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Serotonin and neuropeptide F have opposite modulatory effects on fly aggression

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Both serotonin (5-HT) and neuropeptide Y have been shown to affect a variety of mammalian behaviors[1–3], including aggression[4,5]. Here we show in *Drosophila melanogaster* that both 5-HT and neuropeptide F, the invertebrate homolog of neuropeptide Y, modulate aggression. We show that drug-induced increases of 5-HT in the fly brain increase aggression. Elevating 5-HT genetically in the serotonergic circuits recapitulates these pharmacological effects, whereas genetic silencing of these circuits makes the flies behaviorally unresponsive to the drug-induced increase of 5-HT but leaves them capable of aggression. Genetic silencing of the neuropeptide F (*npf*) circuit also increases fly aggression, demonstrating an opposite modulation to 5-HT. Moreover, this neuropeptide F effect seems to be independent of 5-HT. The implication of these two modulatory systems in fly and mouse aggression suggest a marked degree of conservation and a deep molecular root for this behavior.

5-HT has long been implicated in aggressive behavior in a wide variety of vertebrate[4] and invertebrate species[6]. It has also been shown to influence a spectrum of other behaviors in species as different as humans and flies[1,7,8]. Similarly, neuropeptide Y (NPY) and its invertebrate homolog neuropeptide F (NPF) are known modulators of an array of behaviors in different species[2,3,9–11]. Recently, the receptor for NPY was shown to affect territorial aggression in mice, an effect apparently mediated by 5-HT[5]. Here we explore whether these two modulators also affect aggression in *Drosophila melanogaster*. 5-HT has been reported to have no effect on aggression in *D. melanogaster*[12] or in another insect species, *Gryllus bimaculatus*[13]. No effect has been described for neuropeptide F (NPF), nor is anything known about its interaction with 5-HT. We first addressed the role of 5-HT by asking two basic questions: can 5-HT affect aggression in *D. melanogaster*, and what is the nature of that effect? We answered these questions using pharmacology and genetics. We used drugs first, in order to test previously generated selected lines that had strongly diverged in aggressive behavior through selection for escalated fighting[14] (**Supplementary Fig. 1** online). Because these lines represented a wide phenotypic range of aggression, we could easily assess increases and decreases in the behavioral response to drug treatments. Moreover, our previous molecular analysis of these lines did not show any differences in expression in genes related to 5-HT function in the aggressive (Aggr) versus the neutral (Neutr) lines. This suggested to us that 5-HT has no effect on aggression or that aggression can be markedly affected without altering 5-HT function. Alternatively, changes might have occurred in these genes that did not affect their expression levels. We started investigating these possibilities at generation 38, at which point the neutral and aggressive lines showed a five- to sevenfold difference in fighting frequency, as measured in our previously described arena assay[14] (**Supplementary Fig. 1** and **Fig. 1a**; Kruskal-Wallis analysis of variance (ANOVA), $P < 0.001$). We first confirmed the array results obtained at generation 21 by measuring the expression of genes involved in 5-HT function using quantitative RT-PCR on fly heads from generation 38. None of the changes in gene expression significantly correlated with both neutral or both aggressive selected lines in any of these genes, consistent with the previous array results (**Fig. 1b** and **Supplementary Table 1** online).

We then measured 5-HT in the heads of the selected populations and did not find any significant differences between the lines (and these levels were consistent with published results[15]) (ANOVA, $P = 0.26$), further suggesting that global changes in 5-HT level do not have a role in the selected response. To test for a direct effect of 5-HT on aggression, we pharmacologically manipulated 5-HT levels in the selected flies by feeding them drugs that act either as a precursor or inhibitor of 5-HT metabolism (**Supplementary Fig. 2** online). First, we generated a dose-response curve for 5-HT levels in the heads of flies that were fed on different concentrations of the 5-HT precursor 5-hydroxytryptophan (5-HTP) or the 5-HT synthesis inhibitor alpha-methyltryptophan (αMTP) (**Fig. 1c**,**d**). Flies treated for 4 d with 50 mM of the precursor 5-HTP show roughly 15- to 20-fold increases in 5-HT over baseline levels. Flies treated with 20 mM (or 50 mM) of the inhibitor αMTP showed an approximately twofold drop in 5-HT levels. We also tested the time course for these 5-HT changes by treating flies for 1–4 d with 50 and 20 mM of these drugs, respectively (**Fig. 1e**,**f**). Maximum effects were reached after 3 and 4 d of treatment with the precursor and the inhibitor, respectively.

