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Authors

Huang, Jia
Liu, Weiwei
Qi, Yi-Xiang
et al.

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Neuromodulation of courtship drive through tyramine-responsive neurons in the Drosophila brain

Jia Huang[1,2,4,5], **Weiwei Liu**[2,4], **Yi-xiang Qi**[1], **Junjie Luo**[2,3], and **Craig Montell**[2,5]

[1]Institute of Insect Sciences, Zhejiang University, Hangzhou 310058, China

[2]Neuroscience Research Institute and Department of Molecular, Cellular and Developmental Biology University of California Santa Barbara, Santa Barbara, CA, 93106, USA

[3]The Johns Hopkins University School of Medicine, Baltimore, MD 21205

SUMMARY

Neuromodulators influence the activities of collections of neurons, and have profound impacts on animal behavior. Male courtship drive is complex, and is subject to neuromodulatory control. Using the fruit fly, *Drosophila melanogaster*, we identified neurons in the brain (inferior posterior slope; IPS) that impact courtship drive, and were controlled by tyramine—a biogenic amine related to dopamine, whose roles in most animals are enigmatic. We knocked out a tyramine-specific receptor, TyrR, which was expressed in IPS neurons. Loss of TyrR led to a striking elevation in courtship activity between males. This effect occurred only in the absence of females, as the $TyrR^{Gal4}$ mutant males exhibited a wild-type preference for females. Artificial hyperactivation of IPS neurons caused a large increase in male-male courtship, while suppression of IPS activity decreased male-female courtship. We conclude that TyrR is a receptor for tyramine, and suggest that it serves to curb high levels of courtship activity through functioning as an inhibitory neuromodulator.

INTRODUCTION

Neurotransmitters and neuromodulators that are derived from tyrosine are evolutionarily conserved, and are critical mediators of animal behavior. Dopamine and the related catecholamine, norepinephrine, are synthesized through a simple pathway that begins with conversion of tyrosine into DOPA (Figure 1A). Tyrosine is also a substrate for production of octopamine, which is structurally similar to norepinephrine. Octopamine is produced in both mammals and invertebrates [1], although its role as a neuromodulator and neurotransmitter is best characterized in insects, where it promotes an array of behaviors. These range from

[5]Correspondence: huangj@zju.edu.cn or craig.montell@lifesci.ucsb.edu.

[4]These authors contributed equally to this work.

AUTHOR CONTRIBUTIONS

J.H. and C.M. designed the experiments, interpreted the data and wrote the manuscript. J.H. and W.L. performed most of the experiments. Y.Q. and J.L. helped perform some immunohistochemistry and molecular biology experiments.

male aggression [2, 3] to learning and memory in flies [4], female post-mating behaviors [5, 6], sleep [7], foraging [8] and others.

The biosynthesis of octopamine is initiated by decarboxylation of tyrosine to produce tyramine, which is present at low levels in many mammalian tissues, including the brain [9]. Due to its concentration in trace amounts, it has long been thought to serve primarily as a biosynthetic precursor of octopamine, and not as a neuroactive chemical in its own right. Nevertheless, the discovery of a specific family of G-protein coupled receptors (GPCRs), some members of which are activated primarily by tyramine, raises the possibility that tyramine may function independently as a neuromodulator [10]. Indeed, the concentration of tyramine is altered in a variety of human neurological disorders, including schizophrenia, Parkinson's disease, attention deficit hyperactivity disorder (ADHD), Tourette syndrome, and phenylketonuria [11]. Nevertheless, the functions of tyramine are enigmatic, especially in mammals.

The brains of the fruit fly harbor populations of neurons that produce tyramine, and not octopamine [12, 13], arguing against a trivial role for tyramine exclusively as a metabolic intermediate. A few experiments in insects address this possibility. For example, a Drosophila mutation affecting a receptor for both octopamine and tyramine (Oct-TyrR) results in reduced odor avoidance [14]. However, it is unclear whether the phenotype reflects a role for octopamine or tyramine since Oct-TyrR is activated by tyramine and octopamine with similar potency. Application of tyramine to Drosophila tissue, or injections of tyramine into the blowfly or moth produce a variety of physiological responses [15, 16]. However, the tyramine might be metabolically converted to other biogenic amines that elicit function. At this time, there is no clear genetic evidence indicating a role for tyramine as an independent neuromodulator in *Drosophila*. Despite the presence of tyramine in the brains of animals that include mammals and insects, *C. elegans* is the only organism for which genetic evidence supports a role of tyramine as a neuromodulator [17–20].

The Drosophila genome encodes multiple GPCRs that are activated by biogenic amines, one of which (TyrR) is activated specifically by tyramine but not by other biogenic amines tested, including octopamine, dopamine, serotonin and histamine [21]. Here, we generated a null mutation in *TyrR* and found that the mutant males displayed a profound increase in male-male courtship, but no change in gender preference. We found that TyrR was expressed and functioned in a set of tyramine-responsive neurons in the Drosophila brain called the inferior posterior slope (IPS). Genetic hyperactivation of IPS neurons induced a significant elevation in male-male courtship, similar to the mutant males. Conversely, inactivation of these neurons decreased male-female courtship. We conclude that basal IPS activity is required to permit sufficient levels of sexual drive for male-female courtship. In addition, through activation of TyrR, we suggest that tyramine serves as an inhibitory neuromodulator to reduce sexual drive.

RESULTS

Expression of the *TyrR*-reporter in a small subset of neurons in the brain

Seven *Drosophila* GPCRs are receptors for octopamine and/or tyramine. However, TyrR (CG7431) is the only receptor that is potently and specifically activated by tyramine, and not other biogenic amines [>1000-fold; 21, 22]. To dissect the physiological role of TyrR, we generated a *TyrR* knock-out allele by ends-out homologous recombination ($TyrR^{Gal4}$). We deleted ~0.7 kb encoding the N-terminal region and the first two transmembrane domains of TyrR. In addition, we introduced a *Gal4* gene reporter at the site of the normal *TyrR* translation initiation codon (Figure 1B). We confirmed the *TyrR* knockout and *Gal4* knock-in by PCR (Figures 1B and 1C).

We analyzed the expression pattern of the *TyrR* reporter ($TyrR^{Gal4}/+$) in the brain and ventral nerve cord (VNC) using *UAS-mCD8-GFP* and detected GFP staining in 20–25 cells per brain hemisphere (Figure 1D). These neurons were mainly in four regions of the brain [23]: the superior medial protocerebrum (SMP), the posteriorlateral protocerebrum (PLP), the inferior posterior slope (IPS) and the gnathal ganglia (GNG). There were also several GFP-positive neurons in each ganglia of the VNC (Figure 1E). However, we did not detect *TyrR* reporter expression in the peripheral nervous system.

Loss of TyrR increased male-male courtship, but did not alter gender preference

We found that the $TyrR^{Gal4}$ mutant flies were healthy and fertile. However, $TyrR^{Gal4}$ males displayed a large increase in male-male courtship. We quantified this behavior by introducing 8–10 $TyrR^{Gal4}$ males into a small Petri dish, and found that they chased each other and formed chains of courting males, resulting in a strikingly high chaining index (Figures 2A and 2B; Movie S1). This behavioral phenotype was due to loss of *TyrR* since we phenocopied the increased male-male courtship by knocking down *TyrR* by RNAi ($UAS\text{-}TyrR^{RNAi}$ and $TyrR^{Gal4}/+$) or by placing the $TyrR^{Gal4}$ mutation *in trans* with a deficiency (Df) that uncovered the gene (Figure 2A). Single $TyrR^{Gal4}$ males also showed strong courtship toward single w^{1118} target males (Figure 2C). We suppressed the increased male-male courtship by introducing either a wild-type genomic transgene or a *UAS-TyrR* transgene under the control of the *TyrR* reporter ($TyrR^{Gal4}$; Figures 2A and 2C). Thus, *TyrR* was necessary to inhibit high levels of male-male courtship.

