NEUROSCIENCE

A sperm peptide enhances long-term memory in female *Drosophila*

L. Scheunemann, A. Lampin-Saint-Amaux, J. Schor, T. Preat*

Can mating influence cognitive functions such as learning and memory in a permanent way? We have addressed this question using a combined behavioral and in vivo imaging approach, finding that aversive long-term memory performance strongly increases in *Drosophila* females in response to sperm transfer following mating. A peptide in the male sperm, the sex peptide, is known to cause marked changes in female reproductive behavior, as well as other behaviors such as dietary preference. Here, we demonstrate that this sex peptide enhances memory by acting on a single pair of serotonergic brain neurons, in which activation of the sex peptide receptor stimulates the cyclic adenosine monophosphate/protein kinase A pathway. We thus reveal a strong effect of mating on memory via the neuromodulatory action of a sperm peptide on the female brain.

INTRODUCTION

Pregnancy and the postpartum period in mammalian females are associated with noticeable physiological and behavioral adaptations, along with fundamental changes in the hormonal regulation of brain functions (*1*). More generally, reproduction in all animals, including insects, is associated with the energetic costs of fetus or egg development and behavioral changes that serve the survival of the offspring (*2*, *3*). Nevertheless, the impact of mating on cognitive functions remains poorly studied. One long-debated hypothesis states that women face a decline in cognitive functions such as memory and attention during pregnancy (*4*). Because fetus development demands high energy resources, costly brain functions in women could be sacrificed to realize a trade-off in energy supply between the mother and fetus during pregnancy (*1*). On the other hand, it has been proposed that the physiological and morphological adaptations of the brain during pregnancy and early motherhood lead to a selective decline in brain functions, while those adaptations serving maternal and fetal survival are promoted (*1*, *5*, *6*). These promoted behaviors could include spatial orientation and the ability to remember the location of food and water sources, as well as a decrease in risk-taking behavior (*5*, *7*). However, the mechanisms underlying these behaviors remain to be shown.

After copulation, females of the fruit fly *Drosophila melanogaster* undergo a variety of behavioral changes (*3*), including the rejection of subsequent mating partners (*8*), an increase in egg production and oviposition (*9*), and changes in sleep and activity patterns (*10*) as well as dietary preferences (*11*). All these changes are mediated by a sperm-bound peptide, the sex peptide (SP), which is transferred into the female's reproductive tract during mating (*9*, *12*). While the sensory pathway of SP signaling in *Drosophila* females has been intensively studied (*12*, *13*), a full picture of how higher-order neurons in the brain coordinate the sensory integration of the postmating response is still pending (*13*, *14*).

Once transferred to females, the SP acts via the SP receptor (SPR), a G protein–coupled receptor that is coupled to the inhibitory subunit α_i and/or α_o (*15*). The described postmating switch is mediated by SPR-expressing cells, specifically a subpopulation in the fly uterus called SP-sensing neurons (SPSNs) (*12*, *14*). The SPR is also widely expressed within the central nervous system (CNS), and SP is proposed to circulate within the hemolymph in mated females and to cross the blood-brain barrier (*9*). Nevertheless, a specific function has not yet been attributed to circulating SP within the CNS. Furthermore, myoinhibitory peptides (MIPs), neuromodulators also known as allatostatin B, have been shown to activate the SPR in an interaction that mediates mating-dependent, but also mating-independent, functions such as food intake in the brain (*16*–*18*). Until now, the question of whether mating influences cognitive functions such as learning and memory has not been addressed. Here, we bring closure to these uncertainties and demonstrate that the reproductive state in female *Drosophila* acts on long-term memory (LTM) mechanisms via the direct neuromodulatory activity of SP and MIP.

RESULTS

Mating allows aversive LTM formation by SP transfer

We found that virgin females have a strong deficit in aversive LTM, whereas memory performance increases after mating (Fig. 1A). To test memory performance in *Drosophila*, we used classical associative conditioning in which an aversive olfactory memory is generated by pairing an odor with electric shocks (*19*). As in most other species, LTM formation in *Drosophila* is dependent on de novo protein synthesis. This is restricted to the most salient information, and it is induced only through repeated and spaced training cycles (*20*). Repeated, massed training cycles induce another form of consolidated memory called LT-ARM, which is formed independently of protein synthesis (*19*). Here, LT-ARM performances were indistinguishable between virgin and mated females (fig. S1). We also checked that virgin females responded normally to the sensory cues presented during training and found no differences in their responses to odors or electric shocks, as compared to mated females (table S1). Because this demonstrates that LTM performance specifically increases through mating, we wondered whether SP was responsible for this effect. Females mated to mutant SP^0 males that do not produce SP displayed low LTM scores, similar to that of virgin females (Fig. 1B) (*9*). Moreover, injecting virgin females with synthetic SP into the hemolymph increased memory performances at 24 hours to a level that was indistinguishable from that of mated females (Fig. 1C). Hence, the SP present in the sperm increases aversive LTM in females.

Genes and Dynamics of Memory Systems, Brain Plasticity Unit, ESPCI Paris, PSL Research University, CNRS, 10 rue Vauquelin, 75005 Paris, France.
*Corresponding author. Email: thomas.preat@espci.fr

