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Figs. S1 to S19
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Sexual Deprivation Increases Ethanol Intake in *Drosophila*

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The brain's reward systems reinforce behaviors required for species survival, including sex, food consumption, and social interaction. Drugs of abuse co-opt these neural pathways, which can lead to addiction. Here, we used *Drosophila melanogaster* to investigate the relationship between natural and drug rewards. In males, mating increased, whereas sexual deprivation reduced, neuropeptide F (NPF) levels. Activation or inhibition of the NPF system in turn reduced or enhanced ethanol preference. These results thus link sexual experience, NPF system activity, and ethanol consumption. Artificial activation of NPF neurons was in itself rewarding and precluded the ability of ethanol to act as a reward. We propose that activity of the NPF–NPF receptor axis represents the state of the fly reward system and modifies behavior accordingly.

Natural rewards and abused drugs affect the function of the brain's reward systems, and abnormal function of these brain regions is associated with addictive behavior (*1–3*). Some aspects of drug reward can be modeled in the genetically tractable fruit fly, *Drosophila melanogaster*. Flies exhibit complex addiction-like behaviors, including a lasting attraction for a cue that predicts ethanol intoxication (*4*) and a preference for consuming ethanol-containing food, even if made unpalatable (*5*). Here, we extend studies in the *Drosophila* model to incorporate the effect of social experiences, which can have long-lasting effects on behavior (*6*, *7*).

We used two distinct sexual experiences to generate two cohorts of male flies. One cohort, rejected-isolated, was subjected to courtship conditioning (*8*); they experienced 1-hour sessions of sexual rejection by mated females, three times a day, for 4 days (Fig. 1A). Such conditioning suppresses future male courtship behavior, even toward receptive virgin females (*9*) (fig. S1). Flies in the mated-grouped cohort experienced 6-hour sessions of mating with multiple receptive virgin females (ratio 1:5) for 4 days. Flies from each cohort were then tested in a two-choice preference assay (*10*), in which they voluntarily choose to consume food with or without 15% ethanol supplementation (*11*). Results for the two cohorts differed markedly. The value of the ethanol preference index (which when positive signifies attraction) was consistently higher for the rejected-isolated cohort (Fig. 1B). The experiences did not alter food consumption when tested in the absence of ethanol (fig. S2).

The rejected-isolated and mated-grouped cohorts differ in several respects in addition to sexual deprivation (lack of copulation) per se, including individual versus group housing, exposure to the social experience of rejection, and exposure to aversive chemosensory cues found on mated females. Several experiments were designed to determine which of these was the predominant contributor to the enhanced ethanol preference seen in rejected-isolated males (*11*). First, we compared males that differed in sexual experience but not in housing conditions—that is, mated and virgin males that were both group-housed. The virgin males showed higher ethanol preference, although in general not quite as high as rejected-isolated males. This argues that isolation is not the major explanation for the enhanced ethanol preference.

We next investigated ethanol preference in males that were sexually deprived (blocked from copulating) but not exposed to the social experience of rejection. For this purpose, males were exposed individually to decapitated virgin females on the same schedule as the rejected-isolated cohort, using a protocol that results in courtship suppression (*9*). These males, which experience neither rejection nor copulation, showed enhanced ethanol preference when compared to the mated-grouped cohort (Fig. 1C); the preference index was similar to that displayed by the rejected-isolated cohort. These results point to sexual deprivation per se, rather than rejection, as the major factor influencing ethanol preference.

Finally, we sought to establish whether there was a role for the repellant chemosensory cue *cis*-vaccenyl acetate (cVA), which is found on the cuticle of mated but not virgin females (*12*). We compared males trained with either mated females (rejected-isolated) or decapitated virgin females (*11*). Both groups endured sexual deprivation (lack of copulation), but only the former was exposed to cVA. There was no difference in ethanol preference between these two cohorts (fig. S3A). There was also no difference between males exposed individually to biologically relevant concentrations of cVA (*13*) and vehicle-exposed controls (fig. S3B) (*11*). Together, these experiments point to sexual deprivation per se, rather than other factors, as the major contributor to enhanced ethanol preference.