To address whether $TyrR^{Gal4}$ flies exhibited a change in gender preference, we performed choice assays. We placed a decapitated wild-type female and a decapitated wild-type male into a chamber that contained a $TyrR^{Gal4}$ mutant or a control male. We found that gender preference was unchanged in the mutant flies (Figure 2D). We repeated the gender preference experiments using live targets. Because fru^{M} null ($fru^{LexA}/fru^{\Delta 4-40}$) males do not court females, we allowed the $TyrR^{Gal4}$ males to choose between one female and one fru^{M} male. We found that the $TyrR^{Gal4}$ males showed the same strong bias for female flies as did the controls (Figure 2E). However, when presented with fru^{M} males alone, the $TyrR^{Gal4}$ males elicited strong courtship to these males as well (Figure S1A). We then paired sexually naive mutant males with isogenic wild-type control (w^{1118}) virgin females, and compared their behavior to heterozygous control males. Both mutant and control males displayed

similar initiation latencies and copulation durations (Figure S1B and S1C). In addition, the enhanced male-male courtship behavior was not likely due to a general increase in arousal since the *TyrR*^{Gal4} males did not show elevated aggression or locomotion. Rather, these behaviors were decreased relative to the controls (Figure S1D and S1E).

TyrR mutant males exhibited increased courtship towards females

To determine whether *TyrR*^{Gal4} males exhibited increased courtship towards females, we initially tested 4–10 day old males and 10–12 day old females. However, since the vast majority of control (*TyrR*^{Gal4}/+) males courted these females, there was insufficient sensitivity to discern whether or not the *TyrR*^{Gal4} mutant males increased courtship towards females (Figure 2F). Therefore, we performed additional male-female courtship assays using females that ranged in age. The control males displayed lower courtship behavior towards young (2–3 day old) and older females (30–32 and 40–42 days old) relative to the mid-aged (10–12 and 20–22 day old) females (Figure 2F). However, *TyrR*^{Gal4} males retained nearly maximal courtship indices towards females regardless of their age (Figure 2F). In contrast to the increased courtship activity of the mutant males, *TyrR*^{Gal4} female flies exhibited normal post-mating behaviors, such as decreased receptivity and remating and increased egg laying (Figure S1F-S1H).

Hyperactivating *TyrR*-expressing neurons recapitulates the *TyrR* mutant phenotype

Because the *TyrR*^{Gal4} mutant showed strong male-male chaining, we tested whether artificial inactivation of *TyrR*^{Gal4} neurons would induce similarly robust behavior. To silence the *TyrR*^{Gal4} neurons, we expressed the tetanus toxin light chain (*UAS-TNT*) or the inwardly rectifying K^+ channel (*UAS-Kir2.1*) under control of the *TyrR-Gal4* (*TyrR*^{Gal4}/+). Surprisingly, introduction of these transgenes did not induce male chaining behavior (Figure 3A). Rather, inactivation of *TyrR*^{Gal4}-expressing neurons with *UAS-TNT* suppressed the dramatic elevation in chaining behavior exhibited by the *TyrR*^{Gal4} mutant males (Figure 3A). Moreover, silencing these neurons with *UAS-TNT* also significantly decreased the courtship indices toward females (Figure 3B). These phenotypes were not due to any obvious effects on neurite architecture resulting from expressing *TNT* (Figure S2A and S2B), although we cannot formally exclude any developmental phenotypes.

To determine the effects on courtship behavior resulting from hyperactivation of the *TyrR*-expressing neurons, we used a transgene expressing NaChBac to chronically raise the resting potential and thereby increase both the spontaneous and induced firing rates. We also used a *trpA1* transgene to acutely hyper-stimulate these neurons by thermally activating TRPA1. We found that expression of *NaChBac* in *TyrR*^{Gal4} neurons greatly increased male courtship toward individual target males, and generated strong male-male chaining behavior (Figures 3C and 3D). We obtained similar results by expressing TRPA1 in *TyrR*-neurons, and shifting the animals to a 30°C enviro nment to conditionally hyperactive these neurons (Figure 3E). However, activation of *trpA1* in *TyrR*^{Gal4} mutant males did not cause a further increase in courtship behavior (Figure S2C and S2D).

Fruitless (Fru) and Doublesex (Dsx) are two transcriptional factors that impact male-specific behaviors. Activation of FruM or Dsx neurons in solitary males induces courtship behaviors

[24]. However, we did not detect any overlap between the *TyrR* reporter and either anti-FruM or anti-DsxM staining (Figure S2E and S2F), indicating that FruM and DsxM are expressed in different subsets of neurons in the brain from TyrR. We also tested the possibility that the $TyrR^{Gal4}$ neurons transiently expressed *fru* during development using a lineage tracing approach (*fru-FLP* and *UAS>stop>mCD8-GFP*), but did not detect GFP signals.

We then used the GRASP technique (GFP reconstitution across synaptic partners) to test whether *TyrR*-positive neurons might contact fru^{M} neurons to affect courtship behaviors, since fru^{M} function is critical for building the potential for nearly all aspects of male sexual behavior [25, 26]. The GRASP method employs two complementary fragments of GFP ($spGFP^{11}$ and $spGFP^{1–10}$) tethered to the membranes of different cells [27, 28]. If the cells are in contact, GFP is functionally reconstituted. Since GRASP requires dual expression systems (e.g. *LexA/LexAop* and *GAL4/UAS*), we used the fru^{LexA} and the $TyrR^{Gal4}$ [29] to drive *LexAop-mCD4-spGFP*^{11} and *UAS-mCD4-spGFP*$^{1–10}$, respectively. Brains expressing just one or the other GFP fragment did not exhibit fluorescent signals (Figure 3F). In contrast, flies expressing both GFP fragments displayed GFP fluorescence in several regions of the brain including the SCL (superior clamp), AVLP (anterior ventrolateral protocerebrum), CRE (crepine), AL (antennal lobe) and PENP (periesophageal neuropils) (Figure 3G). The positive GRASP signals suggest that there are synaptic contacts between *TyrR* and fru^{M} neurons, and that they may function within a common neural circuit to regulate courtship.

Hyperactivation of cholinergic IPS neurons stimulated male chaining behavior

The $TyrR^{Gal4}$ was expressed in the brain and the VNC. To address whether hyperactivation of *TyrR*-positive neurons in the brain contributed to inducing high levels of male-male courtship, we eliminated most *TyrR* expression in the VNC by introducing a Gal4 repressor (Gal80) in the VNC (*Tsh-Gal80*) [30]. This *Gal4* suppressor removed *TyrR* reporter expression in the VNC, except for a pair of neurons between the first and second ganglion (Figures 4A and 4B). We found that thermal activation of TRPA1 in this remaining subset of *TyrR*-expressing cells caused a similarly high level of male-male chaining as did TRPA1 activation of the full set of *TyrR*-expressing cells (Figure 4C). We then used the *VGluT-Gal80* [31] to suppress the *TyrR-Gal4* in the brain, except for the IPS neurons and very weak expression in the GNG (Figure 4D). Thermal activation of TRPA1 in these remaining *TyrR*-positive brain neurons induced male-male chaining (Figure 4C), indicating the important contribution of IPS or GNG neurons in controlling male-male courtship.