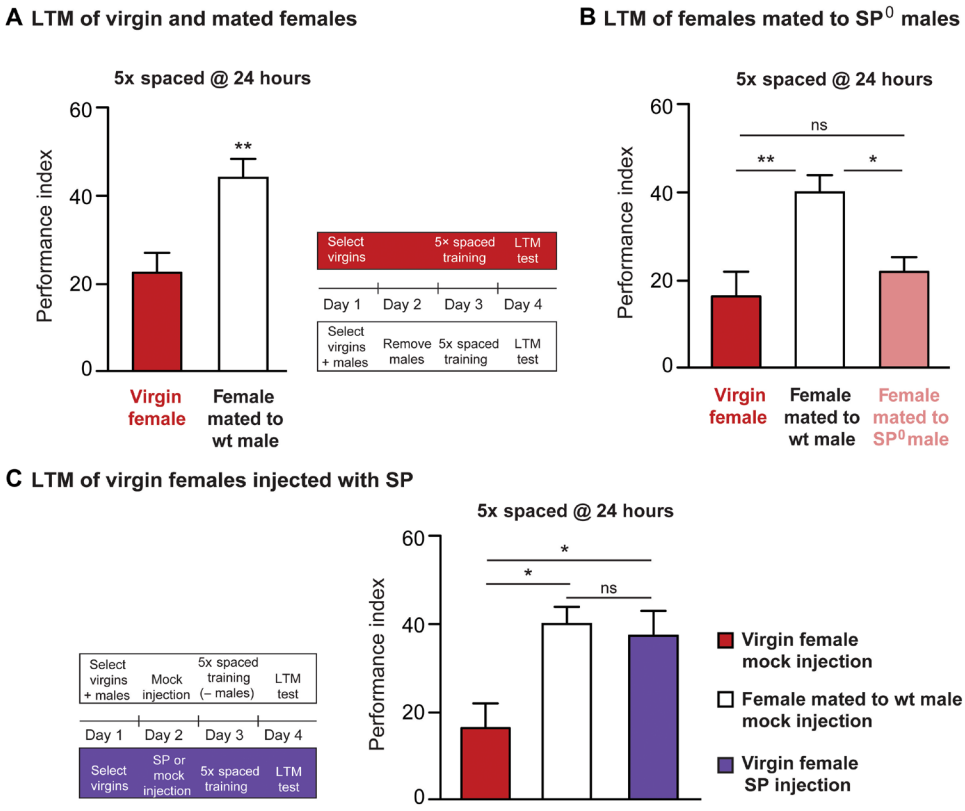

Fig. 1. Aversive LTM is impaired in virgin females. (**A**) Left: LTM performance at 24 hours after 5× spaced training cycles of 3-day-old wild-type (wt) virgin females is significantly decreased in comparison to mated females of the same age (*t* test, $t_{43} = 2.8$, $P = 0.006$; $n = 19$ to 25). Right: Scheme to illustrate the time points of fly selection, mating, and the memory test for virgin and mated female groups. (**B**) Females mated to SP^0 mutant males fail to increase their LTM performance. Memory scores at 24 hours after 5× spaced training are similar to those of virgin females and significantly different from females mated to wt males [one-way analysis of variance (ANOVA), $F_{2,34} = 6.57$, $P = 0.004$; $n = 11$ to 13]. Fly selection, mating, and the memory test were all performed as in (A). (**C**) Left: Scheme to illustrate the time point of fly selection, SP injection, and memory testing for virgin and mated female groups. Right: Injection of virgin females with synthetic SP rescues the LTM defect of virgins injected with Ringer's solution (mock group). The memory performance of SP-injected virgins is indistinguishable from Ringer's-injected females mated to wt males (one-way ANOVA, $F_{2,30} = 5.5$, $P = 0.009$; $n = 11$). Data are presented as means ± SEM. *$P < 0.05$; **$P < 0.01$; ns, not significant. Asterisks indicate the results from a two-tailed unpaired *t* test or the least significance level in a Newman-Keuls post hoc comparison of indicated groups.

SPR mediates the memory effect in a pair of serotonergic neurons

Next, we asked whether SPR-expressing SPSN neurons in the female uterus mediate the LTM effect of SP after mating, as is the case for other postmating behaviors. Unexpectedly, SPR knockdown in SPSN neurons using RNA interference (RNAi) had no effect on LTM (Fig. 2A).

LTM is gated by a series of neuronal events, and its early stages involve the inhibition of Dunce (Dnc) phosphodiesterase (PDE), a cyclic adenosine monophosphate (cAMP)–degrading enzyme found in a pair of serotonergic neurons (SPNs) (Fig. 2B) (*21*–*23*). This inhibition induces increased activity of the cAMP/protein kinase A (PKA) pathway and activation of the SPN (Fig. 2B) (*22*). The SPN is targeted by a GAL4 line, VT057280-Gal4, that drives expression through an enhancer sequence located near the *spr* gene (Fig. 2B). This observation suggested to us that the SPR is expressed in the SPN. Using an anti-SPR antibody, we observed that SPR is expressed in the SPN (Fig. 2C) and that knockdown of SPR in the SPN significantly reduces anti-SPR staining (Fig. 2C). To address the role of the SPR in LTM, we performed a transient knockdown of the SPR in the SPN and tested the LTM of these flies. We used two different lines, VT026326-Gal4 and GH298-Gal4 (*22*), to drive expression of SPR^{RNAi} in the adult SPN by taking advantage of the temperature-sensitive tubulin-$Gal80^{ts}$ promoter (tub-$Gal80^{ts}$) (Fig. 2D). Inhibition of SPR in the SPN induced a strong LTM defect (Fig. 2D). We reproduced the same defect using a second SPR^{RNAi} (fig. S2A). Noninduced controls performed normal LTM, and memory after massed training was not affected by the RNAi induction (fig. S2, B and C). Moreover, the sensory acuity of induced flies was normal (table S2). In addition, we used the SPNsplit-Gal4 line to specifically target the SPN (fig. S2D) (*22*). Using this line to express the SPR knockdown, we observed once again a defect in LTM performance of mated females (Fig. 2E). Green fluorescent protein (GFP) labeling of the SPN using SPNsplit-Gal4 revealed staining in the ventral nerve cord (VNC) (fig. S2D). Thus, SPR activation could take place in the VNC and/or the brain. LT-ARM and sensory acuity were normal after SPR knockdown using SPNsplit-Gal4 (fig. S2E and table S3). Together, these results demonstrate that SPR signaling specifically in the SPN is indispensable for LTM formation but independent of the SP signaling pathway in the SPSNs.

SPR controls LTM via Dnc PDE inhibition

How do SPRs in the SPN control LTM? Previously, we demonstrated that Dnc PDE is a molecular checkpoint that inhibits SPN activity