We next evaluated the effect of these altered 5-HT levels on the behavior of the different fly lines. To this end, we subjected 3-d-old flies from all the lines with the treatment regimen described above after confirming that this treatment had the expected effect in these

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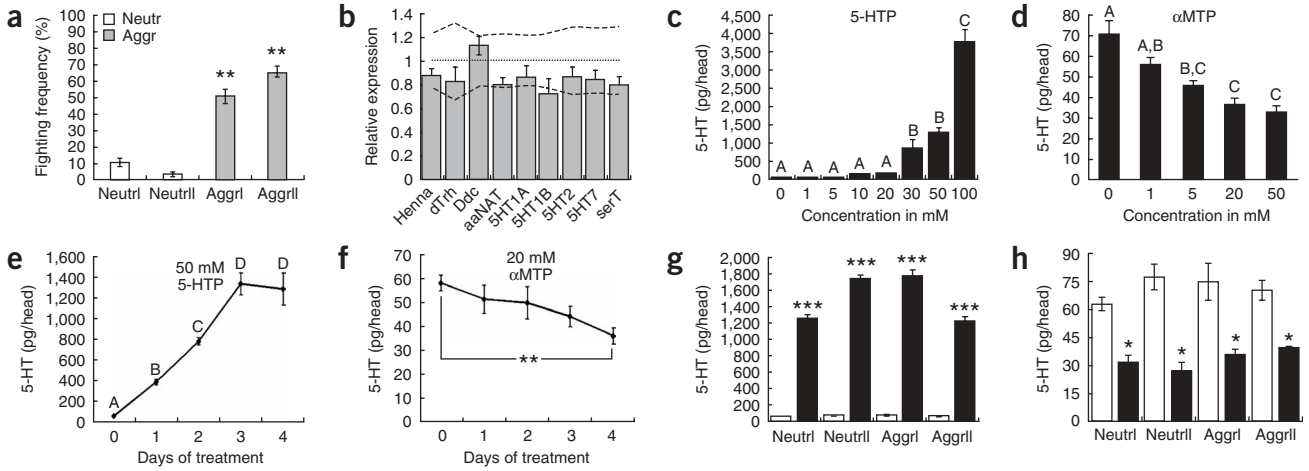

Figure 1 Fighting frequency and pharmacological manipulation of 5-HT levels in fly heads from generation 38. (**a**) Fighting frequencies of the neutral (Neutr; open bars) and aggressive (Aggr; filled gray bars) lines at Gen38. Aggressive lines have significantly higher fighting frequencies than the neutral lines (Kruskal-Wallis ANOVA, $P < 0.001$). (**b**) Expression analysis of nine genes involved in 5-HT function averaged for both aggressive lines (filled gray bars) and normalized against both neutral lines (dotted line), set to the unit value. The dashed lines represent the 95% confidence interval for the neutral lines. Error bars represent the 95% confidence interval for the aggressive lines. None of the genes shows significantly different expression levels in the averaged aggressive lines compared with the averaged neutral lines. (**c**) Dose-response curve for 5-HTP (4-d treatment). Letters above each bar indicate significantly different groups (ANOVA, $P < 0.0001$). (**d**) Dose-response curve for αMTP (4-d treatment). Letters above each bar denote significance as in **c** (ANOVA, $P < 0.0001$). (**e**) Time course for 5-HTP (50 mM). Letters above each point indicate significance as in **c** and **d** (ANOVA, $P < 0.001$). (**f**) Time course for αMTP (20 mM). Untreated levels are significantly higher than after 4 d of treatment (ANOVA, $P < 0.01$). (**g**) All the selected lines (filled black bars) have significantly higher levels of 5-HT compared with the baseline (open bars) after 4 d of treatment with 50 mM 5-HTP (Student's t test, $P < 0.0001$). (**h**) All the selected lines (filled black bars) have significantly lower levels of 5-HT after 4 d of treatment with 20 mM αMTP compared with baseline (open bars) (Student's t test, $P < 0.05$). Baseline 5-HT levels in heads of the different lines are not significantly different (ANOVA, $P = 0.26$).

