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The neuropeptide drosulfakinin enhances choosiness and protects males from the aging effects of social perception

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The motivation to reproduce is a potent natural drive, and the social behaviors that induce it can severely impact animal health and lifespan. Indeed, in *Drosophila* males, accelerated aging associated with reproduction arises not from the physical act of courtship or copulation but instead from the motivational drive to court and mate. To better understand the mechanisms underlying social effects on aging, we studied male choosiness for mates. We found that increased activity of insulin-producing cells (IPCs) of the fly brain potentiated choosiness without consistently affecting courtship activity. Surprisingly, this effect was not caused by insulins themselves, but instead by drosulfakinin (DSK), another neuropeptide produced in a subset of the IPCs, acting through one of the two DSK receptors, CCKLR-17D1. Activation of *Dsk*$^+$ IPC neurons also decreased food consumption, while activation of *Dsk*$^+$ neurons outside of IPCs affected neither choosiness nor feeding, suggesting an overlap between *Dsk*$^+$neurons modulating choosiness and those influencing satiety. Broader activation of *Dsk*$^+$ neurons (both within and outside of the IPCs) was required to rescue the detrimental effect of female pheromone exposure on male lifespan, as was the function of both DSK receptors. The same broad set of *Dsk*$^+$ neurons was found to reinforce normally aversive feeding interactions, but only after exposure to female pheromones, suggesting that perception of the opposite sex gates rewarding properties of these neurons. We speculate that broad *Dsk*$^+$ neuron activation is associated with states of satiety and social experience, which under stressful conditions is rewarding and beneficial for lifespan.

mate choice | condition dependence | cholecystokinin | reward

Significance

Unmet social expectations have been shown to be harmful for animal health and lifespan. This study establishes Drosulfakinin (*Drosophila* homolog of human satiety neuropeptide Cholecystokinin) as a regulator of male choosiness among available mates as well as a rewarding signal and a promoter of male lifespan under stressful social conditions. Our finding that broad activation of *Dsk*$^+$ neurons is both rewarding to males and rescues reduced lifespan under social stress suggests therapeutic application of rewarding experiences.

Recent times have seen a shift in thinking about how reproduction modulates aging. It has long been assumed that reproduction exerts large metabolic demands that accelerate aging (1, 2). However, in certain circumstances, metabolic costs of reproduction are relatively low, especially in controlled laboratory environments, while detriments to health and aging that arise from perceptive systems can be substantial (3–6). This seems to be particularly true for males, where central processing of premating interactions reduces lifespan and stress resistance, especially if these interactions do not result in mating (7, 8). The mechanisms underlying these effects are largely unknown.

Premating behaviors include searching and fighting for mates, courtship, and, often, engaging in decisions of choice between alternative mating partners. While other types of activities have been extensively studied, decision making and what makes an animal choosy have lacked systematic attention. For both sexes, picking a healthy and vigorous mate can significantly increase individual fitness, and males gain substantially from choosing more fecund females in species with high variance in female fecundity like *Drosophila* (9–12). Choosiness represents a notable aspect of premating interactions, as it has the potential to affect the evolution of female attractiveness and male sensory traits (9, 13–15).

It is unknown how choosiness relates to other pre-mating behaviors and whether similar underlying mechanisms that affect choosiness are involved in aging effects of female perception (3, 4). In *Drosophila,* for example, when male flies are housed with females they experience reductions in starvation survival, fat storage, and lifespan. If females do not produce pheromones or if males are genetically altered such that they cannot perceive them, then these effects do not occur (8). Loss of the rewarding neuropeptide F (NPF), or inhibition of neurons that release it, abrogates the detrimental effects of female pheromone perception as does controlled copulation, suggesting that costs of reproduction in male flies are influenced by neural circuits governing reproductive expectation and reward (7, 8).

Several lines of evidence support the notion that molecular pathways associated with feeding and satiety may be influential modulators of choosiness. Evolutionary analyses indicate that, across taxa, male body condition is associated with choosiness: Males of higher condition who are well fed and larger tend to exhibit increased choosiness (16). Most of these studies are correlative or phenomenological, and little has been done to

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identify the underlying mechanisms. Insulin/insulin-like growth factor signaling is arguably one of the best-studied nutrient sensing pathways, and in *Drosophila,* neurohemal insulin-producing cells (IPCs), part of the *pars intercerebralis* (PI) region of the brain (17), naturally release their insulins and other peptides into fly circulation, increasingly so after a meal (18, 19). They are also known to modulate growth and social behaviors and, therefore, may provide links among nutrient sensing, body condition, and neuronal processing of sexual interactions (20). The monoamines dopamine, serotonin, and octopamine are other candidates given their known effects on feeding and metabolism (21–23), motivational behaviors and reinforcement learning (21, 24), and sensory perception of the opposite sex and of food (25–29).

We sought to investigate choosiness as a part of male pre-mating social behaviors in *Drosophila melanogaster*, with the goals of identifying molecular components of this behavior and assessing the role of these components in modulating the effect of social perception on aging. We found that the neuropeptide Drosulfakinin (DSK), which is well known for its role in promoting satiety (30, 31), produced in a subset of IPCs and likely acting through one of its two putative receptors, CCKLR-17D1, was required for male flies to exhibit choosy behavior. We found that these same *Dsk*$^+$ neurons regulated feeding behavior, while modulation of *Dsk*$^+$ neuron activity outside of IPCs did not affect choosiness or feeding, suggesting a functional link between choosiness and satiety. Activation of a broader set of *Dsk*$^+$ neurons was required to render male lifespan unaffected by female pheromones—manipulation of IPCs alone or non-IPC *Dsk*$^+$ neurons alone was not sufficient. Importantly, this effect was abolished by mutations in either of the two DSK receptors, indicating that DSK effects on aging involve broader peptide release and receptor activation than is involved in choosiness. Interestingly, we found that wider activation of *Dsk*$^+$ neurons is also sufficient for behavioral reinforcement but only following exposure to female pheromones. Activation of the broader *Dsk*$^+$ neural circuit therefore has rewarding properties that are gated by social experience suggesting that compensatory reward may mitigate the health consequences of social stress.

Results

Male Choosiness Is Potentiated by Increased Activity of IPCs Independent of Insulins. To study male choosiness, we devised an assay that would present individual male flies with a choice between two females of differential attractiveness (Fig. 1*A*). Previous studies in *D. melanogaster* have established that old females are less attractive than young females. This is due to a systematic age-related change in the composition of cuticular hydrocarbons, which becomes progressively shifted toward heavy-chain species (32). Although similar compositional shifts and changes in attractiveness have been observed with manipulations of diet and insulin signaling (14, 15, 33), the aging effect was more reproducible in our hands, with male flies consistently preferring young females over old females when given a choice, although there was substantial individual variation (32). We hypothesized that individual variability in choosiness among males might stem from variation in male motivational state, potentially linked to the animal's own perceived nutritional condition (34, 35). We also sought to evaluate the consequences of changes in neural states while avoiding developmental abnormalities that might influence non-specific mating behaviors by, for example, significantly reducing overall activity. We therefore focused our initial investigation on candidate pathways and signaling molecules known to be involved in these processes (e.g., insulin, dopamine, serotonin, and octopamine), and we evaluated the effects of acute (48 h to 72 h) manipulations of the activity of their corresponding neurons prior to the assay rather than chronic

