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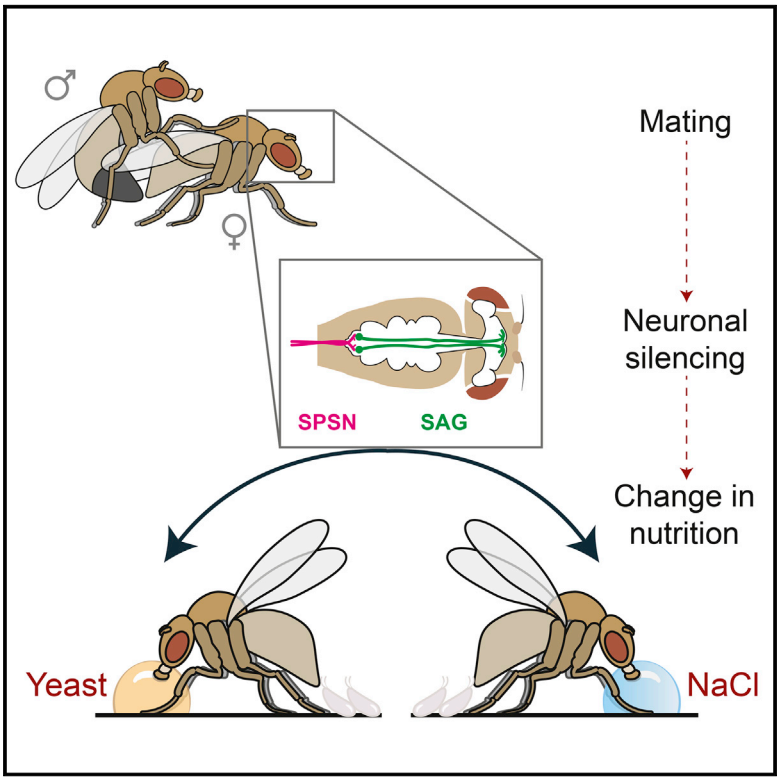
Current Biology

Postmating Circuitry Modulates Salt Taste Processing to Increase Reproductive Output in *Drosophila*

Graphical Abstract



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In Brief

How animals match nutrient intake to their needs is unclear. Walker et al. show that mating induces a salt appetite in *Drosophila*, resembling that seen during reproduction in many species. This appetite is induced by a feedforward change in taste processing, driven by a male-derived signal acting on female postmating circuitry.

Highlights

- Dietary salt supports egg production
- Mating induces a salt appetite by increasing the gustatory response to sodium
- Salt appetite is independent of egg production rate
- Sex Peptide Receptor circuitry drives postmating appetites for both salt and yeast

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Postmating Circuitry Modulates Salt Taste Processing to Increase Reproductive Output in *Drosophila*

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SUMMARY

To optimize survival and reproduction, animals must match their nutrient intake to their current needs. Reproduction profoundly changes nutritional requirements, with many species showing an appetite for sodium during reproductive periods. How this internal state modifies neuronal information processing to ensure homeostasis is not understood. Here, we show that dietary sodium levels positively affect reproductive output in *Drosophila melanogaster*; to satisfy this requirement, females develop a strong, specific appetite for sodium following mating. We show that mating modulates gustatory processing to increase the probability of initiating feeding on salt. This postmating effect is not due to salt depletion by egg production, since abolishing egg production leaves the sodium appetite intact. Rather, the salt appetite is induced need-independently by male-derived Sex Peptide acting on the Sex Peptide Receptor in female reproductive tract neurons. We further demonstrate that postmating appetites for both salt and yeast are driven by the resultant silencing of downstream SAG neurons. Surprisingly, unlike the postmating yeast appetite, the salt appetite does not require octopamine, suggesting a divergence in the postmating circuitry. These findings demonstrate that the postmating circuit supports reproduction by increasing the palatability of specific nutrients. Such a feedforward regulation of sensory processing may represent a common mechanism through which reproductive state-sensitive circuits modify complex behaviors across species.

INTRODUCTION

To maintain homeostasis, animals must select suitable nutrients and consume them in appropriate quantities. Moreover, while an environmental resource could be useful to an animal in one physiological state, under different conditions that same resource could be detrimental to its fitness [1]. Thus, animals must adapt their behavioral responses to sensory cues depending on their internal state. This can be achieved by modulating the attractiveness of an external cue depending on its usefulness in the current internal state, a process termed ''alliesthesia'' [2]. Despite an increasing body of knowledge on neuronal circuit mechanisms underlying homeostatic motivated behaviors [3, 4], we lack a comprehensive understanding of the mechanistic basis of alliesthesia. To achieve such an understanding, it would be necessary to characterize the neurons that detect an external stimulus, to identify how the internal state is sensed, and to elucidate how these signals are integrated to produce state-appropriate behaviors.

Intake of specific nutrients can be adapted to the needs in the current state through two alternative mechanisms: need-dependent, where an internal deficit of a particular nutrient induces an appetite for that nutrient through negative feedback to restore homeostasis; and need-independent, where, in contrast, an internal state signal itself induces a feedforward appetite for a nutrient that is usually necessary in that state, independently of the internal levels of that nutrient [5]. In spite of a wealth of study on the feedback mechanisms underlying homeostatic behaviors, the relative contribution and mechanisms of feedforward modulation to nutrient homeostasis remains unclear.

During reproduction, animals' nutritional requirements drastically change, with females generally investing large quantities of resources into producing progeny. To meet this nutritional demand, many species show specific behavioral adaptations to increase their intake of particular nutrients during reproductive episodes. In *Drosophila melanogaster*, for example, mating increases females' preference for protein-rich yeast, ensuring adequate protein for egg development [6, 7]. Another nutrient that has particularly profound effects on reproductive success is sodium [8–11]. In order to ensure sufficient sodium is acquired to maximize reproductive output, many species, including humans, show a specific appetite for sodium during the reproductive period [12–16]. However, the neuronal mechanisms through which animals' reproductive states drive this salt appetite remain obscure.

Upon mating, *Drosophila* females dramatically change their behavior [17, 18]. The long-term postmating behavioral switch can be attributed largely to the action of the male-derived Sex Peptide (SP), transferred to the female during copulation, on the neuronal Sex Peptide Receptor (SPR) [19–22]. At the circuit level, recent years have seen the emergence of a ''canonical'' postmating neuronal pathway mediating the postmating switch, pinpointing the action of SP to a small population of Sex Peptide Sensory Neurons (SPSNs) in the reproductive tract [23–25]. SP binding silences the activity of SPSNs, and thereby silences

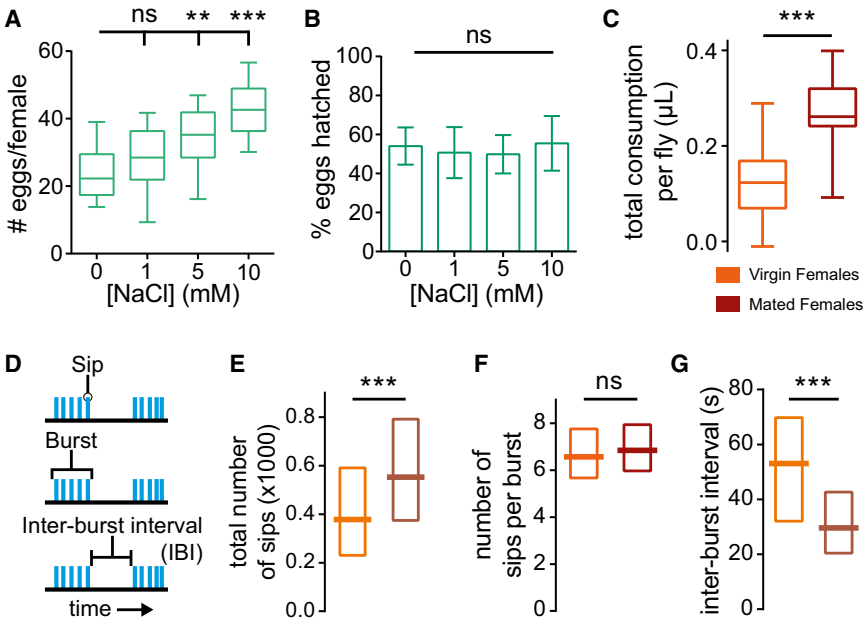

Figure 1. Dietary Salt Stimulates Egg Laying, and Mating Drives a Salt Appetite

(A) Number of eggs laid per female after feeding on standard medium with varying concentrations of added NaCl (n = 14). (B) Percentage of eggs hatched 48 hr after removal of females fed on standard medium with varying concentrations of added NaCl (n = 14). See also Figure S1A.