lines (**Fig. 1g,h**; Student's t test, $P < 0.0001$ for 5-HTP treatment and $P < 0.05$ for αMTP treatment). We then behaviorally tested the treated flies in the arena assay and found that all the lines treated with 5-HTP showed significantly higher fighting frequencies than untreated flies (**Fig. 2a**, Kruskal-Wallis ANOVA, $P < 0.001$ for all lines). In contrast, flies treated with the 5-HT synthesis inhibitor had only modestly lower fighting frequencies compared with their untreated controls (although not significantly so) (**Fig. 2a**), suggesting that 5-HT may not be necessary for aggression. We also found that 5-HTP affected the intensity of fighting as seen in the increased frequencies of escalations in the arena assay in all but one of the lines (**Fig. 2b**). These results clearly demonstrate that 5-HT has at least an enhancing role in fly aggression, affecting all aspects of aggressive behavior (latency and index are also changed significantly upon 5-HTP treatment (data not shown)) and suggesting that the effect is central. Moreover, all the lines responded similarly to the drug feeding, as shown by the linear regression of the treated versus untreated fighting frequencies for both drugs (**Fig. 2c,d**, $P < 0.01$). Thus, over a

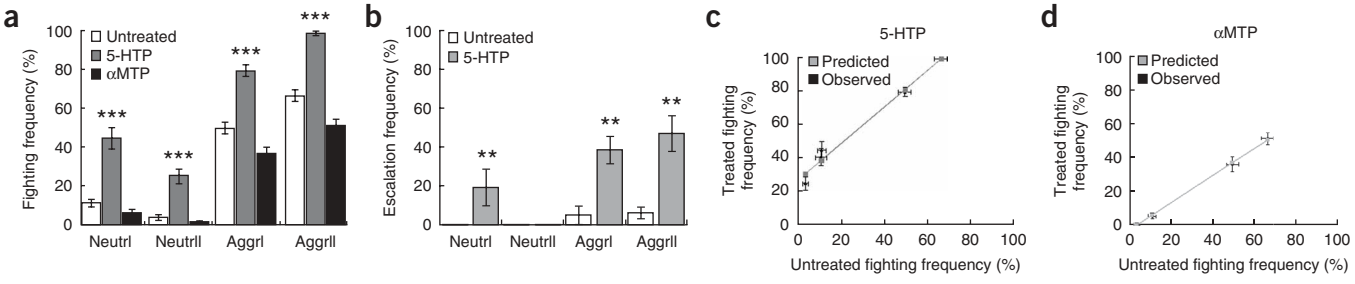

Figure 2 5-HTP increases aggression in a linear manner in the selected lines. (**a**) Mean fighting frequencies in flies from the selected lines from generations 37–38, as assessed by 15-min arena assay. Open bars represent untreated flies; filled gray bars represent flies treated for 4 d with 50 mM 5-HTP; filled black bars represent flies treated for 4 d with 20 mM of αMTP. Error bars represent s.e.m. Asterisks denote statistically significant increases in fighting frequency compared with untreated and αMTP-treated flies (Kruskal-Wallis ANOVA, $P < 0.001$ for all lines, n = minimum of 70 pairs). (**b**) Mean escalation frequencies in untreated and 5-HTP–treated flies from the selected lines, as assessed by 15-min arena assay. Open bars represent untreated flies that engaged in escalations in 15-min arena assay; filled gray bars represent flies treated for 4 d with 50 mM of 5-HTP. Error bars represent s.e.m. Asterisks denote statistically significant increases in fighting frequency compared with untreated flies (Kruskal-Wallis ANOVA, $P < 0.01$ for all