To further test the strength of this conclusion, we divided a cohort of rejected-isolated males into two subgroups, one of which was left undisturbed, and the other of which was allowed to mate with virgin females for 2.5 hours immediately before testing. Ethanol preference was markedly lower in the rejected, then mated subgroup (Fig. 1E) compared to the subgroup that had only experienced rejection. Thus, the effects of sexual deprivation can be reversed by copulation, which is consistent with sexual deprivation being the major contributor to ethanol preference.

We focused on *Drosophila* neuropeptide F (NPF) as a potential mediator of the effects of sexual experience. The mammalian NPF homolog, neuropeptide Y [NPY (*14*)], regulates ethanol consumption (*15*), the NPF–NPF receptor (NPFR) system regulates acute ethanol sensitivity in *Drosophila* (*16*), and the *Caenorhabditis elegans* NPY receptor homolog NPR-1 regulates ethanol behaviors (*17*). Intriguingly, stressful experiences regulate mammalian NPY levels. These include restraint stress and early maternal separation in rodents and post-traumatic stress disorder in humans (*18–20*). However, a direct connection between social experience, NPY, and ethanol-related behaviors has not been established.

To investigate whether NPF mediates ethanol preference in *Drosophila*, we first compared NPF transcript levels in heads of males subjected to different sexual experiences: rejected-isolated, virgin-grouped, and mated-grouped (*11*). Rejected-isolated males showed the lowest transcript levels, virgin-grouped males showed higher levels, and mated-grouped males showed the highest

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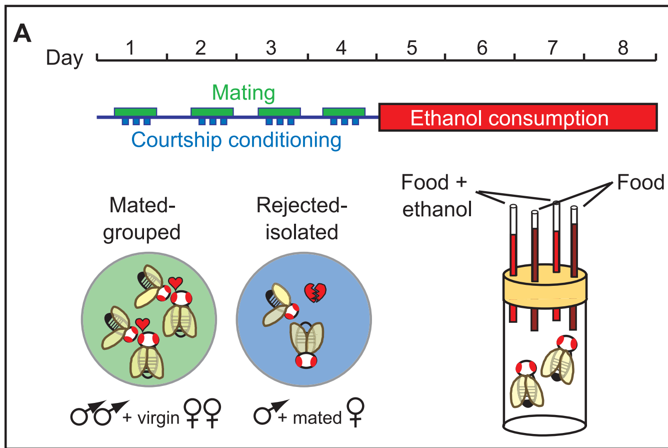