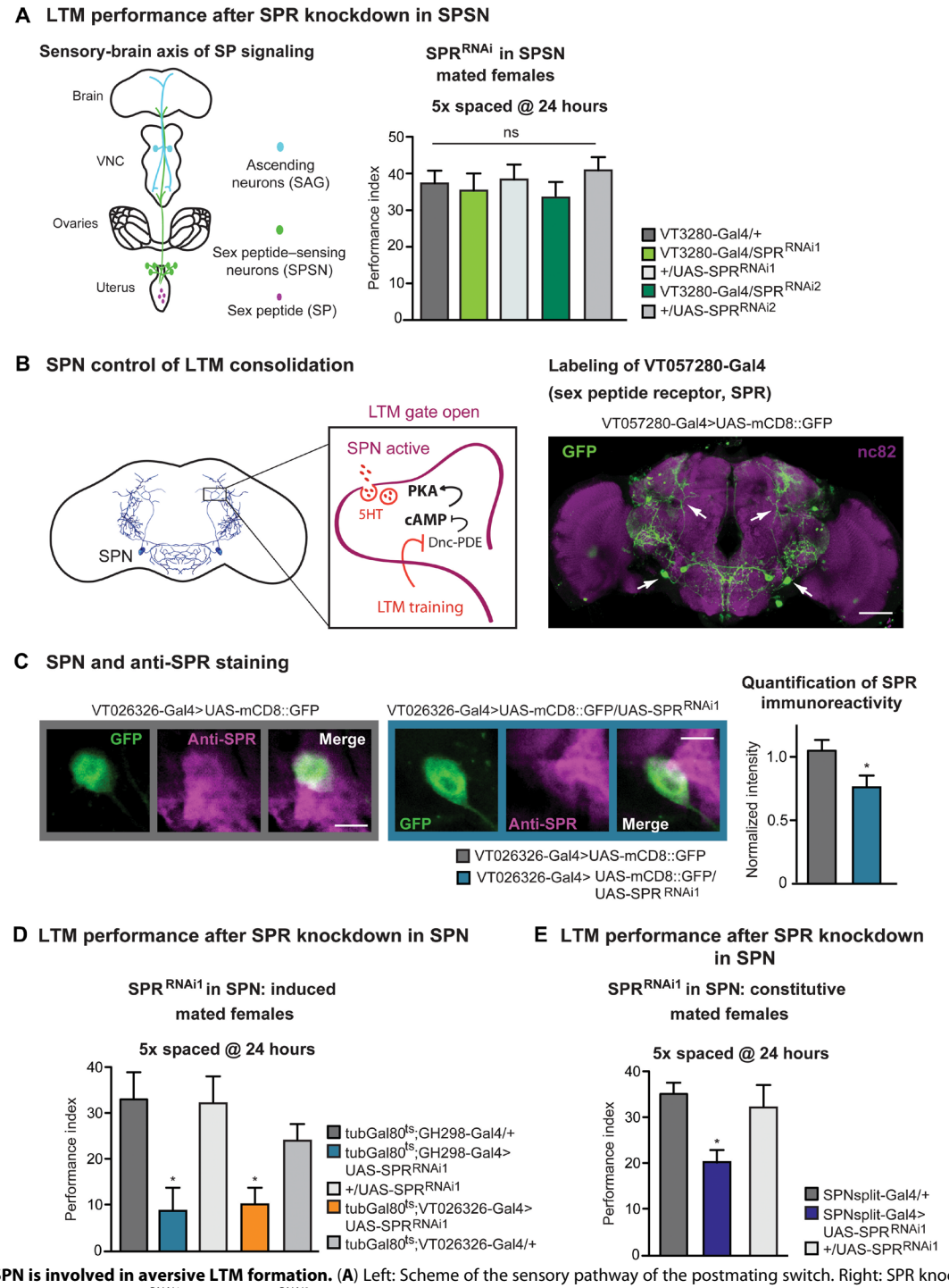

Fig. 2. SPR in the SPN is involved in aversive LTM formation. (**A**) Left: Scheme of the sensory pathway of the postmating switch. Right: SPR knockdown in the SPSN driven by *VT003280-Gal4* using *UAS-SPR^{RNAi1}* and *UAS-SPR^{RNAi2}* has no effect on LTM performances (one-way ANOVA, $F_{4,37} = 0.12$, $P = 0.91$; $n = 8$). (**B**) Left: Scheme to illustrate SPN anatomy in the brain. The inset illustrates the control of LTM consolidation: After LTM training, Dnc PDE default activity is inhibited, PKA levels rise, and serotonin [5-hydroxytryptamine (5HT)] signaling from the SPN allows downstream consolidation processes. Right: Immunolabeling of *VT057280-Gal4* (SPR-Gal4) flies driving *UAS-mCD8::GFP* shows expression in the SPN (white arrows), as revealed by anti-GFP staining (green). Scale bar, 50 μm. (**C**) The cell body of the SPN visualized with anti-GFP staining (green) of *VT026326-Gal4>UAS-mCD8::GFP* flies colocalizes with a marker for SPR (Anti-SPR; magenta). Simultaneous knockdown of SPR using *UAS-SPR^{RNAi1}* driven by *VT026326-Gal4* reduces SPR signals in the SPN cell body. The images represent a single 1-μm *z*-stack. Scale bars, 10 μm. Quantification of the normalized intensity of anti-SPR staining reveals a significant decrease in the SPN after SPR knockdown using *VT026326-Gal4>UAS-SPR^{RNAi1}* as compared to controls (*t* test, $t_{10} = 2.4$, $P = 0.03$; $n = 6$). (**D**) SPR knockdown in the SPN of adult flies with 3 days of induction using *UAS-SPR^{RNAi1}* driven by either *tub-G80^{ts};GH298-Gal4* or *tub-G80^{ts};VT026326-Gal4* impairs LTM performances (one-way ANOVA, SPR-RNAi^{1}: $F_{4,73} = 5.27$, $P = 0.0009$; $n = 13$ to 18). (**E**) SPR knockdown in the SPN using UAS-SPR^{RNAi1} driven by SPNsplit-Gal4 impairs LTM performances (one-way ANOVA: $F_{2,51} = 4.28$, $P = 0.023$; $n = 18$). Data are presented as means ± SEM. *$P < 0.05$. Asterisks indicate the results from a two-tailed unpaired *t* test or the least significance level in a Newman-Keuls post hoc comparison of indicated groups.