lines, n = minimum of 70 pairs). (**c**) Linear regression of fighting frequencies of untreated versus 5-HTP precursor–treated flies ($P < 0.01$). Gray line connects the predicted values (gray squares). Black dots represent the actual data (± s.e.m.). All lines respond similarly to the 5-HTP treatment. (**d**) Linear regression of fighting frequencies of untreated versus αMTP inhibitor–treated flies ($P < 0.001$). Gray line connects the predicted values (gray squares). Black dots represent the actual data (± s.e.m.).

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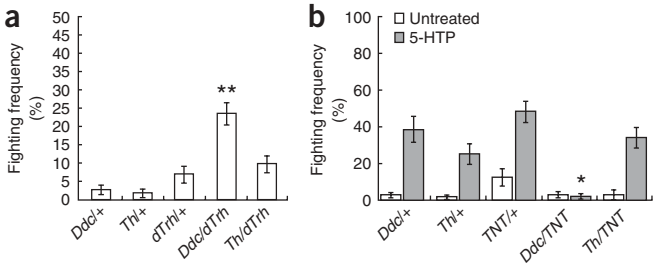

Figure 3 Genetic elevation of 5-HT increases aggression, and genetic silencing of the 5-HT circuit makes flies unresponsive to 5-HTP. (**a**) Fighting frequencies in flies expressing *UAS-dTrh* (to elevate 5-HT in the circuit) in cells expressing *Ddc* (serotonergic and dopaminergic) compared with neurons expressing *Th* (dopaminergic) and control lines containing only one component of binary expression system (*Ddc-GAL4/+*, *Th-GAL4/+* and *UAS-dTrh/+*, $n =$ minimum of 100 pairs). Asterisks denote statistically significant higher means (Kruskal-Wallis ANOVA, $P < 0.01$). (**b**) Fighting frequencies of flies expressing *UAS-TNT* (to silence evoked synaptic release in the circuit) in neurons expressing DDC (5-HT and DA) or TH (DA) and control lines containing only one component of binary expression system (*Ddc-GAL4/+*, *Th-GAL4/+* and *UAS-TNT/+*, $n =$ minimum of 70 pairs). Open bars represent the mean fighting frequencies of untreated flies. Filled gray bars represent the mean fighting frequencies of 5-HTP–treated flies (4 d at 50 mM, $n = 35$ pairs). Asterisk shows the only group that does not respond to the treatment (Kruskal-Wallis ANOVA, $P < 0.01$).

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40-fold range around normal 5-HT concentrations (0.5-fold to 20-fold), all of the selected lines respond in a predictable linear fashion. This suggests that the aggression differences between the lines depend on another mechanism within these flies that has undergone a selected change that can be further enhanced by 5-HT. This would predict that shutting down 5-HT in the brain would leave that mechanism (and thus the potential for aggressive behavior) intact, as suggested by the inhibitor experiment (**Fig. 2a**).

