

# A requirement for the neuromodulators octopamine and tyramine in *Drosophila melanogaster* female sperm storage

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**Female sperm storage is common among organisms with internal fertilization. It is important for extended fertility and, in cases of multiple mating, for sperm competition. The physiological mechanisms by which females store and manage stored sperm are poorly understood. Here, we report that the biogenic amines tyramine (TA) and octopamine (OA) in *Drosophila melanogaster* females play essential roles in sperm storage. *D. melanogaster* females store sperm in two types of organs, a single seminal receptacle and a pair of spermathecae. We examined sperm storage parameters in females mutant in enzymes required for the biochemical synthesis of tyrosine to TA and TA to OA, respectively. Postmating uterine conformational changes, which are associated with sperm entry and accumulation into storage, were unaffected by the absence of either TA or OA. However, sperm release from storage requires both TA and OA; sperm were retained in storage in both types of mutant females at significantly higher levels than in control flies. Absence of OA inhibited sperm depletion only from the seminal receptacle, whereas absence of both OA and TA perturbed sperm depletion from both storage organ types. We find innervation of the seminal receptacle and spermathecae by octopaminergic-tyramineric neurons. These findings identify a distinct role for TA and OA in reproduction, regulating the release of sperm from storage, and suggest a mechanism by which *Drosophila* females actively regulate the release of stored sperm.**

reproductive tract | physiology | insects

In organisms with internal fertilization, female sperm storage is an essential process required for efficient gamete use and the subsequent maintenance of fertility. In female organisms that mate multiply, the storage of sperm from a previous mating allows for sperm competition between rival males and/or sperm preference by females. Sperm storage is a multistep process that includes sperm entrance and accumulation into, maintenance within, and regulated release from the sites of storage (1). Sperm are typically retained in specific regions of the female reproductive tract (RT) usually associated with the epithelium of the RT lumen (e.g., the oviductal reservoir in mammals) or in specialized, blind-ended structures that maintain sperm until they are used for fertilization (e.g., sperm storage tubules in birds and insects). Among different species, the duration that sperm remain in storage varies greatly, ranging from hours to several years (2–7). In animals as diverse as mammals and insects, the disruption of sperm storage has detrimental effects on fertility (8, 9). However, the physiological mechanisms required in females for sperm storage remain unclear.

There has been emphasis on the male contributions that promote sperm storage, most notably the action of seminal fluid proteins (SFPs). SFPs are transferred in the ejaculate during mating and constitute one group of factors required for successful sperm storage in numerous organisms (reviewed in 10, 11). For example, in cows, the bovine seminal plasma family of proteins coat sperm and enable them to bind oviductal epithelium, leading to the formation of the oviductal reservoir and extending sperms' motile life (12). In the honey bee, *Apis mellifera*, and the leaf-cutter

ant, *Atta colombica*, seminal secretions from the male accessory glands promote sperm viability in storage (13, 14). In *Drosophila melanogaster*, genetic and transgenesis tools have allowed for the dissection of the roles of SFPs in general, and of individual SFPs, in sperm storage events. Specific SFPs function at each step of the process and are required for the efficient use of sperm stored in *Drosophila* female sperm storage organs (SSOs; these organs are the single seminal receptacle and the paired spermathecae) (15) (Fig. S1). Disruption of any identified step drastically reduces the fertility of a mating pair (9, 16–19).

In contrast to the ample evidence that highlights the importance of SFPs in sperm storage, we know little about the molecular contributions of females to this process. Several lines of evidence suggest that females do control aspects of sperm storage. First, females of many insect species are able to regulate the depletion of their sperm stores. Indirect evidence of this includes species with long-term sperm storage capability, such as the leaf-cutter ant, *A. colombica*, and the honey bee, *A. mellifera* ( $\geq 6$  y) (20, 21), and the high efficiency of fertilization in some species, such as wild *Drosophila pseudoobscura* ( $>99\%$ ) (22). Direct evidence of this is observed in *D. melanogaster* females, who retain significantly more sperm in storage than normal when presented with unsuitable egg-laying substrates (23, 24). Second, secretions from female RTs have positive effects on sperm viability in the honey bee, *A. mellifera*, and in *D. melanogaster* (13, 25). Third, in *Drosophila*, sperm continue to be maintained and released long after most SFPs are no longer detected in the female RT. Fourth, when incapacitated with anesthesia shortly after mating, females of several insect species fail to accumulate normal quantities of sperm in storage (26–28). Finally, studies in numerous species indicate that in polyandrous situations, females can exert preference or choice over which male's sperm fertilize their eggs (29).