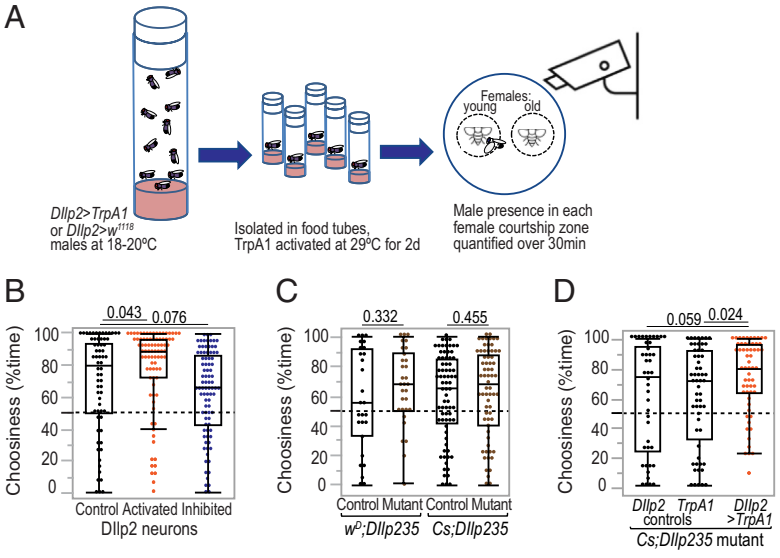

Fig. 1. Male choosiness is potentiated by increased activity of IPCs independent of insulins. (*A*) Schematic representation of experimental design for measuring male choosiness. Flies were raised in 18 to 21 °C to avoid activation of TrpA1, and 5 to 10 d after eclosion, individual males were isolated into Eppendorf food tubes and placed at 29 °C for 2 d to activate IPC neurons in *Dllp2 > TrpA1* flies. *Dllp2 > w*^{1118} control flies experienced the same treatment but lacked the *TrpA1* transgene and thus did not experience neuronal activation. Male behaviors were recorded for 30 min at 25 °C and two frames per second and subsequently analyzed for choosiness based on the fraction of time spent in the vicinity of young vs. old, immobilized females in two-choice chambers. (*B*) Modulation of *Dllp2*$^+$ neurons influences male choosiness. TrpA1-mediated activation significantly increased choosiness (orange vs. black) while inhibition via expression of *Kir2.1* showed a trend to reduce it (purple vs. black). Following normalization of the data, a one-way ANOVA revealed a significant effect of genotype ($P = 0.0003$). Displayed *P*-values reflect contrast analysis using least-squares means (LSM). (*C*) Loss of *Dllps 2, 3, and 5* does not affect male choosiness. Mutant males for which all three genes were deleted exhibited similar choosiness levels as respective control flies for two genotypes, one carrying the *white* mutation. *P*-values are based on chosen LSM contrasts analysis after two-way ANOVA with Dllp235 mutation and genetic background as main factors, neither of which nor their interaction having a significant effect. (*D*) *Dllps 2, 3, and 5* are not required for increased choosiness following activation of insulin-producing neurons. Flies in which TrpA1 was used to activate IPCs (orange) exhibited increased choosiness relative to genetic controls (black). *P*-values represent LSM contrasts between each control group and the neuron activation treatment after ANOVA on transformed data, that included Gal4-UAS genotype as a main factor ($P = 0.049$), and trial as block effect.

manipulation or manipulation only during assay itself. We used transgenic expression of the temperature-sensitive transient receptor potential cation channel, TrpA1, or the light-sensitive cation channel, CsChrimson, to increase neural activity (36). To decrease neural activity, we used targeted expression of the inwardly rectifying potassium channel $Kir^{2.1}$, combined with temperature-sensitive Gal80 for temporal control.

Of the neuron populations tested, we found that modulation of the activity of IPCs of the brain (specified by *DIlp2-Gal4*) for 2 d immediately prior to the assay significantly affected male choosiness (Fig. 1*B* and *SI Appendix*, Table S1). Male flies that expressed the TrpA1 channel and were exposed to an activating temperature of 29 °C for 2 d prior to testing exhibited a significant increase in choosiness in favor of young vs. old females compared to genotypic controls that did not express the channel (Fig. 1*B*, compare black and orange points). Conversely, when we silenced IPCs by expressing *$Kir^{2.1}$/GAL80* in an analogous manner, males exhibited a reduced preference for young females compared to control males, although this effect was only marginally significant (Fig. 1*B*, compare black vs. purple points). Importantly, the total duration of courtship directed to both females was not affected by these manipulations in the same directions as choosiness (*SI Appendix*, Fig. S1*A* and Table S2), establishing that differences in choosiness did not result from changes in overall courtship activity or mating motivation, per se. Increased choosiness upon IPC neuronal activation proved to be a phenotype that was robust with respect to genetic background (e.g., following insertion of a wild-type X-chromosome into the *DIlp2-Gal4* driver strain, see *SI Appendix*, Fig. S1*B*) and to the method of activation (e.g., optogenetic activation of IPCs with CsChrimson also increased choosiness, *SI Appendix*, Fig. S1*B*). We therefore chose to focus our subsequent investigation on mechanisms required for IPC neuronal activation to increase male choosiness, reasoning that this phenotype was less susceptible to pleiotropic effects that might result in decreased choosiness.

The neurohemal IPC cells targeted by *DIlp2-GAL4* are well known to release three out of eight *D. melanogaster* insulin-like peptides (DIlps 2, 3, and 5) into fly circulation in a pulsatile fashion, increasingly so after a meal (18, 19). They also regulate different aspects of nutrient sensing (37). We therefore asked whether loss of these three insulin-like peptides affected choosiness. Surprisingly, we found that it did not. When we measured choosiness in male flies carrying a *DIlp2,3,5* triple deletion (38), we observed that their preference for young females was not significantly different from control males (Fig. 1*C*): This was true regardless of whether we maintained the original *white* mutation in deletion and control strains or substituted in wild-type X chromosomes to ensure against potential *white* effects on mating behaviors (39). Next, using a strain in which *DIlp2,3,5* deletion was combined with the *DIlp2-GAL4* driver and *TrpA1* transgene, we observed that IPC activation was still able to significantly increase choosiness (Fig. 1*D* and *SI Appendix*, Fig. S1*C*). Taken together, these experiments with *DIlp235* mutants demonstrate that the insulin-like peptides 2, 3, and 5 do not influence choosiness in normal conditions and are not responsible for increased choosiness following activation of *$DIlp2^{+}$* neurons. Some other IPC-localized peptide must be involved.