To test this directly, we evaluated whether 5-HT circuits are required for aggressive behavior in *D. melanogaster*. We performed these experiments outside the context of the selected lines. We targeted the serotonergic circuits in the fly brain by means of the binary GAL4/UAS system[16]. First, we genetically recapitulated the drug-induced increase in aggression by expressing the rate-limiting enzyme in 5-HT synthesis in the relevant neurons of the brain. To do this, we used the *Dopa decarboxylase* driver line (*Ddc-GAL4*[17]). Dopa decarboxylase is expressed in both 5-HT- and dopamine-producing neurons as it catalyzes the final conversion of both 5-HT[18] (**Supplementary Fig. 2**) and dopamine[19] in the fly. The rate-limiting step in 5-HT synthesis is catalyzed by tryptophan hydroxylase, which converts tryptophan into 5-HTP[18]. This genetic manipulation results in a change in 5-HT levels that, compared with the effects of precursor treatment, is more in the physiological range, as 5-HT levels in the heads of these flies differ from levels in their control counterparts by less than twofold[7]. Driving *UAS-dTrh* in the serotonergic and dopaminergic neurons with *Ddc-GAL4* significantly increased fighting frequency compared with fighting frequency in controls that carried only one component of the binary expression system (*Ddc-GAL4* or *UAS-dTrh*, **Fig. 3a**, Kruskal-Wallis ANOVA, $P < 0.01$). To exclude that this effect is due to the dopaminergic component of the *Ddc* circuit, we expressed *UAS-dTrh* exclusively in the dopaminergic neurons using the *Tyrosine hydroxylase* driver (*Th-GAL4*), which drives expression of *GAL4* in the *Th*-expressing cells that are known to be dopaminergic[20]. These flies did not show any significant increase in fighting frequency over controls (**Fig. 3a**).

Next, we evaluated the effect of silencing these same circuits (*Ddc* and *Th*) by expressing tetanus toxin light chain (*UAS-TeTxLC* or *UAS-TNT*), which has been shown to silence neurons through the inhibition of evoked synaptic release[21]. Males derived from *Ddc-GAL4* × *UAS-TNT* crosses showed very low levels of aggression, although not significantly lower than controls (**Fig. 3b**, Kruskal-Wallis ANOVA, $P = 0.15$). However, when these *Ddc-GAL4/UAS-TNT* flies were treated with 5-HTP precursor, their fighting frequencies did not increase significantly, whereas fighting frequencies of all the other lines did increase (**Fig. 3b**, Kruskal-Wallis ANOVA, $P < 0.05$). It is not likely that this lack of responsiveness is due to the simultaneous silencing of the 5-HT and dopamine circuits, as flies treated with the dopamine synthesis inhibitor 3ITY still strongly respond to 5-HTP precursor (data not shown). These data further support that 5-HT is indeed capable of modulating aggression. Moreover, the fact that *Ddc-GAL4/UAS-TNT* flies still fight, albeit at low frequency, demonstrates that 5-HT is not required for aggression. The low remaining fighting frequency is not due to ineffective silencing of the circuit, because the *Ddc-GAL4/UAS-TNT* flies were the only ones in this study that did not respond to treatment with 5-HTP, demonstrating that the circuit is indeed silenced (**Supplementary Fig. 3** online).