Since the IPS neurons (2–3 cells per hemisphere) project extensive processes to SCL, AVLP and PENP, while the GNG neurons project outside the brain (Figure 4D), we propose that the IPS neurons are key neurons in regulating male courtship. We found that a Janelia *Gal4* (*61A01-Gal4*) also labeled IPS but not other *TyrR* neurons (Figures 4E-4H). To determine the effects of loss of *TyrR* in IPS neurons, we used the *61A01-Gal4* to drive *UAS-TyrR-RNAi* and found that these animals also showed strong male-male courtship behaviors (Figure 4I), while leaving male-female courtship intact (Figure 4J). Taken together, our data

indicate that activation of *TyrR*-expressing IPS ($TyrR_{IPS}$) neurons is sufficient to stimulate male-male courtship, and that stimulation of TyrR inhibited the activity of these neurons.

To identify candidate neurotransmitters or neuromodulators that may be expressed and function in $TyrR_{IPS}$ neurons to promote courtship we performed whole-mount immunostaining using a panel of antibodies against acetylcholine transferase (ChAT), serotonin (5-HT), tyrosine hydroxylase (TH) and tyrosine decarboxylase 2 (TDC2), which is the only *tdc* gene expressed in neurons [32]. We found that the $TyrR_{IPS}$ neurons stained with anti-ChAT (Figure 5A), but not with the other antibodies tested (Figure S3A-3C). The *61A01-Gal4* used a promoter region for the gene encoding the neuropeptide myoinhibiting peptide (Mip). $TyrR_{IPS}$ neurons also expressing MIP (Figure 5B and Figure S3D). We knocked down *ChAT* and *Mip* in *TyrR*-expressing neurons using RNAi and found that suppressing *ChAT* but not *Mip* decreased the male-female courtship index (Figure 5C), even though Mip^{RNAi} was effective in knocking down expression of MIP (Figure S3E and S3F). We obtained similar results after knocked down *ChAT* with the *61A01-Gal4* (Figure 5C). However, RNAi-mediated knockdown of *ChAT* or *Mip* had no significant effect on increasing male-male courtship (Figure 5D).

To test whether $TyrR_{IPS}$ neurons form contacts with FruM neurons in the male brain, we used GRASP. We drove the two halves of a split-GFP on the external cell membranes using the fru^{NP21}-*Gal4* [33] and the *61A01-LexA*. The *61A01-LexA* driver labeled the IPS neurons and neural projections in the SCL, AVLP and PENP, which are derived mainly from IPS neurons (Figures 4F and 4H). Expression of one half of the split-GFP under the control of either driver did not yield GFP-positive signals (e.g. Figure 5E). In contrast, upon expression of both halves of the GFP, we detected reconstituted GFP signals in SMP, SCL, AVLP and PENP (Figures 5F-5H). This was not due to expression of *fru* in *61A01* neurons since we did not detect a GFP signal in an intersectional experiment using *UAS>stop>mCD8-GFP*/Cyo;fru^{FLP}/TM6b and *61A01-GAL4*, the latter of which labels the same neurons as the *61A01-LexA*, in additional to several others (Figure 4E and 4F). Thus, we suggest that a portion of GRASP signal observed in these regions reflects synaptic contact between IPS and fru^{M} neurons.

TyrR neurons respond to tyramine specifically

TyrR is activated by tyramine and not octopamine *in vitro* [21], suggesting that *in vivo* TyrR is receptor for tyramine but not octopamine. To address this proposal, we performed genetic and Ca^{2+} imaging analyses. Tyrosine is biosynthetically converted to tyramine, which in turn is the precursor for octopamine. These biogenic amines are produced through the sequential action of tyrosine decarboxylase (TDC) and tyramine β-hydroxylase (TbH), respectively (Figure 1A). We found that the $Tdc2^{RO54}$ mutant flies, which are missing the enzyme needed for generating tyramine, showed elevated male-male courtship (Figure 6A). However, a null mutation that eliminates the tyramine β-hydroxylase (Tbh^{nM18}) that is essential for octopamine biosynthesis [34] did not cause an increase in male-male courtship (Figure 6A).

To test whether tyramine activates TyrR in the brain, we performed Ca^{2+} imaging using the Ca^{2+} indicator GCaMP6f [35]. We isolated fly brains and found that application of tyramine

drove a significant increase in Ca^{2+} signals in *TyrR*-expressing neurons (Figure 6B). The effect using the whole mount preparation embedded in gel required much higher concentrations of tyramine than used *in vitro*, in which the receptors are overexpressed and directly exposed to chemicals [21]. In some experiments, we observed Ca^{2+} oscillations (Figure S4A, Movie S2), a frequent consequence of activation of Gq-coupled GPCRs, which is attributable to repeated Ca^{2+} release and uptake from intracellular stores. Since IPS neurons were critical for regulating male courtship behaviors, we focused on these cells. Application of 30 mM octopamine did not stimulate a significant Ca^{2+} response in IPS neurons (Figures 6C and 6D). By contrast, application of either 3 or 30 mM tyramine induced a rise in Ca^{2+} (Figures 6C and 6D). This tyramine-induced Ca^{2+} response depended on TyrR, since it was eliminated in the $TyrR^{Gal4}$ mutant (Figure 6C). Thus, it appears that TyrR is activated *in vivo* by tyramine but not octopamine, consistent with *in vitro* studies [21].

DISCUSSION

Nearly all wild-type Drosophila males court and mate with females. However, among wild-type flies the frequency of male-male courtship is low. Nevertheless, there are multiple mutations that increase male-male courtship [reviewed in 36]. The changes in behavior are typically due to deficits in identifying males, such as occurs upon elimination of male pheromones or the corresponding receptors [36]. We found that $TyrR^{Gal4}$ mutant males exhibit a dramatic increase in male-male sexual activity. In contrast to previous mutations that increase male-male courtship [37–39], the $TyrR^{Gal4}$ flies discriminate between male and females. When provided a choice between the two genders, the $TyrR^{Gal4}$ mutants select females at the same high proportion as wild-type males. These results suggest that the strong male-male courtship activity was not due a deficit in sensing repulsive male pheromones. Furthermore, $TyrR^{Gal4}$ males also exhibited increased courtship towards young and aged females. These phenotypes were due to loss of *TyrR* and not potential effects of the *mini-white* transgene since we rescued the *TyrR* phenotype with a wild-type *TyrR* transgene. Moreover, heterozygous control males harboring the *mini-white* gene (*TyrR*/+) display wild-type levels of courtship behavior. Furthermore, we recapitulated the increased male-male courtship by RNAi knockdown of *TyrR*. Consistent with the conclusion that tyramine modulates male courtship activity, *Tdc2* but not *Tβh* mutant males show elevated levels of male-male courtship.

We propose that the *TyrR*-expressing neurons control overall male sexual drive. In support of this concept, suppressing the normal activity of *TyrR*-expressing neurons in wild-type males significantly reduced male-female courtship behavior. This manipulation slightly reduced male-male courtship behavior in wild-type. However, the effect was not statistically significant since basal male-male courtship activity was very low. Nevertheless, silencing $TyrR^{+}$ neurons in $TyrR^{Gal4}$ mutant males eliminated the high male-male courtship activity. Conversely, when we artificially activated the $TyrR^{+}$ neurons in wild-type males, the animals displayed a strong elevation in male-male courtship behavior. Thus, the dramatic increase in male-male courtship reflected an increase in overall sexual activity, rather than an increase in same-sex preference. Based on GRASP studies, we suggest that the $TyrR^{+}$ neurons function through the FruM neural circuits. Thus, the normal low activity of TyrR-positive neurons is

permissive for male-female courtship. Higher activity stimulates greater courtship drive such that the animals will also court males, but only if females are not present since even at artificially elevated levels of activity, the males still prefer females if both gender targets are available. This role for the TyrR-expressing neurons differs from P1 neurons, which promote distinct behaviors, aggression and courtship, at low and high activity levels, respectively [40].

