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1 **Full Title:** Histamine signaling *via* the metabotropic receptor *Trapped in endoderm 1* regulates

2 courtship initiation in *Drosophila melanogaster*.

3

4 **Short Title:** Histamine regulates *Drosophila* Courtship Behavior

5

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15 **Abstract**

16 Understanding the role of genes in directing behavior is one of the primary goals of
17 neuroscience. Mating behavior in *Drosophila* is controlled by male-specific splicing of the
18 master regulatory gene *fruitless* (*fru*), and the male-specific splice form, $fru^{M}$, is both necessary
19 and sufficient for all aspects of the courtship ritual. We have previously described the role of
20 *Trapped in endoderm 1* (*Tre1*) in courtship behavior. *Tre1* encodes an orphan G-protein-coupled
21 receptor that is essential for normal courtship behavior in male flies. We previously found that
22 feminizing *Tre1*-expressing cells in males via expression of the female-specific splicing factor
23 Transformer ($Tra^{F}$) resulted in rapid courtship initiation. Here we confirm that Tre1 is required
24 in neurons for normal courtship behavior, and present genetic evidence that Tre1 acts through
25 the downregulation of the E-cadherin Shotgun, and that the neurotransmitter histamine is the
26 likely Tre1 ligand. Our findings are the first evidence for metabotropic histamine receptors in
27 *Drosophila*, and the first to demonstrate a role for histamine in courtship.

28

29 **Introduction**

30 Genetic regulation of behavior is a vital topic in neuroscience—understanding gene-
31 controlled behaviors will help to elucidate the intricacies of the central nervous system (CNS).
32 The common fruit fly *Drosophila melanogaster* is an ideal model organism for the study of the
33 genetic control of behavior. *Drosophila* has been used as a genetic model organism for over 100
34 years and provides a vast array of genetic manipulation techniques, complex and instinctive
35 innate behaviors, and simple methods for observation of neuronal activity.

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36 Male *Drosophila* carry out a complex courtship ritual involving a specific sequence of
37 steps which depend on the processing of sensory cues by the CNS. First, the male fly orients
38 himself toward the female fly and follows her. He then taps her with his forelegs, which contain
39 gustatory receptors that he uses to taste her. Next, he performs a species-specific wing song
40 where he extends and vibrates one wing at a time towards her. Next, the male fly licks the
41 genitalia of the female to open up the vaginal plate and further assess pheromonal cues, and
42 lastly attempts copulation by curling up his abdomen (EBBS and AMREIN 2007). Mating and
43 reproduction depends on the correct performance of this ritual. Each of these courtship
44 behaviors is easily observable, and thus courtship makes for an ideal behavioral model.

45 We previously reported the identification of *Trapped in endoderm* 1 (*Tre1*) as a novel
46 gene controlling courtship initiation in *Drosophila* (LUU *et al.* 2015). *Tre1* encodes an orphan G-
47 protein-coupled receptor (GPCR) that is required for both germ cell migration and
48 establishment of cell polarity (KUNWAR *et al.* 2008; YOSHIURA *et al.* 2012), and is closely related
49 to a family of GPCRs in vertebrates that respond to hormones and neurotransmitters, including
50 melatonin and histamine (YOSHIURA *et al.* 2012). We identified a set of neurons that express the
51 transgene *Tre1-GAL4,* which, when genetically feminized either through expression of the
52 female-specific splicing factor Transformer ($Tra^F$), or expression of an RNAi construct targeted
53 to the male-specific transcripts of the *fruitless* gene ($Fru^M$), result in males displaying unusually
54 rapid courtship initiation (TRAN *et al.* 2014; LUU *et al.* 2015). We also found that feminization of
55 *Tre1*-expressing cells led to a competitive reproductive advantage, and that *Tre1-GAL4* is
56 expressed in a sexually dimorphic fashion in the olfactory organs and adult CNS (LUU *et al.*
57 2015).

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58 Tre1 can signal through multiple heterotrimeric G-proteins. In establishment of cell
59 polarity, the current model suggests that upon detection of an extrinsic signal, Tre1 activates
60 the G-protein $G_o\alpha$, which then recruits Pins, which subsequently recruits the entire polarity
61 complex (YOSHIURA *et al.* 2012). In germ cell migration, Tre1 signals through the G-proteins Gγ1
62 and Gβ13f (KUNWAR *et al.* 2008). Additionally, Tre1 directs the redistribution of the *D.*
63 *melanogaster* E-cadherin, encoded by *shotgun (shg),* resulting in its polarized downregulation.
64 Here we further characterize the role of the Tre1 cells in courtship behavior. We confirm
65 that *Tre1* function is required in neurons, and that genetic silencing of *Tre1-GAL4* expressing
66 neurons results in rapid courtship initiation. We further identify histamine as the likely ligand of
67 the Tre1 GPCR in courtship, and provide behavioral evidence for the negative regulation of
68 *shotgun* by Tre1 during courtship. This is the first demonstration for a role of histamine in
69 courtship behavior, as well as the first evidence for the existence of metabotropic histamine
70 receptors in *Drosophila*.
71
72 **Materials and Methods**
73 **Fly Strains and Genetics**
74 Fly stocks were maintained at 25° C on standard cornmeal/ yeast/ molasses medium.
75 With the exception of RNAi strains from the Transgenic RNAi Project (TRiP), all mutant alleles
76 and transgenes were introgressed for five generations into our standard lab background [$w^{1118}$;
77 Wild Type Berlin (*w*; WTB)]. *UAS-Tra*$^{F}$, *UAS-mCD8-GFP*, *UAS-shgRNAi*, *UAS-Gγ1RNAi*, UAS-
78 *Gβ13fRNAi, UAS-ctaRNAi, Hdc*$^{MB07212}$, *Hdc*$^{JK910}$, *UAS-NaChBac*, and *UAS-Tre1RNAi* were all
79 obtained from the Bloomington Drosophila Stock Center (Bloomington, Indiana) (stock nos.