We next asked whether *neuropeptide F* (*npf*), the fly homolog of mammalian neuropeptide Y (*NPY*), might also affect fly aggressive behavior. Several reasons prompted us to consider *npf*. First, like its mammalian homolog *NPY* and like 5-HT, *npf* has been implicated in the modulation of a variety of physiological processes and behaviors[1–3,7–11]. Second, a knockout of one of the receptors for mammalian *NPY* (*NPYR1*) was shown to significantly increase territorial aggressive behavior in mice, an effect that appeared to be mediated by 5-HT[5]. Finally, *D. melanogaster npf* has a sex-specific expression pattern[11], and aggression in *D. melanogaster*, as in other species, has been shown to have both qualitative and quantitative gender differences[22,23]. We first measured the effect of synaptically silencing the *npf* circuit by crossing *npf-GAL4* (ref. 9) flies to the *UAS-TNT* flies described above. Males derived from this cross showed significantly higher fighting frequencies than either control (*npf-GAL4/+* and *UAS-TNT/+*) (**Fig. 4a**, Kruskal-Wallis ANOVA, $P < 0.001$). We next verified whether the small subset of male-specific *npf*-expressing neurons was important for this response. Others have shown that feminization of the *npf* circuit eliminates *npf* expression in a small cluster of male-specific *npf*-positive cells in the lateral and dorsal brain[11]. To eliminate male-specific *npf* from these cells, we crossed *npf-GAL4* flies to flies containing a female-specific *transformer* transgene downstream of the UAS promoter (*UAS-tra*^{F}). Driving expression of this construct has been shown to feminize the affected circuit cell autonomously and dominantly[24]. Males derived from this cross showed significantly higher fighting frequencies than controls (*npf-GAL4/+* and *UAS-tra*^{F}*/+*) (**Fig. 4a**, Kruskal-Wallis ANOVA, $P < 0.05$). Finally, we evaluated whether this effect is dependent on 5-HT, as has been suggested in mice[5]. To this end, we fed 5-HTP precursor to the males derived from the *npf-GAL4* crosses to evaluate whether (and to what extent) this would increase their fighting frequencies. All the lines responded similarly to the precursor treatment (**Fig. 4b**), demonstrating that the effects of NPF and 5-HT are additive and thus are likely to be independent. This is more clearly illustrated on a linear regression plot of the fighting frequencies of the fed and unfed flies (**Fig. 4c**, $P < 0.01$).

Our results here suggest that there are two discrete circuits in the fly brain that act independently and in opposite directions to modulate

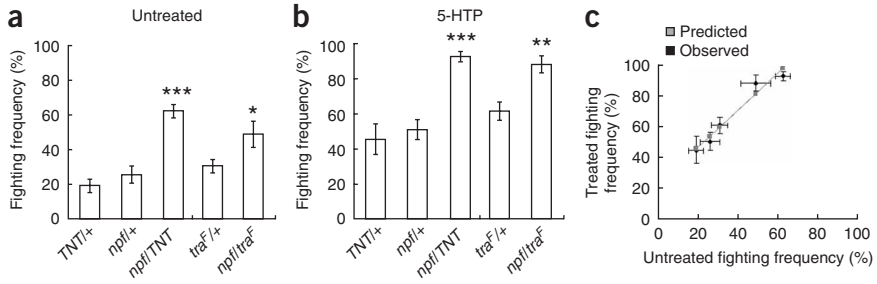

Figure 4 Genetic silencing of the *npf* circuit increases aggression independently of 5-HTP–induced aggression. (**a**) Fighting frequencies in flies expressing *UAS-TNT* (silenced) or *UAS-tra*^{F} (feminized = loss of male-specific *npf*) *npf* circuits compared with controls expressing only one component of the UAS/GAL4 system (*UAS-TNT*/+, *npf-GAL4*/+, *UAS-tra*^{F}/+, n = minimum of 70 pairs). Asterisks denote significance compared with controls (Kruskal-Wallis ANOVA: ***, $P < 0.001$; *, $P < 0.05$). (**b**) Fighting frequencies in flies from the same genotypes treated with 50 mM 5-HTP for 4 d (n = minimum of 49 pairs). Asterisks denote significance compared with controls (Kruskal-Wallis ANOVA, ***, $P < 0.001$; **, $P < 0.01$). (**c**) Linear regression of fighting frequencies from **a** compared with **b** ($P < 0.01$). Gray line connects the predicted values (filled gray squares). Filled black dots represent the actual data (± s.e.m.).