The *TyrR*^{Gal4} phenotype was due to a requirement for tyramine for controlling courtship behavior since we found that *TyrR*-expressing neurons were activated by tyramine but not octopamine, consistent with *in vitro* data indicating that the TyrR is activated specifically by tyramine [21]. Tyramine is most likely acting as a neuromodulator, rather than as a neurotransmitter, since TyrR is a GPCR rather than an ionotropic receptor. In further support of this model, we did not detect any GRASP signals using the *Tdc2-LexA* and *TyrR*^{Gal4}, suggesting that the tyramine-producing neurons are not in direct contact with the TyrR-expressing neurons. However, a caveat is that the *Tdc2-LexA* recapitulates only a subset of the Tdc2 neurons (Figure S4B).

A neuromodulator can either be excitatory or inhibitory, depending on the receptor that is activated. Our data suggest that as a consequence of activating TyrR, tyramine serves an inhibitory rather than excitatory neuromodulator, which curbs sexual activity. In favor of this proposal are the genetic activation and inactivation experiments. Artificial stimulation of *TyrR*-expressing neurons increased male-male courtship, while inhibition of these neurons reduced male-female courtship.

The model that TyrR is an inhibitory neuromodulator receptor *in vivo* is consistent with *in vitro* studies showing that tyramine reduces the amplitude of EJP in neuromuscular junctions. [41, 42]. Our *in vivo* Ca^{2+} imaging results, as well as an *in vitro* analysis [21] indicate that TyrR is coupled to G_q, which typically leads to neuronal activation [43]. However, loss of G_q/G_{11} signaling can increase neuronal activity as well [44]. This could potentially occur through inhibiting glutamate release, gating of a Ca^{2+}- activated K^+ channel or through promiscuous coupling of a G_q/G_{11}-coupled receptors to $G_{i/o}$ G-proteins [44, 45]. In support of this latter possibility, two related Drosophila catecholamine receptors are coupled to both G_q and G_i proteins [46, 47].

We found that TyrR activity was required in a small group of neurons ($TyrR_{IPS}$) in the brain for controlling courtship drive. Tyramine-induced inhibition of $TyrR_{IPS}$ neurons was strictly dependent on TyrR since the response was eliminated in *TyrR*^{Gal4} mutant brains. Based on our findings using the GRASP technique, we propose that $TyrR_{IPS}$ neurons may form synaptic connections with FruM neurons, which regulate courtship. We propose that courtship behavior is enhanced by release of acetylcholine from basal or highly activated $TyrR_{IPS}$ neurons. In support of this proposal, $TyrR_{IPS}$ cells expressed acetylcholine transferase (ChAT), and knockdown of ChAT in these cells reduced courtship behavior.

In conclusion, our findings show that in Drosophila tyramine is not simply a biosynthetic intermediate for octopamine. Rather, it has an important function in the neuromodulation of male courtship drive through its specific receptor, TyrR. However, it does not affect gender

preference. Given that the presence of tyramine as a trace monogenic amine in the mammalian brain [48], the question arises as to whether tyramine also functions in mammals as a neuromodulator of behavior.

EXPERIMENTAL PROCEDURES

Animals

Flies were maintained on conventional cornmeal-agar-molasses medium under a 12 h light: 12 h dark cycle at 25°C and ambient humidit y. Specific details regarding the strains used in the experiments can be found in the Supplemental Experimental Procedures.

Generation of the *TyrR^{Gal4}* mutant and transgenes

We generated the *TyrR^{Gal4}* allele by ends-out homologous recombination. See Extended Experimental Procedures for details.

Behavioral assays

Unless indicated otherwise, the behavioral assays were carried out 3 h before the onset of darkness. Tester males were raised in isolation for 4–10 days post-eclosion. Target *w^{1118}* males and virgin females were segregated by sex, and group-housed for 4–10 days old. For age-dependent courtship assays, *w^{1118}* virgin females were aged in groups for intended days. A detailed protocol is included with the Supplemental Experimental Procedures.

Immunohistochemistry

For details on procedures and antibodies used see the Extended Experimental Procedures.

Statistical analyses

Statistical analyses were performed using Prism6 (GraphPad Software). We used nonparametric tests to analyze all data, except for Figure S1E and S1F (Fisher's exact test). We performed the Mann-Whitney test for two groups of data. For comparison of three or more sets of data, we performed Kruskal-Wallis test and Dunn's *post hoc* test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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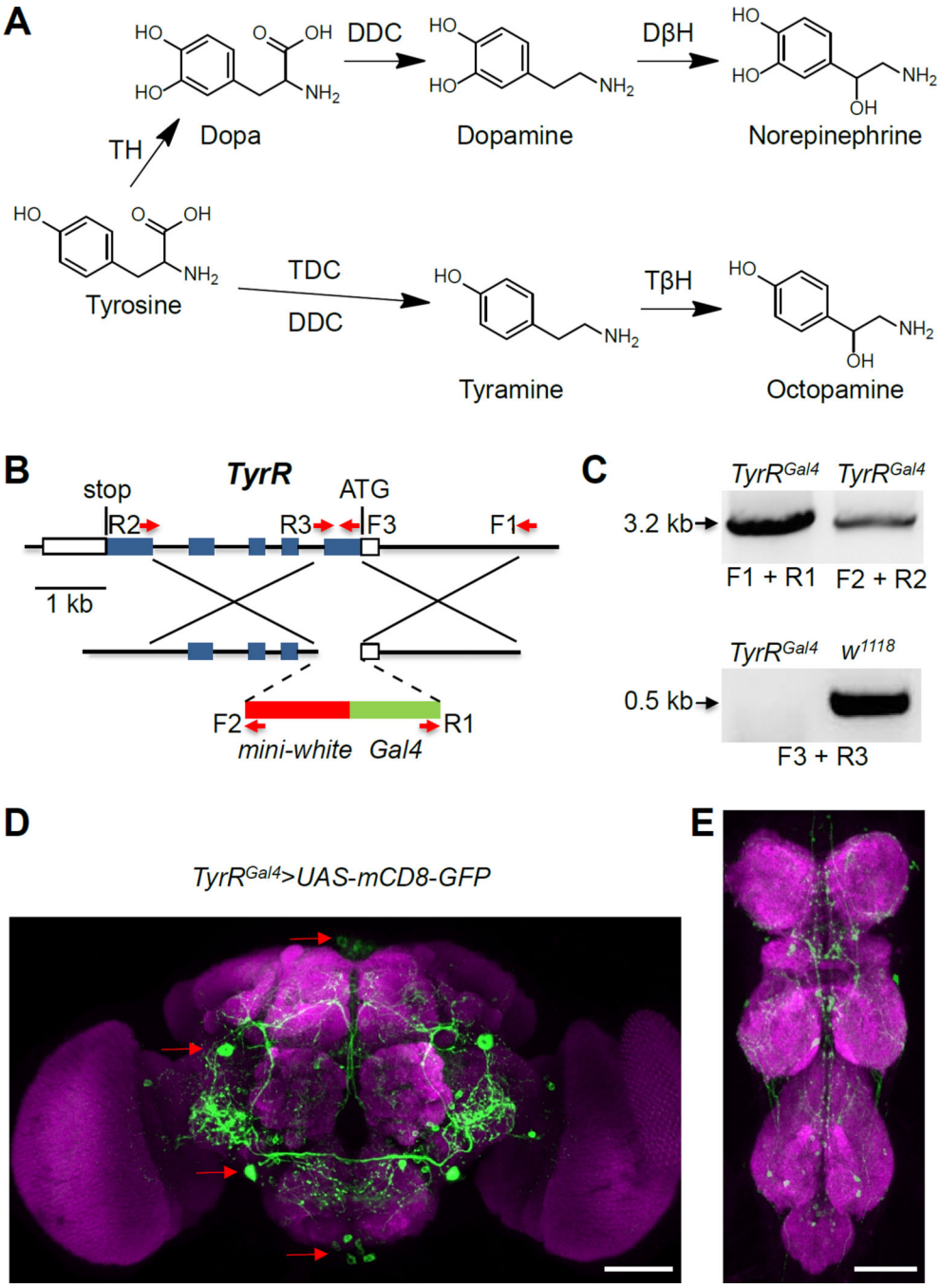