(C) Consumption of 100 mM NaCl per fly in 1 hr for virgin and mated females, as measured in the CAFE assay (n = 14–15). Groups compared by unpaired two-tailed t test.

(D) The microstructure of feeding in *Drosophila*. Single contacts of the proboscis with the food ("sips") are grouped into feeding bursts separated by inter-burst intervals.

(E) Total number of sips, (F) number of sips per feeding burst, and (G) mean inter-burst interval of single virgin and mated females feeding from 100 mM NaCl on the flyPAD (n = 72–80).

(A and B) Groups compared by one-way ANOVA, followed by post hoc multiple comparisons tests comparing each group to the 0 mM control group, with Bonferroni correction. (E–G) Groups compared by Wilcoxon rank-sum test. In (A) and (C), boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. In (B), bar represents mean, and error bars represent SD. In (E)–(G), boxes represent upper and lower quartiles with median.

Not significant (ns) = $p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the downstream SP abdominal ganglion (SAG) neurons, which project into the central brain to bring about increased egg laying and decreased receptivity to remating [26]. Furthermore, octopamine is a key modulator of postmating behavioral adaptations, presumably acting through a small set of octopaminergic neurons located in the abdominal ganglion [27]. Therefore this "canonical" postmating neuronal circuit, consisting of the SPSN/SAG/octopamine components, stipulates that SP acts on one small set of neurons that then convey this information to different sensorimotor systems. This view stands in contrast to early work on SP, which proposed that postmating changes could be elicited by SP acting directly on sensory neurons that detect cues such as nutrients and male pheromones, modulating the sensory responses of these neurons and thus the behavioral responses to sensory cues [17]. How the postmating circuit alters female behaviors and if these behavioral changes are mediated by modulation of sensory processing still remains to be elucidated, particularly in the context of postmating changes in nutritional homeostasis.

In this study, we show that, as in many animals, salt intake positively affects reproductive output in *Drosophila melanogaster*, and, in order to acquire this resource, flies develop a specific sodium appetite following mating. This appetite can be attributed to an increased probability of initiating feeding on salt, which is driven by increased attractiveness of salt taste. Further, we show that the salt appetite is induced independently of salt requirements for egg laying. Instead, the postmating salt appetite is driven need-independently by Sex Peptide (SP) transferred in male seminal fluid. SP acts through the canonical postmating circuitry to enhance the behavioral response to attractive salt taste. Likewise, the yeast appetite following mating is driven through the canonical postmating circuitry. Surprisingly, unlike other postmating behaviors including yeast appetite, the postmating salt appetite does not require octopamine. Thus, a central internal state-sensitive neuronal pathway drives alliesthesia, enhancing the attractiveness of salt taste in order to increase consumption of salt, which is useful for egg production. These results highlight reproduction as critical modulator of taste processing and bring new insight into the mechanistic basis of this state-dependent nutritional modulation.

RESULTS

NaCl Enhances Reproductive Output

Salt intake has been shown to impact reproductive success in several species [8, 9, 11, 15, 28]. To assess whether this was also the case in *Drosophila melanogaster*, we supplemented the diet of adult females with varying concentrations of NaCl for 3 days, before measuring their egg laying capacity. This treatment resulted in a dose-dependent increase in eggs laid within the range of concentrations tested (Figure 1A). This could be due either to an effect of sodium itself supporting egg production or to the phagostimulatory power of sodium increasing total food intake, which, in turn, increases egg production. Importantly, the supplement had no effect on egg viability (Figure 1B), so that ultimately females with higher dietary sodium produced more offspring (Figure S1A).

Mating Drives Increased Salt Intake

In *Drosophila*, mating leads to a change in the behavioral repertoire of females aimed at increasing offspring production [17].

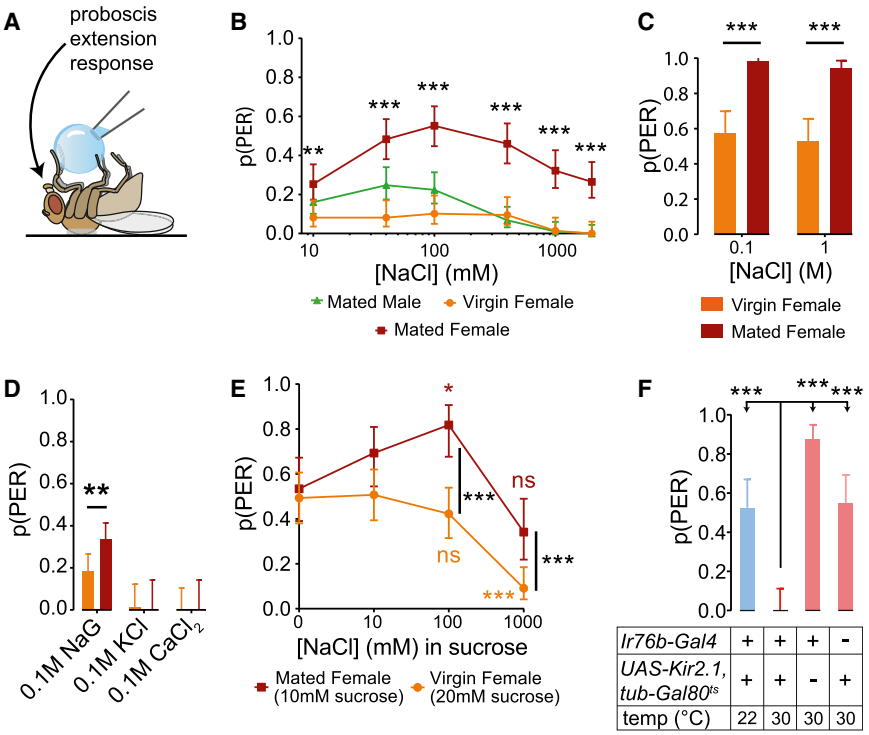

Figure 2. Mating Modulates Gustatory Response to Sodium

(A) Proboscis extension response (PER) assay reports probability of behavioral response upon gustatory stimulation.

(B and C) Probability of responding with proboscis extension (p(PER)) following stimulation of the tarsi (B) or labellum (C) with varying concentrations of NaCl for virgin and mated females (n = 53–87) and mated males (n = 103). Response of mated females is greater than that of mated males at all concentrations except 10 mM.

(D) p(PER) following stimulation of the tarsi with NaG (n = 114–163), KCl (n = 29–41), and $CaCl_2$ (n = 29–41). NaG is sodium gluconate, $NaC_6H_{11}O_7$.

(E) p(PER) following stimulation of the tarsi with NaCl at varying concentrations mixed with sucrose at the indicated concentration (n = 44–71). Sucrose concentrations were chosen to give similar p(PER) when presented alone, based on sucrose dose-response curves for virgin and mated females (data not shown). Flies were starved for 24 hr to elicit sucrose responses.

(F) p(PER) of mated females of the indicated genotypes in response to 100 mM NaCl presented to the tarsi. Flies were kept for 24 hr at the indicated temperature before assays, which were performed at room temperature (n = 19–40).