How females exert control over sperm storage is not understood. There is evidence that the female's nervous system is important in the process, suggesting active control by the female. Innervation of the female RT potentially allows for control over specific regions, as well as for the coordination of sperm movement within the RT. In the African migratory locust, *Locusta migratoria*, nervous system-mediated contractions of the spermatheca are hypothesized to trigger sperm release (30–32). In *D. melanogaster*, females with incapacitated or masculinized central nervous systems store significantly fewer sperm than controls, with the distribution of sperm stored within the SSOs perturbed, suggesting that a functional

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nervous system is required to manage sperm entry into storage as well as sperm distribution between the SSOs (33, 34). Thus, female contributions to sperm storage can serve two important functions: (i) promoting the maintenance, nourishment, or stabilization of sperm (13, 25, 35, 36) and (ii) modulating the mobilization of sperm within the female RT through nervous system inputs.

Here, we explored one aspect of nervous system inputs, using *Drosophila* as a model. We identified roles for the biogenic amines tyramine (TA) and octopamine (OA) in sperm storage. TA and OA are potent neuromodulators, considered to be the invertebrate counterparts of vertebrate adrenergic transmitters. Both molecules have been implicated in a multitude of physiological processes and overt behaviors, exerting their actions through G-coupled protein receptors (reviewed in 37). In *Drosophila*, TA and OA are synthesized from tyrosine precursors. Mutation of the enzymes responsible for their biochemical synthesis results in female sterility, reportedly attributable to abnormal egg retention; specifically, mutant females are unable to lay eggs (38–40). Sterility of females lacking OA is attributable to a defect in ovulation: mature eggs are not released from the ovary (39). Females lacking TA ovulate, but the mature oocytes released from the oviduct do not normally transit through the RT and do not reach the uterus (38). These studies suggest that OA and TA have distinct and separate activities in the egg production pathway. Because of their presence in the RT and their functions in ovulation and egg laying, OA and TA are also candidates to modulate sperm storage. However, this distinct role has not yet been examined.

Measuring an early process required for sperm entry into storage and assessing the overall number of sperm stored over time allowed us to determine whether sperm accumulation into and depletion from the SSOs are affected by the absence of OA, TA, or the OA receptor OAMB (OA receptor in Mushroom Bodies). We found that the absence of TA, OA, or OAMB did not affect sperm accumulation into storage. However, sperm were retained at significantly higher levels in storage in the absence of TA, OA, and OAMB, suggesting that these molecules are required for efficient sperm depletion from storage. Further, the SSOs appear to be differentially affected by the absence of these molecules: The lack of OA only inhibited sperm release from the seminal receptacle, whereas the absence of both OA and TA suppressed sperm release from both storage organ types. The sperm retention defect is distinct from the effects of TA and OA on ovulation and egg laying, because similar egg-laying mutants depleted their total sperm stores faster than controls. Finally, we show direct and distinct innervation of both the seminal receptacle and the spermathecae by neurons that release OA and TA. These results support a newly identified role for OA and TA in *Drosophila* reproduction, in addition to their roles in ovulation and egg laying. Our results also identify a physiological mechanism by which females manage stored sperm.

## Results and Discussion

**TA and/or OA Is Not Required for the Accumulation of Sperm into Storage in Females.** The events required for the efficient storage of sperm in *Drosophila* begin shortly after the onset of copulation. First, a series of changes in uterine conformation are initiated during mating and continue after mating ends (41). These changes, in which the lumen of the uterus opens from its tightly compacted, predated state, require male SFPs from the accessory gland for initiation (41). Failure of these changes to complete results in submaximal sperm storage, suggesting that the changes in uterine conformation are necessary for the efficient accumulation of sperm into storage (42).

If these changes in uterine shape reflect the contraction of the circular muscles surrounding the uterus (43), it is possible that the biogenic amines OA and TA might have a role in their initiation or modulation. OA and TA are synthesized in neurons that innervate the female RT and are required for contraction of