Figure 1. Synthesis of tyramine and knockout and expression of *TyrR*

(A) Biosynthetic pathway of biogenic amines derived from tyrosine. DβH, dopamine β-hydroxylase; DDC, Dopa decarboxylase; TDC, tyrosine decarboxylase; TβH, tyramine β-hydroxylase; TH, tyrosine hydroxylase. Drosophila DDC is also capable of converting tyrosine to tyramine *in vitro* [49].

(B) Schematic of the *TyrR* locus (90C2-90C3) and the targeting construct used to generate the $TyrR^{Gal4}$ allele. The boxes represent exons and the coding regions are shown in blue.

Indicated are the translational start codon (ATG), stop codon and the forward (F) and reverse (R) PCR primers.

(C) PCR confirmation of the deletion in *TyrR*^{Gal4} and the replacement of the *mini-white* and *Gal4* in *TyrR*^{Gal4}. We prepared genomic DNA and performed PCR using the primer pairs indicated in (B).

(D) *UAS-mCD8-GFP* expression driven under the control of the *TyrR*^{Gal4}/+ in the brain. The red arrows indicate the location of some of the neurons expressing the *TyrR* reporter. SMP, superior medial protocerebrum; PLP, posteriorlateral protocerebrum; IPS, inferior posterior slope; GNG, gnathal ganglia. MB, mushroom body; AL, antennal lobe; OL, optical lobe.

(E) *TyrR*-reporter expression in the ventral nerve cord. TG, thoracic ganglion; AG, abdominal ganglion.

The scale bars represent 50 μM.

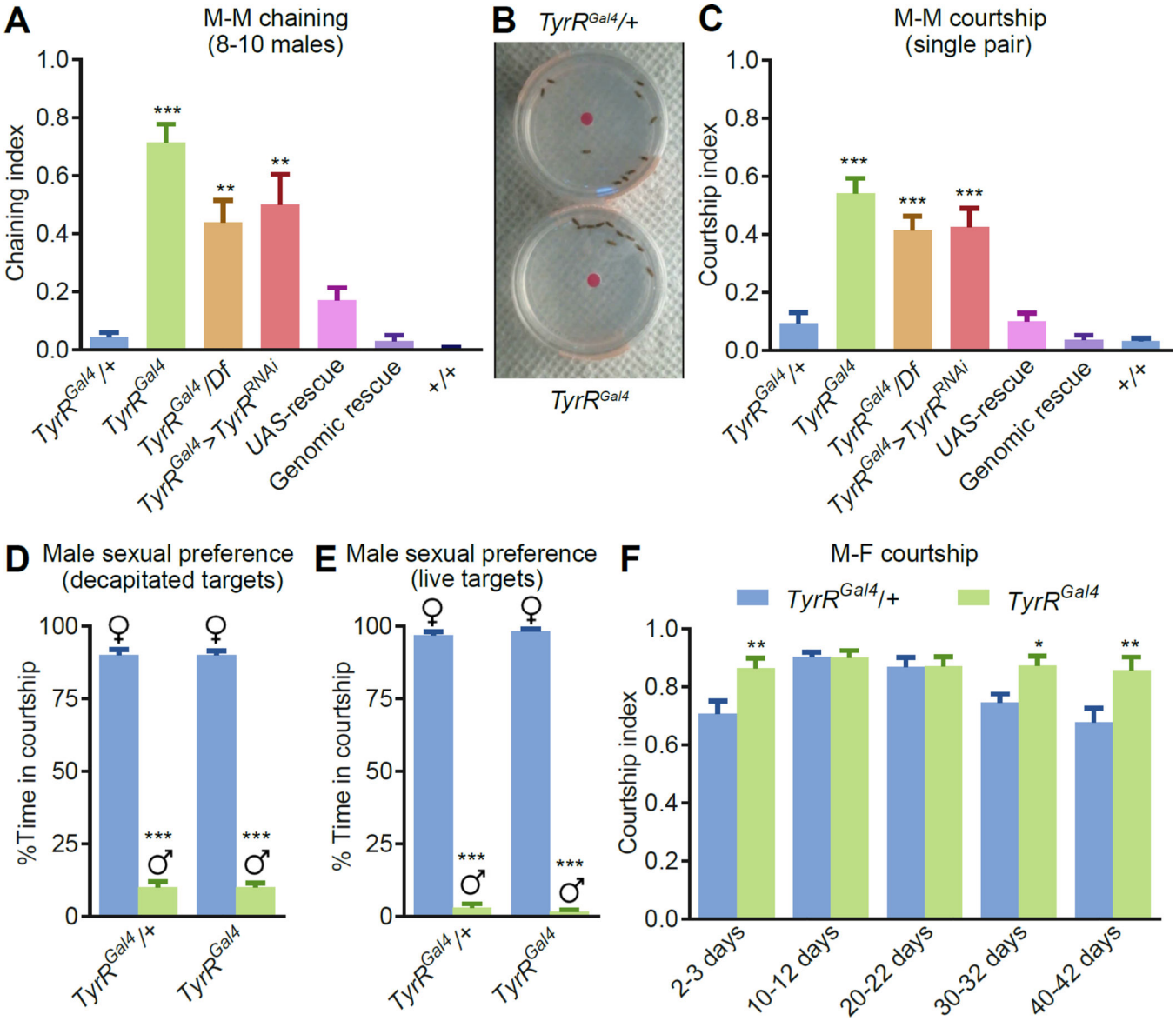

Figure 2. *TyrR*^{Gal4} mutant showed enhanced courtship behavior

(A) Chaining indices for groups of males of the indicated genotypes. The "wild type" +/+ flies used here and in other panels are w^{+} flies that have been outcrossed to w^{1118} for five generations. p<0.01; p<0.001 compared to control. n=7–10.

(B) A snapshot from Movie S1 showing male-male chaining exhibited by *TyrR*^{Gal4} (bottom) but not the heterozygous control (top).

(C) Courtship indices using one target (w^{1118}) male and one tester male of the indicated genotypes. p<0.001 compared to the *TyrR*^{Gal4}/+ control. n=24–29 trials/genotype.

(D) Testing sexual preference. Single *TyrR*^{Gal4}/+ and *TyrR*^{Gal4} males were tested for courtship behavior towards a single decapitated female and a single decapitated male. p<0.001 compared to the other gender. n=24.