Error bars show 95% confidence interval (CI). In (B), asterisks represent comparison between virgin and mated females. All comparisons were performed using 2 × 2 Fisher's exact test. In (E), colors indicate comparisons to corresponding 0 mM NaCl response, and black asterisks represent comparison between virgin and mated responses.
ns = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

While virgin *Drosophila* lay few eggs, mating causes a rapid increase in egg production and laying [29, 30]. Since dietary salt supports increased egg production, we hypothesized that mating could drive an increased appetite for this nutrient. Accordingly, we compared the NaCl intake of virgin and mated females using the CAFE assay [31]. Indeed, mated females consumed more salt (Figure 1C). To identify the behavioral strategies underlying the increase in salt intake, we turned to the fly-PAD technology, which allows us to identify individual "sips" as well as the organization of these sips into feeding bursts by measuring capacitance changes upon contact of the fly with food ([32]; Figure 1D). As expected from the CAFE assay, mated females had a higher number of sips from NaCl compared to virgins (Figure 1E). Flies could increase their salt intake by increasing the probability of initiating a feeding burst or by increasing the duration of these bursts or both. However, analysis of the feeding microstructure showed that mating does not affect the number of sips in each feeding burst, but decreases the interval between these bursts (Figures 1F and 1G). This strongly suggests that mated females increase their salt intake by increasing the probability of initiating feeding.

Mating Increases the Gustatory Response to Sodium

The probability of initiating feeding is thought to be strongly dependent on the detection of food by the gustatory system [33–35]. Thus, increased salt intake could be achieved by modulating the initial response to the gustatory salt stimulus, opening the intriguing possibility that mating directly alters taste processing. To directly address whether mating changes the gustatory response to salt, we turned to the proboscis extension response (PER) assay, which assesses the behavioral response to gustatory stimulation alone, in the absence of consumption ([33, 36]; Figure 2A). We measured the PER to stimulation of tarsal gustatory receptor neurons (GRNs) by salt and found that mating consistently increased the probability of proboscis extension in response to NaCl (Figure 2B). This increased gustatory response was observed both when tarsal and labellar GRNs were stimulated, and across all concentrations tested (Figures 2B and 2C). The strong salt response was clearly sexually dimorphic, since mated male flies showed a weaker response to NaCl (Figure 2B); and it was independent of hunger state, since starvation had no effect on salt responses (Figure S1B). Furthermore, this postmating appetite was specific to sodium: a similar increase was seen for sodium gluconate, whereas flies failed to respond to other Cl^- salts (Figure 2D). Interestingly, behavioral responses to sodium gluconate are lower than those to NaCl, suggesting that, as in mammals, the anion may contribute to salt taste, with larger anions decreasing salt palatability [37]. Thus, mating leads to an increased gustatory response to sodium. The resultant increased probability of initiating feeding from salt sources is likely to underlie the observed increase in salt intake, and thus to support the high rate of egg production following mating.

Just as in mammals, sodium is detected by *Drosophila* using the gustatory system, with low concentrations activating gustatory receptor neurons (GRNs) that drive attraction, and high concentrations recruiting an additional aversive pathway [38]. To assess the contribution of these two components, we used a mixture of sucrose and salt. We chose a sucrose concentration

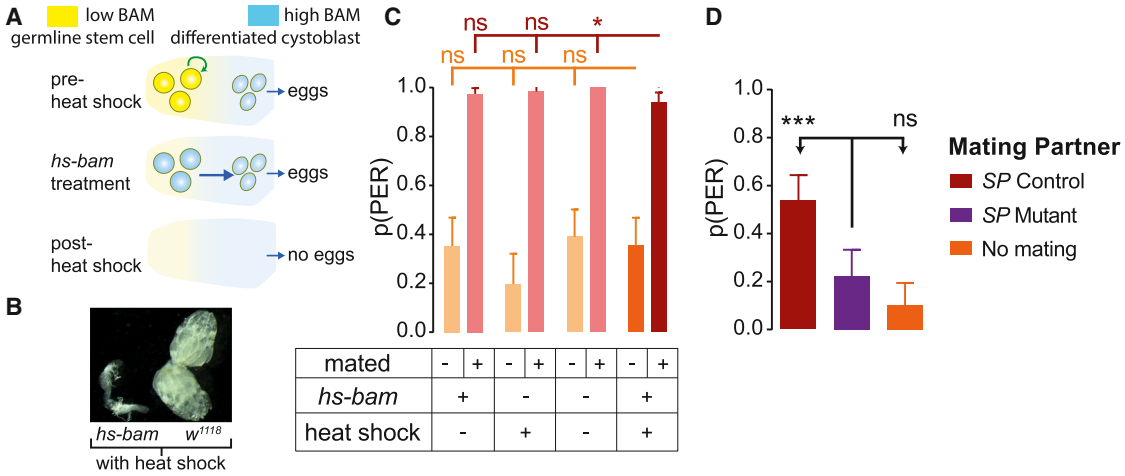

Figure 3. Postmating Salt Appetite Is Driven Need-Independently by Sex Peptide

(A) Schematic diagram of germline manipulation. Upon heat-shock treatment, *bam* overexpression induces differentiation of germline stem cells, leading to a loss of egg production.

(B) Ovaries dissected from *hs-bam* (left) and w^{1118} (right) females following heat shock.

(C) p(PER) of females of the indicated genotypes and conditions in response to 100 mM NaCl presented to the tarsi (n = 56–79).

(D) p(PER) of wild-type virgin females and females mated to males with or without *Sex Peptide*, in response to 100 mM NaCl presented to the tarsi (n = 73–99).

(C and D) Error bars show 95% CI. Significance tested using 2 × 2 Fisher's exact test.

ns = $p > 0.05$, *$p < 0.05$, ***$p < 0.01$

that elicited an intermediate probability of proboscis extension. In mated females, addition of 100 mM salt increased the probability of proboscis extension while a higher concentration inhibited the response compared to sucrose alone (Figure 2E). Interestingly, we found that the attractive response to low salt was largely absent in virgin females, while the aversive effect at higher concentrations (>100 mM) was still clearly visible (Figure 2E). Furthermore, mating also shifts the peak p(PER) to a higher concentration of NaCl (Figure 2E). These results suggest that mating increases the palatability of attractive concentrations of salt while leaving the aversive effect of high concentrations intact. The attractive response to salt taste has been shown to depend on the Ir76b receptor [38]. Indeed, we found that adult-specific silencing of Ir76b-expressing neurons abolished the behavioral response of mated females to salt, indicating that these neurons form the basis of the attractive salt response that is modulated by mating (Figure 2F).

Postmating Salt Appetite Is Driven in a Need-Independent Manner by Sex Peptide

Animals can regulate their nutrient intake using either need-dependent or need-independent mechanisms [5]. Since mating increases the rate of egg production, and salt intake positively affects egg production, we hypothesized that mating could deplete sodium levels, leading to a homeostatic salt appetite to restore these levels in a need-dependent manner. To test this hypothesis, we genetically abolished egg production by driving the differentiation factor Bam under the control of a heat-shock promoter, and exposing flies to heat-shock treatment during larval development, causing premature differentiation of all germline stem cells and hence a loss of egg production ([39]; Figure 3A). Examination of the ovaries from these flies revealed a loss of all stages of egg chambers (Figure 3B). If the postmating salt appetite is driven by egg production, this treatment should abolish the salt appetite. In fact, we found that blocking egg production had no effect on this appetite, with germ-less females increasing their response to salt after mating in the same way as all control lines (Figure 3C). Therefore, egg production does not itself influence salt-taste responses.

If the postmating salt appetite is not caused by feedback from salt depletion, it could be driven in a feedforward, need-independent manner. Mating drastically changes the behavior of female flies, with many of these behavioral effects induced by Sex Peptide (SP), a male-derived peptide transferred to the female during copulation in the seminal fluid [19–21]. Indeed, it has been shown that SP drives the postmating switch in female nutrient preference toward protein-rich yeast, independently of its effect on egg production [6]. We therefore speculated that SP could drive the postmating salt appetite. To test this, we mated wild-type females to males that lack the *Sex Peptide* gene and found that these males failed to induce the postmating salt appetite induced by mating to control males (Figure 3D). Sex Peptide transferred from the male during copulation therefore induces salt appetite in females upon mating.