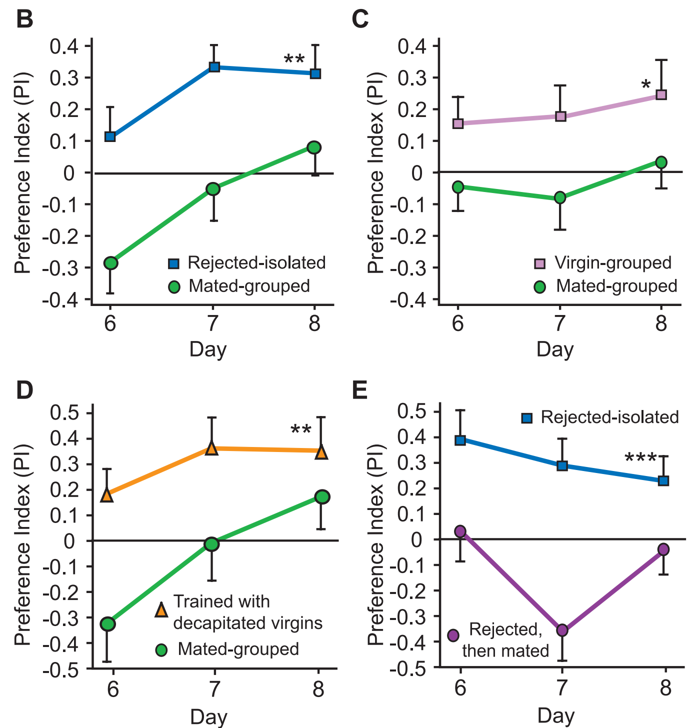

Fig. 1. Mating and chronic sexual deprivation have opposite effects on voluntary ethanol consumption. (**A**) Schematic of the behavioral assay. Virgin wild-type males were allowed to mate with virgin females (groups of 4 males and 20 females) for 6 hours daily ("mated-grouped"; green blocks) or were subjected to courtship conditioning for 1 hour, three times daily ("rejected-isolated"; blue squares). Training was repeated for 4 days, after which males were placed in vials where they could choose to feed from capillaries containing food solutions with (red) or without (brown) 15% ethanol (*10*). Ethanol consumption was measured on days 6 to 8. (**B**) Rejected-isolated males exhibited higher ethanol preference than mated-grouped males (** $P < 0.005$, $n = 12$). (**C**) Mated-grouped males showed lower ethanol preference than "virgin-grouped" males (* $P < 0.05$, $n = 12$). (**D**) Males conditioned with decapitated virgins showed enhanced ethanol preference compared to mated-grouped males (** $P < 0.01$, $n = 12$). (**E**) Mating reversed the effects of rejection on ethanol preference. Rejected-isolated males that were allowed to mate after the end of the last conditioning session showed lower ethanol preference than similarly conditioned males that were left undisturbed (** $P < 0.001$, $n = 8$). Statistical analysis was carried out by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post tests; comparisons are between treatment groups across all days of the assay. Data shown are the mean + SEM or mean – SEM.

(Fig. 2A and fig. S4). Rejected-isolated males also showed markedly lower NPF protein levels than mated-grouped males by immunohistochemistry (Fig. 2, B to D).

To determine whether the inverse correlation between NPF levels and ethanol preference reflects a cause-and-effect relationship, we manipulated the NPF-NPFR system genetically. We first tested the effect of NPFR down-regulation by expressing an NPFR-specific short interfering RNA (*UAS-NPFR^{RNAi}*) pan-neuronally (using *elav-GAL4*). This manipulation significantly reduced ethanol preference in mated males, which have elevated NPF levels, but not in virgin males (Fig. 3, A and B). Second, we tested the effect of artificial activation of NPF neurons by expressing the heat-activated cation channel dTRPA1 (*21*) under *NPF-GAL4* control (*22*). There was no effect on ethanol preference when virgin males were tested at 20°C, when the channel is inactive, but there was aversion to ethanol-supplemented food at 29°C, when the channel is active (Fig. 3, C and D). An intermittent dTRPA1 activation protocol that more closely mimics our conditioning protocol produced similar aversion (fig. S5). These data suggest a causal relationship between sexual experience, NPF levels, and ethanol preference.

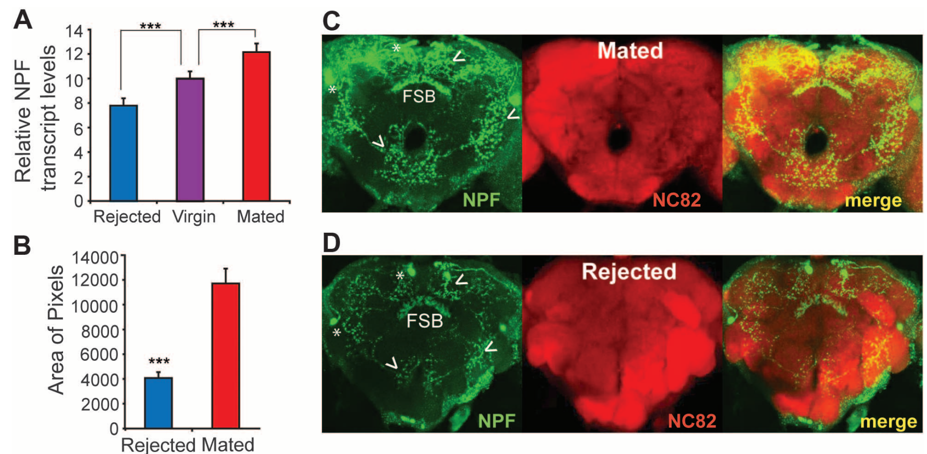

Fig. 2. Sexual experience regulates levels of NPF and NPF mRNA. (**A**) Total RNA extracted from heads of virgin, rejected, and mated males was analyzed for NPF mRNA levels by quantitative polymerase chain reaction (qPCR). NPF mRNA levels were reduced by sexual rejection and increased by mating (*** $P < 0.001$ compared to virgin control, Dunnett's test, $n = 3$ independent experiments). NPF transcript levels were normalized *to rp49* mRNA. (**B** to **D**) Effect of rejection on NPF protein abundance as determined by immunohistochemistry. (B) Quantitative analysis of overall NPF staining intensity in brains of rejected and mated males (*** $P < 0.001$, *t* test). (C and D) Differential NPF staining in rejected and mated males was observed in all major regions of NPF expression (arrowheads). Asterisks denote the positions of NPF-expressing cell bodies (which are obscured by high levels of expression in mated males). FSB, fan-shaped body.