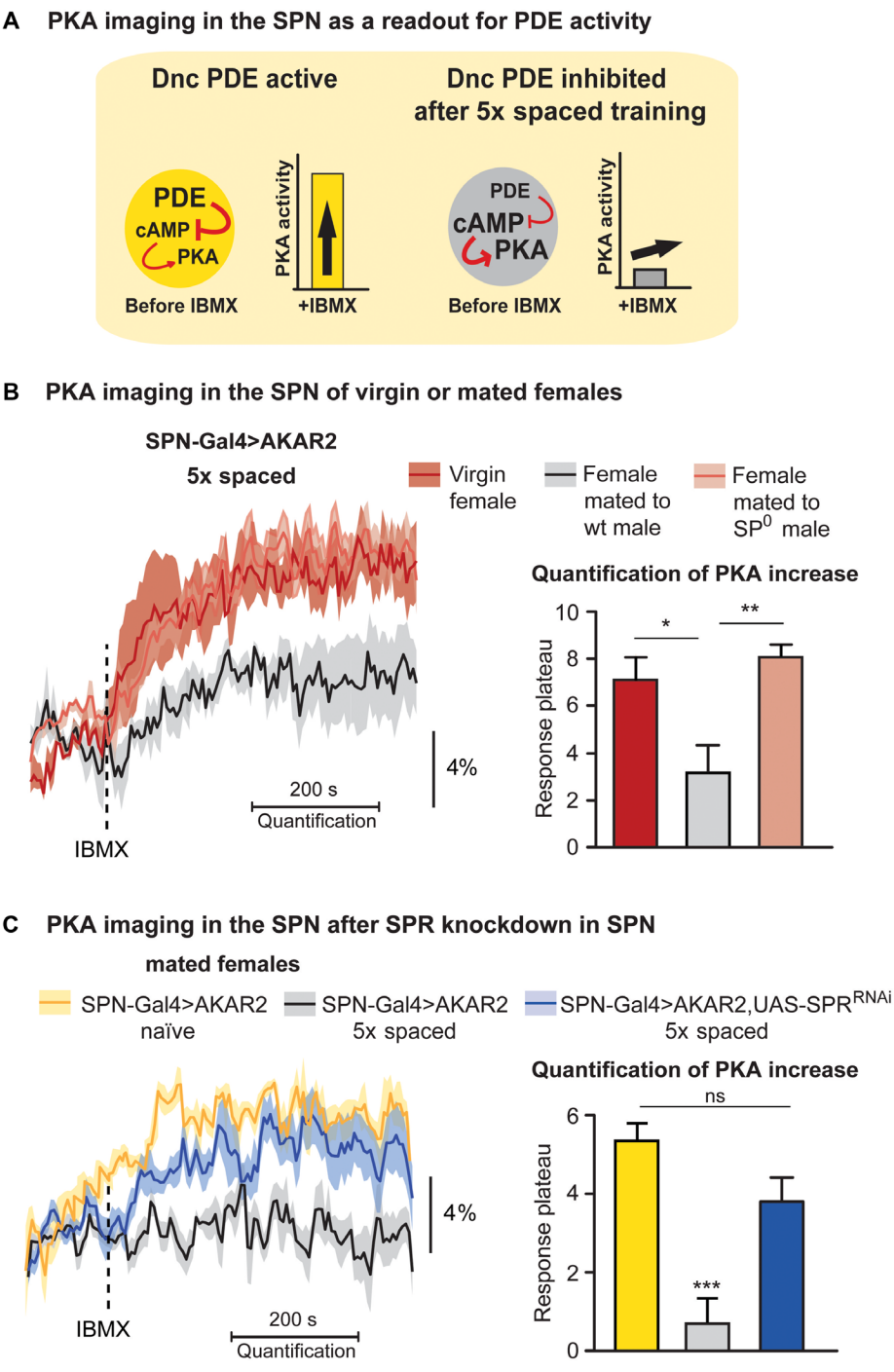

Fig. 3. SPR in the SPN inhibits Dnc PDE. (**A**) Schematic illustration of the Dnc PDE activity readout using PKA imaging. Left: In the event of high Dnc PDE activity, intracellular PKA activity level is low. After 3-isobutyl-1-methylxanthine (IBMX) injection to inhibit PDE, PKA levels are no longer restricted by Dnc PDE and consequently rise. In contrast, if Dnc PDE activity is inhibited in the SPN after spaced training, injection of IBMX does not lead to any further PDE inhibition and PKA levels show little change. (**B**) Dnc PDE is not inhibited in virgin females or females mated to SP^0 males after 5× spaced training. In vivo PKA imaging was conducted on flies expressing *UAS-AKAR2* in the SPN using *VT057280-Gal4* (SPR-Gal4). Time traces of PKA activity are shown, including the point at which IBMX is applied on the brain (dashed lines) to inhibit PDE. After 5× spaced training, mated females displayed a weak increase in PKA activity in response to IBMX, because Dnc PDE is inhibited by spaced training. In both virgin females and females mated to SP^0 mutant males, IBMX evoked PKA activity in the SPN after 5× spaced training, which was significantly higher than in wt mated controls (one-way ANOVA: $F_{2,26} = 6.8$, $P = 0.004$; $n = 9$ to 11). (**C**) SPR is involved in Dnc PDE inhibition after LTM training. PKA imaging reveals that Dnc PDE activity is high in naïve flies and inhibited after 5× spaced training. Under SPR knockdown conditions using *UAS-SPR*^{RNAi1} driven by *VT057280-Gal4*, IBMX evoked an increase in PKA activity after 5× spaced training in mated females that was significantly greater in comparison to the trained wt control and indistinguishable from naïve flies (one-way ANOVA: $F_{2,21} = 17.1$, $P < 0.0001$; $n = 7$ to 9). Data are presented as means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$. Asterisks indicate the results from a two-tailed unpaired *t* test or the least significance level in a Newman-Keuls post hoc comparison of indicated groups.