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80 4590, 5130, 32904, 25934, 31134, 31132, 25260, 64203, 9469, and 34956). *Tre1-GAL4* was
81 isolated using meiotic recombination from the strain *GAL4*$^{9\text{-}210}$ (LUU *et al.* 2015). The strains
82 *GAL4*$^{9\text{-}210}$, *w*$^{1118}$*; UAS-TNT(II)*, and *UAS-Elav-GAL4[3E1]* were gifts from Ulrike Heberlein.

83 **Courtship Assays**

84 Courtship assays were performed according to published protocols (VILLELLA *et al.* 1997).
85 Virgin males were collected and kept in isolation for 1-4 days. After this period of isolation, each
86 male was presented with a single 1-to-4-day-old *w*$^{1118}$;WTB virgin female. Custom plexiglass
87 chambers were used to contain the flies during the courtship assays, with each chamber being
88 10 mm in diameter and 6 mm in height. One male and one female were placed in each
89 chamber, separated by a plastic transparency. After an acclimation period of 2-3 hours in the
90 dark, plastic transparencies were removed to initiate contact between the pairs. Courtship
91 behavior was recorded in infrared light for 20 minutes.

92 **Immunofluorescence**

93 The CNS and peripheral tissue were dissected and fixed according to (WU and LUO 2006)
94 with the following modifications: tissues were fixed for 1 hr, incubated with NGS block for 24
95 hr, stained in primary antibody for 4 days, and secondary for 2 days. NC82 mouse anti-Brp was
96 used at 1:50 (Developmental Studies Hybridoma Bank, AB 2314866). Rabbit anti-GFP was used
97 at 1:750 (Life Technologies, A6455). Alexa Fluor 488 goat anti-rabbit was used at 1:1000
98 (Jackson Immunoresearch, 111-545-144). Alexa Fluor 594 goat anti-mouse was used at 1:1000
99 (Jackson Immunoresearch, 705-586-147). Images were taken using a Zeiss LSM700 confocal
100 microscope.

101 **Statistics**

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102 An $\alpha$ level of 0.05 was used in all experiments. Data are represented as boxplots where the
103 median is the middle line, and the 1st and 3rd quartiles are the lower and upper edges of the box,
104 respectively. Whiskers represent the lowest and highest data points still within 1.5x interquartile range,
105 while open dots represent data points outside of this range. Statistics were performed on log-
106 transformed means of wing song latency. Data were analyzed using one-way analysis of
107 variance (ANOVA), followed by Tukey's HSD *post hoc* test. No statistical tests were used to
108 predetermine sample sizes, but our sample sizes are consistent with those reported in previous
109 publications (TRAN *et al.* 2014; LUU *et al.* 2015).

110 **Reagent and Data Availability**

111 All strains and reagents are available upon request. The full dataset will be uploaded as a
112 supplementary file upon publication.

113

114 **Results**

115 ***Tre1* is required in neurons for normal courtship behavior**

116 As we have previously shown, (LUU *et al.* 2015), *Tre1* is required for normal courtship,
117 and is expressed in neurons in both the periphery and CNS. To demonstrate that neuronal
118 expression of *Tre1* is required for its role in courtship behavior, we used the pan-neuronal
119 driver *elav-GAL4*$^{c155}$ to drive expression of *Tre1-RNAi* (Figure 1A). When we knocked down *Tre1*
120 in neurons, male flies displayed rapid courtship initiation relative to background controls.
121 Average time to courtship initiation in *elav-GAL4/Y; UAS-Tre1RNAi/+* males was 19 seconds,
122 compared with 30-43 seconds in heterozygous genetic background controls (Figure 1A). These

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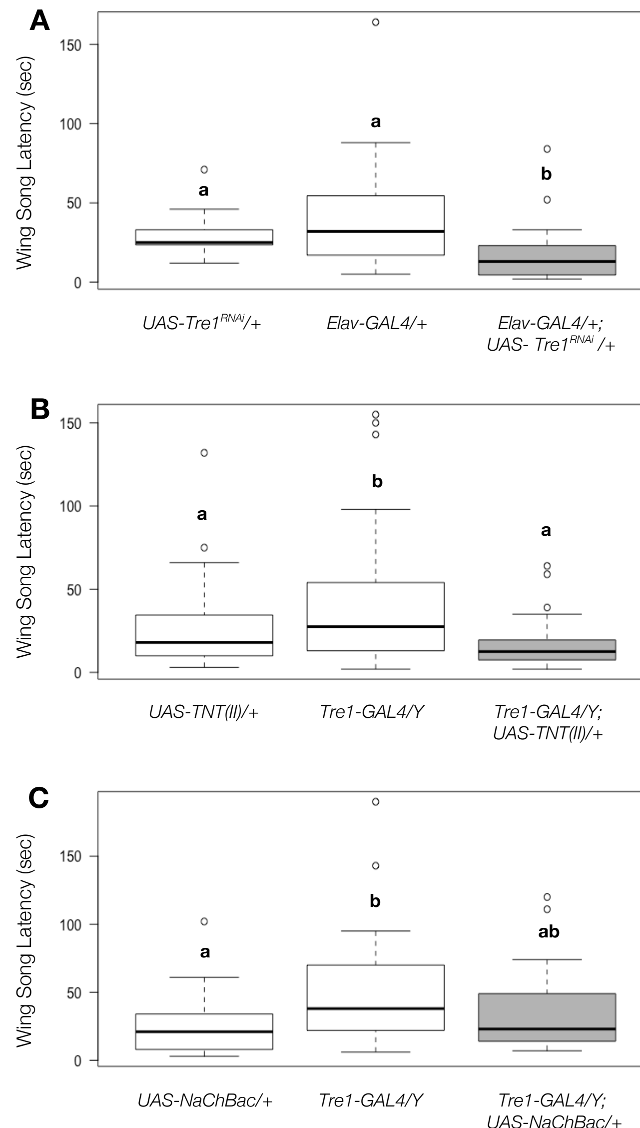
123 results demonstrate that *Tre1* expression is required specifically in neurons to establish a