Sex Peptide Receptor Inhibits the Postmating Circuitry to Drive Postmating Appetites

Sex Peptide drives a host of behavioral changes following mating. It has been proposed that these changes could be elicited by Sex Peptide acting directly on chemosensory neurons and thus modifying the behavioral responses to sensory cues [17]. On the other hand, SP increases egg laying and decreases receptivity to remating solely through its action on a small population of Sex Peptide Sensory Neurons (SPSNs) in the reproductive tract [23–25]. Thus, SP could drive a salt appetite either by acting on salt-detecting GRNs, or through the canonical

postmating circuitry, to change the behavioral response to salt detection. Since we can isolate the sensory channel responsible for salt detection, we can disambiguate these two possibilities.

To test whether SP induces appetites toward specific nutrients by acting on sensory neurons or through the canonical postmating circuitry, we knocked down the expression of *SPR* in these two neuron types and measured the effect on postmating appetites. We employed RNAi to knock down *SPR* expression in salt-detecting GRNs using *Ir76b-Gal4*, and in SPSNs using *VT3280-Gal4* (Figure 4C). To control for effects of genetic background on salt responses, we calculated the difference between the probability of proboscis extension response of mated females and virgins, Δp(PER), and compared this value between lines. Using this approach, we found that SPR knockdown using *Ir76b-Gal4* had no effect on the magnitude of the postmating change in labellar salt responses (Figure 4D). In contrast, SPR knockdown in SPSNs, using *VT3280-Gal4*, reduced the magnitude of this postmating effect on salt, and this change in magnitude is due specifically to a decrease in the response of mated females (Figures 4D and S2A). These results suggest that mating influences salt intake through the same molecular and neuronal pathway that modulates egg laying and receptivity. Further, SPR is also required for the postmating increase in yeast feeding [6]. SPR is known to be required in neurons expressing *ppk-Gal4* [23, 24], but whether these are chemosensory neurons or SPSNs is unknown. Thus, we used *VT3280-Gal4* to knock down *SPR* specifically in SPSNs and measured the effect of mating on feeding from yeast and sucrose sources (Figures 4B and 4E). We found that this manipulation decreased the yeast feeding of mated, but not virgin, flies deprived of protein for 3 days, whereas it had no effect on sucrose feeding, suggesting that SPR preferentially increases feeding on resources that are important for reproductive output, namely salt and yeast (Figures 4E and S2B). We thus conclude that Sex Peptide acts on its receptor, SPR, in Sex Peptide sensory neurons of the reproductive tract to increase females' feeding on sodium and yeast following mating.

SP binding is thought to result in silencing of SPSNs [23, 24, 26]. To investigate whether this silencing also causes the postmating salt and yeast appetites, we artificially silenced SPSN activity using Kir2.1 (Figure 4F). Indeed, virgins in which SPSNs were silenced showed increased salt responses compared to control virgins when stimulated on the labellum, whereas the response of mated females was unaffected (Figure 4G). Likewise, the yeast feeding behavior of virgins in which SPSNs were silenced was increased to a level close to that of mated females, whereas sucrose intake was unaffected compared to controls (Figures 4H and S2C). Thus, silencing of SPSN activity is sufficient to induce the postmating salt and yeast appetites, mimicking the effect of mating.

To elucidate the circuitry downstream of SPSNs that alters food preference following mating, we silenced the activity of SAG neurons, the neurons postsynaptic to SPSNs that bring this signal into the central brain to modulate receptivity and egg laying, by expressing Kir2.1 under the control of *VT50405-Gal4* (Figure 4I). We found that silencing the activity of SAG neurons caused virgin females to develop a salt appetite, fully recapitulating the effect of mating on salt responses (Figure 4J). Similarly, silencing SAG neurons caused virgin females to increase their yeast feeding as if they were mated, without affecting the intake of mated females (Figure 4K). This effect was nutrient specific, since the same manipulation had no effect on virgins' sucrose feeding (Figure S2D). To ensure that the observed effects were due to silencing of SAG neurons, we further restricted Kir2.1 expression to these neurons using split-Gal4 combinations [26]. These more precise manipulations recapitulated all observed effects (Figures S2E and S2F). These results demonstrate that SP transferred from the male acts on SPR in SPSNs, and the resultant silencing of downstream SAG neurons drives an increased appetite for both salt and yeast, independently of the fly's salt and protein requirements for egg production.

In order to elucidate the behavioral strategies and neuronal mechanisms underlying the mating-induced increase in yeast intake of protein-deprived flies, we looked into the microstructure of yeast feeding. In contrast to its effect on salt feeding, we found that in yeast-deprived females mating resulted in both a decrease in the inter-burst interval and an increase in the number of sips per feeding burst for all control lines feeding on yeast, including *Canton-S* (Figure S3). Manipulating SPSNs or SAG neurons had a clear effect on the inter-burst interval, mirroring the effect on the total number of sips (Figures S3A, S3C, S3E, and S3G). The influence of these manipulations on the number of sips per burst, however, was less clear: while the normal postmating increase in number of sips per burst was lost in the manipulated group, comparisons of each condition with the control genotype did not yield a clear effect (Figures S3B, S3D, S3F, and S3H). Thus, the postmating yeast appetite in protein-deprived females is induced by both a change in the probability of feeding initiation, as seen for salt, and an increase in yeast sips per feeding burst. While the SPR pathway has a strong effect on the inter-burst interval of yeast feeding, it may also contribute to the change in burst length.

Octopamine Is Required for Postmating Yeast but Not Salt Appetite

Octopamine has been proposed to be an important mediator of postmating responses. Mutation of the gene encoding the octopamine biosynthetic enzyme TβH decreases the magnitude of the postmating change in receptivity [27]. The action of octopamine on postmating behaviors has been proposed to be mediated by a small population of octopaminergic dsx^{+} $Tdc2^{+}$ neurons in the female abdominal ganglion. Thus, we tested the effect of abolishing octopamine production on the magnitude of the postmating change in salt responses, Δp(PER) (Figure 4L). In contrast to its effect on receptivity, we found that a deficit of octopamine had no effect on the magnitude of the postmating increase in salt responses (Figure 4M). Interestingly, we also found that flies lacking octopamine had a generally reduced level of response to salt, suggesting that while octopamine is dispensable for the postmating salt appetite, it may be involved in the regulation of salt-taste processing by other physiological factors such as hemolymph osmolarity (Figure S2G). In contrast, flies lacking *TβH* failed to increase their yeast feeding following mating (Figure 4N). This effect on yeast feeding was due to an insensitivity to mating status and not to an insensitivity to protein deprivation, since the mutation did not decrease the intake of male flies deprived of protein for 10 days (Figure S2H). Similarly