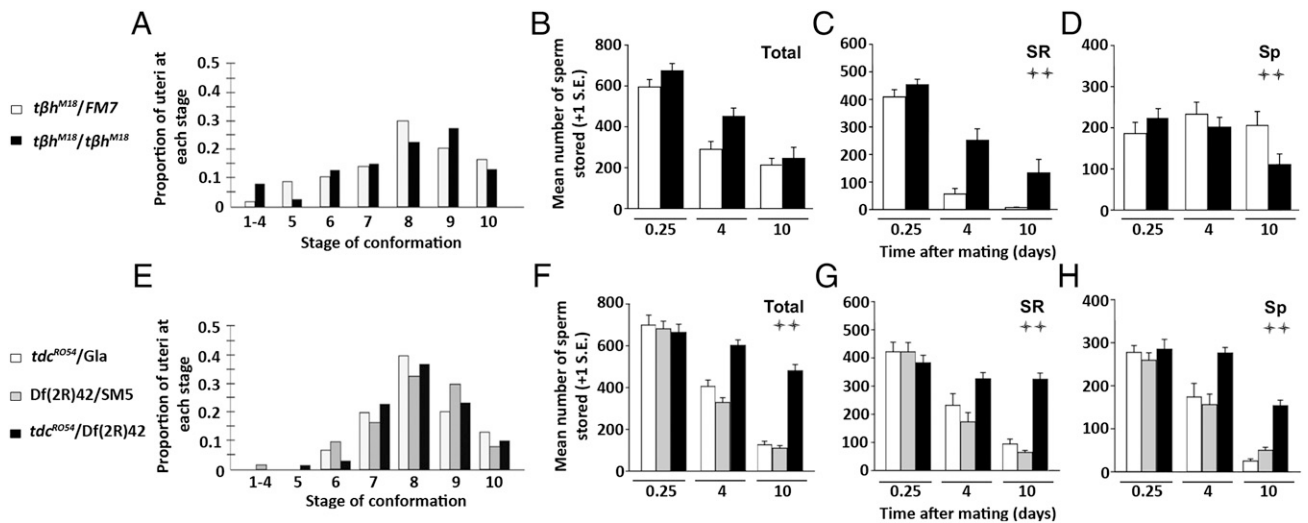
the oviduct (34, 44). To address the potential involvement of OA and TA in the uterine conformational changes and subsequent accumulation of sperm into storage, we used fly lines mutant in genes that encode the enzymes that synthesize TA from a tyrosine precursor [*tyrosine decarboxylase 2 (tdc2)*] and OA from a TA precursor [*tyramine  $\beta$ -hydroxylase (t $\beta$ h)*], generating adult females lacking both TA and OA (*tdc2<sup>ROS4</sup>*) or only OA (and with excess TA; *t $\beta$ h<sup>M18</sup>*) (38). We performed uterine conformation assays in both types of females following mating to a wild-type (WT) male (41, 42).

In the absence of OA, or both TA and OA, we detected no difference in the progression of the uterine conformational changes. OA-less and TA/OA-less mutant females progressed through the uterine stages at similar rates to control females (Fig. 1 *A* and *E*; Wilcoxon test (Rank sums),  $P = 0.88$  and  $P = 0.92$ , respectively). Consistent with this, the total number of sperm stored in OA-less and TA/OA-less females at 6 h postmating, when sperm entry into storage is complete and females have begun to lay eggs, did not differ from that of the controls (Fig. 1 *B* and *F*; *t $\beta$ h<sup>M18</sup>*:  $t = -1.61$ ,  $df = 26$ ,  $P = 0.12$ ; *tdc2<sup>ROS4</sup>*:  $F_{2,22} = 0.135$ ,  $P = 0.87$ ). Additionally, sperm numbers in the individual SSO types did not differ between mutant and control females at this time point (Fig. 1 *C* and *G*; *t $\beta$ h<sup>M18</sup>*:  $t = -1.36$ ,  $df = 26$ ,  $P = 0.19$ ; *tdc2<sup>ROS4</sup>*:  $F_{2,22} = 0.440$ ,  $P = 0.65$ ; Fig. 1 *D* and *H*; *t $\beta$ h<sup>M18</sup>*:  $t = -1.06$ ,  $df = 26$ ,  $P = 0.30$ ; *tdc2<sup>ROS4</sup>*:  $F_{2,22} = 0.521$ ,  $P = 0.60$ ). These results suggest that neither TA nor OA has a role in uterine conformational change or in the accumulation of sperm into storage.

**TA and OA Are Required for the Depletion of Sperm from Storage in Females.** Although no significant differences were observed in uterine conformation or sperm accumulation within storage in the absence of OA or TA/OA, sperm release from storage was perturbed in the absence of each neuromodulator. Examination of the total number of stored sperm in OA-less females did not reveal differences in sperm retention relative to their controls over time (Fig. 1*B*;  $F_{1,47} = 0.146$ ,  $P = 0.71$ ). However, a retention phenotype became obvious when the SSOs were examined separately. OA-less females retained significantly more sperm in the seminal receptacle than did the controls (Fig. 1*C*;  $F_{1,47} = 11.94$ ,  $P = 0.001$ ). In contrast, although control females maintained a similar number of stored spermathecal sperm over 10 d, OA-less females depleted nearly half of their spermathecal sperm, resulting in a statistically significant difference between the two groups (Fig. 1*D*;  $F_{1,47} = 12.24$ ,  $P = 0.001$ ). Together, these results suggest that either OA is necessary for normal sperm depletion from the seminal receptacle or that the elevated TA levels present in *t $\beta$ h* mutants inhibit sperm depletion there.