124 normal courtship initiation time.




125 **Figure 1** *Tre1* is required in neurons for normal courtship behavior and inactivation of Tre1

126 neurons results in rapid courtship initiation. **A.** *elav-GAL4/Y; UAS-Tre1RNAi*/+ males initiate

127 courtship in 19 seconds, on average, compared with 30-43 seconds in controls (N=11-16, One-

128 Way ANOVA with Tukey's HSD posthoc analysis. **B.** *Tre1-GAL4/Y; UAS-TNT(II)/+* males initiate

129 courtship in 17 seconds, on average, compared with 42 seconds in *Tre1-GAL4/+* controls and 25

130 seconds in *UAS-TNT/+* controls. (N=42-44, One-Way ANOVA with Tukey's HSD posthoc analysis).

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131 **C.** Expression of *UAS-NaChBac* in *Tre1-GAL4* neurons has no effect on the speed of courtship
132 initiation relative to controls (N=21-24, One-Way ANOVA with Tukey's HSD posthoc analysis).
133 Boxplots represent the median as the middle line, and the 1st and 3rd quartiles as the lower
134 and upper edges, respectively. Whiskers represent the lowest and highest data points still
135 within 1.5x interquartile range. Open dots represent outliers (data points outside 1.5x
136 interquartile range). Boxes sharing the same letter do not differ significantly, while boxes with
137 different letters are significantly different ($p \leq 0.05$).
138
139 **Silencing of *Tre1* cells results in rapid courtship initiation**
140 We have previously shown that feminization of *Tre1* cells in males via expression of the
141 female splicing factor Transformer results in rapid courtship initiation (Luu *et al.* 2015). This is
142 an unusual phenotype, as feminization of neurons generally leads to delayed or absent
143 courtship. We therefore considered the possibility that developmental feminization of the *Tre1*
144 neurons results in an unusual gain-of-function effect that was not representative of the gene's
145 true function. To address this question, we used genetic techniques to examine the effects of
146 silencing, and activation, of the *Tre1*-expressing neurons. Silencing the *Tre1-GAL4* neurons via
147 expression of tetanus toxin (*UAS-TNT(II)*) resulted in males with wing song latency significantly
148 shorter than that of *Tre1-GAL4*/Y background controls (average time to courtship initiation was
149 17 seconds for *Tre1-GAL4/Y; UAS-TNT(II)/+* and 42 seconds in *Tre1-GAL4/Y* controls) and these
150 males were also somewhat faster than *UAS-TNT(II)/+* males (25 seconds to initiate courtship),
151 though this result did not achieve statistical significance, due at least in part to the fact that
152 *UAS-TNT(II)/+* males also display somewhat rapid courtship initiation (Fig. 1B). We attempted to

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153 silence the *Tre1-GAL4* neurons using a variety of alternative transgenes, but found that, in most
154 cases, the result was lethality, or flies with profound locomotor defects that would impact
155 courtship behavior in a nonspecific fashion (not shown).

156 In order to activate the *Tre1*-expressing neurons, we drove expression of a voltage-
157 gated bacterial sodium channel, *UAS-NaChBac* (HODGE 2009) with *Tre1-GAL4*. There was no
158 significant difference in wing song latency in *Tre1-GAL4/Y*; *UAS-NaChBac/+* males relative to
159 either of the background controls (average time to courtship initiation for *Tre1-GAL4/Y*; *UAS-*
160 *NaChBac/+* males was 36 seconds, compared with 27-52 seconds for heterozygous background
161 controls; Figure 1C). These experiments suggest that feminizing the *Tre1* neurons results in loss
162 of normal neuronal function, which is supported further by our previously-published
163 experiments showing that a loss-of-function mutation in Tre1 (*Tre1*$^{EP496}$) phenocopies
164 feminization of the *Tre1* neurons (LUU *et al.* 2015).

