucial component of male courtship in this subset of fru neurons. The observed courtship reduction in GH146/4395-RNAi males suggests that some of these neurons are projection neurons.

The hector protein is one of the few molecules identified that are specifically and functionally required in fru neurons. In support of
this, we also find that the feminization of hector expressing cells leads to courtship defects, demonstrating the sexually dimorphic nature of these neurons. However, feminization of these neurons in adult males does not affect courtship. This is in agreement with earlier genetic studies that suggested that the basic competence for male courtship is established in late pupal stages [44,45]. hector does not appear to be required to establish the male competence of fra neurons during development, since conditional expression of hector in adult males using the 4395-Gal4 as well as the fra-Gal4 driver was capable of completely rescuing the mutant phenotype. These results indicate that hector signaling is required to mediate adult male-specific signaling that is required for normal courtship. These signals may come from the fra ORN that project to these glomeruli, or from interneurons or other projection neurons. A fairly large number of neurotransmitters and neuropeptides has been found in the antennal lobes [46], and it remains to be seen what the ligand is for hector, and what signaling pathways lie downstream.

**Materials and Methods**

**Fly strains**

All flies strains were reared on standard corn meal/sugar-based medium at room temperature, except for Gal80 flies that were grown at 18°C and induced as adults at 32°C as indicated. The CG4395 mutants PBacCG4395[f04274] and PBacCG4395[f06077] were obtained from the Exelixis Drosophila Stock Collection at the Harvard Medical School. tra-F is w^{1118}, P[UAS-tra.F]2077 [30]. w^{1118}/CS and w; GH146-Gal4 were a gift from Gregg Roman, University of Houston. fra-Gal4 [12] was a gift from Barry Dickson, IMP, Austria. fraP1;lexA [31] is a gift from David Mellert and Bruce Baker, Janelia farms. LexAop-myr Cherry [32] was a gift from Gregg Roman, University of Michigan.

Cambridge and Erin Savner, Columbia University. UAS-4395 RNAi is DVRD stock no. 7223. desat1^{2727} [23] was a gift from Jean-François Ferveur, Université de Bourgogne, Dijon, France. w^{1119}; P[ub-P-Gal80]20; TM2/TM6B, Tb (stock no. 7019), w^{1118}; P[UAS-lacZ.NZ]f312 (stock no. 3956), y^{1} w^{*}; P[UAS-mCD8::GFP.P]1155 (stock no. 5137), w^{1118}; P[UAS-FGFP.nls]14, w^{1118}; lexA-2×hrGFP.nls (chs 2, stock no. 29954), w^{1118}; UAS-lacZ.NZ 20b (chs 2, stock no. 3955), and w^{1118}; [GH146-QF.P]53 P[QUAS-mdTomato-3×HA]244 (stock no. 30037) [48] were obtained from the Bloomington stock center.

**Courtship assay and Short term activity assay**

Assays were performed as described [37]. Tested males were 4–6 days old. For experiments with mature virgin females, females were collected 2–3 hours after eclosion and aged in groups for 4 days before the courtship assay. Male–male courtship was performed as described in [23]. For experiments with removed antennae, antennae of newly eclosed virgin males were removed with a razor blade. The flies were then put in individual vials and tested for courtship 4–5 days later.

**Immunohistochemistry**

The brains of 4–5 day-old male flies were dissected in 1× PBS and fixed in freshly prepared 4% paraformaldehyde in PBSH (PBS containing 1 M NaCl) for 20 min at room temperature. All subsequent procedures were performed at room temperature, except for antibody incubations. Brains were washed three times in 1× PBH5/0.5% Triton for 15 min each and then washed three times in 0.1 M Tris-HCl/0.3 M NaCl (pH 7.4), containing 0.5% Triton X-100 (TNT) for 15 min each. Blocking was performed in TNT solution containing 4% normal goat serum (blocking buffer) for 1.5 h. The primary antibody was applied in blocking solution and incubated overnight at 10°C. The brains were rinsed six times with PBH5/0.5% Triton/0.3 M NaCl and incubated in blocking buffer for 1 h before second antibody incubation.