DSK in IPCs Modulates Male Choosiness via CCKLR-17D1 Receptor. DSK is another neuropeptide produced in and released from a subset of the IPCs (30, 40). DSK is a fly homolog of the mammalian gut–brain neuropeptide hormone, cholecystokinin (CCK), with conserved functions of suppressing satiety and modulating social behaviors (40, 41). DSK also affects larval feeding and the development of neuromuscular junctions (30, 42), and for that reason, we decided to restrict loss of function spatially and temporally. We first tested whether DSK signaling modulates choosiness by using *DIlp2-Gal4-GeneSwitch* to express *Dsk-RNAi* (see also ref. 30) only in adult IPCs to knock down expression in those cells. Driving *Dsk-RNAi* with this driver in the presence of the Geneswitch ligand RU486 (mifepristone) in adult food for 72h prior to testing resulted in significantly decreased male choosiness; RU486 had no such effect in control flies (Fig. 2*A*). On the other hand, over-expression of DSK in IPCs using the same driver increased choosiness (Fig. 2*B* and *SI Appendix*, Fig. S2*A*), as did feeding synthetic DSK peptide (Fig. 2*C*). To assess whether DSK is

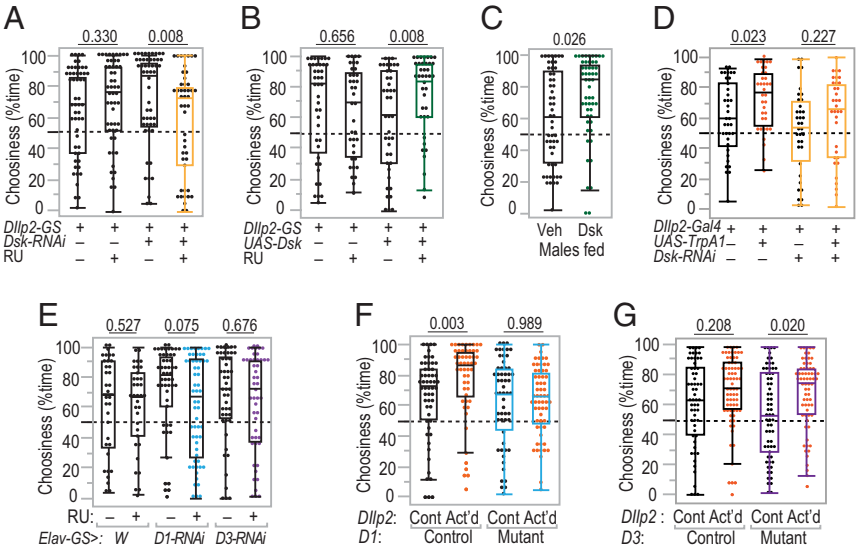

Fig. 2. DSK in IPCs modulates male choosiness via CCKLR-17D1 receptor. (*A*) Conditional knockdown of *Dsk* in *$DIlp2^{+}$* neurons decreases male choosiness. Induction of RNAi-mediated knockdown of *Dsk* expression by feeding flies RU486 significantly reduced male choosiness toward young females (compare right two boxes). RU486 feeding had no effect on the control genotype (left two boxes). Two-way ANOVA revealed a significant effect of genotype by RU interaction ($P = 0.0095$). Displayed *P*-values are least squares means (LSM) contrasts for the effect of RU feeding within each genotype. (*B*) Conditional overexpression of *Dsk* in *$DIlp2^{+}$* neurons increases male choosiness. RU486-mediated overexpression of *Dsk* in IPCs significantly increased choosiness, while RU486 had no such effect on the control genotype. Two-way ANOVA on normalized choosiness revealed a significant effect of genotype by RU interaction ($P = 0.030$). Displayed *P*-values are LSM contrasts for the effect of RU feeding within each genotype. (*C*) Feeding synthetic DSK peptide increases male choosiness. One-way ANOVA on normalized choosiness revealed a significant effect of DSK feeding. (*D*) DSK is required for IPC activation to influence choosiness. Activation of IPCs with TrpA1 significantly increased choosiness in genetic control flies but had no such effect when *Dsk* was simultaneously knocked down in the same neurons using RNAi. There was no significant difference between the *DIlp2-Gal4* only control group and either *DIlp2 > Dsk-RNAi* ($P = 0.253$) or *DIlp2 > Dsk-RNAi + TrpA1* ($P = 0.224$). *P*-values are based on LSM contrasts after ANOVA, which returned a significant effect of genotype ($P = 0.007$). (*E*) Pan-neuronal conditional knockdown of DSK receptor *CCKLR-17D1*, but not *CCKLR-17D3*, decreases choosiness. Displayed *P*-values are LSM contrasts for the effect of RU feeding within each genotype. (*F*) Loss of DSK receptor CCKLR-17D1 blocks increased choosiness upon IPC activation with *TrpA1*. Displayed *P*-values are LSM contrasts comparing *UAS-TrpA1* alone controls (no activation) to *DIlp2 > TpA1* (activation) within W vs. D1 mutant backgrounds. (*G*) Loss of DSK receptor CCKLR-17D3 does not suppress increased choosiness upon IPC neuronal activation with TrpA1. Displayed *P*-values are LSM contrasts comparing *DIlp2-Gal4* alone controls (no TrpA1 activation) to *DIlp2 > TpA1* (activation) within genetic backgrounds. See also *SI Appendix*, Fig. S2*C*.

required for increased choosiness upon IPC activation, we expressed *Dsk-RNAi* in IPC neurons while simultaneously activating them with *TrpA1*. *Dsk* knockdown suppressed the increase in male choosiness normally observed upon IPC activation (Fig. 2*D*). We concluded that increased choosiness upon IPC activation is mediated by DSK peptide produced in these neurons.

We next sought to investigate the receptors through which DSK peptide influences choosiness. DSK putatively binds two known receptors, CCKLR-17D1 (hereafter referred to as D1) and CCKLR-17D3 (hereafter D3), and we began by targeting each with inducible, adult-specific RNAi knockdown. Here, if DSK effects on choosiness are mediated predominantly by one of the receptors, we would expect to observe decreased choosiness, as was the case with inducible *Dsk-RNAi* (Fig. 2*A*). We observed such a decrease following D1, but not D3, knockdown, although this effect was marginally significant (Fig. 2*E*). To address whether either of these receptors is required for increased choosiness following IPC activation, we activated IPCs in flies that also carried mutant alleles for each receptor. To test D1 involvement, we used a short deficiency, *Df(1)Exel9051*, which removes 40 kb containing *D1* and four non-essential genes (flybase.org/reports/FBab0047359) thereby achieving a complete loss of D1 function, while preserving D3 (*SI Appendix*, Fig. S2*B*). To interrogate D3, we used a recently created and validated loss-of-function D3 mutant (43, 44). Because both *D1* and *D3* genes are located on the X chromosome, hemizygous male progeny of mutant mothers carries the receptor disruption. Taking advantage of this situation, we created male flies carrying *DIlp2-Gal4*, *UAS-TrpA1*, and a single receptor mutation. Upon measuring choosiness following IPC activation, we observed that loss of D1, but not D3, abrogated increased male choosiness (Fig. 2 *F* and *G* and *SI Appendix*, Fig. S2*C*). Incidentally, the DSK and cognate receptor manipulations had no consistent effect on overall courtship activity (*SI Appendix*, Table S2). Together, these data indicate that DSK signaling through the CCKLR-17D1 receptor is an important modulator of male choosiness in *Drosophila*, and they suggest that choosiness is genetically distinct from total courtship activity and is likely regulated independently.