165

166 **Feminization of *Tre1* cells results in the loss of *Tre1-GAL4* expression**

167 We have previously shown that *Tre1-GAL4* is expressed in a sexually dimorphic pattern
168 in the brain, with more expression in the male brain than the female brain (LUU *et al.* 2015). We
169 hypothesized that feminization of the *Tre1*-expressing cells would lead to the loss of this
170 sexually dimorphic expression pattern. To test this hypothesis, we used *Tre1*-GAL4 to drive
171 simultaneous expression of UAS-*Tra*$^{F}$ and UAS-mCD8-*GFP* (Figure 2). Consistent with our
172 hypothesis, *Tre1-GAL4/Y; UAS-Tra*$^{F}$*/+; UAS-mCD8-GFP/+* males have little to no GFP expression
173 compared with *Tre1-GAL4/Y; UAS-mCD8-GFP/+* males (Figures 2A, B). These results provide

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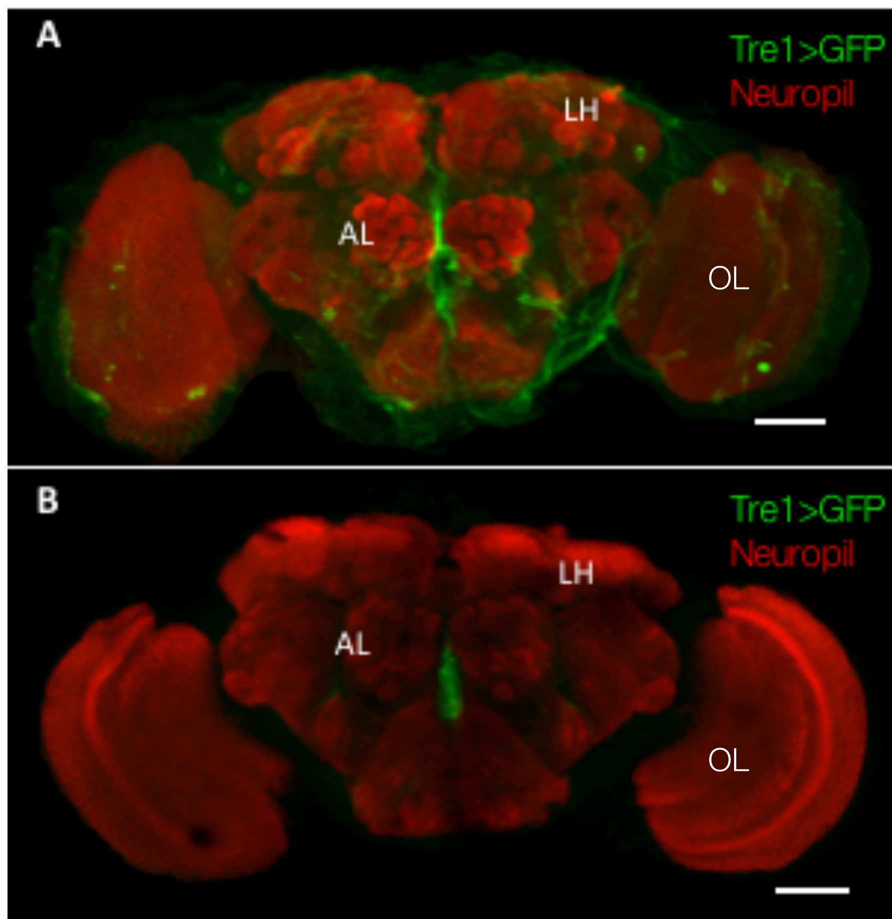
174 further evidence that feminization of the *Tre1*-expressing neurons results in a loss-of-function

175 effect.




176

177 **Figure 2** *Tre1-GAL4* expression in male brains is lost when *Tre1* cells are feminized. **A-B**.

178 Representative confocal images of brains from male flies expressing GFP in *Tre1*-expressing

179 cells; scale bars: 50 µM **A.** In *Tre1-GAL4>UAS-GFP* male brains, *Tre1* is expressed in a variety of

180 regions, including the antennal lobe (AL), lateral horn (LH), and optic lobes (OL). **B.** In *Tre1-*

181 *GAL4>UAS-Tra$^F$, GFP* male brains, *Tre1-GAL4>UAS-GFP* expression is lost.

182

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183 **Shotgun/E-cadherin is involved in regulating courtship initiation**

184 The Tre1 GPCR has been studied in other contexts, and some downstream components
185 of the G-protein signaling pathway are known in those systems (KUNWAR *et al.* 2008; YOSHIURA *et*
186 *al.* 2012). To begin to characterize the signal transduction pathway downstream of Tre1 in
187 courtship, we screened known downstream effectors of Tre1 for aberrant courtship initiation.
188 Based on the findings of other groups, we tested a host of candidate genes including: *Gγ1*,
189 *Gβ13f*, the $G_{\alpha12,13}$ subunit encoded by *concertina*, and the E-cadherin Shotgun; of these, only
190 knockdown of *shotgun* resulted in aberrant courtship behavior (Supplementary Figure and
191 Figure 3A).

192 *shotgun* (*shg*) encodes *D. melanogaster* E-cadherin, which is responsible for cell-cell
193 adhesion, and mutations in *shg* result in the opposite effect of mutations in *Tre1* on germ cell
194 migration (KUNWAR *et al.* 2008). Knocking down *shg* in *Tre1* cells via expression of *shg-RNAi*
195 resulted in a significantly slower time to courtship initiation compared with control males. *shg*
196 knockdown males took, on average, 127 seconds to initiate courtship compared to 59 and 43
197 seconds in background controls (Fig. 3A). These results demonstrate that *shg*/*E-cad* is involved
198 in the regulation of courtship initiation time, and suggest that E-cadherin is a downstream
199 component regulated by *Tre1* signal transduction.