We propose that the activity of the NPF-NPFR system may be a neural representation of the state of the *Drosophila* reward system. If so, experiences that change NPF-NPFR activity should promote behaviors that restore the system to its normal state. In this model, sexual deprivation would create an NPF deficit that increases reward-seeking behavior such as ethanol consumption. Conversely, successful copulation would create a NPF surfeit that reduces reward seeking. This model predicts that mating and ethanol consumption should be rewarding (*4*, *5*), that activa-

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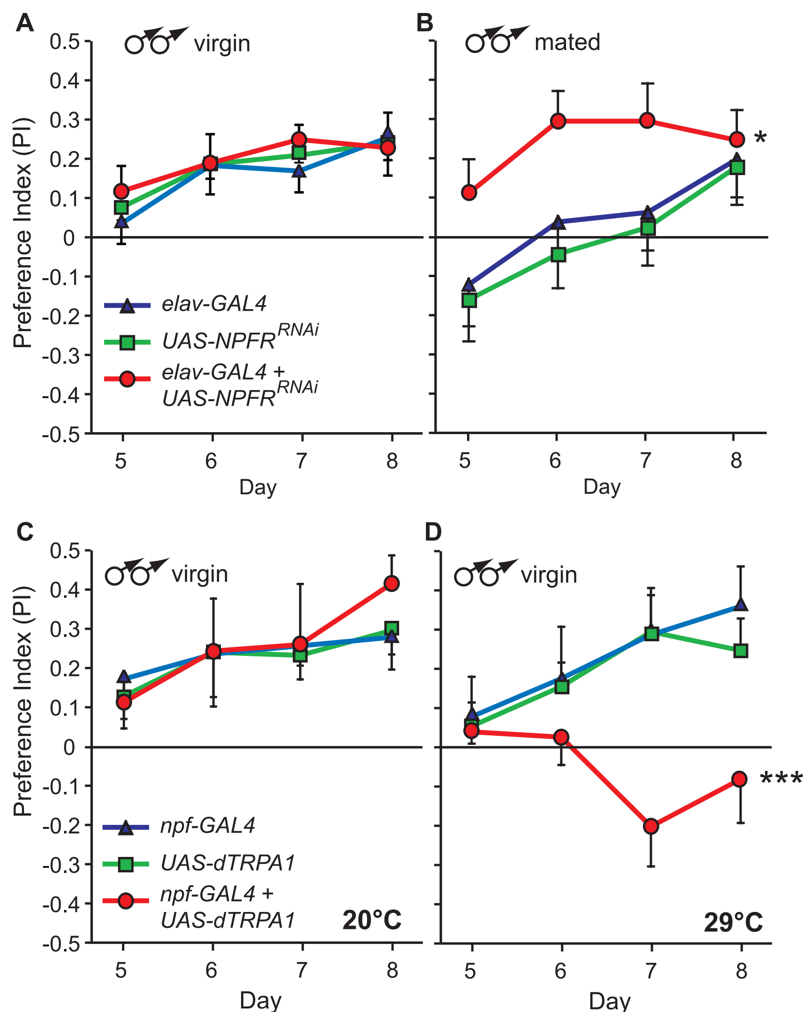

Fig. 3. NPF signaling regulates ethanol preference. (**A** and **B**) Expression of an NPFR RNA interference (RNAi) transgene (*UAS-NPFR*^{RNAi}) using a pan-neuronal driver (*elav-GAL4*) increased ethanol preference in mated males compared to the genetic controls carrying either transgene alone (B) (*$P < 0.05$, $n = 12$), but not in virgin males (A) ($P > 0.5$). (**C** and **D**) Activating NPF neurons reduced ethanol preference. Virgin males expressing dTRPA1 in NPF neurons (*NPF-GAL4* + *UAS-dTRPA1*), and the genetic controls carrying either transgene alone, developed similar levels of ethanol preference at 20°C (C) when dTRPA1 is not active ($P > 0.05$, $n = 8$), but developed aversion to ethanol containing food at 29°C (D), when dTRPA1 is active (***$P < 0.001$, $n = 8$). Statistical analysis was carried out by two-way repeated-measures ANOVA with Bonferroni post tests; comparisons are between treatment groups across all days of the assay. Data are the mean + or mean − SEM (for clarity purposes).

tion of the NPF-NPFR pathway is rewarding per se, and that artificial activation of the NPF circuit will diminish ethanol reward-seeking behavior.