Choosiness and Feeding Are Exclusively Affected by the PI Subset of DSK Neurons. While *Dsk* is expressed in roughly half of the IPCs targeted by *DIlp2-Gal4* (30, 45, 46) (but see refs. 44 and 47 that do not find such expression), it is also expressed more broadly (40, 45). To better understand which Dsk^{+} neurons influence choosiness, we obtained two available *Gal4* drivers that express in a larger portion of Dsk^{+} neurons (RRID: BDSC_51981 and _84630). Dsk^{51981}*-Gal4* was generated by combining the putative regulatory region of *Dsk* with *Gal4* (https://flybase.org/reports/FBrf0222772), while Dsk^{84630}*-Gal4* was generated by inserting a *Gal4* element at the 3′ end of the endogenous *Dsk* gene, with a linking sequence replacing the stop codon of the gene (43). In our hands, Dsk^{51981}*-Gal4* drives expression in the median protocerebrum MP1 and MP3 Dsk^{+} neurons as well as in IPCs, while Dsk^{84630}*-Gal4* does not express in IPCs but does express in MP1/MP3 neurons (Fig. 3*A*). When *TrpA1* was expressed in Dsk^{51981} neurons and males were exposed to activating temperature of 29 °C for 2 d, they exhibited increased choosiness toward young females compared to genetic controls exposed to the

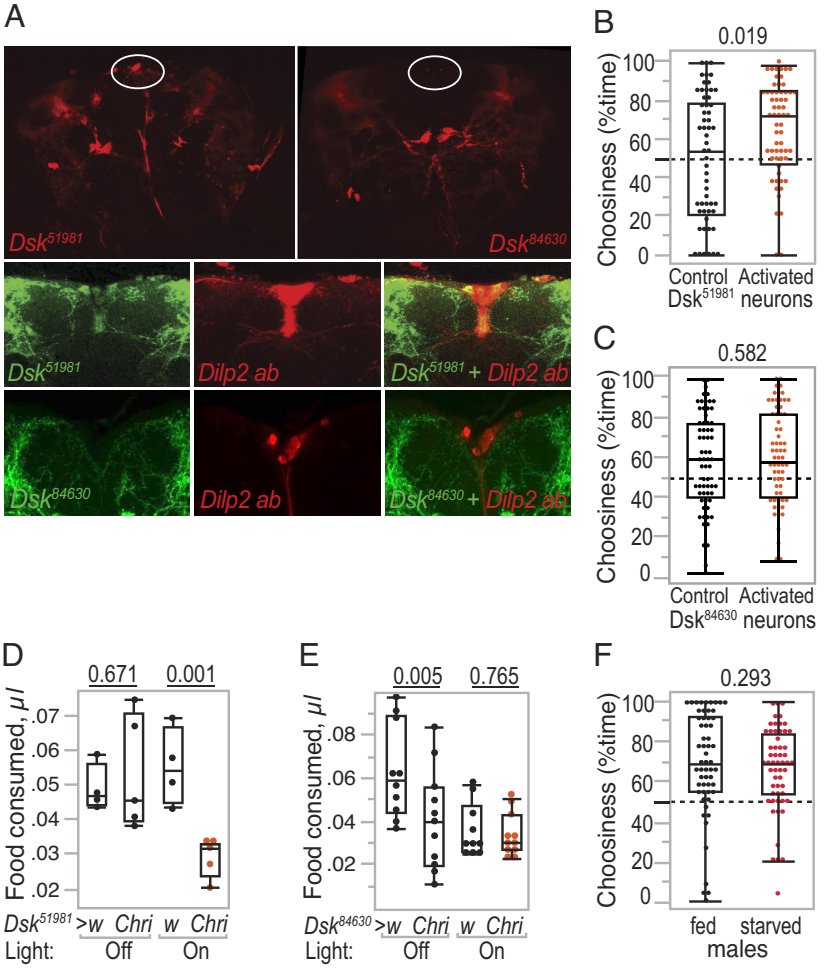

Fig. 3. Choosiness and feeding are exclusively affected by the PI subset of DSK neurons. (*A*) The two *Dsk-Gal4* drivers show different expression patterns with respect to IPCs. *Gal4* expression is visualized by crossing to *UAS-cd4tdTomato*. No fluorescence is detected in IPCs when using Dsk^{84630}*-Gal4* (white ellipse in the top right panel), while it is consistently detected in IPCs in Dsk^{51981}*-Gal4* (*Top Left*). Co-localization of *GFP* and antibody staining for DIlp2 indicates that Dsk^{51981}*-Gal4* is expressed in IPCs (*Middle*), while Dsk^{84630}*-Gal4* is not (*Lower*). (*B*) Activation of Dsk^{51981} neurons increases choosiness. Following 2 d at an activating temperature of 29 °C flies in which Dsk^{51981} neurons are activated (Dsk^{51981} > *TrpA1)* exhibit significantly increased choosiness relative to control males (Dsk^{51981} > w^{1118}). The *P*-value is from one-way ANOVA on normalized choosiness. (*C*) Activation of Dsk^{84630} neurons using *TrpA1* does not increase choosiness. Choosiness is measured after 2-d exposure to activating temperature of 29 °C for both no-activation control (Dsk^{84630} > w^{1118}) and for Dsk^{84630} > *TrpA1* males. The *P*-value is from one-way ANOVA on normalized choosiness. (*D* and *E*) Activation of Dsk^{51981} neurons suppresses feeding, while activation of Dsk^{84630} neurons does not. Control (*Dsk* > *wCs*) males are compared to activation genotypes (*Dsk* > *CsChrimson*) after exposure to dark or activating red light during 24-h blue food assay. Two-way ANOVA revealed a significant genotype by activation interaction effect for Dsk^{51981} > *CsChrimson* ($P = 0.007$, panel *D*), but not for Dsk^{84630} > *CsChrimson* males ($P = 0.068$, panel *E*). Displayed *P*-values are least squares means contrasts for the effect of genotype within each activation regimen (lights off and light on). (*F*) Starvation is not sufficient to affect choosiness. The choosiness of males starved for 18 to 24 h is statistically indistinguishable from fed controls. The *P*-value reflects one-way ANOVA on normalized choosiness.

same temperature but lacking *TrpA1* (Fig. 3*B*). These results were not surprising given our previous data establishing *DIlp2*$^{+}$/IPC activation as sufficient for increased choosiness, although they do implicate the subset of IPCs that express *Dsk*. However, when we activated *Dsk*^{84630} neurons by expressing either *TrpA1* (Fig. 3*C*) or *CsChrimson* (*SI Appendix*, Fig. S3*A*), it did not affect choosiness, establishing that *Dsk*$^{+}$ neuron activation outside the IPCs is not sufficient to influence this behavior.