To distinguish between these two possibilities, we examined sperm depletion in *tdc2* mutants, which lack both TA and OA. TA/OA-less females retained significantly more sperm than controls (Fig. 1*F*;  $F_{2,66} = 116.1$ ,  $P < 0.0005$ ; least significant difference (LSD) post hoc test, mutant < controls,  $P < 0.0005$ ), and this effect was seen in both types of SSO (Fig. 1 *G* and *H*; seminal receptacle:  $F_{2,68} = 57.15$ ,  $P < 0.0005$  and LSD post hoc test, mutant < controls,  $P < 0.0005$ ; and spermathecae:  $F_{2,66} = 22.97$ ,  $P < 0.0005$  and LSD post hoc test, mutant < controls,  $P < 0.0005$ ). These results are consistent with the idea that OA is necessary for normal sperm depletion from storage. Differences between *t $\beta$ h<sup>M18</sup>* and *tdc2<sup>ROS4</sup>* females in sperm storage dynamics indicate that TA may also influence sperm retention.

To compare sperm storage between these two groups more directly, we normalized sperm storage values of mutant females by their controls and examined depletion over time. A significant genotype  $\times$  time interaction ( $F_{1,45} = 10.19$ ,  $P = 0.003$ ) was further examined to determine whether or not *t $\beta$ h<sup>M18</sup>* and *tdc2<sup>ROS4</sup>* females differed at 4 d and 10 d after mating. At each time point, females lacking OA but producing TA retained relatively fewer sperm than females lacking both OA and TA (4 d:



**Fig. 1.** OA and TA do not contribute to early sperm storage events but are required for sperm depletion from storage. Sperm storage events are shown in OA-less females (A–D) and in OA/TA-less females (E–H). Distribution of uterine conformational stages, shown as proportions, at 35 min after the start of mating in *tβh<sup>M18</sup>* (A) and *tdc<sup>ROS4</sup>/Df(2R)42* mutant females (E) compared with their sibling controls (A:  $N_{M18} = 40$ ,  $N_{FM7} = 50$ ; E:  $N_{ROS4/Df(2R)42} = 30$ ,  $N_{Gla} = 30$ ,  $N_{SM5} = 42$ ). Total sperm stored in the SSOs (B and F), only in the seminal receptacle (C and G), and only in the spermathecae (D and H) in *tβh<sup>M18</sup>* and *tdc<sup>ROS4</sup>/Df(2R)42* mutant females and their sibling controls. Differences between/among female genotypes in the depletion of stored sperm over time were analyzed using two-factor ANOVA. The significance of the genotype factor, indicating differences in sperm depletion between/among mutant and control females, is reported in the figure as follows: ++ =  $P < 0.005$  (an additional explanation of the statistical analysis is provided in *Materials and Methods*). Sp, spermathecae; SR, seminal receptacle. Sample sizes for sperm counts range from  $n = 7$ –20 (Table S1).

separate variances  $t$  test,  $t = 3.64$ ,  $df = 20.41$ ,  $P = 0.002$ ; 10 d:  $t = 7.01$ ,  $df = 16.0$ ,  $P < 0.0005$ ). Thus, the retention phenotype we observe is significantly more pronounced in TA/OA-less females than in OA-less females within the seminal receptacle (ANOVA mutant genotype effect:  $F_{1,45} = 8.83$ ,  $P = 0.005$ ) and spermathecae ( $F_{1,45} = 91.75$ ,  $P < 0.0005$ ). The sperm retention is not attributable to the lack of egg laying in our mutants. Total sperm release from storage in an egg-retention mutant (*logjam*) (45) was faster than in controls (Fig. S2). In this mutant, sperm release from the seminal receptacle occurred at a significantly greater rate than in controls. Sperm depletion from the spermathecae of *logjam*-mutant females was significantly greater than in controls only on day 10 postmating. These results suggest that the defects in sperm release in OA and OA/TA-less females is not simply a consequence of egg retention but, rather, that both OA and TA have roles in promoting sperm depletion from storage.

**Female OAMB Mutants Retain Sperm.** To dissect the role of OA and TA in sperm release further, we examined sperm storage parameters in females mutant for *oamb* (*oamb<sup>286</sup>*), the G protein-coupled receptor responsible for mediating OA's effects on ovulation in the female RT (46). OAMB has also been shown to bind TA, but cAMP accumulation occurs ~100-fold more efficiently when OA acts as the ligand (47). Several TA and OA receptors have been identified (48–52), but OAMB is the only receptor known thus far to affect female fertility. OAMB activity is required for ovulation: Mated *oamb* mutant females retain mature eggs in their RT (46). We expected that if OA and/or TA mediates its actions on sperm release through OAMB, female *oamb* mutants should recapitulate the sperm retention phenotypes of *tβh<sup>M18</sup>* mutants, and perhaps *tdc<sup>ROS4</sup>* mutants.