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aggression. The 5-HT results in the selected lines argue that the changes that have occurred over the course of roughly 40 generations of selection did not involve the 5-HT circuits or the sensitivity to modulation by these circuits, as both aggressive and neutral lines respond equivalently to precursor and inhibitor treatment. This suggests that the fly brain harbors some regulatory center(s) that normally regulate(s) the aggressive behavioral output and that can be influenced by 5-HT. This interpretation is further bolstered by the fact that 5-HT does not seem to be necessary for aggressive behavior. In contrast, the aggression-inducing effect of silencing the *npf* circuit suggests that NPF normally acts as a brake on the animal's aggressive output. This male-specific modulation can be induced by elimination of *npf* expression from just a few male-specific *npf*-expressing neurons in the brain. Females have low levels of aggression compared with males and do not express *npf* in these neurons, suggesting that decreasing NPF by itself is not sufficient to induce more aggressive behavior. This further supports the existence of a circuit that is required for aggression and that is inhibited by NPF. Moreover, others have shown that silencing the *npf* circuit (or just its male-specific component) decreases normal courtship[11]. This result, in conjunction with our current findings, suggests that decreasing NPF can simultaneously increase aggressive behavior and suppress courtship behavior. Territorial males often switch rapidly between these two behaviors depending on who invades their territory: when a male intruder enters the territory, the territory holder will almost invariably quickly chase him off, but when a female enters, the territorial male will generally switch to courtship behavior in an attempt to mate with her. We propose that small fluctuations in neuropeptide secretion from the *npf* circuit could represent the neuronal mechanism that allows for this flexibility in behavioral output. Finally, it is notable that both modulators of aggressive behavior identified here also affect aggression in mammals, because it suggests that the molecular roots for this complex social behavior are of ancient evolutionary origin.

METHODS

Stocks and breeding conditions. The selected lines and the selection procedure have been described previously[14]. The selected lines were all derived from the same starting population and kept separate throughout the selection. They were named AggrI, AggrII, NeutrI and NeutrII. The following stocks were standardized against our wild-type laboratory strain Canton S as previously described[14]: *Ddc-GAL4/TM3, Ser* and *Th-GAL4/TM3, Ser* (provided by J. Hirsch, University of Virginia), *UAS-TNT* (provided by U. Heberlein, University of San Francisco), *UAS-tra*^{F} and *UAS-dTrh*. The latter stock was made with a sequence-verified construct generated by directionally cloning the *dTrh* cDNA (clone GH12537, corresponding to *CG9122* obtained from Research Genomics) into the *pUAST* vector using *Eco*RI and *Xho*I restriction sites. Transformants were generated by T. Stone (University of California, San Diego) as previously described[25]. The *yw; npf-GAL4/CyO* stock was provided by P. Shen (University of Georgia). This stock was not background standardized but was outcrossed against Canton S or Canton S standardized lines in all experiments. All flies were reared in plastic bottles on yeast, dark corn syrup and agar food at room temperature (22.5 ± 0.5 °C) on a 12-h light/12-h dark cycle.

Drug treatment and serotonin measurement. For 5-HT measurements in the heads of males from the untreated selected lines, a minimum of ten replicates of 20–40 5- to 7-d-old males of each line were frozen in liquid nitrogen and their heads separated from their bodies on dry ice using a sieve, as previously described[26]. Heads were then analyzed for 5-HT levels by HPLC as previously described[27,28]. Males were tested over several generations (generations 36–39).

For dose-response curves, 3-d-old males were put on our fly food recipe described above, with drugs mixed in at concentrations ranging from 1–100 mM for 5-HTP and 1–50 mM for αMTP (25 mg ascorbic acid was added per 100 ml food as a stabilizer). Flies fed 100 mM 5-HTP have 5-HT increases of roughly 50-fold, but at this concentration the drug crystallizes into salt clumps on the food surface (H.A.D., unpublished observation). A minimum of four replicates of 20–40 males were tested for each concentration. After 4 d of treatment, the flies were killed and analyzed as above. For time courses, males were treated with 50 mM 5-HTP and 20 mM αMTP for 1–4 d. A minimum of four replicates of 20–40 males were tested for each time point. All males were sacrificed at 7 d of age and analyzed as above. Dose-response curves and time courses were generated based on measurements on heads from the NeutrI line. To verify that all the selected lines responded similarly to these treatments, a minimum of four replicates of 20–40 3-d-old males of each line were fed 50 mM 5-HTP and 20 mM αMTP for 4 d and were sacrificed and analyzed as above. For behavioral experiments, 3-d-old males were put on food with 50 mM 5-HTP or 20 mM αMTP for 3 d in groups of 10–20. The day before testing, they were isolated in individual tubes with food containing the same concentration of each drug.