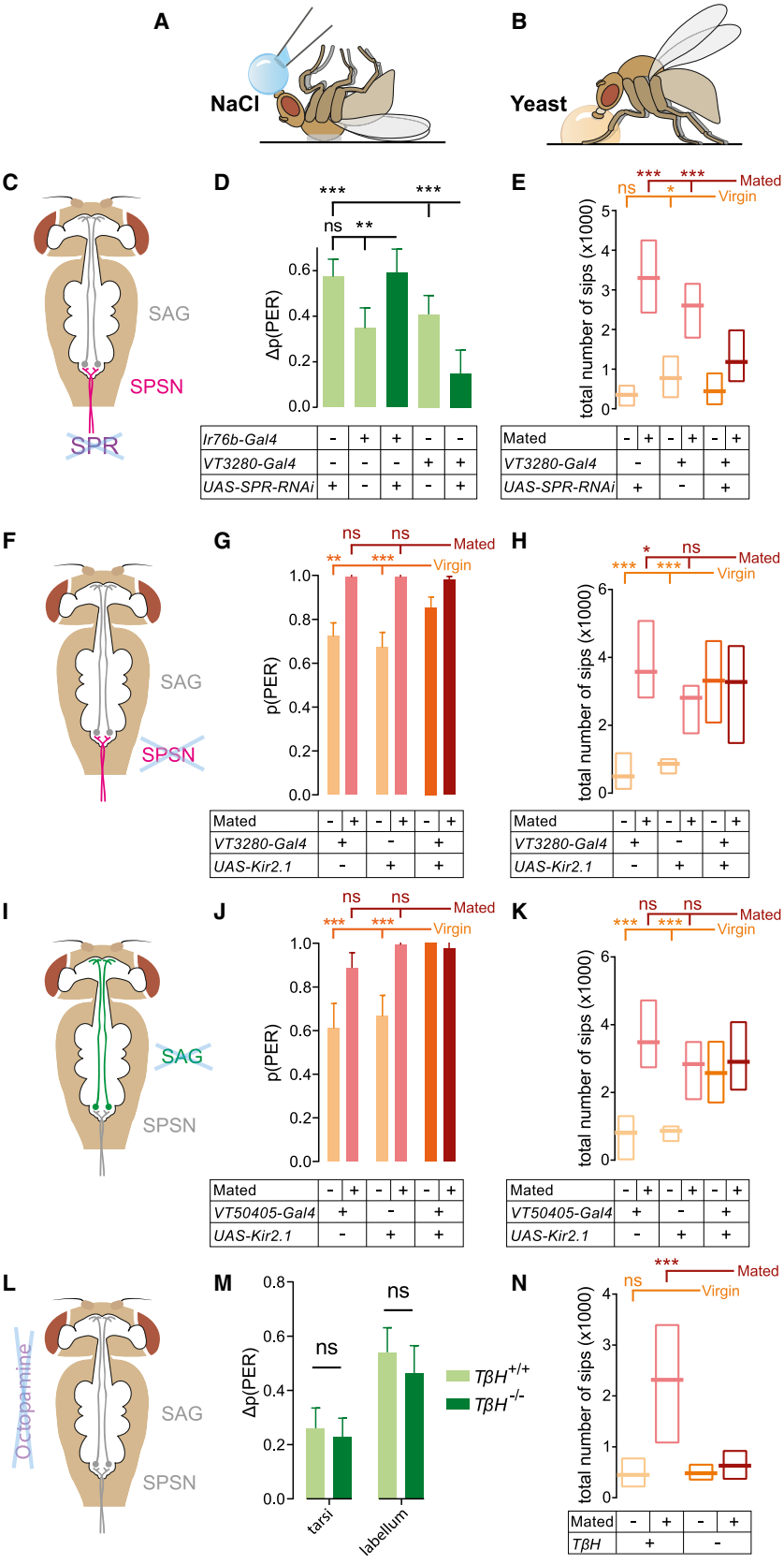

Figure 4. SPR Silences Postmating Circuitry to Drive Salt and Yeast Appetites

(A and B) Diagrams of assays used. (A) PER to NaCl; (B) feeding from yeast on flyPAD during sucrose-yeast choice experiment.

(C, F, I, and L) Diagram representing manipulations of postmating molecules and circuitry.

(D) Δp(PER), the difference between mated and virgin p(PER), for females with or without *SPR* knockdown in salt GRNs (*Ir76b-Gal4*) or SPSNs (*VT3280-Gal4*) (n = 79–158).

(E) Number of sips from 10% yeast in 1 hr by females with or without *SPR* knockdown in SPSNs (*VT3280-Gal4*) (n = 29–35).

(G and H) Effect of silencing SPSNs (*VT3280-Gal4*) in virgin and mated females on p(PER) to 100 mM NaCl presented to the labellum (G; n = 134–177) and on number of sips from 10% yeast on the flyPAD (H; n = 40–49).

(J and K) Effect of silencing SAG neurons (*VT50405-Gal4*) in virgin and mated females on p(PER) to 100 mM NaCl presented to the labellum (J; n = 39–78) and on number of sips from 10% yeast on the flyPAD (K; n = 32–49).

(M and N) Effect of *TβH* mutation on Δp(PER) in response to 100 mM NaCl presented to the tarsi or labellum (M; n = 174–215) and on number of sips from 10% yeast by virgin and mated females on the flyPAD (N; n = 52–57).

(D, G, J, and M) Error bars, 95% CI.

(D and M) Groups compared by weighted least-squares statistic.

(E, H, K, and N) Boxes represent median with upper/lower quartiles. Groups compared by Wilcoxon rank-sum test.

(G and J) Groups compared by 2 × 2 Fisher's exact test.

ns = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

to the effect of manipulating the postmating circuitry, the lack of octopamine strongly affects the interval between yeast feeding bursts specifically in mated females, whereas the effect on the number of sips per burst is less clear (Figures S3I and S3J). Therefore, octopamine is required to induce the postmating yeast appetite as well as other postmating behavioral changes, but not the postmating salt appetite. This suggests a possible divergence point in the CNS of the postmating signals modulating distinct postmating behaviors, including appetites for different nutrients.

DISCUSSION

Animals' nutritional requirements vary over their life cycle, and this necessitates specific behavioral mechanisms to adapt their food choices to their current internal state. Here, we show that similarly to the previously characterized switch in feeding preference toward high-protein yeast, *Drosophila* also develop a specific appetite for sodium following mating. This appetite is adaptive for the female since, like protein, salt is important for reproductive success: we demonstrate here that dietary sodium levels positively impact the rate of offspring production. Salt could increase reproductive output in two ways: it could support egg production by providing ions required for the osmotic balance within the newly created eggs, or the phagostimulatory power of sodium could result in increased total food intake and hence an increase in egg production. Irrespective of the exact mechanisms, our results show that dietary sodium clearly affects the rate of offspring production. The postmating salt appetite is due primarily to an increase in the probability of initiating feeding from salt, which can be attributed to an increased gustatory attraction to sodium. Mating not only elevates the gustatory response to all concentrations of salt, but also results in a shift in the peak response toward higher concentrations. This shift would allow mated females to regulate their salt consumption to a different intake target from virgins, without requiring nutrient-specific feedback to operate within the fly [40]. Indeed, neither the postmating salt nor yeast appetites are driven by feedback from depletion of internal nutrient stores by egg production [6]. While we cannot exclude the possibility that physiological processes induced by mating, other than egg production, could consume salt or protein, our data indicate that a feedforward signal in the male seminal fluid, Sex Peptide, directly drives salt and yeast appetites. Sex Peptide binds to SPR in SPSNs, whose silencing results in silencing of SAG neurons [22–24, 26]. This leads to appetites for both salt and yeast, in addition to the previously described changes in receptivity and egg laying [26]. These results suggest that the intake of reproductive nutritional resources is controlled by a common regulatory logic, whereby the signal of mating is detected by local uterine neurons and changes nutrition in a feedforward, anticipatory manner. It will be interesting to explore to what extent feedforward regulation is employed to control specific behavioral strategies used to acquire nutrients depending on different internal state signals.

Our data are consistent with the current view that the signal of mating status is brought into the central brain through a common pathway, the SPSN-SAG axis, to regulate the full set of postmating responses including egg laying, remating, and nutrition. Given the diverse set of behaviors regulated by mating, one would expect the circuit to diverge downstream. However, the point of divergence is currently unknown. Octopamine is known to be required for ovulation [41] and is required for the full reduction in receptivity that normally follows mating [27]. In agreement with these results, we found that octopamine is also required for the postmating increase in yeast intake in protein-deprived females, while it is dispensable for sensing internal amino acid deficiency. However, while octopamine does influence the overall level of salt responses, our results show that it is not necessary for the postmating change in salt response. These data suggest that octopamine may represent such a divergence point in the postmating circuit, with the previously characterized dsx^{+} $Tdc2^{+}$ neurons being likely neuronal candidates mediating this divergence. It has, however, been proposed that octopamine may act genetically upstream of SP [26, 42]; this could be compatible with our results if the salt appetite is relatively insensitive to small changes in SP levels. Regardless, this result hints at distinct circuitry controlling the different behavioral changes elicited by mating, which could aid in the future elucidation of how a specific internal state signal could coordinate changes in a wide range of different behaviors.