Our findings of the influence of male *Dsk*$^{+}$/IPC neurons on choosiness of females echoed published results, which demonstrated that knockdown of *Dsk* in these neurons decreased both satiety and food choosiness (30). It seemed plausible that satiety and choosiness for either mates or food might share, at least in part, similar mechanisms. We therefore asked whether the same subset of *Dsk*$^{+}$/IPC neurons that exclusively affects male choosiness for females is also exclusive in influencing male feeding/satiety. Using an assay that quantifies the amount of food consumed by measuring excretion of a blue dye tracer, the consumption–excretion assay (48), we found that optogenetic activation of *Dsk*^{51981} neurons, but not *Dsk*^{84630} neurons, reduced male food consumption by roughly half compared to control flies in activating red light (Fig. 3 *D* and *E*). We also observed a similar, but less prominent, effect of *DIlp2*$^{+}$/IPC neuronal activation on feeding (*SI Appendix*, Fig. S3*A*). It was formally possible that total food intake in the days prior to testing, or possibly the extent to which flies are hungry/sated during this time, was a causal influence for choosiness. If so, one might predict that a sustained period of reduced food intake alone, in the absence of neuronal manipulation, would be sufficient to affect choosiness. To test this prediction, we starved males on 2% agar for 18 to 24 h before behavioral testing and found that this manipulation, while decreasing total courtship activity (*SI Appendix*, Table S2), had no effect on choosiness (Fig. 3*F*). This finding suggests that the effect of *Dsk*^{51981} neuron activation on choosiness works through mechanisms that are, at least in part, independent of feeding itself.

Broad *Dsk*$^{+}$ Neuronal Activation Rescues Male Lifespan Shortened by Female Pheromone Exposure. Our observation that increased DSK signaling from IPCs enhanced male choosiness suggested that it may also influence the effects of social interactions on aging. Recent studies have shown that sensory perception of females in the environment, and the consequent pre-mating social interactions are particularly costly for males with respect to their health and lifespan, but the mechanisms of these costs are largely unknown (3–6). To determine whether *Dsk*$^{+}$ neurons are involved in the ability of sexual perception to modulate lifespan, we employed a previously developed paradigm that compares experimental males that are exposed to donor males who are engineered to develop normal or feminized pheromone-producing cells. The latter donor males express female pheromone profiles (7). They are perceived as females and actively courted, but unable to mate with experimental males. Exposure to female pheromones in this manner robustly accelerates male aging compared to sibling experimental males that are exposed only to male pheromone from control donor flies (7). Using this paradigm, we observed that activation of *Dsk*^{51981} neurons protects experimental males from the deleterious effects of female pheromone exposure by extending lifespan to the extent observed for males exposed to only male pheromone (Fig. 4*A*). Neither activation of *Dsk*^{84630} neurons nor of *DIlp2*$^{+}$ neurons alone had this effect (Fig. 4 *B* and *C*). Notably, combined activation of both *Dsk*^{84630} and *DIlp2*$^{+}$ neuronal subsets

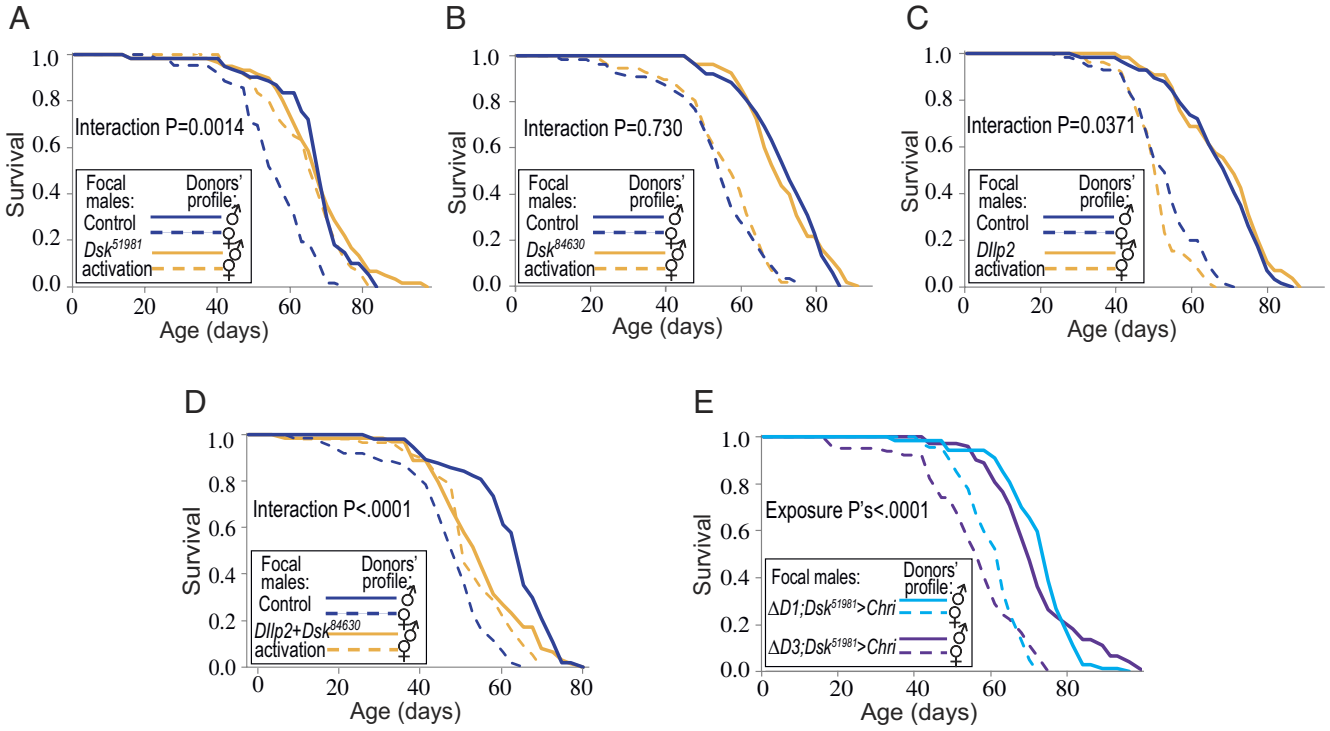

Fig. 4. Broad *Dsk*$^{+}$ neuronal activation rescues male lifespan shortened by female pheromone exposure. (*A*) Optogenetic activation of *Dsk*^{51981} neurons (*Dsk*^{51981} > *CsChrimson*) ameliorated the effect of female pheromone exposure on male lifespan. Control males (*Dsk*^{51981} > *wCs,* shown in purple) responded normally (see also ref. 7). (*B*) Activation of *DIlp2*$^{+}$ neurons (*DIlp2* > *CsChrimson*) has no effect on the ability of female pheromone exposure to modulate male lifespan. (*C*) Activation of *Dsk*^{84630} neurons (*Dsk*^{84630} > *CsChrimson*) exerts no effect on the ability of female pheromone exposure to modulate male lifespan. (*D*) Activation of both *Dsk*^{84630} and *DIllp2* neurons (*DIlp2*>, *Dsk*^{84630} > *CsChrimson*), however, abrogates the effect of female pheromone exposure on male lifespan, resembling the effect of activation of *Dsk*^{51981} neurons (see panel *A*). (*E*) Loss of either DSK receptor (indicated as *ΔD1* or *ΔD3*) is sufficient to reverse the protective effect conferred by activation of *Dsk*^{5198} on lifespan in the presence of female pheromone (as seen in *A*). All lifespan experiments were conducted by exposing males to 2 min of activating red light out of every 10 min during the 12 h of each normal day–night cycle, with no red-light exposure during the 12 h of night.

abolished the effect of feminized male exposure on lifespan (Fig. 4*D*), resembling the effect of Dsk^{51981} neuronal activation, indicating that both *Dsk*$^{+}$ subsets are involved in the response to the exposure. Furthermore, activation of *Dsk*^{51981} neurons combined with either of the single DSK receptor mutants failed to protect males from the lifespan-shortening effect caused by female pheromone exposure, establishing that signaling through both receptors is required (Fig. 4*E*).