Aggression assay. All aggression analysis was done in the arena assay that we have previously described[14]. Briefly, we used an arena chamber (1.3-cm-thick rectangular plate (15.2 × 10.8 cm) with 35 evenly spaced cells (1.6 cm diameter, 1.1 cm high), arranged in 5 × 7 rows[14] (**Supplementary Fig. 1**). The insides of the cells were coated with Fluon (Northern Products) to prevent the flies from walking on the walls of the cell. The chamber was placed on a bed of 2% agarose and covered with a plastic lid. One pair of 5- to 7-d-old males was introduced in each arena through a loading hole in the cover plate that was plugged with a small cotton plug. When all the males were loaded, the cover plate was gently moved up so that the loading holes now aligned just above each arena, and the cotton plugs were removed. The chamber was then filmed for 15 min after a 5-min adjustment phase. All males were collected on the day of eclosion and were isolated 1 d before analysis, because isolation has been shown to increase subsequent aggressive behavior[29]. A minimum of 70 pairs of males were tested for every line for each drug treatment. For the genetically manipulated flies, 70–100 pairs were analyzed for each untreated line and 35–49 pairs for the treated lines. Four parameters were used for quantification

in the arena assay as previously described[14]. Here we focus mostly on fighting frequency, which represents the percentage of pairs that engage in any fighting in the 15-min observation period. We also quantified escalation frequency, which is the percentage of pairs that engaged in the highest level of fighting during the 15-min observation period. For escalations, we considered only boxing, wrestling and tussling, not holding, because it does not involve reciprocation of the opponent (**Supplementary Fig. 1**). We scored only unambiguous offensive fighting elements, which included wing threat, charging or lunging, holding, tussling and boxing.

Quantitative RT-PCR. Quantitative RT-PCR was done with TaqMan probes for *Henna*, *dTrh*, *Ddc*, *aaNAT*, *5-HT1A*, *5-HT1B*, *5-HT2*, *5-HT7* and *serT* according to the manufacturer's protocols. Data were analyzed with the ΔΔCT method using the ABI 7300 System Software (V.1.3.1), as described by ABI and normalized to endogenous reference genes (*Gapdh* and *B52*). Gene expression was compared between the neutral lines as a group and the aggressive lines as a group. A minimum of two replicate reverse transcription reactions followed by a minimum of three replicate PCR reactions were performed for each gene for each subgroup (that is, Neutr I, AggrI, etc.). The neutral group was chosen as the calibrator sample, and the average expression level of the replicate neutral samples was set to 1. The average and s.e.m. were calculated for the aggressive group compared with the calibrator and plotted.

Statistical analysis. Statistical analysis was done in MATLAB (Mathworks). Normally distributed data were analyzed using a two-tailed Student's *t* test for single comparisons and ANOVA for multiple comparisons, followed by Tukey-Kramer's honestly significant difference (HSD) test to identify groups that were statistically significantly different. Aggression data are typically not normally distributed, and for these data, medians were statistically compared using the nonparametric Kruskal-Wallis ANOVA, followed by Tukey-Kramer's HSD test. All parametric data are presented as bar graphs representing the mean ± s.e.m. Population proportions (percentages) are also plotted as bar graphs showing the mean (bar) ± s.e.m., because proportions are more clearly visualized by the mean than the median. Linear regressions were calculated for comparisons of fighting frequencies of untreated flies and fighting frequencies of drug-treated flies. The predicted points were calculated based on the optimal linear function that fits the observed data using Excel. Statistical significance was set at $\alpha = 0.05$.

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

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AUTHOR CONTRIBUTIONS

This study was designed by H.A.D. and R.J.G. H.A.D. performed the experiments, analyzed the data and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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