Salt has been shown to be one of the most limiting nutritional resources in many ecosystems [28, 43–45]. Our results provide insights into the physiological regulation of salt intake, which until now has remained unexplored in *Drosophila*. The postmating sodium appetite we demonstrate here is intriguing in the light of the specific sodium appetite seen during pregnancy and lactation in various mammalian herbivores, and even humans [12–16]. As in *Drosophila*, these species show an increased gustatory attraction to salt following mating [16, 46]. While the mechanism used to detect mating in these species is different, the feedforward, need-independent nature of the salt appetite is likely to be similar. In rats, this appetite is induced within a few days after mating [12] and is present even if the animal has access to sufficient salt in its diet [47]; furthermore, a salt appetite can be induced in rabbits by administration of a mixture of reproductive hormones in the absence of mating [48]. Thus, the detection of mating by the nervous system and the subsequent feedforward increase in response to salt taste is likely to be a common feature of many non-carnivorous species, making alliesthesia a likely universal mechanism driving reproductive salt appetites. While much is known about the regulation of salt intake in mammals [49, 50], the mechanisms through which reproduction affects salt appetite remain poorly understood in any species. Functional genetic circuit analysis combined with activity imaging in *Drosophila* offer the unique opportunity to understand the circuit mechanisms through which this internal state signal can modulate taste processing in the brain, and thus bring about an adaptive change in food preference [51]. To achieve this, three possibilities exist. Mating could modulate the response of sensory neurons to salt taste, as demonstrated in the olfactory pheromone system of moths [52]. In a similar way, GRN responses are modulated by starvation [53–55], and the sensitivity of pheromone-sensitive olfactory receptor neurons in mice is modulated across the estrus cycle [56]. Alternatively, mating could alter higher-order taste processing. Finally, mating state could lead to a combination of modulation at the receptor neuron level and modification of higher-order processing. Identifying

how alliesthesia is implemented at the circuit level will represent a unique opportunity to understand how internal state changes affect sensory processing to mediate adaptive behaviors.

EXPERIMENTAL PROCEDURES

Fly Stocks

Flies were reared at 25°C, 70% relative humidity on a 12-hr-light-dark cycle. All experimental and control cohorts were matched for age and husbandry conditions. The fly medium contained, per liter, 80 g cane molasses, 22 g sugar beet syrup, 8 g agar, 80 g corn flour, 10 g soya flour, 18 g yeast extract, 8 ml propionic acid, and 12 ml nipagin (15% in ethanol). *Canton-S* was used as the wild-type strain in this study. *UAS-Kir2.1* (II) was obtained from the Bloomington *Drosophila* Stock Center (#6596). *VT3280* and *VT50405* were obtained from the Vienna *Drosophila* Resource Center (#200327 and #200652, respectively). Other stocks used in this study included *Ir76b-Gal4* [57]; *tub-Gal80^{ts}*; *SP* mutant *SP^{0}/Δ130* and *SP* control *SP^{+}/Δ130* [20]; *UAS-SPR-IR2* [22]; *TβH^{nM18}* [41]; *hsp70-bam* [39]; and *SAG-1* and *SAG-2* split-Gal4 combinations [26]. For detailed genotypes and sources, see Table S1. For experiments with *UAS-SPR-RNAi*, all genotypes contained a first chromosomal *UAS-Dcr2* transgene.

Egg Laying and Viability Assays

Canton-S flies were collected into vials of 16 females and six males on the day of eclosion and aged for 6 days. At this point, they were transferred to a standard food medium with an added 0, 1, 5, or 10 mM NaCl. After 3 days on this supplemented food, flies were transferred to apple juice agar plates (per liter 250 ml apple juice, 19.5 g agar, 20 g sugar, 10 ml nipagin [15% in ethanol]), where they were allowed to lay eggs for 24 hr. At this point, flies were removed and counted, and eggs were counted. Egg laying was calculated by dividing the number of eggs by the number of females on each plate. The plates were then kept at 25°C for a further 48 hr, and the number of remaining unhatched eggs were counted. Egg viability was calculated as (total number of eggs – number of unhatched eggs) / total number of eggs.

CAFE Assays

Female flies of the indicated genotypes were collected upon eclosion into vials of 20 virgins. After 3–7 days of aging, they were sorted into vials of either 20 virgins or 10 virgins + 10 *Canton-S* males and aged for a further 3 days. To construct the CAFE setup, three holes were bored into the lid of a *Drosophila* bottle, into which three 5-μl capillaries were inserted, each containing 100 mM NaCl. The bottle also contained a tissue soaked with 50 mM sucrose to ensure satiation with sugar and hydration. 16 females were inserted into each bottle by mouth aspiration, at which time the level of each capillary was marked. After 1 hr at 25°C, 70% relative humidity, the level of each capillary was marked again and the distance between these marks converted into a volume consumed per fly. In addition, three bottles were set up in the same way but without flies, and the mean volume change from these capillaries subtracted from the capillaries with flies, to control for the effect of evaporation.

flyPAD Assays

Flies of the indicated genotypes were collected upon eclosion into vials of 20 virgins. After 3–7 days of aging, they were sorted into vials of either 20 virgins or 10 virgins + 10 *Canton-S* males. For salt assays, flies were aged for a further 3 days before testing on the flyPAD with a single source of 100 mM NaCl in 1% agarose. For sucrose-yeast choice assays, flies were aged for 1 day and then transferred to vials containing a tissue soaked with 100 mM sucrose for 3 (females) or 10 (males) days of protein deprivation and then tested on the flyPAD with two food sources: 10% yeast and 20 mM sucrose, each in 1% agarose. Flies were individually transferred to flyPAD arenas by mouth aspiration and allowed to feed for 1 hr at 25°C, 70% relative humidity. flyPAD data were acquired using the Bonsai framework [58] and analyzed in MATLAB (MathWorks) using custom-written software, as described [32].

PER Assays

Flies of the indicated genotypes were collected upon eclosion into vials of 20 virgins. After 3–7 days of aging, they were sorted into vials of either 20 virgins or 10 virgins + 10 *Canton-S* males and aged for a further 3 days. For experiments involving starvation, flies were kept on a tissue soaked with water for the final 24 hr before mounting. For inducible expression of Kir2.1, flies were kept at 30°C or 22°C for the final 24 hr before mounting. Flies were then gently anaesthetized using CO_2 and affixed by the dorsal thorax to a glass slide using No More Nails (UniBond) in groups of ~20. Flies were allowed to recover for 2 hr at 25°C in a humidified box and then moved to room temperature (~22°C). They were first allowed to drink water until they no longer responded to a 5-s stimulation, and then a droplet of test stimulus was presented for 3 s on the tarsi (Figures 2 and 3) or labellum (Figure 4). Flies were checked for water satiation between every two to three different stimuli. Flies were scored as 1 (full extension), 0.5 (partial extension), or 0 (no extension), and each fly was treated as a single data point for each stimulus. For Figure 2C, flies were immobilized in a pipette tip rather than a glass slide, to avoid contact of the legs with the taste stimulus.

Germline Manipulation

After crossing parental flies on day 0, progeny were subjected to a heat shock treatment on days 6 and 9 to induce expression of *bam*: vials were submerged in a water bath at 37°C for 1 hr, followed by 1 hr recovery, and a further 1 hr at 37°C. Ovaries were dissected in PBS from flies in the same batch as PER assays.

Statistics

Egg laying and viability values were compared using one-way ANOVA, and each group compared to the 0 mM control using a post hoc multiple comparisons test with Bonferroni correction. CAFE data were compared using a two-tailed Student's t test. flyPAD parameters were compared using Wilcoxon rank-sum test. PER data were compared using a 2 × 2 Fisher's exact test, appropriate for categorical data, and 95% confidence intervals were computed using the modified Wald method [59]. Δp(PER) was calculated as $p(PER)_{mated}$-$p(PER)_{virgin}$, and these values were compared using the weighted least-squares statistic to test for heterogeneity of risk difference, appropriate for comparing effect sizes in categorical data [60]. 95% confidence intervals for Δp(PER) were calculated using the Newcombe-Wilson method without continuity correction [61]. Statistical tests were performed using Microsoft Excel and GraphPad Prism 6, which was also used to plot graphs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.08.043.