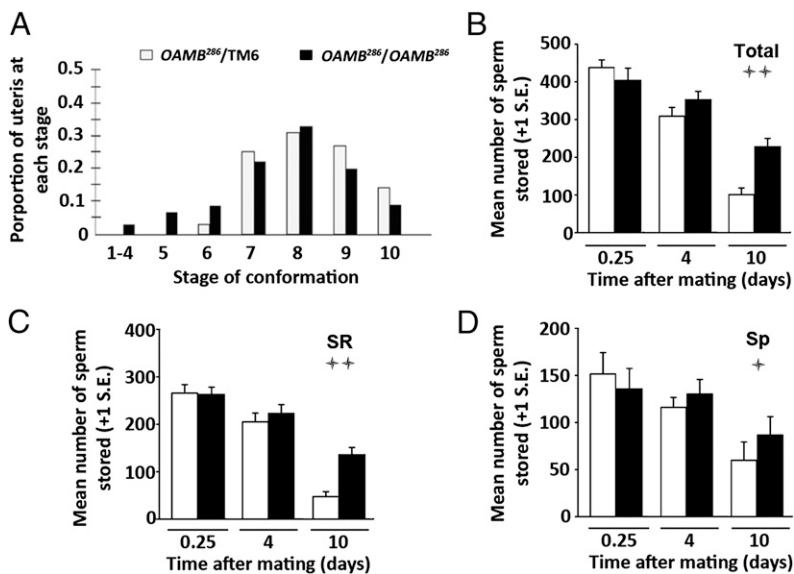
As in females lacking OA and TA/OA, uterine conformation [Fig. 2A; Wilcoxon test (Rank sums),  $P = 0.12$ ] and subsequent sperm accumulation into storage (Fig. 2B–D; total sperm: separate variances  $t = -0.832$ ,  $df = 17.36$ ,  $P = 0.42$ ) were unaffected by the absence of OAMB. Sperm stored in the seminal receptacle and spermathecae in *oamb<sup>286</sup>* females were similar to

those in controls at 6 h postmating (Fig. 2C and D;  $t = -0.051$ ,  $df = 28$ ,  $P = 0.96$  and  $t = -0.48$ ,  $df = 23$ ,  $P = 0.634$ , respectively). However, defects in sperm depletion from storage were observed (Fig. 2B;  $F_{1,43} = 30.58$ ,  $P < 0.0005$ ), with sperm in the seminal receptacle and spermathecae retained at significantly higher numbers than in controls at 10 d postmating (Fig. 2C and D;  $F_{1,51} = 12.34$ ,  $P = 0.001$  and  $F_{1,43} = 4.88$ ,  $P = 0.033$ , respectively). These results further support a role for OA (and possibly TA) in the efficient release of sperm from storage. The magnitude of sperm retention in *oamb* mutants is less severe than that seen in *tdc2* and *tβh* mutants. This may be because OA has several receptors (49, 50, 52), only one of which was non-functional in these experiments. It is also possible that the milder phenotype of *oamb* mutant females relative to *tβh* and *tdc2* mutant females reflects OA and/or TA exerting its effects through alternative receptors in the nervous system. Additionally, TA may exert its RT effects through specific TA receptors. A role for these receptors in *Drosophila* female fertility has yet to be examined.

**Innervation of the SSOs by *tdc* Neurons.** Because our genetic analysis uncovered a role for OA, TA, and OAMB in the release of sperm from storage, we examined whether the SSOs are innervated by motor neurons that release OA and TA and determined OAMB expression patterns in these tissues. To address SSO innervation, we expressed a mouse CD8-GFP (mCD8-GFP) reporter construct using a *tdc2*-GAL4 driver, which allowed for the visualization and identification of putative octopaminergic-tyramineric nerve terminals (38). Neurotransmitter release occurs at boutons, axonal swellings that contain synaptic release machinery. OA and TA are released from a subset of boutons, morphologically discernible by their small size and “wandering” appearance, indicative of type II nerve terminals (53).

In the seminal receptacle, we noted innervation by type II terminals in 8 of 8 preparations using only GFP fluorescence and in 24 of 27 preparations combining GFP fluorescence and anti-GFP immunostaining (*Materials and Methods* and Fig. 3A and B). Innervation by type II terminals was limited to compartments





**Fig. 2.** OAMB is not required for sperm entry into storage but is required for the depletion of sperm from the seminal receptacle and spermathecae. (A) Distribution of the uterine conformational stages at 35 min after the start of mating in *oamb*<sup>286</sup> mutant females compared with their sibling controls ( $N_{OAMB} = 36$ ,  $N_{TM6} = 36$ ). Total sperm stored in the SSOs (B), in the seminal receptacle (C), and in the spermathecae (D) in *oamb*<sup>286</sup> mutant females compared with their sibling controls. The difference between female genotypes in the depletion of stored sperm over time was analyzed using two-factor ANOVA. The significance of the genotype factor, indicating differences in sperm depletion between *oamb*<sup>286</sup> mutant and control females, is reported in the figure as follows: + =  $0.005 < P < 0.05$  or ++ =  $P < 0.005$  (an additional explanation of the statistical analysis is provided in *Materials and Methods*). Sp, spermathecae; SR, seminal receptacle. Sample sizes for sperm counts range from  $n = 11$ – $15$  (Table S1).

of the folded seminal receptacle most proximal to the uterus. It is possible the limited innervation selectively activates specific compartments of seminal receptacle, such as the proximal half of the receptacle, which is morphologically distinct from the distal half and contains the organ's entrance (43, 54).