To test these predictions, we used a series of conditioning assays in which male flies were trained to associate the proposed rewarding experiences (mating, ethanol exposure, or NPF circuit activation) with one of two neutral odor cues. After 24 hours, flies were tested for their odor preference; development of a preference for the odor associated with these experiences would imply that flies found the events rewarding. To test if mating is rewarding, males were exposed sequentially for 30 min to two odorants [ethyl acetate (EA) or isoamyl alcohol (IAA)], one in the absence and the other in the presence of virgin females, and tested for odor preference 24 hours later in the absence of females. A conditioned odor preference index (CPI) for mating was calculated by averaging preference indices for reciprocally trained groups of flies. Positive CPI values indicate conditioned preference, negative values indicate aversion. Males displayed a strong preference for the mating-associated odor (Fig. 4A). We have separately shown that flies exhibit conditioned preference for an odor associated with ethanol intoxication in a similar assay (*4*). Together, these results indicate that both mating and ethanol intoxication, the latter of which is likely achieved in the two-choice consumption assay (*5*), are indeed rewarding experiences to male flies.

To test whether activation of the NPF-NPFR pathway is rewarding per se, we trained virgin males to associate artificial activation of NPF neurons with either EA or IAA. Males expressing dTRPA1 in NPF neurons (*NPF-GAL4* + *UAS-dTRPA1*) and the genetic controls each carrying only one of the two transgenes were trained for three 1-hour sessions at 29°C, with dTRPA1 active, interspersed with three 1-hour rest periods at 18°C, with dTRPA1 inactive (Fig. 4B). When tested 24 hours later, males in the experimental group demonstrated strong preference for the odor associated with NPF neuron activation. The genetic controls, which did not undergo NPF neuron activation, but were exposed to the same training protocol, developed no odor preference (Fig. 4C). Other controls, which underwent NPF neuron activation but were not exposed to the training protocol, similarly developed no odor preference (fig. S6C). Thus, activation of the NPF-NPFR system is in itself rewarding to flies.

We next tested whether artificial activation of the NPF-NPFR system diminishes ethanol reward seeking. Flies were trained to associate EA or IAA with a moderately intoxicating exposure of ethanol vapor (three 10-min training sessions spaced by 1 hour) as described before (*4*). Wild-type flies normally show conditioned aversion to ethanol (negative CPI) when tested 30 min after training, and conditioned preference 24 hours later (*4*). We used this assay to compare virgin male flies that expressed dTRPA1 in NPF neurons (*NPF-GAL4* + *UAS-dTRPA1*) with genetic controls that did not. Artificial activation of NPF cells, which occurs at 30°C but not 22°C, had no effect on the initial aversion (fig. S6, A and B), but abolished conditioned preference for ethanol (Fig. 4, D and E). Thus, NPF neuron activation, which is in itself rewarding to flies, interferes with the ability of flies to form a positive association between ethanol intoxication and an odor cue, which is reflected in lower ethanol consumption.

If the NPF-NPFR system were to function generally to signal the state of the *Drosophila* reward system, NPF levels should be increased by rewarding experiences other than mating, such as exposure to intoxicating levels of ethanol. To test this hypothesis, we exposed virgin males to ethanol vapor using an exposure paradigm previously shown to be rewarding (three 10-min exposures spaced by 1 hour) (*4*). NPF transcript levels increased 1 hour after exposure and returned to basal levels 24 hours later (Fig. 4F). Because the ethanol-induced changes in NPF levels are transient, whereas the memory of ethanol reward lasts for several days, it is possible that ethanol-induced changes in NPF levels set in motion a process, likely involving dopaminergic systems (*4*, *22*), that modifies the fly reward system. Indeed, the activity of the NPF circuit could remain altered long after the levels of NPF had returned to baseline. Regardless of the exact mechanism, these data suggest that activity of the NPF system is regulated by at least two rewarding experiences, mating and ethanol intoxication.