AUTHOR CONTRIBUTIONS

S.J.W. and C.R. conceived and designed the project, interpreted data, and wrote the manuscript. S.J.W. performed experiments and data analysis. V.M.C.-C. performed initial characterization of *TβH* mutants in nutrient choice.

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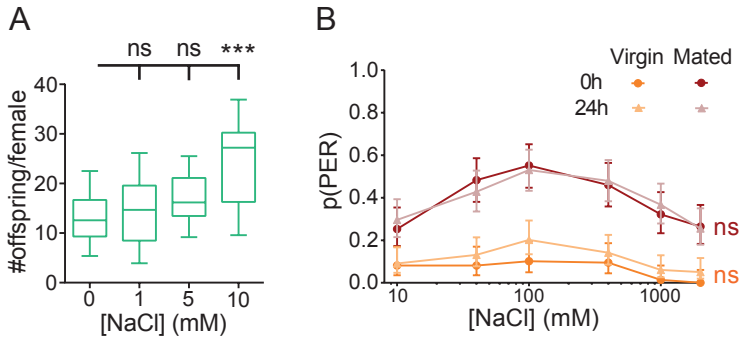
Current Biology

Supplemental Information

Postmating Circuitry Modulates Salt Taste Processing to Increase Reproductive Output in *Drosophila*

Samuel James Walker, Verónica María Corrales-Carvajal, and Carlos Ribeiro

Figure S1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20
10
0
0
1
5
10
[NaCl] (mM)
B
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Mated
0h
24h
p(PER)
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0.6
0.4
0.2
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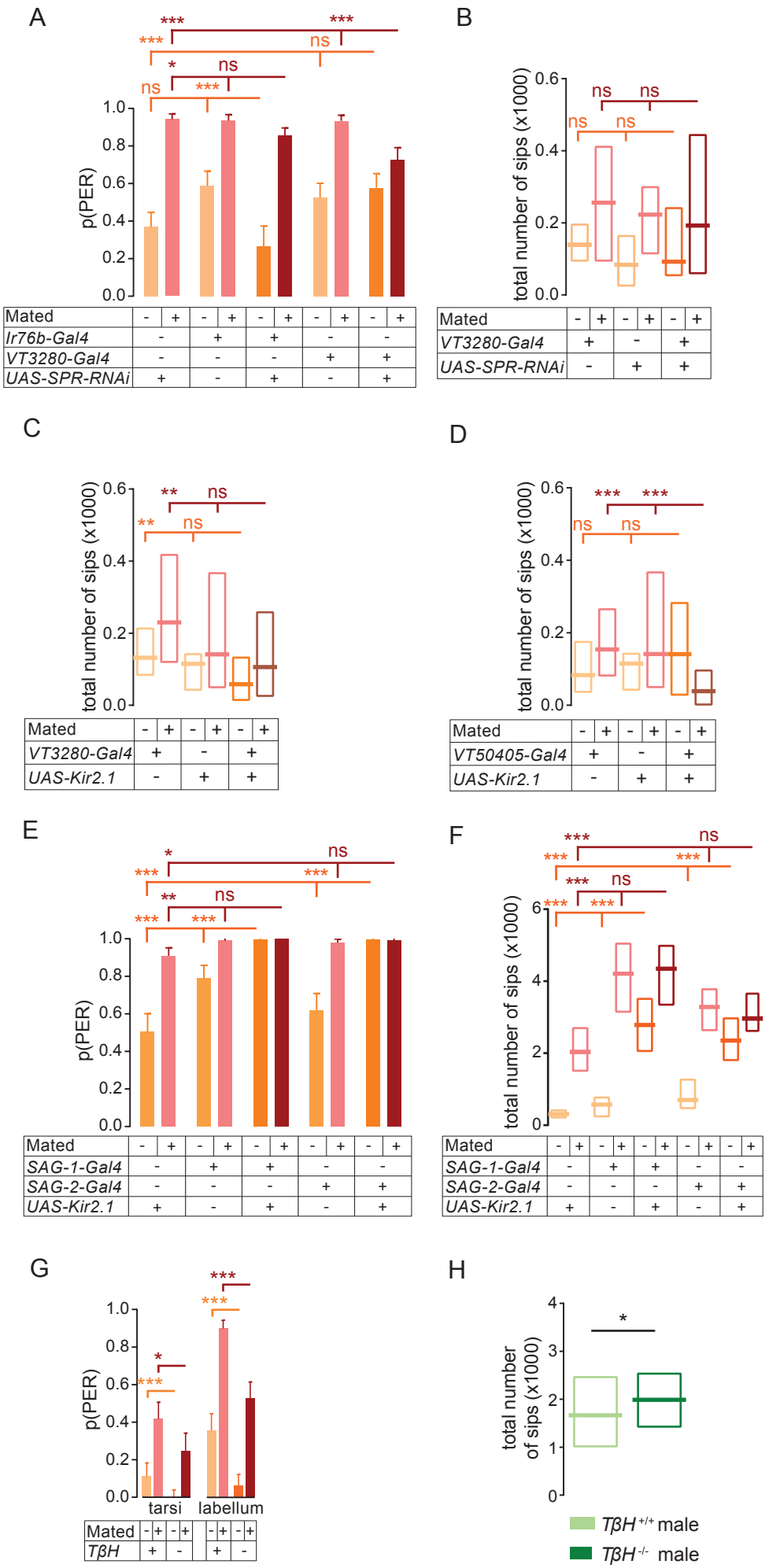
ns
ns
10
100
1000
[NaCl] (mM)

Figure S2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p(PER)
Mated
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VT3280-Gal4
UAS-SPR-RNAi
B
total number of sips (x1000)
Mated
VT3280-Gal4
UAS-SPR-RNAi
C
total number of sips (x1000)
Mated
VT3280-Gal4
UAS-Kir2.1
D
total number of sips (x1000)
Mated
VT50405-Gal4
UAS-Kir2.1
E
p(PER)
Mated
SAG-1-Gal4
SAG-2-Gal4
UAS-Kir2.1
F
total number of sips (x1000)
Mated
SAG-1-Gal4
SAG-2-Gal4
UAS-Kir2.1
G
p(PER)
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Mated
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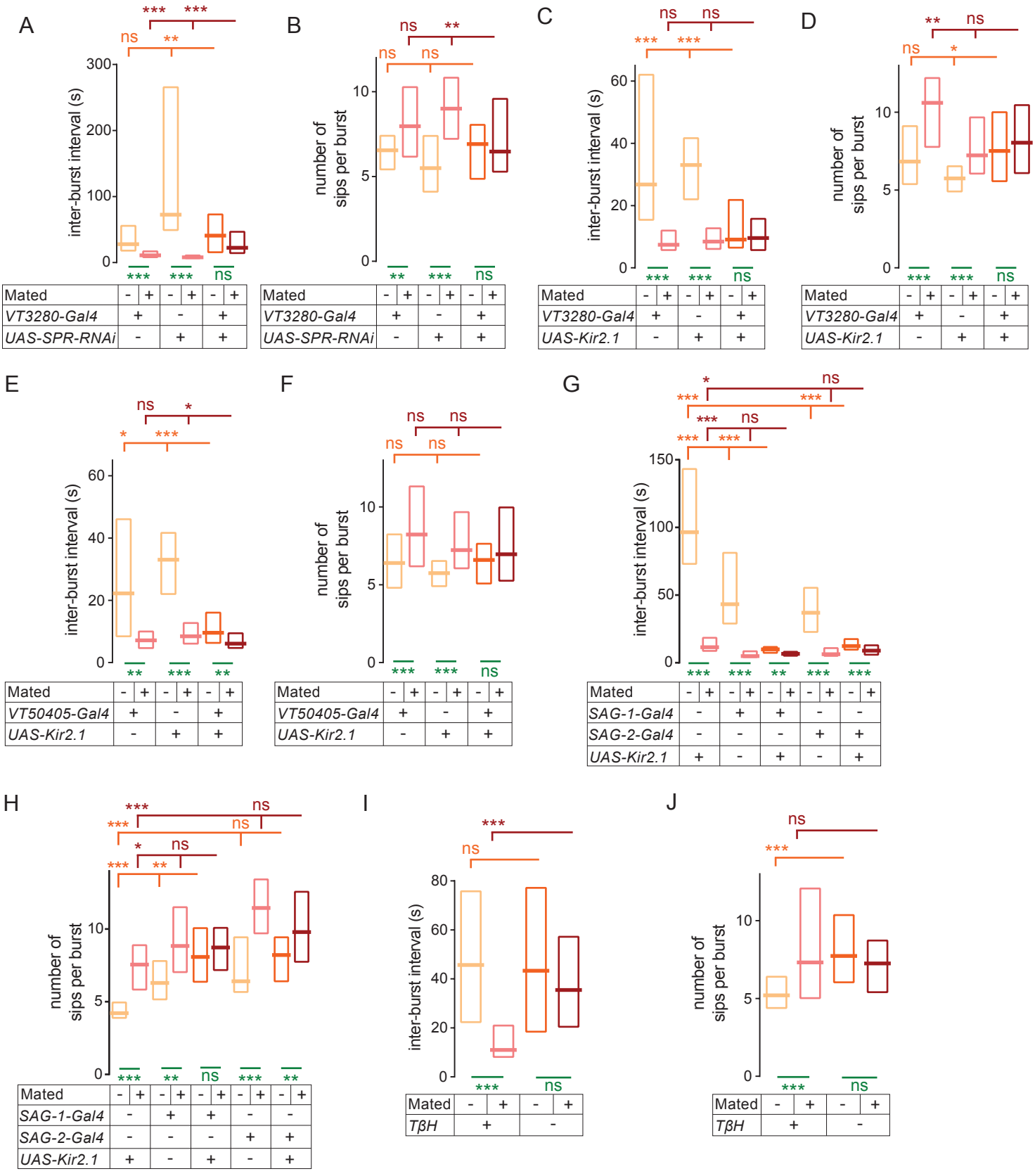
TβH+/+ male
TβH-/- male