(E) Single $TyrR^{Gal4}$/+ and $TyrR^{Gal4}$ males were tested for courtship behavior towards a single w^{1118} female and a single fru^{M} null (fru^{LexA}/fru^{4-40}) male. $p<0.001$ compared to the other gender. n=24.

(F) Courtship indices using different age groups of w^{1118} females and heterozygous or $TyrR^{Gal4}$ homozygous males. The ages of the target females are indicated. n=13–15 trials/genotype.

The error bars depict the means ±SEMs. All analyses of two samples employed Mann-Whitney tests. To analyze multiple samples we used the Kruskal-Wallis and Dunn's *post hoc* test. **p<0.01. ***p<0.001.

See also Figure S1.

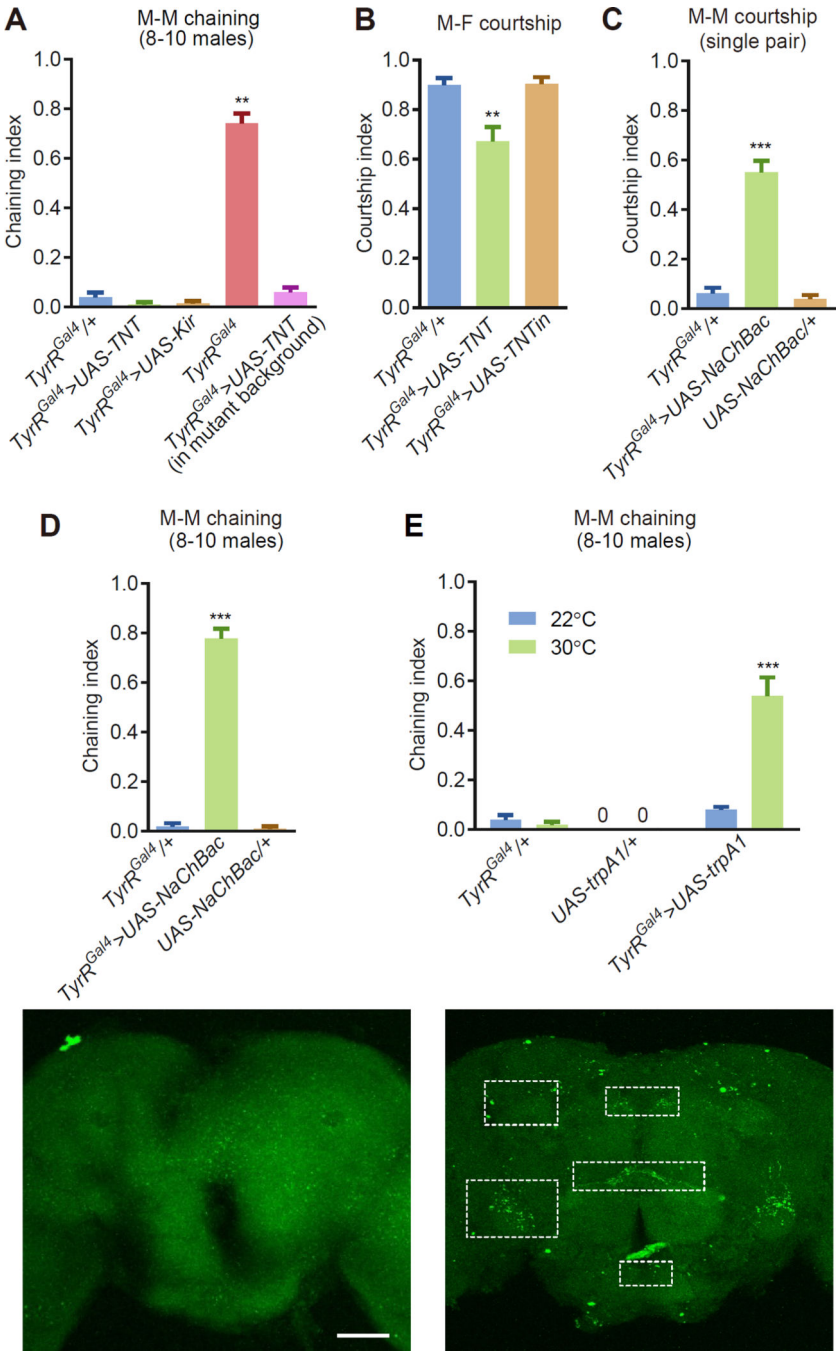

Figure 3. Activating or silencing *TyrR* neurons affected courtship behavior

(A) Chaining indices using groups of 8–10 males. To silence the *TyrR* neurons, we expressed *UAS-TNT* or *UAS-Kir2.1* under control of the *TyrR-Gal4* (*TyrR*^{Gal4}/+). *TyrR*^{Gal4}>*UAS-TNT* flies contain two copies of the *TyrR*^{Gal4} insertion, and are therefore mutants. Statistical significance was determined by comparing the data to the *TyrR*^{Gal4}/+ control. n=5–8.

(B) Single pair male-female courtship after silencing *TyrR*-expressing neurons by expressing *UAS-TNT* in the males. We used males of the indicated genotypes and control (w^{1118}) virgin females. Changes were compared to the $TyrR^{Gal4}$/+ control. n=24 trials/genotype.
(C) Male-male courtship indices in males with hyperactivated *TyrR*-expressing neurons. We combined one male expressing *UAS-NachBac* in *TyrR* neurons ($TyrR^{Gal4}$/+) in combination with one control (w^{1118}) male. Controls expressing just the *Gal4* or *UAS* transgene are included. n=18–20 trials/genotype.
(D) Chaining indices exhibited by groups of males with *TyrR* neurons constitutively activated by *UAS-NachBac*. n=5–13
(E) Chaining among groups of males in which we thermally hyperactivated *TyrR* neurons with *UAS-trpA1*. n=5–7.
(F and G) Putative synaptic connections between $TyrR^{Gal4}$ and fru^{M} neurons in the brain using the GRASP technique. All GRASP signals represent endogenous GFP fluorescence (no GFP antibodies were used). (F) Representative negative control showing the lack of GFP signal when using one rather than both GRASP components.
(F) GRASP-mediated GFP reconstitution observed in the SCL, AVLP, CRE, AL and PENP regions (dotted rectangles) due to $TyrR^{Gal4}$ neurons expressing $CD4::spGFP_{1–10}$ and fru^{LexA} neurons expressing $CD4::spGFP_{11}$. SCL, superior clamp; AVLP, anterior ventrolateral protocerebrum; CRE, crepine; AL, antennal lobe; PENP, periesophageal neuropils. The scale bars represent 50 μm.
The error bars represent the means ±SEMs. We used the Kruskal-Wallis and Dunn's *post hoc* test. **p<0.01. ***p<0.001.
See also Figure S2.