**Immunostaining**

The brains were rinsed six times with PBH5/0.5% Triton/0.3 M NaCl and incubated in blocking buffer for 1 h before second antibody incubation. The brains were then rinsed six times with PBH5/0.5% Triton/0.3 M NaCl before the second antibody incubation. For visualization of the secondary antibody, the brains were imaged using a Zeiss Axio Observer Z1 microscope equipped with a Zeiss AxioCam MRm camera (Zeiss, Oberkochen, Germany) and dedicated software (Axio Vision 4.7, Zeiss).

**Figures**

**Figure 5. Localization of cells that express both fra and 4395-Gal4.** (A–F) Brains of adult UAS-lacZ^{nls}/lexAop::GFP^{nls}; 4395-Gal4/traF1LexA males were double-stained with anti-β-Gal (magenta) and anti-GFP (green) to identify cells expressing both fra and hector. Although this GFP carries a nuclear localization signal (nls), diffusion of the protein is also seen into the projections. Co-staining is observed in numerous cell bodies in the mb area. (A, D: merged images, B, C, E, F: single images). A–C show the Z-stack of a whole brain, D–F show a representative magnified optical section in the area of the mb calyx. (G–K) Triple staining of UAS-lacZ^{nls}/lexAop::GFP^{nls}; 4395-Gal4/fruP1LexA males with anti-β-Gal (blue), anti-GFP (green) and anti-Dco (red) shows that cells that express both fra and hector are mushroom body cell bodies. Co-staining is shown in G, single channels are shown in M, N, O. (L–Q) Brains of adult UAS-mcd8::GFP/4395-Gal4; lexAop-myr-mCherry/fruP1LexA were co-stained with anti-GFP (magenta) and anti-RFP (green). The projections of the neurons can be seen. In contrast to 4395-Gal4, fra, in addition to expression in the α, β and γ lobes, is also expressed in the α’ and β’ lobes. (L’) shows a magnification at the level of the β and γ lobes that visualizes overlap as well as close proximity of the labeled projections. (O–Q) Colocalization of fra and 4395-Gal4 staining in glomeruli of the antennal lobes. 4395-Gal4 projections are visible in all fra positive glomeruli.

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**Figure 6. Reduction of CG4395 RNAi in GH146 neurons reduces courtship.** Graphs show the courtship index CI (fraction of time males spend courting during the observation period) ± SEM of the indicated genotypes, N = 10. Expression of 4395-RNAi in GH146 projection neurons reduces male courtship in both white and red light (A, B). (C) Optical sections showing co-expression of UAS-mcd8::GFP/4395-Gal4 (magenta) and UASQ-tomato::GH146-Q (green) in antennal glomeruli. (D) CG4395^{f06077} mutant males missing the third antennal segment court less than control males.

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for 15 min each and five times for 30 min each in TNT. The secondary antibody was diluted in blocking solution and incubated overnight at 10°C. Brains were washed six times for 15 min each. Fluorescently labeled brains were mounted with Vectashield mounting medium (Vector Laboratories, CA). For double-staining experiments, antibody stainings were performed sequentially using the procedure described above.

**Antibodies used**

Chicken anti-β-galactosidase (abcam ab3961) 1:500; Mouse anti-β-galactosidase (Sigma G8021) 1:100; Rabbit anti-β-Galactosidase Rabbit anti-GFP (abcam, ab62341) 1:100, Rabbit anti-GFP (Invitrogen 673782) 1:100; Mouse anti-GFP (Roche 11814460001) 1:50, Mouse nc82 (Developmental Studies Hybridoma Bank, Univ. of Iowa) 1:20, Rabbit anti-Dco (Skoulakis et al., 1993) 1:100, Alexa Fluor 635 goat anti-mouse (Invitrogen A31575) 1:200, DyLight 405 goat anti-chicken (Jackson Immuno Research 1993) 1:100, Alexa Fluor 635 goat anti-mouse (Invitrogen A31575) 1:200, Alexa Fluor 555 goat anti-rabbit (Invitrogen A21429) 1:200.

**Confocal microscopy**

Fluorescent preparations were viewed using an Olympus FV1000 confocal microscope, Images were processed using Adobe Photoshop.

**References**

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