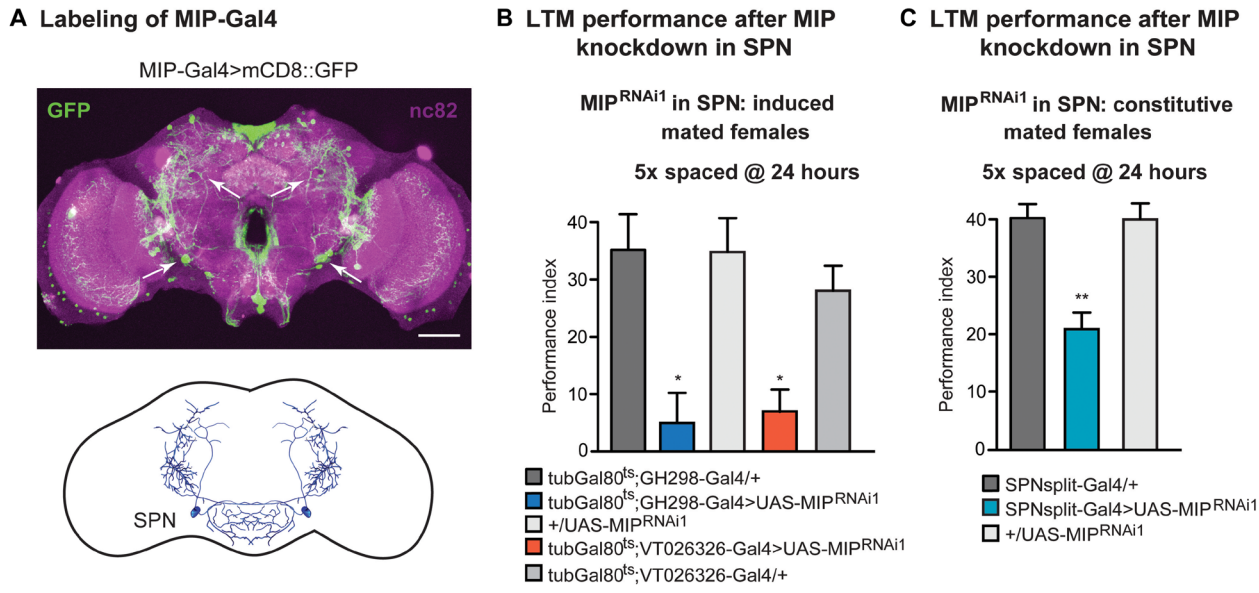

Fig. 4. MIP signaling from the SPN is involved in aversive LTM formation. (**A**) Immunolabeling of *MIP-Gal4* flies driving *UAS-mCD8::GFP* shows expression in the SPN (white arrows), as revealed by anti-GFP staining (green). Scale bar, 50 μm. (**B**) MIP knockdown in the SPN of adult flies with 3 days of induction using *UAS-MIP^{RNAi1}* driven by either *tub-G80^{ts}; GH298-Gal4* or *tub-G80^{ts}; VT026326-Gal4* impairs LTM performances (one-way ANOVA: $F_{4,75} = 6.15$, $P = 0.0002$; $n = 13$ to 19). (**C**) MIP knockdown in the SPN using *UAS-MIP^{RNAi1}* driven by *SPNsplit-Gal4* also impairs LTM performances (one-way ANOVA: $F_{2,33} = 7.59$, $P = 0.002$; $n = 12$). Data are presented as means ± SEM. *$P < 0.05$; **$P < 0.01$. Asterisks indicate the results from a two-tailed unpaired *t* test or the least significance level in a Newman-Keuls post hoc comparison of indicated groups.

and LTM formation as a default state (Fig. 2B) (*22*). We addressed the possibility that mating could act on Dnc PDE inhibition in the SPN via the SPR. Molecularly, Dnc inhibition activates cAMP, which, in turn, activates PKA. We thus used the fluorescence resonance energy transfer (FRET) sensor AKAR2 to image PKA activity in the SPN in vivo (*22*, *24*). In our previous work, we showed that Dnc is the main PDE active in the SPN (*22*). This demonstrates that an increase in the AKAR2 FRET signal following treatment with a PDE inhibitor can be used as a proxy for Dnc activity (Fig. 3A) (*22*). Our results confirm that Dnc activity was low in the SPN of normal mated females after LTM training. However, we observed strong Dnc PDE activity after LTM training in virgin females, as well as females mated to SP^0 males (Fig. 3B). To demonstrate that Dnc PDE inhibition upon LTM formation is mediated via SP activation of SPR in the SPN, we imaged PKA activity in flies with reduced SPR levels in the SPN. First, we confirmed that Dnc PDE activity was high in naïve mated flies and low after spaced training (Fig. 3C) (*22*). However, flies with reduced SPR still displayed high Dnc PDE activity after spaced training (Fig. 3C). This reveals that Dnc PDE is not inhibited after LTM formation in mated females expressing SPR^{RNAi} in the SPN (Fig. 3C). Thus, SPR in the SPN is directly involved in the process of Dnc PDE inhibition after LTM training. Our data therefore indicate that LTM is not formed in virgin females, because the default inhibition of the SPN by Dnc PDE cannot be released because of a lack of SP and consequently SPR activation. We also tested whether SP transfer by mating alone could inhibit Dnc PDE. Comparing naïve virgin and mated females revealed a slight but significant inhibition of Dnc PDE in mated females (fig. S3). This suggests that the strong inhibition of Dnc PDE in the SPN after 5× spaced training of mated females involves the effect of SP transfer, as well as the additional role of another effector.

MIP is involved in LTM formation

Along with SP, MIPs are known ligands of SPR (*16*). The SPN itself expresses MIP (Fig. 4A) (*18*). We therefore asked whether MIP signaling in the SPN could be important for normal LTM formation. By targeting RNAi-mediated knockdown of MIP to the SPN in adult flies, as previously carried out for SPR^{RNAi}, we observed a strong LTM defect (Fig. 4B). This effect was reproducible using a second RNAi, whereas the LTM of noninduced controls as well as LT-ARM were normal after MIP knockdown in the SPN (fig. S4, A to C). Furthermore, sensory acuity was not affected (table S4). We also tested whether MIP is needed specifically in the SPN for LTM formation. We were able to induce a significant decrease in LTM performance by using split-Gal4 to express MIP^{RNAi} in the SPN (Fig. 4C). Once again, LT-ARM and sensory acuity were not affected (fig. S4D and table S5). We therefore conclude that MIP signaling from the SPN represents a crucial step in generating LTM, probably in a cell-autonomous way via SPR activation in the SPN itself.

Inhibition of PDE in the SPN rescues LTM in virgin females

Last, we wondered whether forcing Dnc PDE inhibition in the SPN could rescue LTM performances in virgin females. When Dnc^{RNAi} is targeted to the SPN during adulthood, LTM performances in virgin females are indistinguishable from those of mated females (Fig. 5A). The same LTM rescue was achieved by exclusively targeting Dnc PDE knockdown to the SPN using the SPNsplit driver, demonstrating the specificity of the SPN effect (Fig. 5B). Thus, the mating status controls an LTM checkpoint in the SPN of female brains by switching the SPN into an activable state via the sperm peptide SP (Fig. 5C). Because SPR knockdown in the SPSN did not have any effect on memory performances, we hypothesize that this effect is mediated by SP circulating in the female hemolymph, acting directly on SPRs in the SPN (Fig. 5C).