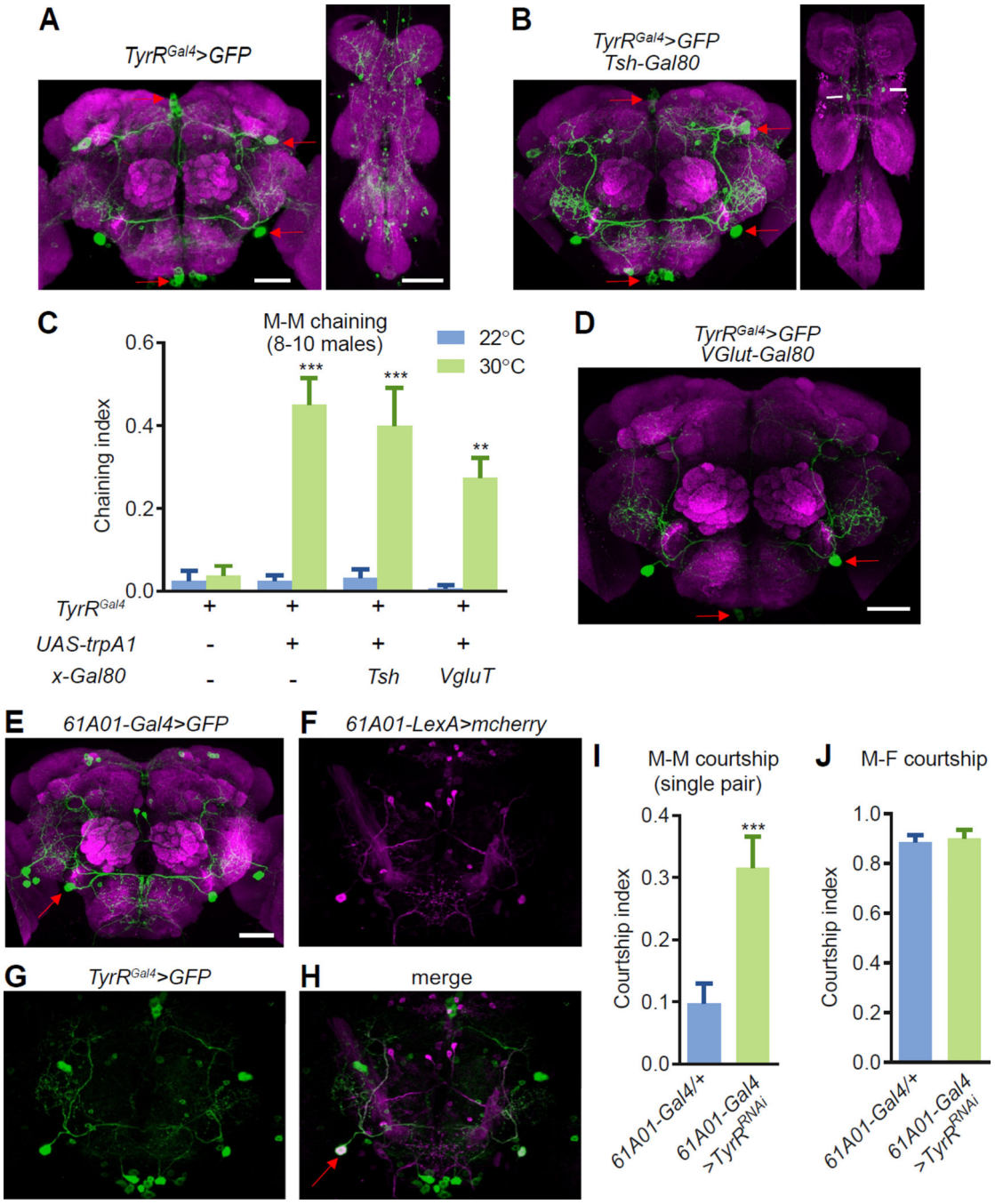

Figure 4. TyrR controls courtship behavior through IPS neurons

(A) Staining of an adult male brain (left) and VNC (right) using the *TyrR-Gal4* reporter (*TyrR*$^{Gal4/+}$) in combination with *UAS-mCD8-GFP*. Anti-GFP (green) and anti-nc82 (panneuropil marker, magenta).

(B) Elimination of *TyrR*-reporter expression in most cells in the VNC using a *Gal4* repressor *(Gal80): Tsh::Gal80*. Anti-GFP (green) and anti-nc82 (pan-neuropil marker, magenta).

(C) Male chaining resulting from thermal hyperactivation of defined subsets of *TyrR*-expressing neurons using two *Gal80* transgenes. To determine statistical significance, we

compared the chaining indices at 22°C (no TRPA1 activation) and 30°C (TRPA1 activated). n=4–7 trials/genotype.

(D) *VGlut::Gal80* restricted $TyrR^{Gal4}$ expression to the IPS and GNG regions of the male brain. The image was obtained from an animal maintained at room temperature.

(E) *UAS-mCD8-GFP* expressed under the control of the *61A01-Gal4*. The red arrow indicates the IPS neurons in the brain. Anti-GFP, green; anti-nc82, magenta.

(F-H) IPS neurons co-expressed the $TyrR^{Gal4}$ and the *61A01-LexA*. The brains contained the following transgenes: *61A01-LexA/UAS-mCD8-GFP;*$TyrR^{Gal4}$*/LexAop2-mCherry*. Anti-GFP, green; anti-DeRed, magenta.

(I) Male-male courtship indices after RNAi knockdown of *TyrR* in *61A01-Gal4* neurons. n=20–22.

(J) Male-female courtship indices after RNAi knockdown of *TyrR* in *61A01-Gal4* neurons. n=15–19.

The immunohistochemistry experiments were performed at room temperature. The scale bars represent 50 μm. The error bars indicate the means ±SEMs. Mann-Whitney test were performed. **p<0.01. ***p<0.001.