Broad *Dsk*$^{+}$ Neuronal Activation Is Rewarding to Males Exposed to Female Pheromones. Copulation is also known to protect males from the effects of female pheromone exposure on lifespan (8). It has been postulated that it may serve as a rewarding influence that opposes the consequences of increased motivation driven by pheromone perception (49). To determine whether *Dsk*$^{+}$ neuron activation might be similarly rewarding, we used the Fly Liquid Interaction Counter, FLIC (50), coupled with optogenetic stimulation. For this paradigm, we coupled red light stimulation precisely to coincide with the moment when individual flies interact with a normally aversive solution in a closed-loop configuration. The solution contains water and denatonium, which is bitter and non-nutritive. It is generally aversive to wild-type flies. If neuronal activation is rewarding, then paired activation is expected to reinforce interactions with liquid, stimulating flies to overcome the aversive bitter input and to exhibit an increase in interactions. Genetically identical flies that are exposed to matching patterns of activating red light that are decoupled from interaction with the liquid serve as controls for the effects of general neuronal activation. Using this paradigm, we found that males exposed for 72hr to feminized donors exhibit significantly increased interactions with denatonium water upon paired, closed-loop activation of *Dsk*^{51981} neurons compared to decoupled control flies (Fig. 5*A*). The rewarding effect of activation of these neurons was gated by female pheromone exposure because there was no difference in denatonium interactions between paired- and unpaired-activation in males pre-exposed only to male pheromone (Fig. 5*A*, blue points). Activation of *Dsk*^{84630} or *Dilp2* neurons in a similar closed-loop paradigm generally decreased interactions with liquid compared to uncoupled controls indicating more localized activation is aversive regardless of pheromone exposure (Fig. 5 *B* and *C*).

Discussion

Although mate choice has been examined in the context of sexual selection theory as an evolutionary driver for traits such as elaborate bird plumage, intricate flower shapes, and complex sexual behaviors (51), much less attention has been directed to understanding the phenotype of choosiness itself and, specifically, to the mechanisms underlying what makes individuals more or less choosy toward the opposite sex. Many association studies provide hints. In vertebrates, for example, female choosiness has been linked to the peptides arginine, vasopressin, and oxytocin, which are involved in pair bonding, and to dopamine activity, which is correlated with feelings of romantic attraction in humans (52, 53). In nature, animals in better condition, including those that are better fed or larger, tend to exhibit increased choosiness, which has drawn attention to conserved nutrient sensing and metabolic pathways as modulators of sexual behaviors (14, 16, 54). We found that the neuropeptide DSK in *Drosophila* males potentiates choosiness toward more attractive females. Furthermore, a subset of *Dsk*$^{+}$ neurons, specifically those co-expressing insulins in the PI, are responsible for this effect, which is also mediated by one of the two putative DSK receptors, CCKLR-17D1. DSK is the homologue of the vertebrate CCK, which is well documented as an influential modulator of feeding behaviors, although its roles in anxiety and aggression in several settings have also been noted (28, 55, 56).

As an important neuroendocrine center in flies, the PI orchestrates a complex response to nutrient consumption that includes the release of DSK peptide as a feedback satiety signal (19, 30, 40). Perhaps prolonged neuronal activation specifies an extended state of satiety, which signals good condition and promotes increased choosiness. In support of this idea, we found that DSK influences both choosiness and feeding through its release from subsets of neurons overlapping in the PI region. Activation of these neurons, targeted by either the *Dilp2* or *Dsk*^{51981} drivers, increased choosiness and suppressed feeding. On the other hand, *DSK*+ neurons outside of the PI, targeted by the *Dsk*^{84630} driver, did not affect either choosiness or feeding. The former manipulations promoted choosiness despite reduced food consumption, and modest starvation had no effect on choosiness (see also ref. 57). Choosiness is, therefore,

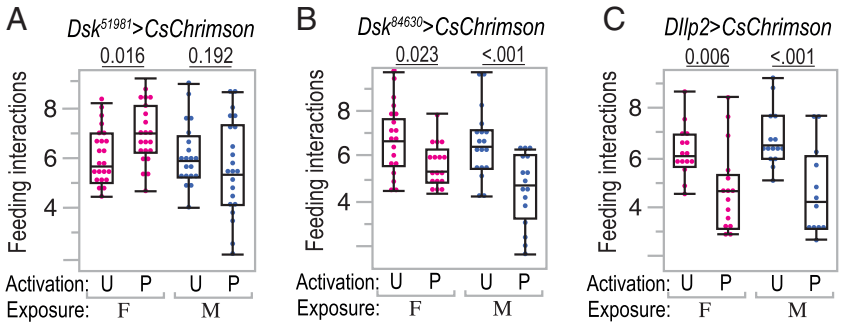

Fig. 5. Broad *Dsk*$^{+}$ neuronal activation is rewarding to males exposed to female pheromones. (*A*) Activation of *Dsk*^{51981} neurons (*Dsk*^{51981} > *CsChrimson)* reinforces male feeding behavior following exposure to female pheromones. Males pre-exposed for 3 d to feminized males (F) exhibit increased interactions with an aversive denatonium solution when such interactions are paired in a closed-loop paradigm with an optogenetic activation of *Dsk*^{51981} neurons (indicated as "P") compared to flies who received the identical light treatment that was not paired with feeding activity ("U"). No such effect was observed when males were pre-exposed only to male pheromones. Interactions with liquid are expressed as the number of "feeding interaction" to the degree of 0.25 (to normalize distribution) measured using the FLIC (*Materials and Methods*). Two-way ANOVA revealed a significant effect of pheromone exposure-by-light interaction ($P = 0.0098$). Displayed *P*-values are least squares means contrasts for coupled vs. uncoupled light regimens within each donor exposure treatment. (*B*) Activation of *Dsk*^{84630} neurons (*Dsk*^{84630} > *CsChrimson)* is aversive. Males pre-exposed for 3 d to either feminized males or control males significantly decreased interactions with a denatonium solution when these interactions were paired in a closed-loop paradigm with optogenetic activation of *Dsk*^{84630} neurons ("P") compared to flies who received the identical light pattern that was not paired with feeding activity ("U"). Two-way ANOVA revealed a significant effect of light pairing ($P < 0.001$) but a non-significant pairing-by-pheromone exposure interaction ($P = 0.101$). Displayed *P*-values are least squares means contrasts for paired vs. unpaired light regimens within each donor exposure treatment. (*C*) Activation of *Dilp2* neurons (*Dilp2* > *CsChrimson)* is aversive. Males pre-exposed for 3 d to either feminized males or control males significantly decreased interactions with a denatonium solution when these interactions were paired in a closed-loop paradigm with optogenetic activation of *Dilp2* neurons ("P") compared to flies who received the identical light pattern that was not paired with feeding activity ("U"). Two-way ANOVA revealed a significant effect of effect of pairing ($P < 0.001$) but a non-significant pairing-by-pheromone exposure interaction ($P = 0.422$). Displayed *P*-values are least squares means contrasts for paired vs. unpaired light regimens within each donor exposure treatment.

apparently distinct from nutritional state, per se, and perceived satiety might directly influence an individual's assessment of their condition, perhaps by simulating a neural state normally brought about by high levels of nutrition. Since PI neurons release neuropeptides into the hemolymph to induce systemic effects (37), a post-prandial ligand such as DSK might influence neurons that control choosiness along with its traditional satiety-inducing targets. This idea is consistent with the observation that DSK receptors are expressed widely in the brain, including mushroom bodies and neurons that control courtship and aggression (43, 44, 46).