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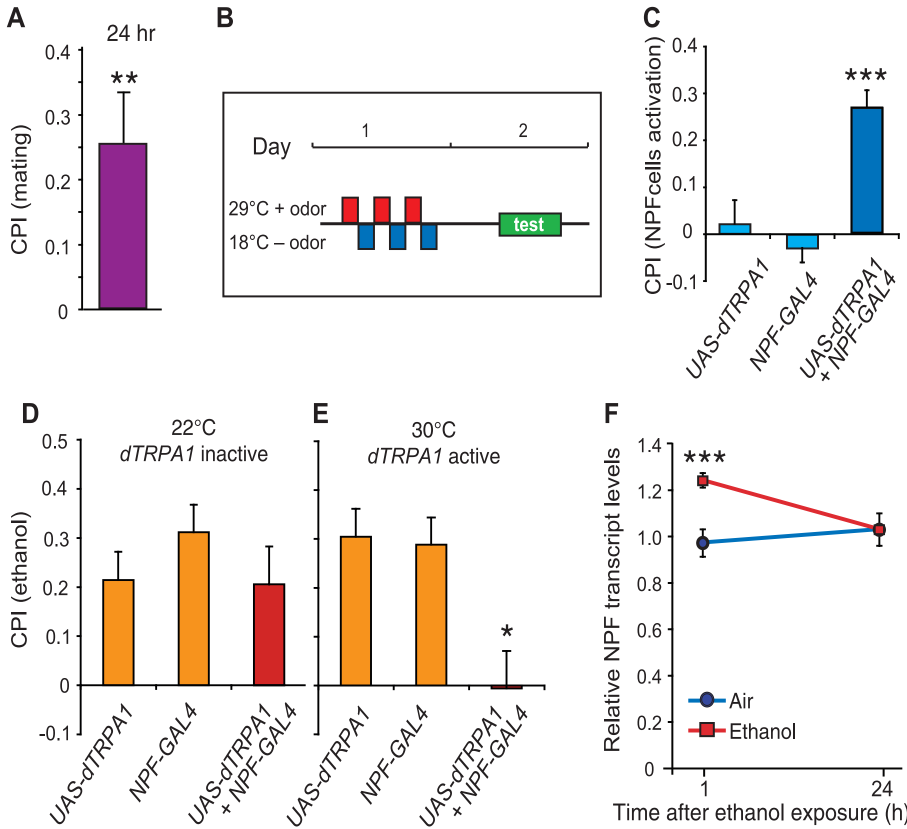

Fig. 4. Mating and NPF cell activation are rewarding and reduce ethanol reward. (**A**) Mating is rewarding to male flies. Males trained to associate an odor with mating (presence of virgin females) develop preference for that odor. *P* values were calculated by Wilcoxon analysis against zero. Mating against zero was $^{**}P = 0.001$; each reciprocal group against zero was $P = 0.004$ for one odor (IAA) plus mating and $P = 0.02$ for the reciprocal odor (EA) plus mating. CPI, conditioned preference index (calculated by averaging the odor preference indexes for reciprocally trained males). (**B** and **C**) NPF cell activation is rewarding. Males expressing dTRPA1 in NPF neurons (*NPF-GAL4* + *UAS-dTRPA1*) and the genetic controls carrying either transgene alone were exposed to three 1-hour training sessions at 29°C in the presence of odor [red rectangles in (B)] that were spaced by 1-hour rest periods at 18°C in the absence of odor [blue rectangles in (B)]. Testing for odor preference was performed 24 hours after training at 21°C. Experimental males, but not the genetic controls, showed preference for the odor that was associated with dTRPA1 activation in NPF neurons. Data are averages of three independent experiments. Statistical analysis was carried out by two-way ANOVA with Bonferroni post tests; comparisons are between treatment groups ($^{**}P < 0.001$, $n = 24$). (**D** and **E**) Activation of NPF neurons abolishes ethanol reward. Activation of NPF neurons using dTRPA1 (*NPF-GAL4* + *UAS-dTRPA1*) eliminated conditioned ethanol preference compared to the singly transgenic controls when tested 24 hours after training ($^{*}P < 0.01$, one-way ANOVA with Wilcoxon/Kruskal-Wallis post-hoc tests, $n = 22$). (**F**) NPF transcript levels are induced by ethanol intoxication. Males were exposed to moderately intoxicating levels of ethanol vapor (three 10-min ethanol exposures spaced by 1 hour), collected, and frozen 1 or 24 hours later. NPF mRNA levels, measured by qPCR, were elevated 1 hour after ethanol exposure and returned to basal level after 24 hours ($^{**}P < 0.001$ compared to air-exposed controls, Dunnett's test, $n = 3$ independent experiments with 30 males each).