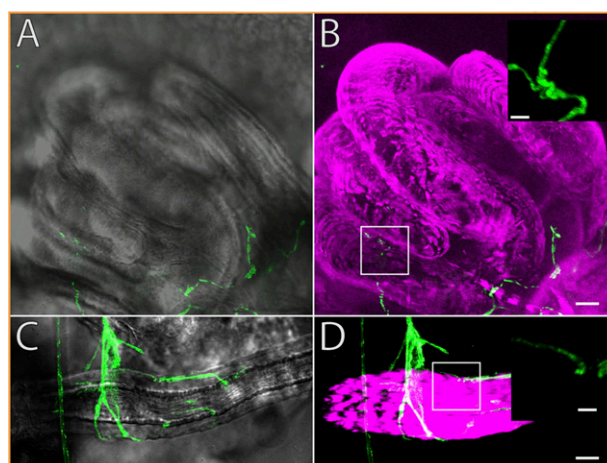
We observed spermathecal innervation by type II terminals in 10 of 11 preparations using GFP fluorescence and in 26 of 27 preparations altogether (Fig. 3 C and D). Most branching was observed in the distal one-third of the stalk, although processes were sometimes observed further from the stalk base. Longitudinal muscle fibers, visualized using phalloidin staining, were observed to stretch the entire length of the stalk. No circular fibers were observed, suggesting that neural-evoked muscle contraction may allow “pumping” of the spermathecae to extrude sperm or the products from secretory cells associated with the organ (55). Our results identify octopaminergic-tyraminerbic innervation at SSO neuromuscular junctions, but it is currently unknown whether the SSOs also receive input from other neurotransmitters (e.g., glutamate).

Because we identified a role for OA and TA in sperm storage, we examined the expression pattern of OAMB in the lower female RT. We expressed mCD8-GFP using an *oamb*-GAL4 driver (46). Surprisingly, we were unable to find substantial *oamb*-driven fluorescence in tissues of the spermathecae or seminal receptacle (Fig. S3 A and B), although the occasional spermathecal epithelial cell showed a low level of expression (Fig. S3 A and B). Interestingly, we detected high levels of OAMB expression in the parovaria (Figs. S1 and S3C). Assuming that *oamb*-GAL4 faithfully recapitulates OAMB expression, these results suggest that OAMB may be expressed at low levels in SSO tissues (beyond our detection limit) or that OA is signaling through an as yet unidentified alternative receptor. Additionally, OAMB function may be required not in the female RT but, rather, upstream in the central nervous system. Alternatively, OAMB expression from the parovaria may be influencing sperm storage. Although the function of these structures remains ambiguous, mutations that perturb development of the spermathecae in *Drosophila* also affect parovaria formation (55, 56).

**SSOs Differ in Their Sperm Storage Dynamics.** We noted striking differences between the SSOs in the magnitude of sperm retention in the absence of OA and/or TA. The retention phenotype, when normalized by the appropriate control, was consistently greater in the seminal receptacle than in the spermathecae in females lacking OA and TA/OA at 4 d postmating (paired *t* test:  $t = 9.42$ ,  $df = 14$ ,

$P < 0.0005$  and  $t = 2.84$ ,  $df = 8$ ,  $P = 0.022$ , respectively) and 10 d postmating ( $t = 3.81$ ,  $df = 10$ ,  $P = 0.003$  and  $t = 4.00$ ,  $df = 13$ ,  $P = 0.002$ , respectively). These findings are consistent with the role of the seminal receptacle as the primary SSO (57). Surprisingly, we observed a greater degree of octopaminergic-tyraminerbic innervation of the spermathecae, a result that may suggest greater female control over organs that not only store sperm but secrete molecules essential to manage stored sperm in both types of SSO (25, 55, 56).