The perception of conspecifics affects health and aging in *Drosophila* as well as in species as divergent as nematode worms and mice (3, 4, 58). Certain mating behaviors, including copulation itself, can reduce or eliminate these effects, and in *Drosophila*, conserved neuropeptides involved in mating decisions, such as NPF (the homolog of vertebrate neuropeptide Y) and corazonin (the homolog of vertebrate adipokinetic/gonadotropin-releasing hormones) are important mediators of the perceptive response (8). We found that broad *Dsk*$^+$ neuron activation significantly extended male lifespan in the presence of female pheromones, effectively eliminating the effect of pheromone exposure on lifespan. Notably, activation of neither the satiety-related PI *Dsk*$^+$neuronal subset nor the non-PI *Dsk*$^+$ subset alone abrogated effects on lifespan, and both DSK receptors were also required. Furthermore, activation of the same broad set of *Dsk*$^+$ neurons was required to reinforce normally aversive feeding interactions, but only after exposure to female pheromones, indicating that perception of the opposite sex gates their rewarding properties. Activation of the non-PI *Dsk*$^+$neuronal subset has been shown to influence male social interactions, including acute suppression of courtship, increased aggression, and perceived social dominance (40, 44, 46, 56). We speculate that broader *Dsk*$^+$ neuron influences a perception of satiety and social status, which may be rewarding under otherwise stressful conditions. Perhaps male flies exposed to female pheromones in the absence of mating benefit from this alternative reward, as was suggested when rejected males increased ethanol consumption or upregulated rewarding NPF signaling (59).

CCK, the vertebrate homologue of DSK, was originally described as a gastrointestinal peptide hormone (60). It was later found to be abundant in the nervous system and subsequently studied for its role in behavior, such as panic and anxiety, mating, addiction, and reward (61–63). Our study adds male mate choosiness as another motivational behavior modulated by this neuropeptide, and it introduces its function in reversing the consequences of social interactions on aging. Given our results, it is perhaps not surprising that mammalian studies have also revealed a complexity in CCK action. For example, CCK injections induce anxiety and panic attacks (64), yet have also have been shown to exert a protective effect in response to emotional and physical stress (65). From this perspective, exposure of male flies to female pheromones without mating might be viewed as a psychological stress, which manifests in decreased fat storage, decreased survival under starvation, and ultimately, decreased lifespan. Further investigation of how DSK signaling in distinct regions of the fly brain modulates behavior and health-related phenotypes could inform studies of CCK therapeutic applications.

Materials and Methods

Fly Stocks and Husbandry. The following fly stocks were obtained from Bloomington Drosophila Stock Center: *Canton-S*, two *Dsk-GAL4* lines (51981, 84630), *DIlp2-GAL4* (37516), *CCKLR-17D1* null deficiency line *Df*(1)*Exel9051* (7762), *CCKLR-17D3* mutant (84463), *CCKLR-17D3*^{RNAi} (28333), *UAS-CsChrimson* (55135), *TubP-GAL80ts* (7017), *OK72-GAL4* (6486), UAS-CD4-tdTomato (35837), and UAS-mcd8-GFP (32194), while *UAS-Dsk*^{RNAi} (14201) and *CCKLR-17D1*^{RNAi} (100760) were purchased from the VDRC stock center. The following stocks were kindly provided by the following individuals: *UAS-TrpA1* by P. Garrity, *UAS-Kir2.1* by R. Baines, *UAS-tra* by B. J. Dickson, *Elav-G.S.-GAL4* by Osterwalder (66), *DIlp2,3,5* mutant and its *W-Dahomey* control by S. Grönke (38), *DIlp2-G.S.-GAL4* (41) by H. Jasper, and *UAS-Dsk* by B. Ganetsky. For the purposes of the study, some of these transgenes were combined in the same fly stock following standard *Drosophila* genetics methods. All reported transgenic genotypes were tested as heterozygotes unless stated otherwise. Control lines for transgenic stocks were made by back-crossing transgenic flies into their control lines for at least 10 generations, thereby homogenizing potential genetic background differences. For all experiments, larvae were cultured in cornmeal-sugar-yeast "larval" media, and virgin adults were collected shortly after eclosion, and kept on 10% sugar/yeast (SY) food. Flies were maintained at 25 °C and 60% relative humidity in a 12:12-h light:dark cycle. Fresh food was provided every 2 or 3 d. Detailed media recipes can be found in Poon et al. (67).

Transgene Activation Procedures and Synthetic DSK Peptide Feeding. For time-controlled neuronal activation at adult stage, we have used 3 methods: 1) *GAL4* drivers with gene switch (*G.S.*) construct, which is activated by feeding flies RU486 (mifepristone)-spiked food; 2) temperature-sensitive *UAS* constructs *UAS-TrpA1 and UAS-Kir2.1,GAL80ts*; and 3) *UAS-CsChrimson* which is activated by red light. *GAL4-G.S.* transgenic crosses were placed on 10% SY food with RU486 (200 μM) to activate transgene expression (treatment) or with 80% ethanol vehicle (control) for 2 to 3 d before experiments. *UAS-TrpA1* and *UAS-Kir2.1,GAL80ts* crosses were kept at 18 to 20 °C during development and early adulthood and moved to activating temperature of 29 °C for ~2 d before experiment. *UAS-CsChrimson* crosses were kept in the dark throughout development and were activated by controlled red light-emitting diode (LED) light inside specially designed enclosure devices. Notably, control flies were exposed to the same temperature or light regimen but contained a single copy or either Gal4 or UAS, so that their neurons could not be activated. This was done to control for the known effect of temperature and light on behaviors. For synthetic DSK feeding, we used a sulfated and amidated octopeptide FDDY(SO3)GHMRF-NH2 (NovoPro Bioscience Inc). The lyophilized peptide was freshly diluted in 10% sucrose water to make 20 μM DSK solution for immediate use. Experimental Canton-S males were placed in 0.7 μL microfuge tubes with a drop of DSK sucrose solution or sucrose solution alone (control) for 2 d before behavioral trials.

Measuring Male Choosiness. We designed our male choosiness assay to quantify behavioral choice between one young and one old female, which were standardized by genotype and rearing conditions. To rear standardized density-controlled females, *Canton-S* eggs laid over a 1-d period were washed off a grapejuice agar egg-laying medium with PBS and a standard volume of egg suspension (about 250 eggs in 32 μl) was pipetted into cornmeal media bottles. Virgin Canton-S females were collected from such controlled larval density bottles upon eclosion and kept in SY food vials until 3 to 4 d of age (=young) or between 35 to 50 d of age (=old). The age spread for each category was experimentally determined to give consistent results in choice trials. Young and old female ages were kept consistent within the trials. Variability among replicate experiments was accounted for in all analyses by including replicate as a random factor.