NPF has been shown to influence several complex behaviors in flies, including larval intake of noxious food (*23*), a switch in feeding behavior (*24*), and responses to physical stressors (*25*) and ethanol (*16*). In addition, NPF neurons modulate the effect of satiety on sugar reward memory (*22*). In our paradigm, NPF appears to play a different role: Its expression is regulated by sexual experience and ethanol intoxication, and activation of NPF neurons acts as a reward signal, thereby abolishing ethanol reward and the enhanced ethanol consumption observed after sexual deprivation. It is likely that the effects of NPF we describe here are mediated by a different set of NPFR-expressing neurons than those mediating NPF's role in sugar reward memory.

Mammalian NPY has several distinct behavioral functions that are mediated by different brain regions, including roles in feeding (*26*, *27*), anxiety, stress (*28*), sleep regulation (*29*), sexual motivation (*30*), and ethanol consumption (*15*, *31*). Stressors also regulate NPY levels (*18*–*20*). In addition, injection of NPY into the nucleus accumbens of rats is rewarding (*32*), and NPY administration relieves the negative affective states of drug withdrawal and depression (*33*, *34*).

Our findings are thus not only consistent with known functions of mammalian NPY and its mode of regulation, but also provide evidence for NPF functioning as a key molecular transducer between social experience and drug reward. *Drosophila* is a useful and accessible model system in which to decipher the mechanisms by which social experiences interact with reward systems.

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Materials and Methods
Figs. S1 to S6
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SNARE Proteins: One to Fuse and Three to Keep the Nascent Fusion Pore Open

Lei Shi,[1] Qing-Tao Shen,[2] Alexander Kiel,[1] Jing Wang,[1] Hong-Wei Wang,[2] Thomas J. Melia,[1] James E. Rothman,[1]* Frédéric Pincet[1,3]*

Neurotransmitters are released through nascent fusion pores, which ordinarily dilate after bilayer fusion, preventing consistent biochemical studies. We used lipid bilayer nanodiscs as fusion partners; their rigid protein framework prevents dilation and reveals properties of the fusion pore induced by SNARE (soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor). We found that although only one SNARE per nanodisc is required for maximum rates of bilayer fusion, efficient release of content on the physiologically relevant time scale of synaptic transmission apparently requires three or more SNARE complexes (SNAREpins) and the native transmembrane domain of vesicle-associated membrane protein 2 (VAMP2). We suggest that several SNAREpins simultaneously zippering their SNARE transmembrane helices within the freshly fused bilayers provide a radial force that prevents the nascent pore from resealing during synchronous neurotransmitter release.

Efficient synaptic transmission requires the fast release of neurotransmitters from synaptic vesicles that fuse with the presynaptic plasma membrane upon entry of calcium ions (*1*). Membrane fusion necessarily implies a fusion pore that opens between the vesicle and its partner membrane at the instant of fusion. The conductance properties of such nascent fusion pores suggest that their typical diameters are in the range of ~2 nm, although considerable variability exists (*2*–*5*). Neurotransmitter is released from synaptic vesicles [diameter ~40 nm (*6*, *7*)] by diffusion through the nascent pore in the first 100 to 200 μs, even before appreciable dilation of the pore occurs (*4*). The transient and variable nature of the fusion pore has severely limited biochemical and physical chemical studies.

We suggest that nanodiscs (*8*–*11*) provide an ideal model for such studies because the small amount of disc lipid will suffice to allow pores to open but not expand (Fig. 1 and Fig. 2A) beyond their nascent, physiologically relevant state for neurotransmitter release. Nanodiscs are synthetic lipoprotein particles that contain a small piece of circular lipid bilayer (up to ~17 nm in diameter) wrapped by two copies of membrane scaffold protein (MSP) derived from apolipoprotein A1. In the system we describe here, nanodiscs contain the synaptic vesicle SNARE (v-SNARE) VAMP2 and small unilamellar vesicles [diameter 30 to 60 nm (*12*)] contain the synaptic target membrane SNARE (t-SNARE) complex of syntaxin 1 and SNAP25. SNAREs are the core machinery for this and other cellular membrane fusion processes (*12*–*14*). They assemble between bilayers as a four-helix bundle (*15*) that imparts sufficient force to cause bilayer fusion (*16*).