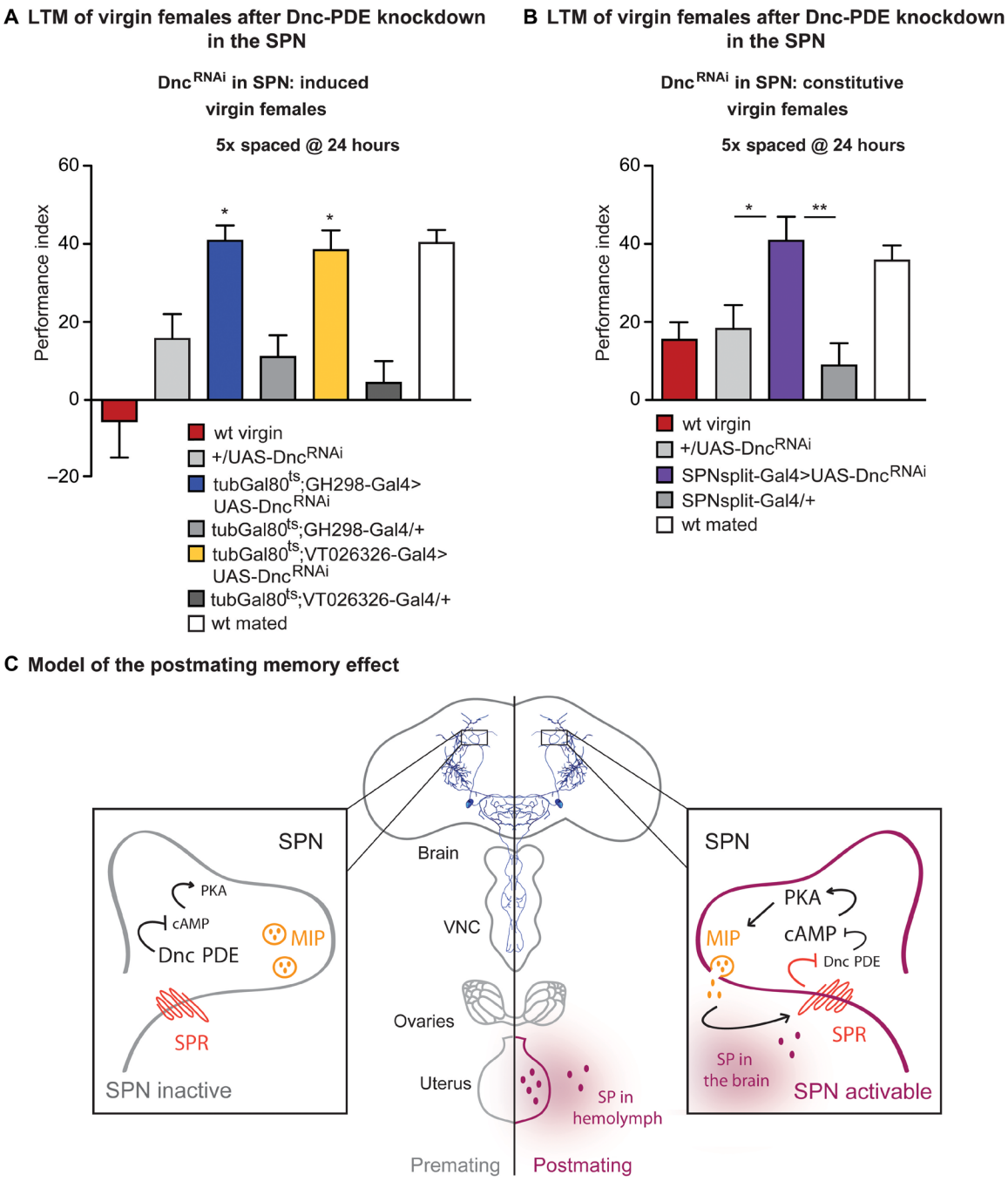

Fig. 5. Dnc PDE knockdown in the SPN of adult flies rescues the LTM performance of virgin females. (**A**) LTM performances of virgin females at 24 hours after 5× spaced training cycles with conditional knockdown using *UAS-Dnc*^{RNAi} in the SPN and driven by either *tub-G80*^{ts}*; GH298-Gal4* or *tub-G80*^{ts}*; VT026326-Gal4* are significantly higher than the performances of the respective genetic control virgin females and are indistinguishable from mated females (one-way ANOVA: $F_{4,50} = 6.31$, $P = 0.003$; $n = 10$ to 12). Note that the memory performances of virgin females and genetic controls are relatively low, which is probably due to the fact that the high temperatures used to induce RNAi also compromise virgin behavior. (**B**) LTM performances of virgin females at 24 hours after 5× spaced training cycles with knockdown using *UAS-Dnc*^{RNAi} driven specifically in the SPN by SPNsplit-Gal4 are significantly higher than the performances of the respective genetic control virgin females and are indistinguishable from mated females (one-way ANOVA: $F_{2,35} = 6.69$, $P = 0.004$; $n = 12$ to 13). (**C**) Schematic illustration of the molecular modulation in the SPN by SP transferred to the female during mating. Thereby, the SPN is switched into an activable state and can be triggered by LTM training. Data are presented as means ± SEM. *$P < 0.05$; **$P < 0.01$. Asterisks indicate the least significance level in a Newman-Keuls post hoc comparison of indicated groups.

DISCUSSION

Why does aversive LTM increase after mating, while it is inhibited in virgin females? Increased aversive LTM after mating could elevate the survival chances of females during egg laying and favor the choice of secure egg deposition sites (*25*). In addition, the phenomenon we describe here could be linked to a context-dependent change in the perception of aversive stimuli and anxiety mechanisms in the brain. Along these lines, a positive value for risk-taking behavior has been observed in starved mammals in which the feeding status can change the evaluation and memorization of aversive stimuli (*26*). Starvation reduces fear and (like in *Drosophila*) inhibits the consolidation of fear memories and facilitates their extinction (*26*, *27*). Thus, while safety concerns predominate in the satiated state, starvation will induce higher-risk actions and promote foraging behavior (*26*). Similarly, a strong incentive for mating could be an evolutionary drive to decrease fear or fear-related memories to facilitate explorative behavior in the search for a mating partner. In mice, different stages of development can be distinguished due to the differential expression of gonadal hormones. Fear memory is inhibited during early and mid-adolescence, at a stage when mice leave the nest and explore new environments while they become sexually mature and search for mating partners (*28*). Thus, modulation of anxiety-related plasticity by the reproductive state could be an evolutionarily conserved mechanism (*7*).