Females were decapitated and immobilized 10 to 20 min before trials, and thus were alive but unable to respond to male courtship. To prevent females from moving around, their legs were submerged into solidifying 2% agar on the opposite sides and 7 to 10 mm off the edge of each 30-mm well in a custom-made 6-well plate with a slide-in transparent lid. The assays were conducted in the middle of the day at 25 °C. Males were aspirated into courtship chambers without anesthesia and their behaviors were videorecorded at 2 frames per second for 30 min, during which time they were generally active. Videos were then analyzed with our custom DTrack software (14, 15, 32). The software tracks individual males and calculates the amount of time spent by the male inside a circle of 3-mm radius centered on each female. We found previously that the time spent within such a circle was highly correlated with courtship toward the female inside (15), and we therefore defined choosiness as the fraction of time a male spent in the young female's circle relative to the total time spent in either circle.

Brain Imaging and Immunostaining. Fly brain dissections were performed in cold 1× PBS (Gibco, Life Technologies). Unfixed brains were cleaned off surrounding fat and trachea, mounted on a glass slide fitted with tape cutout window and

a drop of Vectashield® mounting medium, and sealed with nail polish around the edge. Brains were imaged immediately with an inverted Nikon A1 confocal microscope (University of Michigan BRCF Microscopy Core), using 1.5-µm distance between Z-stack slices. Fiji software (68) was used to obtain a final maximum projection image. Brains undergoing DIlp2 immunostaining were first fixed in 7.4 pH PBS containing 3.7% formaldehyde for approximately 1 h, and then washed with three quick and three 20-min washes in PBS-T (phosphate-buffered saline with 0.1% Triton X-100). After the last wash, the PBS-T solution was removed, and a block solution containing 5% normal goat serum (Cayman Chemical) was added for 30 min. Rabbit anti-DIlp2 antibody (gift of J. Veenstra) was then added at a 1:500 dilution in PBS-T and incubated at 4 °C for 3 d. On day four, the brains were washed in PBS-T four times for 15 min each at room temperature, after which the secondary antibody, Alexa Fluor 633 (Life Technologies), was added for 24 h at room temperature. The next day, the secondary antibody was removed with three quick washes followed by three 20-min washes in PBS-T. After the last wash, the brains were mounted and imaged as described above.

Consumption-Excretion Blue Food Assay. This assay to measure food consumption is described in more detail in ref. 48. Briefly, normal SY10 fly food was melted in a microwave, spiked with 10 g/L of FD&C Blue #1 dye, and poured in a 5-mL cap tightly fitting into the opening of a standard fly vial. Ten male flies were placed in an empty vial and capped with a blue food cap. The flies were kept in the vial for 24 h, allowing collection of their excretions on the walls, and the vial was replaced with a new one if continuous sample collection was required. The flies were placed in an SY10 normal (if using TrpA1 for activation) or SY10 All-Trans-Retinol (ATR, Cayman Chemical) food (if using *CsChrimson* for activation) in between blue food consumption trials if a periodic excretion collection was desired. A known amount of DI water was added to collection tubes and swirled on the shaker to wash the blue off the walls. A specified amount of the wash was then placed in a 96-well plate, and absorbance measured at 630 nm on the BioTek Synergy 2 microplate reader. The amount of blue dye was calculated using a standard curve and transformed into amount of food consumed per fly.

Lifespan Experiments. Equal volumes of eggs from mated females were cultured on a CT diet until adulthood. Newly emerged flies were collected within a 48-h window and placed in SY10 food bottles for 1 to 3 d to allow for mating. Experimental male flies were then placed in vials in groups of 5 on SY10 food spiked with ATR at a concentration of 0.14 g/L. Twelve replicate vials were prepared for each genotype-treatment combination. Twenty "donor" males were added to each vial. Donor males were made by crossing *UAS-transformer* (*UAS-tra*) virgin females to either *OK72-Gal4* (that drives expression in pheromone-producing cells) males or their genetic control W^{1118} (see ref. 7 for details). Vials containing 5 experimental and 20 donor males were placed into a specially made box with board-controlled red LED lights. It was experimentally determined that the best lifespan rescue for Dsk^{51981} neuron activation is achieved by turning LEDs on for 2min every 10 min over 12 "day" hours and kept turned off for 12 h of "night". The light-on regimen we used was light flickering at a frequency of 40 Hz with a 32% duty cycle. Old donor males were substituted for young ones at about 1 mo of age of experimental flies to preserve donor health/ratio through experimental male lifespan. Dead experimental males were censored three times per week.

Measuring Reward in FLIC. To measure reward, we gave flies the ability to activate their own neurons using optogenetics reagents and a modified FLIC device developed in the lab (48). The FLIC measures the number and duration of contacts with liquid when a fly standing on a metal plate completes an electrical circuit by touching liquid with its proboscis or legs. To enable optogenetic activation, such an FLIC device was fitted with a red LED bearing lid that turns the LED on when the fly touches the liquid. The expectation is that if a chosen neuronal activation is rewarding, then the fly will be trying to touch the liquid more often and/or for a longer duration. Since the goal was to measure reward, and not feeding per se, a non-nutritive and bitter 100 µM Denatonium solution in water was used in the assay. A single male, previously kept on ATR food in the dark in groups of five with 20 donors, was placed in a 2-cm round chamber with a 5-mm liquid well in the middle, and its interactions with Denatonium solution were recorded over 12-h period starting at noon. Half of the flies were subjected to the contact-induced light activation regimen (adaptive). For the other half, which represented the control regimen, the red light turned on independently from touching the liquid, whenever the control fly's neighbor male from the adaptive treatment touched the liquid. Therefore, control males received the same amount of optogenetic stimulation as males in the adaptive light regimen, but for them, there was no link between touching the liquid and neuronal activation. This allowed us to control for neuronal activation affecting drinking, mobility, and other phenotypes unrelated to reward.

Statistics. The effects of neuronal and other genetic manipulations on the percentage of time that males courted the younger of the two females was analyzed in JMP® (Version *Pro 16* SAS Institute Inc., Cary, NC, 1989 to 2021) with 2-way ANOVA after transforming data to conform to a normal distribution. Prior to analysis, however, data were screened to remove non-courting/sluggish/potentially damaged males: Thus, males whose total courtship time was less than 200 frames (=100 s) in each 3,600 frames (=30 min) trial were censored before analyses. Since each of our main datasets consists of at least 3 independent replications, we used replication as an independent variable, in addition to the main effect of genotype/manipulation. After running ANOVA, least squares means' contrasts were used to estimate *P*-values for comparisons of interest. The same method was used to analyze food consumption and feeding interaction data. Group and pairwise comparisons among survivorship curves were performed using the DLife software that was developed in our lab (69) and the statistical software R. Log-rank and Cox regression tests were used for pairwise comparisons, as noted. Cox regression was used for interaction studies after verification of proportionality assumptions.

Data, Materials, and Software Availability. All presented data are available from ZENODO public repository. DOI: 10.5281/zenodo.10052093 (70).

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