200 **Histamine is the likely ligand of the Tre1 receptor**

201 Tre1 encodes an orphan G-protein-coupled receptor (GPCR), with an endogenous ligand
202 that has yet to be identified. The Tre1 receptor shares sequence homology with a family of
203 GPCRs that respond to hormones and neurotransmitters including melatonin and histamine, as
204 well as being more distantly related to chemokine receptors such as CXCR4 (KUNWAR *et al.*

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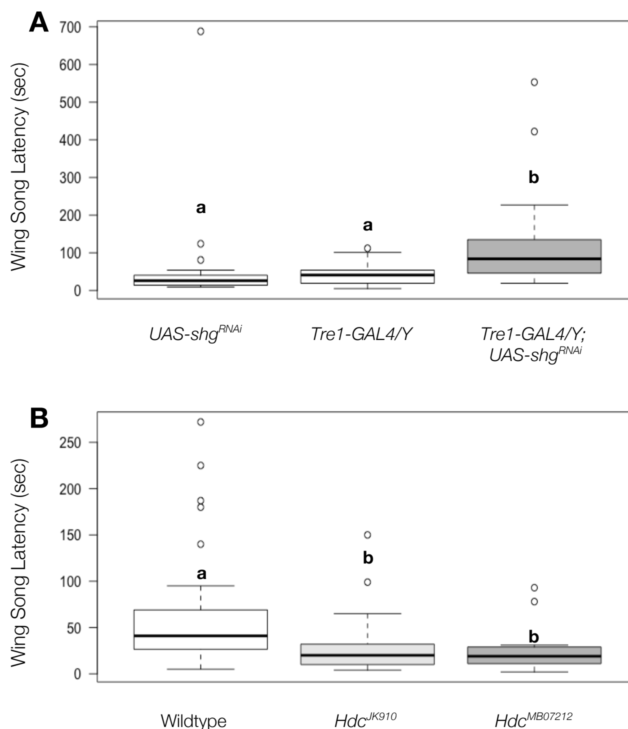
205 2003) In order to identify the Tre1 ligand, we screened mutations in candidate genes for
206 courtship phenotypes and found that male flies mutant for histidine decarboxylase (*Hdc)*
207 displayed rapid courtship initiation (Figure 3B). Histidine decarboxylase catalyzes the
208 decarboxylation of histidine to form the neurotransmitter histamine (BURG *et al.* 1993). We
209 tested two alleles of *Hdc*, $Hdc^{JK910}$ and $Hdc^{MB0721}$, and found that males homozygous for these
210 alleles initiated courtship on average in 31 and 25 seconds, respectively, compared to 61
211 seconds in control flies. These data demonstrate that the neurotransmitter histamine is
212 required for normal courtship behavior, and strongly suggest that histamine is the ligand for
213 Tre1 in courtship initiation.




214 **Figure 3** E-cadherin and histamine are involved in the timing of courtship initiation. **A.**
215 *Tre1GAL4/Y;UAS-shg-RNAi/+* males display a significant delay in wing song latency (127 s)
216 relative to control animals (43-59 s) (N=19-24, One-Way ANOVA with Tukey's HSD post-hoc

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217 analysis). **B.** Histidine decarboxylase mutants, *Hdc*$^{JK910}$ *and Hdc*$^{MB07212}$, display rapid wing song
218 latency (31 s, n=25, P= 2.85 x $10^{-3}$ and 25 s, n= 21, P= 2.04 x $10^{-3}$, respectively) relative to
219 control animals (61 s, n=39). Boxplots represent the median as the middle line, and the 1st and
220 3rd quartiles as the lower and upper edges, respectively. Whiskers represent the lowest and
221 highest data points still within 1.5x interquartile range. Open dots represent outliers (data
222 points outside 1.5x interquartile range). Boxes sharing the same letter do not differ
223 significantly, while boxes with different letters are significantly different ($p \leq 0.05$).
224

225 **Discussion**

226 ***Tre1* is required in neurons for normal courtship behavior**

227 We previously demonstrated that rapid courtship initiation results from both
228 feminization of *Tre1-GAL4*-expressing cells as well as mutation of the GPCR-encoding *Tre1* gene
229 itself (LUU *et al.* 2015). Here we demonstrate that this requirement is specific to neurons—
230 when *Tre1* is knocked down specifically in neurons using the pan-neuronal driver *elav-GAL4*$^{c155}$,
231 males exhibit rapid courtship initiation (Figure 1A). In addition, we provide three lines of
232 evidence that reducing the speed of courtship initiation is a normal function of the *Tre1*-
233 expressing neurons: first, loss-of-function mutations in *Tre1* lead to rapid courtship (LUU *et al.*
234 2015), suggesting that rapid courtship initiation is a result of loss of normal neuronal function.
235 Second, silencing the *Tre1-GAL4* neurons through expression of tetanus toxin results in rapid
236 courtship initiation, similar to that seen with mutation of *Tre1* or feminization of the *Tre1*
237 neurons (Figure 1B). Third, we show that feminizing the *Tre1-GAL4* neurons results in loss of

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238 Tre1 expression (Figure 2B), further suggesting that feminization of the *Tre1-GAL4* neurons
239 results in a reduction of normal function.

240 While silencing of the *Tre1-GAL4* neurons resulted in significantly rapid courtship
241 initiation relative to the *Tre1-GAL4/Y* genetic control, the decrease in courtship initiation speed
242 when compared to the *UAS-TNT(II)/+* control did not achieve statistical significance (Figure 1B).
243 We have three hypotheses to explain this result. First, it is possible that this is due to the fact
244 that we were forced to use a relatively "weak" combination of transgenes for this experiment.
245 We tried a number of approaches to silencing or eliminating the *Tre1-GAL4* neurons, including
246 alternative insertions of the *UAS-TNT* transgene and expression of the cell death protein Reaper
247 (Rpr). We found that, with the exception of the second-chromosomal insertion of *UAS-TNT*,
248 these manipulations resulted in lethality, or, when flies survived, profound locomotor
249 deficiencies. Thus, the experiment may have failed to achieve significance due simply to the
250 relatively weak silencing that was possible with the available tools.