Across different phyla, the reproductive state of females not only changes their behavior but also causes fundamental adaptations to physiological functions and energy demands (*2*, *29*). The reproductive system must monitor the body's energy resources to prevent reproduction in the absence of any energy reserves. In *Drosophila*, females double their food intake after mating, egg production markedly increases overall energy requirements, and mating behavior is inhibited under starvation (*3*). LTM formation is energetically costly, and the initiation of LTM processes is protected by molecular and cellular mechanisms to ensure that only relevant information is consolidated and thus available for future behavioral choices (*21*, *22*, *27*). These mechanisms therefore represent important modulatory targets, such as hormonal regulation, that are used by the reproductive system to manage energy resources and influence behavioral expression in the long term. How changes in metabolic homeostasis after mating are connected to the LTM phenotype must be addressed in more detail by further studies. This includes the question of whether the "memory switch" is specific to females or whether a similar effect takes place in males before and after mating.

In vertebrates, adolescence and the reproductive state control behavioral actions via hormones and neuromodulators governed by hypothalamic regulation (*30*). The *Drosophila* postmating switch (including increased aversive LTM performances) is mediated by the seminal peptide SP, which is transferred to the female after mating (*9*). To date, no substance with such a marked or versatile impact on female behavior has been identified in the seminal fluid of any mammals, including humans. However, several studies in recent decades have identified seminal substances in mammals and humans that could operate in females after copulation to influence their reproductive behavior (*31*). One study specifically identified neural growth factor as an ovulation-inducing factor in the semen of llamas acting directly on the hypothalamo-pituitary-gonadal axis of inseminated females (*32*). Whether such an activation of the hypothalamic axis could have further consequences on brain plasticity is purely speculative at this point. Nonetheless, several studies have proposed that the reorganization of hypothalamic hormonal release could directly influence cognitive functions via the modulation of hippocampal activity (*1*).

Future investigations should aim to address in greater detail how cognitive functions are modulated by hypothalamic neuromodulative actions. Although there is no defined region in *Drosophila* that is homologous to the hypothalamus, there is a neuronal network that executes neuromodulatory activity in the pars intercerebralis and gnathal ganglion that displays a strong functional homology, and has been referred to as a hypothalamic-like center of the *Drosophila* brain (*33*). The SPN and its broad processes are located within this hypothalamic-like region (*22*), and we establish here that it plays a fundamental role in translating reproductive signals into cognitive processes. Furthermore, we demonstrate that SP transferred to *Drosophila* females after mating causes marked changes in their memory performances. It should be noted that this does not necessarily mean that SP transferred to females enhances their cognitive functions in general; rather, it modulates memory systems to allow consolidation of risk-predicting associations.

In addition to the neurotransmitter serotonin, we demonstrate here that the SPN expresses MIP and that MIP signaling from the SPN is important for LTM formation, most likely via cell-autonomous activation of the SPR. These findings indicate that the neuron described here as SPN represents MIP-expressing inferior contralateral interneurons (ICLI) neurons that were previously shown to control a variety of innate behaviors, including reproduction (*34*). MIP as well as its mammalian functional homolog galanin are involved in reproductive behavior (*17*, *35*). Moreover, galanin has been shown to integrate conflicting behaviors, i.e., feeding and mating, under low-energy reserves via direct interactions with neuropeptide Y in the hypothalamus (*35*). Nevertheless, the question of whether MIP is needed only at the point of copulation or whether it is a generic signaling pathway for LTM formation remains to be addressed. Together, these observations suggest that further examination of SPN neuromodulator activity will reveal new mechanisms of memory processes in the fly as well as provide important insights into how internal states modulate memory systems in general.

MATERIALS AND METHODS

Fly strains

D. melanogaster wild-type Canton Special (CS) and mutant flies were raised on standard medium at 18°C and 60% humidity in a 12-hour light/dark cycle. *UAS-SPR*^{RNAi1} and *UAS-SPR*^{RNAi2} were obtained from the Vienna Drosophila Resource Center (VDRC; SPR-RNAi1, ID v330048 and SPR-RNAi2, ID v106804). *UAS-MIP*^{RNAi1} and *UAS-MIP*^{RNAi2} were obtained from the Bloomington Drosophila Stock Center (BDSC; MIP-RNAi1, ID 41680 and MIP-RNAi2, ID 26246). *UAS-Dnc*^{RNAi} was generated as previously described (*36*). Gal4 drivers for expressing the genes of interest in the SPN include *GH298-Gal4*, *VT026326-Gal4* (VDRC, ID 201794), and *VT057280-Gal4* (VDRC, ID 200916) (*22*). For expression in SPSN, *VT003280-Gal4* (VDRC, ID 200327) was used (*14*). *MIP-Gal4* from the Korea Drosophila Stock Centre was provided by Y.-J. Kim. SP mutant males were obtained by crossing *SP*^{0}*/TM3,Sb* flies to Δ*130/TM3,Sb*; these flies are referred to as SP^{0} throughout the text and figures (*9*). Only females were used for the behavioral and imaging experiments. For the memory assay, virgin female and mated control groups were generated by collecting virgins in groups of 30 to 40 from unanesthetized

freshly hatched fly cultures. Seven to 10 CS wild-type or SP^0 mutant males were added to half of the groups, and experiments were then performed with 2- to 3-day-old female flies. If indicated, RNAi expression was specifically induced in adults using the TARGET system (*37*). To achieve RNAi induction, flies were kept at 30°C for 2 to 3 days before conditioning; in addition, flies for LTM were maintained at 30°C until the memory assay. For the imaging experiments, virgin females were collected in groups of 10 from unanesthetized freshly hatched fly cultures. Five CS wild-type or SP^0 mutant males were added to half of the groups, and experiments were then performed with 2-day-old female flies.

Behavioral experiments

Flies were trained using classical olfactory aversive conditioning protocols as previously described (*19*). Training and testing were carried out at 25°C and 80% humidity. Conditioning was performed on samples of 20 to 30 flies with 0.360 mM 3-octanol (99% purity; Sigma-Aldrich) and 0.325 mM 4-methylcyclohexanol (98% purity; Sigma-Aldrich). Odors were diluted in paraffin oil (VWR International), and memory tests were performed using a T-maze apparatus (*38*). Flies were given 1 min to choose between the two arms, with each arm delivering a distinct odor. An index was calculated as the difference in the number of flies in each arm divided by the sum of flies in both arms. The average of two reciprocal experiments yielded the performance index. For LTM analyses, flies were trained with five cycles spaced at 15-min rest intervals and tested 24 hours later. Flies used for odor avoidance tests after electric shock and response to electric shock were treated as previously described (*19*). Briefly, shock avoidance was tested by determining the avoidance index of flies that could freely choose between one tube connected to the electric shock device and another tube lacking any stimulus presentation during 1 min. Flies tested for odor avoidance against 4-methylcyclohexanol were presented 1 min of electric shock paired with 3-octanol. During the avoidance test, flies could freely choose between a tube presenting 4-methylcyclohexanol or air for 1 min. Odor avoidance against 3-octanol was performed in the opposite order.