Figure S3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Mated - + - + - +
VT3280-Gal4 + - +
UAS-SPR-RNAi - + +
B
number of sips per burst
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C
inter-burst interval (s)
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UAS-Kir2.1 - + +
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UAS-Kir2.1 - + +
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inter-burst interval (s)
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** *** **
Mated - + - + - +
VT50405-Gal4 + - +
UAS-Kir2.1 - + +
F
number of sips per burst
10
5
0
ns ns
ns ns
*** *** ns
Mated - + - + - +
VT50405-Gal4 + - +
UAS-Kir2.1 - + +
G
inter-burst interval (s)
150
100
50
0
* ns
*** ***
*** ns
*** ***
*** *** ** *** ***
Mated - + - + - + - + - +
SAG-1-Gal4 - + + - -
SAG-2-Gal4 - - - + +
UAS-Kir2.1 + - + - +
H
number of sips per burst
10
5
0
*** ns
*** ns
* ns
*** **
*** ** ns *** **
Mated - + - + - + - + - +
SAG-1-Gal4 - + + - -
SAG-2-Gal4 - - - + +
UAS-Kir2.1 + - + - +
I
inter-burst interval (s)
80
60
40
20
0
*** ns
*** ns
Mated - + - +
TβH + -
J
number of sips per burst
10
5
0
ns
***
*** ns
Mated - + - +
TβH + -

Figure S1.

(A) Number of viable offspring produced per female, calculated by multiplying the mean number of eggs laid (Figure 1A) by the percentage of eggs hatched (Figure 1B) for each plate (n=14).

(B) p(PER) of fully-fed females and females wet-starved for 24 hours in response to 100mM NaCl presented to the tarsi (n=74-99). Data for fully-fed females is as shown in Figure 2A.

(A) Box shows median and upper/lower quartiles; whiskers show minimum/maximum. Groups compared by one-way ANOVA followed by post-hoc multiple comparisons to the 0mM control, with Bonferroni correction.

(B) Error bars represent 95% CI. Comparisons between fed and starved groups by 2x2 Fisher's exact test.

ns=p>0.05, ***p<0.001

Figure S2.

(A) p(PER) of females of the indicated genotypes/conditions in response to 100mM NaCl presented to the labellum, used to calculate Δp(PER) in Figure 4D.

(B),(C),(D) Number of sips from 20mM sucrose source in 1 hour by females of the indicated genotypes/conditions during corresponding flyPAD sucrose-yeast choice experiments in Figure 4 (n as shown in Figure 4).

Effect of silencing SAG neurons (*SAG-1-Gal4 and SAG-2-Gal4*) in virgin and mated females on p(PER) to 100mM NaCl presented to the labellum (E; n=79-100) and on number of sips from 10% yeast on the flyPAD (F; n=22-40).

(G) p(PER) of females of the indicated genotypes/conditions in response to 100mM NaCl presented to the tarsi or labellum, used to calculate Δp(PER) in Figure 4M.

(H) Number of sips from 10% yeast by 10d yeast-deprived males with or without *TβH* mutation, in flyPAD sucrose-yeast choice experiment (n=103-107).

(A),(E),(G) Error bars represent 95% CI. Groups compared by 2x2 Fisher's exact test. n as shown in Figure 4.

(B),(C),(D),(F),(H) Boxes show median and upper/lower quartiles. Groups compared by Wilcoxon Rank-Sum test.

ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001

Figure S3.

(A),(C),(E),(G),(I) Mean inter-burst interval, and (B),(D),(F),(H),(J) number of sips per feeding burst, of females of the indicated genotypes and conditions feeding on 10% yeast in the flyPAD, during corresponding yeast-sucrose choice experiments shown in Figure 4.

Boxes show median and upper/lower quartiles. Groups compared by Wilcoxon Rank-Sum test. Asterisks above represent comparisons within one condition, between genotypes. Asterisks below (green) represent comparisons within one genotype, between conditions.

ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001

Table S1 – Lines used in this study

Line name	Detailed genotype	Source	References
Ir76b-Gal4	w^{*};P{*Ir76b-GAL4.916*}226.8; +	Richard Benton	[S1]
UAS-Kir2.1	w^{*};P{*UAS-Hsap\KCNJ2.EGFP*}1;+	BDSC stock #6596	
hs-bam	w^{1118};+;P{w^{+}; *hsp70-bam*$^{+}$}^{11d}	Jürgen Knoblich	[S2]
w^{1118}	w^{1118};+;+	Barry Dickson	[S3]
SP^{0}	+;+;SP^{0}/*TM3,Sb*	Barry Dickson	[S4]
Δ^{130}	+;+; Δ^{130}/*TM3,Sb*	Barry Dickson	[S4]
SP^{+}	+;+;SP^{+}/*TM3,Sb*	Barry Dickson	[S4]
VT3280-Gal4	w^{1118};+; P{*VT3280-GAL4*}attP2	VDRC stock #200327	[S5]
VT50405-Gal4	w^{1118};+; P{*VT50405-GAL4*}attP2	VDRC stock #200652	[S5]
SAG-1-Gal4	w^{1118}; P*{50405.p65AD}*attp40*/CyO;* P*{7068.GAL4DBD}*attP2*/TM3ser*	Barry Dickson	[S5]
SAG-2-Gal4	w^{1118};P*{50405.p65AD}*attp40*/CyO;* P*{45154.GAL4DBD}*attP2*/TM3ser*	Barry Dickson	[S5]
UAS-SPR-RNAi	w^{1118};P{*UAS-SPR.IR2*};+	Barry Dickson	[S6]
$T\beta H^{-/-}$	$T\beta H^{nM18}$;+;+	Scott Waddell	[S7]
UAS-Dcr2	w^{1118}, P{*w[+mC]=UAS-Dcr-2*}; +;+	Barry Dickson	[S8]

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