251 Additionally, the time to courtship initiation for the *UAS-TNT(II)/+* controls was relatively
252 rapid compared with typical control speeds, which may contribute to "masking" the effect of
253 silencing the *Tre1-GAL4* cells relative to that control.

254 Finally, it is a formal possibility that, while *Tre1* gene function is required for the timing
255 of courtship initiation, the *activity* of the *Tre1*-expressing cells is not required for the behavior
256 itself. In such a scenario, there might be a requirement for *Tre1* in directing the development of
257 a neural circuit, such that the *Tre1* neurons influence the development of another group of
258 cells, but are not, themselves, required in the adult brain for the expression of the behavior. We

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259 are currently working to test this hypothesis, first, by establishing whether there is a
260 developmental requirement for *Tre1*.
261
262 **Histamine Is Required for Courtship Behavior**
263 In order to investigate possible ligands for the Tre1 receptor, we screened mutations in
264 candidate genes for courtship phenotypes. Because Tre1 has significant sequence similarity to
265 vertebrate histamine receptors (KUNWAR *et al.* 2003), we tested flies mutant for *histidine*
266 *decarboxylase* (*Hdc*). Male flies homozygous for loss-of-function mutations in *Hdc* display the
267 same unusually rapid courtship initiation phenotype we observe in *Tre1* hypomorphs and *Tre1-*
268 *GAL4/Y;UAS-Tra$^F$* /+ males (Figure 3B). Further, histamine is the primary neurotransmitter
269 responsible for photoreception in insects (ELIAS and EVANS 1983; HARDIE 1987; MELZIG *et al.*
270 1996), and Tre1-GAL4 is expressed in the optic lobe in both male and female flies (Figure 2A
271 and data not shown). These data strongly suggest that histamine is the Tre1 ligand.
272 In addition to the requirement for histamine in insect photoreception, histamine is
273 involved in mechanosensation and thermotaxis in flies (BUCHNER *et al.* 1993; MELZIG *et al.* 1996;
274 HONG *et al.* 2006), as well as regulation of sleep-wake cycles in both flies and mammals
275 (WAUQUIER *et al.* 1981; THAKKAR 2011; OH *et al.* 2013).
276 There are four known metabotropic histamine receptors (H1-H4) in humans, all of which
277 are G-protein-coupled receptors, and these regulate various biological processes including:
278 sleep-wake regulation, immune responses, neurotransmission, and chemotaxis (BROWN *et al.*
279 2001; PANULA *et al.* 2015). This work is the first demonstration of histamine's involvement in
280 courtship behavior; moreover, the only characterized receptors for histamine in flies are

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281 histamine-gated chloride channels (ZHENG *et al.* 2002)—ours is the first suggestion of a
282 metabotropic histamine receptor in *Drosophila*, and only the second in invertebrates (ROEDER
283 2003).
284
285 **The Role of E-cadherin In Courtship Behavior**
286 Males in which E-cadherin has been knocked down in the *Tre1-GAL4* neurons display a
287 significant delay in courtship initiation, the opposite phenotype from that seen with loss of *Tre1*
288 Figure 3A). To our knowledge, this is the first demonstration for a role of E-cadherin in
289 courtship regulation.
290 In germ cell migration, *Tre1* expression results in polarized downregulation of Shg/E-cad,
291 and this downregulation is necessary for germ cell dispersal (but not sufficient for
292 transepithelial migration) (KUNWAR *et al.* 2008). Thus, when germ cells were mutant for *Tre1*,
293 they failed to disperse due to continued expression of E-cad, while, when E-cad was mutated,
294 germ cell dispersal occurred prematurely. Moreover, downregulation of E-cadherin promotes
295 neuronal migration in mice (ITOH *et al.* 2013; CHEN *et al.* 2015).
296 E-cadherin is expressed widely in both vertebrate and *Drosophila* nervous systems
297 (FANNON and COLMAN 1996; UCHIDA *et al.* 1996; IWAI *et al.* 1997; BRUSÉS 2000; TEPASS *et al.* 2000;
298 DUMSTREI *et al.* 2003; PRAKASH *et al.* 2005; FUNG *et al.* 2008), and cadherins (both N- and E-
299 cadherin) have been implicated in axon targeting (PRAKASH *et al.* 2005), synaptic partner
300 recognition (SHAPIRO and COLMAN 1999), synaptogenesis (BENSON and TANAKA 1998; TAKAI *et al.*
301 2003; BOZDAGI *et al.* 2004; ARIKKATH and REICHARDT 2008), synaptic adhesion (FANNON and COLMAN
302 1996; BRUSÉS 2000), and synaptic plasticity (TANG *et al.* 1998; ARIKKATH and REICHARDT 2008).