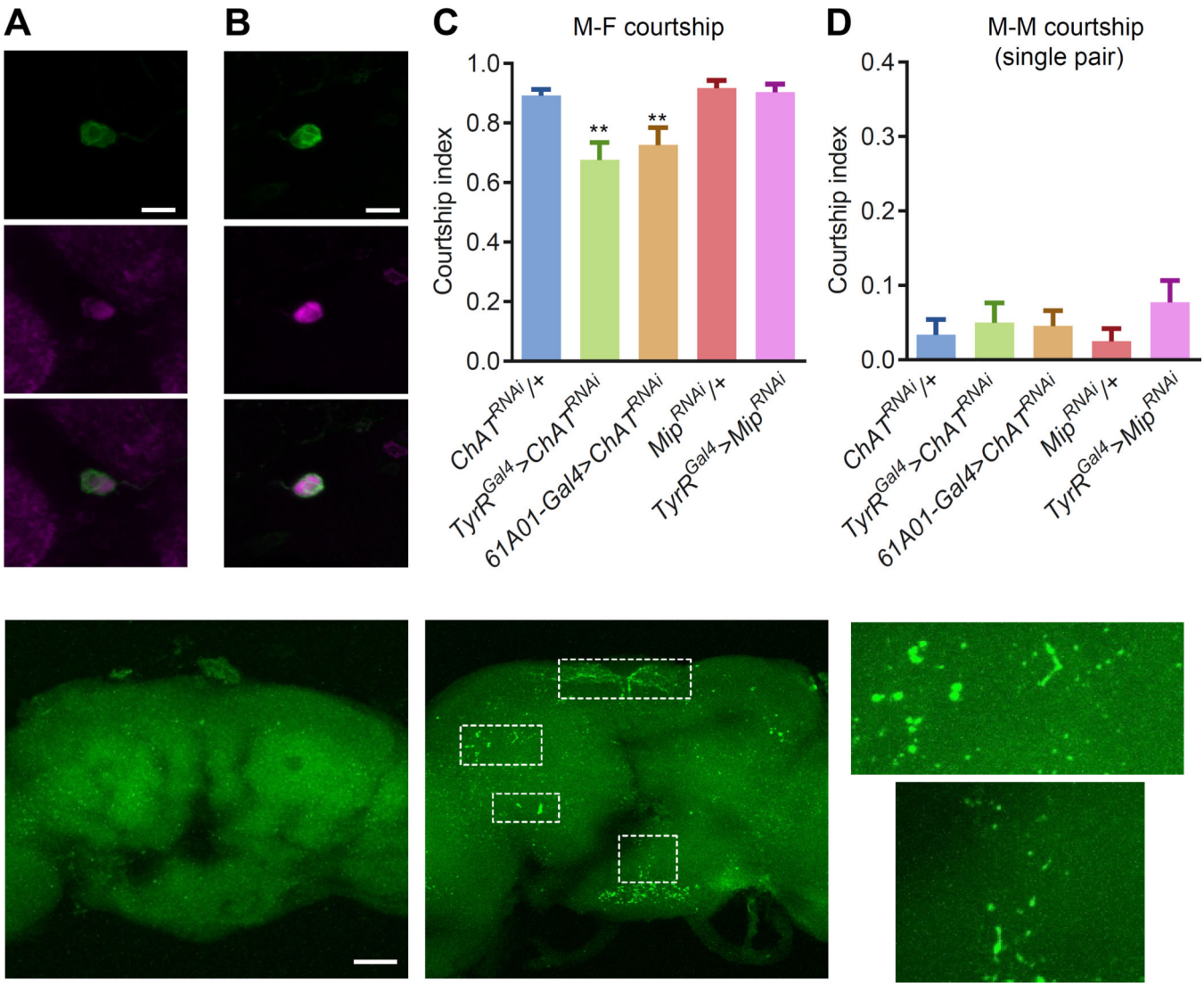

Figure 5. Functional and anatomical analyses of IPS neurons

(A) Double immunostaining using anti-GFP (green; *TyrR*^{Gal4}*>UAS-mCD8-GFP*) and anti-choline acetyltransferase (ChAT, magenta) in IPS neurons.

(B) Double immunostaining using anti-GFP (green) and anti-MIP (magenta) in IPS neurons.

(C) Single-pair male-female courtship indices after RNAi knockdown of *Mip* or *ChAT* in male *TyrR* neurons. The females were *w*^{1118} virgins. n=23–25.

(D) Single-pair male-male courtship indices after RNAi knockdown of *Mip* or *ChAT* in male *TyrR* neurons. n=18–21

(E-H) Images of brains showing putative synaptic connections between IPS and FruM neurons using the GRASP technique. All GRASP signals represent endogenous GFP fluorescence (no GFP antibodies were used). (E) Representative image showing a lack of GPF signal in a brain expression only one of the two GRASP components. (F) GRASP-mediated GFP reconstitution in the SMP, SCL, AVLP and PENP regions (dotted rectangles). The *61A01-LexA* neurons expressed *CD4::spGFP*$_{11}$ and the *fru*^{NP21}*-Gal4* neurons

expressed *CD4::spGFP*$_{1–10}$. (G-H) Close-up images of the boxed regions (SCL and PENP) in F.

The scale bars represent 10 μm in A and B and 50 μm in E and F.

See also Figure S3.

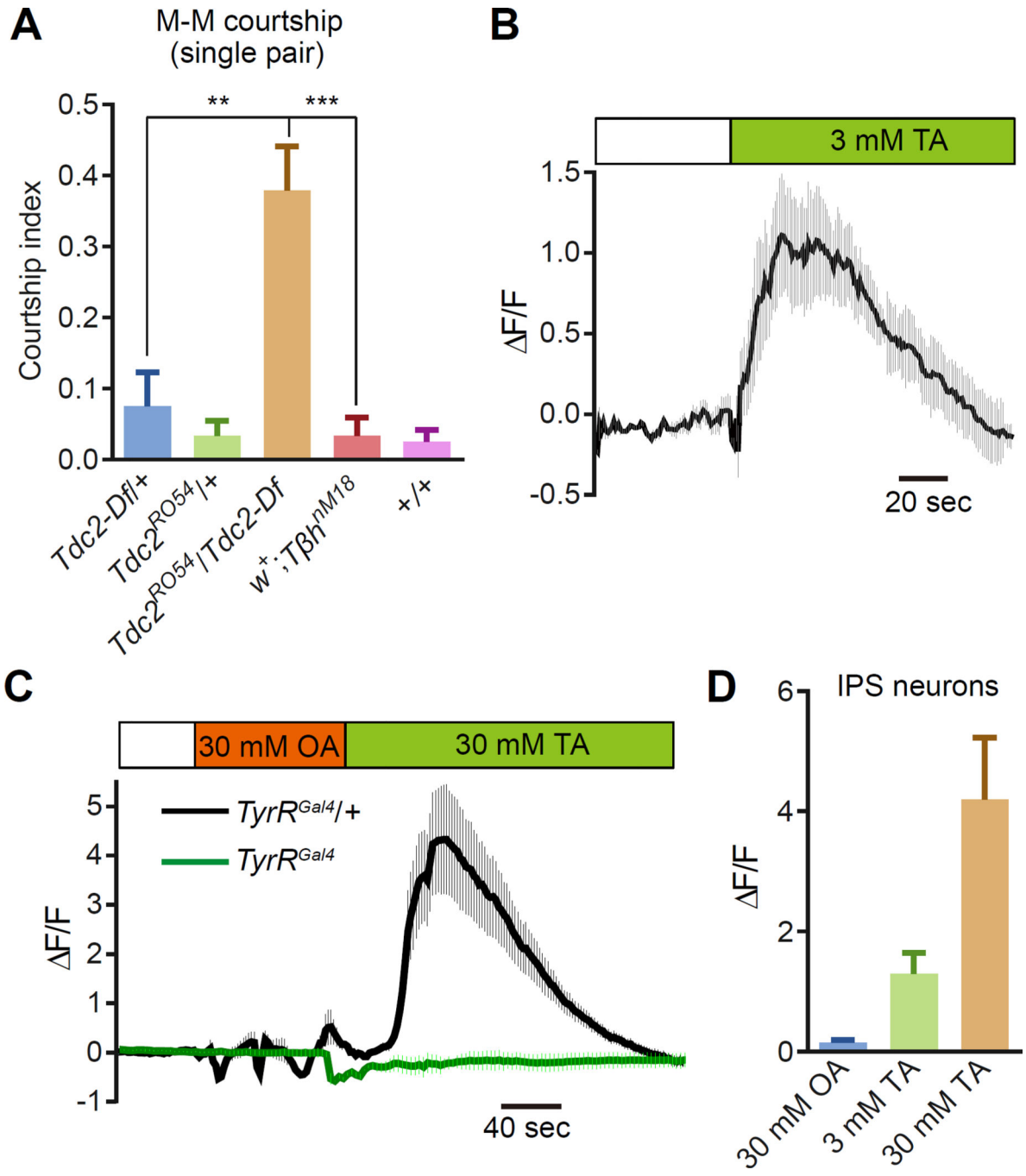

Figure 6. *TyrR* neurons respond to tyramine specifically

(A) Male-male courtship indices using one target (w^{1118}) male and one tester male of the indicated genotypes. n=12 trials/genotype

(B) Ca^{2+} imaging of *TyrR* neurons ($TyrR^{Gal4}$>*UAS-GCaMP6f*) in a brain subjected to 3 mM tyramine (TA). The ΔF/F represents the evoked fluorescence change from the baseline. The traces were averaged from 8 samples. The solid line represents the mean and the shaded areas indicate SEMs.

(C) Ca^{2+} response displayed by IPS neurons upon application of either 30 mM octopamine (OA) or 30 mM TA. Shown are the responses of the homozygous *TyrR* mutant (green trace) and the $TyrR^{Gal4}$ heterozygous control (black trace). (D) Ca^{2+} responses resulting from application of either 3 or 30 mM TA to IPS neurons. The 30 mM OA serves as a negative control. n=9 samples.
See also Figure S4.