Our observation of the differences in neuromodulator roles between the SSOs is of interest in light of previous studies in *D. melanogaster* that reported other differences between the two organ types. For example, although females with masculinized nervous systems accumulate fewer sperm in storage overall, the defect is particularly pronounced in the spermathecae (33). Also, a study using WT flies showed that females retain sperm



**Fig. 3.** Innervation of the SSOs by octopaminergic and tyraminerbic neurons. The seminal receptacle (A and B) and spermathecae (C and D) are innervated by neurons marked with mCD8-GFP (green) driven by *tdc2*-GAL4. Nomarski (A and C) and phalloidin labeling muscles (magenta) (B and D) are shown. (Insets) Higher resolution boutons are illustrated. (Scale bar: main panels, 10  $\mu$ m; Insets, 2  $\mu$ m.) Although arborizations on the seminal receptacle are often limited to the proximal portion, extensive axonal arborizations are visible on the spermathecae.

preferentially within the spermathecae when exposed infrequently to fresh medium (23). The recent identification of *wasted* mutants reported that sperm release is severely defective in these mutants, with sperm rapidly lost during each ovulation. However, sperm depletion from the seminal receptacle occurs much more rapidly than from the spermathecae in these mutants (58). In other cases, only storage in the seminal receptacle is disrupted. Removal from the male ejaculate of the SFP sex peptide, or of a network of SFPs required for sex peptide localization to sperm, results in sperm retention within the seminal receptacle but does not impair sperm release from the spermathecae (17, 19, 59). The results of these studies showcase the differences in sperm use patterns from the individual SSOs. Finally, phylogenetic analysis of SSO use across 113 *Drosophila* species has shown that not all species use both SSO types to store sperm. Although the use of both organs to store sperm represents the ancestral state, loss of sperm storage in one organ, almost always the spermathecae, has occurred multiple times (60). In *Drosophila* species that have lost sperm storage ability in the spermathecae, the organ still exists, likely attributable to the importance of the organ's secretion products in female fertility (55, 61). These results suggest that the SSO types are under different selective pressures, potentially explaining how distinct mechanisms regulating their sperm use patterns may have arisen over time.

Our results show that sperm storage dynamics in the seminal receptacle and spermathecae are modulated differently by the female. This physiological difference is reflected in differences in innervation and is attributable, in part, to the distinct actions of the two neuromodulators studied here.

**Conclusions.** Although organism-level sperm use patterns have been described in vertebrates and invertebrates, much remains to be learned about the physiological mechanisms that control the process. Despite its ubiquity across phyla, few female-derived molecules involved in sperm storage have been identified. The results we report here show that the two neuromodulators, OA and TA, are necessary for the proper release of stored sperm in *D. melanogaster* females, identifying a physiological mechanism by which the female actively modulates sperm release. These newly identified roles for OA and TA differ from their described roles in ovulation and egg laying. Because accelerated egg laying initiates within hours after the start of mating, it is intriguing that sperm release from storage would be influenced by the same molecules required for egg laying, potentially coordinating the two processes and maximizing sperm use efficiency. It is currently unknown how ovulation, egg laying, and sperm storage are coordinated at the neural circuit level, and these processes may indeed involve an identical or overlapping set of neurons. A role for the nervous system in coordinating sperm release and egg laying has been shown in the African migratory locust *L. migratoria*, where egg laying activates sensory neurons of the genital chamber, thereby increasing spermathecal contraction, which is hypothesized to cause sperm release from this organ, a neural loop that couples sperm release to egg presence in the genital chamber (30–32).

A role for both OA and TA has now been shown in two essential processes in *Drosophila* reproduction: egg laying and sperm release. Because changes in the neuronal architecture of the *D. melanogaster* oviduct occur postmating (strengthening of the synapse at neuromuscular junctions) (62), it would be interesting to see if a similar process occurs in the SSO-associated octopaminergic-tyraminergetic neurons we identified here. Because SFP receipt affects vesicle release from neurons of the female RT in *D. melanogaster* (63), SFPs with known roles in sperm storage may mediate changes in axon connectivity at SSO neuromuscular junctions. Another not mutually exclusive possibility is that SFPs

act upstream of octopaminergic-tyraminergetic neuron vesicle release. Both hypotheses await examination.

## Materials and Methods

**Flies.** Matings were carried out by crossing 3- to 5-d-old mutant or control females to 3- to 5-d-old unmated males of the Canton-5 (CS) strain of *D. melanogaster*. Fly lines used in our experiments were provided by the following: Patricia Rivlin [Cornell University, New York, NY (*tβh<sup>M18</sup>/FM7* and *tdc2<sup>ROS4</sup>/Gla*)], Kyung-An Han [University of Texas at El Paso, TX (*oamb<sup>286</sup>/TM6* and *oamb-GAL4*)], Ginger Carney [Texas A&M University, College Station, TX (*loj<sup>00898</sup>/TM3* and *loj<sup>04026</sup>/TM3*)], and the Bloomington Stock Center [Df(2R)42/SM5, *tdc2-GAL4*, and mCD8-GFP; stock nos. 3367, 9313, and 5137, respectively]. All flies were maintained on a standard yeast-glucose medium at room temperature (22 ± 1 °C) and a 12:12 light/dark cycle.