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303 Given our data that loss of both histamine and Tre1 lead to rapid courtship initiation,
304 while *shg/E-cad* mutants have the opposite phenotype, we propose that histamine signals
305 through Tre1 to downregulate E-cad, and that this interaction is required to properly regulate
306 either synaptogenesis or synaptic adhesion between the Tre1 neurons and their targets
307 Both E- and N-cadherin-containing synapses are present throughout the CNS of the
308 adult mouse, and many synapses label with neither antiserum, suggesting that still other
309 cadherins are present at those junctions. Further, the "zones" of E- and N-cadherin expression
310 are largely nonoverlapping, and it has been proposed that cadherins serve to organize the
311 specificity of synaptic junctions as well as provide adhesive connections between neurons
312 (FANNON and COLMAN 1996). N-cadherin is found at both the presynaptic and postsynaptic
313 terminals in cultured rat hippocampal neurons, and clusters together with synaptic markers at
314 developing synapses (BENSON and TANAKA 1998). Later, however, N-cadherin is retained at
315 excitatory synapses, but is lost from inhibitory synapses, while E-cadherin is often found at
316 inhibitory synapses (BENSON and TANAKA 1998; BRUSÉS 2000), suggesting that synapse formation
317 and maintenance may be dependent on developmental regulation and expression of specific
318 types of cadherins.
319 We propose a model in which the Tre1 neurons modulate the speed of courtship
320 initiation, in response to an as-yet-unidentified external cue. Histamine, signaling through the
321 Tre1 receptor, reduces the expression of E-cadherin, and this is necessary for establishment of
322 the correct number (or strength) of synaptic connections between the Tre1 neurons and
323 downstream neurons in the courtship circuit. In neurons deficient for either histamine or Tre1,
324 inappropriate expression of E-cad causes increased numbers or strength of E-cad-containing

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325 synaptic connections, and this imbalance results in rapid courtship initiation. Conversely, when
326 *shg* is mutant, the number or strength of E-cad-containing synapses between Tre1 neurons and
327 their downstream partners is decreased, leading to delayed or absent courtship initiation
328
329 **Conclusions:** We have shown that the GPCR Tre1 is required in neurons to regulate the speed
330 of courtship initiation, and that silencing of Tre1-expressing neurons may lead to rapid
331 courtship initiation. We have also established a role for both E-cadherin and histamine in
332 courtship behavior, and, importantly, have provided the first evidence for metabotropic
333 histamine receptors in *Drosophila*, and, indeed, only the second example of metabotropic
334 histamine receptors in invertebrates. Ongoing and future experiments will further investigate
335 the role of histamine signaling in courtship behavior, as well as examine the expression of E-cad
336 in the *Tre1* neurons and the effects of mutation of *Tre1* and *Hdc* on E-cad expression and
337 synapse formation and distribution.
338

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339 **Literature Cited**

340

341 ARIKKATH J., REICHARDT L. F., 2008 Cadherins and catenins at synapses: roles in synaptogenesis
342 and synaptic plasticity. Trends Neurosci. **31**: 487–94.

343

344 BENSON D. L., TANAKA H., 1998 N-cadherin redistribution during synaptogenesis in hippocampal
345 neurons. J. Neurosci. **18**: 6892–904.

346

347 BOZDAGI O., VALCIN M., POSKANZER K., TANAKA H., BENSON D. L., 2004 Temporally distinct demands
348 for classic cadherins in synapse formation and maturation. Mol. Cell. Neurosci. **27**: 509–21.

349

350 BROWN R. E., STEVENS D. R., HAAS H. L., 2001 The physiology of brain histamine. Prog. Neurobiol.
351 **63**: 637–72.

352

353 BRUSÉS J. L., 2000 Cadherin-mediated adhesion at the interneuronal synapse. Curr. Opin. Cell
354 Biol. **12**: 593–7.

355

356 BUCHNER E., BUCHNER S., BURG M. G., HOFBAUER A., PAK W. L., POLLACK I., 1993 Histamine is a major
357 mechanosensory neurotransmitter candidate in Drosophila melanogaster. Cell Tissue Res. **273**:
358 119–25.

359

360 BURG M. G., SARTHY P. V., KOLIANTZ G., PAK W. L., 1993 Genetic and molecular identification of a
361 Drosophila histidine decarboxylase gene required in photoreceptor transmitter synthesis.
362 EMBO J. **12**: 911–9.

363

364 CHEN D., WU Z., LUO L.-J. J., HUANG X., QIAN W.-Q. Q., WANG H., LI S.-H. H., LIU J., 2015 E-cadherin
365 maintains the activity of neural stem cells and inhibits the migration. Int J Clin Exp Pathol **8**:
366 14247–51.

367

368 DUMSTREI K., WANG F., HARTENSTEIN V., 2003 Role of DE-cadherin in neuroblast proliferation,
369 neural morphogenesis, and axon tract formation in Drosophila larval brain development. J.
370 Neurosci. **23**: 3325–35.

371

372 EBBS M. L., AMREIN H., 2007 Taste and pheromone perception in the fruit fly Drosophila
373 melanogaster. Pflugers Arch. **454**: 735–47.

374

375 ELIAS M. S., EVANS P. D., 1983 Histamine in the insect nervous system: distribution, synthesis
376 and metabolism. J. Neurochem. **41**: 562–8.

377

378 FANNON A. M., COLMAN D. R., 1996 A model for central synaptic junctional complex formation
379 based on the differential adhesive specificities of the cadherins. Neuron **17**: 423–34.

380

381 FUNG S., WANG F., CHASE M., GODT D., HARTENSTEIN V., 2008 Expression profile of the cadherin
382 family in the developing Drosophila brain. J. Comp. Neurol. **506**: 469–88.

bioRxiv preprint doi: https://doi.org/10.1101/150680; this version posted June 15, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