After reconstitution (supplementary text), the nanodiscs containing VAMP2 (v-discs) were separated by gel filtration (Fig. 1, A to C). Each disc contained about 400 lipid molecules wrapped by two MSPs. With a starting VAMP2/MSP ratio of 6:2, we recovered ~7 VAMP2 copies per disc on average after removing VAMP2-free dics. Electron microscopy of v-discs confirmed an average diameter of 16 ± 2 nm (Fig. 1D). Not surprisingly, single VAMP2 proteins on these discs could not be readily distinguished because of their small size and flexible structure. However, addition of the soluble t-SNARE (a complex of syntaxin H3 cytosolic domain and SNAP25N/C helical domains) formed rodlike SNARE complexes that were seen to protrude from the nanodiscs (Fig. 1D). This confirms that VAMP2 on nanodiscs can form SNARE complexes.

We used a well-established lipid mixing assay (*17*) to test whether v-discs can fuse with t-vesicles. Nitro-2-1,3-benzoxadiazol-4-yl-phosphatidylethanolamine (NBD-PE) and rhodamine-PE were included in the v-discs (1.5 mol % each). This surface concentration of rhodamine effectively quenches the NBD fluorescence. However, when a nanodisc fuses with a liposome, NBD fluorescence will greatly increase because of the substantial (>50-fold) lipid dilution as the disc lipid mixes with the massive excess of vesicle lipid, as we observed (Fig. 2B). Little or no increase of NBD signal was observed in control experiments. The slow lipid mixing between nanodiscs and vesicles was limited by the rate of docking (initial SNARE assembly) and not by the rate of fusion (fig. S1), as is the case for vesicle-vesicle fusion systems (*18*). A similar fusion process was observed when the SNAREs were placed in the opposite topology, with v-SNAREs in the vesicle and t-SNAREs in the nanodisc (fig. S2).

To monitor efflux of content via fusion pores that necessarily form at least transiently during the fusion process, we encapsulated calcium (50 mM) in the liposomes, which were then incubated with v-discs in a medium containing a calcium-activated fluorophore, Mag-Fluo-4 (2 μM, K_d for calcium = 22 μM; Invitrogen). When pores open, calcium diffuses through the pores into the exterior buffer, inducing a fluorescence signal. The results (Fig. 2C) clearly show that calcium was released in a SNARE-specific manner. To ascertain that this efflux was due to diffusion through a pore, rather than transient lysis or leakage of the vesicle during fusion, we tested the rate of release of vesicle cargos of different sizes—specifically, the calcium chelator EDTA (Stokes radius, ~0.4 nm) and EGTA (Stokes radius, ~0.5 nm). EDTA release from liposomes was faster than EGTA release from liposomes by a factor of 2.3 (fig. S3). From these data, a pore size of ~2 nm can be calculated (supplementary text), similar in size to the nascent fusion pore size calculated from electrophysiological measurements (*3*).

When vesicles fuse to target membranes, the fusion pore eventually expands and the vesicle is incorporated into the target membrane. With nanodiscs, however, the pore cannot appreciably expand beyond its nascent diameter of ~2 nm, so the only means available to reduce membrane stress (resulting from the extreme curvature inherent in a small pore) is for the pore to eventually reseal. To confirm the prediction that nanodisc-vesicle pores reseal, we introduced dithionite (5 mM) into samples 40 min after beginning the fusion assay. Dithionite quenches all externally accessible NBD (*19*), including NBD on both faces of unreacted nanodiscs and NBD-PE that had diffused into the outer leaflets of liposomes via hemifusion or full fusion. Dithionite is also small enough (Stokes radius, 0.2 to 0.3 nm) to readily diffuse through any 2-nm fusion pores that may remain open and quench the NBD signal on the inner leaflets in the case that the pore remains open, but will not gain access to the interior if the pore has (as predicted) closed off. We observed that some of the NBD dye remained protected against dithionite, and only

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