**Uterine Conformational Assay.** Uterine conformation of mated females was determined as in the study by Avila and Wolfner (42). Briefly, an unmated male and a virgin female were placed together in an empty glass vial containing moistened Whatman filter paper. Their time of mating initiation was recorded, and mating pairs were frozen at –20 °C at 35 min after the start of mating. RTs from mated females were dissected in 0.7% NaCl and visualized using an Olympus SZ61 dissection microscope. Uteri were staged as in the study by Adams and Wolfner (41). Stage distribution of mutant vs. control mates was analyzed by means of a Wilcoxon rank sum test using Jmp-In software (version 5.1.2; SAS Institute).

**Sperm Counts.** The number of sperm stored within the individual SSOs was counted at three time points postmating corresponding to maximal sperm accumulation (6 h) and various levels of storage depletion (4 d and 10 d) (17). Briefly, a null-mutant, virgin female [*tβh<sup>M18</sup>/tβh<sup>M18</sup>, tdc2<sup>ROS4</sup>/Df(2R)42, oamb<sup>286</sup>/oamb<sup>286</sup>, and loj<sup>00898</sup>/loj<sup>04026</sup>, respectively] or a control female [*tβh<sup>M18</sup>/FM7, tdc2<sup>ROS4</sup>/SM5, Df(2R)42/Gla, oamb<sup>286</sup>/TM6, and loj<sup>00898</sup>/TM3, respectively] and a virgin CS male were placed together in a vial containing standard medium until mating occurred. Males were removed shortly after mating to ensure that females mated only once. Females remained alone in their vials for the predetermined time postmating, and were then frozen and processed for sperm counts. To visualize sperm, female RTs were isolated and stained with orcein as previously described (9, 23). Sperm in the SSOs were counted using transillumination microscopy at a magnification of 1,000×. We avoided treatment biases in counting by blind-coding samples before counting sperm. We had a repeatability of >93% for each experiment, based on duplicate counts of a subset of samples.**

To determine whether sperm accumulated normally in mutant females, we used *t* tests or ANOVA to compare the mean number of sperm stored in the SSOs together (total sperm) and separately (seminal receptacle and spermathecae) at 6 h after mating between or among female groups. To determine whether sperm were retained in storage, we calculated the magnitude of sperm depletion: Sperm counts of individual females at 4 d and 10 d were subtracted from the mean number of sperm stored at 6 h after mating for the corresponding female genotype and storage organ. We then analyzed differences in the depletion of stored sperm over time (4 d and 10 d) and among female groups using two-factor ANOVA (we report *F* values from the genotype effects). This approach was preferable to one consisting of a series of paired comparisons (mutant vs. control at each time point for each storage compartment) because it has a higher statistical power for detecting differences in depletion dynamics between mutants and their controls, and because it minimizes type I statistical errors (i.e., false-positive results). To examine retention phenotypes between *th<sup>M18</sup>* and *tdc2<sup>ROS4</sup>* mutant females, we analyzed depletion of control-normalized stored sperm (mean number of stored sperm in control group at time *t* – sperm stored in each mutant at time *t*) over time (4 d and 10 d) and between female genotypes using two-factor ANOVA. The *t* tests were used as planned comparisons between female genotypes at 4 d and 10 d after mating. Assumptions of parametrical statistical tests were not violated. Analyses were performed using SPSS Statistics software (version 18.0.0; IBM SPSS, Inc.). For clarity, absolute numbers of stored sperm at each time point are presented in the figures.

**Histology and Microscopy.** Virgin *w; tdc2-GAL4, UAS-mCD8-GFP* females (*tdc2-GAL4*, stock no. 9313; mCD8-GFP, stock no. 5137; Bloomington Stock Center) or *w; oamb-GAL4/UAS-mCD8-GFP* females (46) were isolated and aged for 5–7 d and mated to 5- to 7-d-old CS males. Females were briefly transferred to ice at 2.5–4 h after mating for processing. Females were dissected open in Ikeda's buffer (64) and fixed in 3.5% paraformaldehyde (wt/vol) in Ikeda's buffer for 20 min at room temperature. Samples were washed six times in PBS and blocked for 2 h with 10% goat serum (vol/vol) in PBS with 0.2% Triton-X



(PBST). Samples were incubated with mouse  $\alpha$ -GFP at 1:500 in block solution for 2 h at room temperature. After washing eight times with PBST, samples were incubated overnight at 4 °C in 1:250 rhodamine-phalloidin and 1:2,000 Alexa 488-conjugated goat  $\alpha$ -mouse in PBST. Samples were mounted and imaged with a Leica confocal microscope at the Cornell University Life Sciences Core Laboratories Center. Reported results of innervation using  $\alpha$ -GFP were further confirmed by observing endogenous GFP fluorescence without immunocytochemistry. In addition to GFP expression, identification of type II motor neuron terminals was based on two further criteria idiosyncratic to type II terminals: wandering axons and swellings  $\leq 2 \mu\text{m}$  (53).

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