383 HARDIE R. C., 1987 Is histamine a neurotransmitter in insect photoreceptors? J. Comp. Physiol.
384 A **161**: 201–13.
385
386 HODGE J. J., 2009 Ion channels to inactivate neurons in Drosophila. Front Mol Neurosci **2**: 13.
387
388 HONG S.-T. T., BANG S., PAIK D., KANG J., HWANG S., JEON K., CHUN B., HYUN S., LEE Y., KIM J., 2006
389 Histamine and its receptors modulate temperature-preference behaviors in Drosophila. J.
390 Neurosci. **26**: 7245–56.
391
392 ITOH Y., MORIYAMA Y., HASEGAWA T., ENDO T. A., TOYODA T., GOTOH Y., 2013 Scratch regulates
393 neuronal migration onset via an epithelial-mesenchymal transition-like mechanism. Nat.
394 Neurosci. **16**: 416–25.
395
396 IWAI Y., USUI T., HIRANO S., STEWARD R., TAKEICHI M., UEMURA T., 1997 Axon patterning requires
397 DN-cadherin, a novel neuronal adhesion receptor, in the Drosophila embryonic CNS. Neuron **19**:
398 77–89.
399
400 KUNWAR P., SANO H., RENAULT A., BARBOSA V., FUSE N., LEHMANN R., 2008 Tre1 GPCR initiates germ
401 cell transepithelial migration by regulating Drosophila melanogaster E-cadherin. J Cell Biology
402 **183**: 157–168.
403
404 KUNWAR P. S., STARZ-GAIANO M., BAINTON R. J., HEBERLEIN U., LEHMANN R., 2003 Tre1, a G protein-
405 coupled receptor, directs transepithelial migration of Drosophila germ cells. PLoS Biol. **1**: E80.
406
407 LUU P., ZAKI S., TRAN D., FRENCH R., 2015 A Novel Gene Controlling the Timing of Courtship
408 Initiation in Drosophila melanogaster. Genetics **202**: 1043–1053.
409
410 MELZIG J., BUCHNER S., WIEBEL F., WOLF R., BURG M., PAK W. L., BUCHNER E., 1996 Genetic depletion
411 of histamine from the nervous system of Drosophila eliminates specific visual and
412 mechanosensory behavior. J. Comp. Physiol. A **179**: 763–73.
413
414 OH Y., JANG D., SONN J. Y., CHOE J., 2013 Histamine-HisCl1 receptor axis regulates wake-
415 promoting signals in Drosophila melanogaster. PLoS ONE **8**: e68269.
416
417 PANULA P., CHAZOT P. L., COWART M., GUTZMER R., LEURS R., LIU W. L., STARK H., THURMOND R. L., HAAS
418 H. L., 2015 International Union of Basic and Clinical Pharmacology. XCVIII. Histamine
419 Receptors. Pharmacol. Rev. **67**: 601–55.
420
421 PRAKASH S., CALDWELL J. C., EBERL D. F., CLANDININ T. R., 2005 Drosophila N-cadherin mediates an
422 attractive interaction between photoreceptor axons and their targets. Nat. Neurosci. **8**: 443–50.
423
424 ROEDER T., 2003 Metabotropic histamine receptors--nothing for invertebrates? Eur. J.
425 Pharmacol. **466**: 85–90.
426

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427 SHAPIRO L., COLMAN D. R., 1999 The diversity of cadherins and implications for a synaptic
428 adhesive code in the CNS. Neuron **23**: 427–30.
429
430 TAKAI Y., SHIMIZU K., OHTSUKA T., 2003 The roles of cadherins and nectins in interneuronal
431 synapse formation. Curr Opin Neurobiol **13**: 520–526.
432
433 TANG L., HUNG C. P., SCHUMAN E. M., 1998 A role for the cadherin family of cell adhesion
434 molecules in hippocampal long-term potentiation. Neuron **20**: 1165–75.
435
436 TEPASS U., TRUONG K., GODT D., IKURA M., PEIFER M., 2000 Cadherins in embryonic and neural
437 morphogenesis. Nat. Rev. Mol. Cell Biol. **1**: 91–100.
438
439 THAKKAR M. M., 2011 Histamine in the regulation of wakefulness. Sleep Med Rev **15**: 65–74.
440
441 TRAN D. H., MEISSNER G. W., FRENCH R. L., BAKER B. S., 2014 A small subset of fruitless
442 subesophageal neurons modulate early courtship in Drosophila. PLoS ONE **9**: e95472.
443
444 UCHIDA N., HONJO Y., JOHNSON K. R., WHEELOCK M. J., TAKEICHI M., 1996 The catenin/cadherin
445 adhesion system is localized in synaptic junctions bordering transmitter release zones. J. Cell
446 Biol. **135**: 767–79.
447
448 VILLELLA A., GAILEY D. A., BERWALD B., OHSHIMA S., BARNES P. T., HALL J. C., 1997 Extended
449 reproductive roles of the fruitless gene in Drosophila melanogaster revealed by behavioral
450 analysis of new fru mutants. Genetics **147**: 1107–30.
451
452 WAUQUIER A., BROECK W. A. VAN DEN, AWOUTERS F., JANSSEN P. A., 1981 A comparison between
453 astemizole and other antihistamines on sleep-wakefulness cycles in dogs. Neuropharmacology
454 **20**: 853–9.
455
456 WU J. S., LUO L., 2006 A protocol for dissecting Drosophila melanogaster brains for live imaging
457 or immunostaining. Nat Protoc **1**: 2110–5.
458
459 YOSHIURA S., OHTA N., MATSUZAKI F., 2012 Tre1 GPCR signaling orients stem cell divisions in the
460 Drosophila central nervous system. Dev. Cell **22**: 79–91.
461
462 ZHENG Y., HIRSCHBERG B., YUAN J., WANG A. P., HUNT D. C., LUDMERER S. W., SCHMATZ D. M., CULLY D.
463 F., 2002 Identification of two novel Drosophila melanogaster histamine-gated chloride channel
464 subunits expressed in the eye. J. Biol. Chem. **277**: 2000–5.
465
466