SP injection

CS virgin female flies were collected in groups of 50 from unanesthetized freshly hatched fly cultures. One-day-old virgins were individually anaesthetized with CO_2, and synthetic SP was injected into the abdomen using an Eppendorf Transjector 5246 microinjector (equipped with Femtotips II needles) with 50 nl containing either 15 pmol of synthetic SP (ProteoGenix) dissolved in *Drosophila* Ringer's solution [46 mM NaCl, 182 mM KCl, 3 mM $CaCl_2$, and 10 mM tris (pH 7.2)] or *Drosophila* Ringer's solution only (mock injection) (*39*). Flies were then separated into groups of 20 females; for mated controls, 10 adult males were added. Conditioning was performed the next day using only females.

In vivo PKA imaging

In vivo two-photon imaging of PKA activity using the AKAR2 sensor was performed according to a previously described protocol (*24*), with the exception that flies homozygous for both *VT057280-Gal4* and *UAS-AKAR2* were used to obtain sufficient signal for two-photon FRET imaging. For RNAi experiments, females were used from a homozygous *UAS-SPR^{RNAi1};VT57280-Gal4,UAS-AKAR2* line. All flies were raised at 25°C. Images were acquired on a Leica TCS SP5 microscope. The AKAR2 sensor was excited at 850 nm using a tunable Mai Tai DeepSee pulsed laser (Spectra-Physics) through a 25× water immersion objective (HCX IRAPO; numerical aperture, 0.95). Images were acquired in a plane showing projections of the SPN neuron on the peduncle region at a rate of one image every 5 s. Stocks of 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) were dissolved and aliquoted in dimethyl sulfoxide at 200 mM and subsequently diluted 100 times in *Drosophila* Ringer's solution on the day of the experiment. Ten microliters of this solution was injected into the 90-µl droplet of *Drosophila* Ringer's solution bathing the fly brain during image acquisition, resulting in a final IBMX concentration of 200 µM. Image analysis was performed using a custom-written MATLAB program. Regions of interest (ROIs) were manually delimited around SPN projections in each hemisphere, and at each time point, the average intensities of the yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) channels were calculated, background-subtracted, and divided to obtain the FRET ratio. FRET ratio time traces were normalized to a baseline value calculated from the 60 s preceding drug application. Plateau responses were measured as the average normalized FRET ratio starting 60 s after drug application and extending over a 120-s duration.

Immunohistochemistry

VT057280-Gal4 female flies were crossed with *UAS-mCD8::GFP* males. Before dissection, whole F1 female flies (3 to 4 days after eclosion at 25°C) were fixed in 4% paraformaldehyde in PBT (phosphate-buffered saline containing 1% Triton X-100) overnight at 4°C. Brains were dissected in Ca^{2+}-free *Drosophila* Ringer's solution and fixed for 1 hour at room temperature in 4% paraformaldehyde in PBT. Samples were then rinsed three times for 20 min in PBT, blocked with 2% bovine serum albumin in PBT for 2 hours, and incubated with rabbit anti-GFP (1:400; Invitrogen Molecular Probes) and mouse anti-nc82 (1:100; Developmental Studies Hybridoma Bank) primary antibodies in the blocking solution overnight at 4°C. Brains were washed three times for 20 min in PBT and then incubated with anti-rabbit secondary antibody conjugated to Alexa Fluor 488 and anti-mouse Alexa Fluor 591 (1:400; Invitrogen Molecular Probes) in the blocking solution overnight. After three washes (20 min), brains were mounted in ProLong Mounting Medium (Life Technologies) for microscopy analysis.

Anti-SPR staining

UAS-mCD8::GFP;VT026326-Gal4 female flies were crossed with wild-type CS or *UAS-SPR^{RNAi1}* males. Before dissection, whole F1 female flies (3 days after eclosion at 25°C) were fixed in 4% paraformaldehyde in PBT at 4°C for 24 hours. Samples were incubated for 48 hours with the following antibodies: rabbit anti-SPR (1:500; a gift from Y.-J. Kim) (*40*) and mouse anti-GFP (1:400; Sigma-Aldrich) (see above for a description of the immunohistochemistry). For secondary antibodies, brains were incubated with a horseradish peroxidase–conjugated goat anti-rabbit antibody (1 : 100; Invitrogen, catalog number T20924) or anti-mouse Alexa Fluor 488 (1:400, Invitrogen) for 3 hours at room temperature. Next, brains were stained using the Tyramide Signal Amplification Kit (Invitrogen) according to the manufacturer's instructions and mounted in ProLong Mounting Medium (Life Technologies). Images were acquired using a Nikon A1R confocal microscope. Confocal *z*-stacks were acquired in 1-µm slices and imported into the National Institutes of Health ImageJ for analyses. To quantify SPR immunoreactivity, an ROI was selected

around the SPN cell body and SPR intensity was normalized to the intensity of the same ROI size at a defined region next to the SPN cell body comprising one *z*-stack of six control brains and six brains with SPR knockdown using *VT026326-Gal4>UAS-SPR*^{RNAi1}.

Quantification and statistical analysis

All data are presented as means ± SEM. Comparisons between two groups were performed using a two-tailed unpaired *t* test; results are provided as the value *t* of the *t* distribution with *x* degrees of freedom obtained from the data. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) followed by Newman-Keuls pairwise comparisons. ANOVA results are given as the value of the Fisher distribution $F_{x,y}$ obtained from the data, where *x* is the numerator degrees of freedom and *y* is the denominator degrees of freedom. Asterisks denote the smallest significant difference between the relevant group and its controls with the post hoc comparisons (*$P < 0.05$; **$P < 0.01$; ns, not significant).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/11/eaax3432/DC1

Fig. S1. The memory defect of virgin females is specific to LTM.
Fig. S2. Controls for SPR knockdown in SPN.
Fig. S3. Dnc PDE is inhibited by mating.
Fig. S4. Controls for MIP knockdown in SPN.
Table S1. Sensory acuity of virgin females.
Table S2. Sensory acuity of flies after SPR knockdown in SPN.
Table S3. Sensory acuity of flies after SPR knockdown with SPNsplit-Gal4.
Table S4. Sensory acuity of flies after MIP knockdown in SPN.
Table S5. Sensory acuity of flies after MIP knockdown with SPNsplit-Gal4.

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