university of groningen

University Medical Center Groningen

University of Groningen

The second sex

Laturney, Meghan Elizabeth

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Laturney, M. E. (2016). *The second sex: Functions and mechanisms of sperm manipulation in female Drosophila melanogaster*. Rijksuniversiteit Groningen.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 29-10-2022

5

Neurogenetics of sperm storage in *Drosophila melanogaster*

Meghan Laturney and Jean-Christophe Billeter

ABSTRACT

Females of many insect species store sperm that they acquire during copulation in specialized sperm storage organs (SSOs), allowing for prolonged fertility. Although sperm storage is under the control of the female nervous system, how genes and neurons influence this process is poorly understood. Here, we performed a neurogenetic screen for neurons that support sperm storage in the two SSOs of *Drosophila melanogaster* females: the long tubular seminal receptacle and the paired mushroom-shaped spermathecae. Using a novel sperm storage scoring system, we determined patterns of storage in both SSOs in females subjected to artificial neuronal activation or silencing in neurons expressing genes associated with female reproductive behaviours. We identified 9 genes, whose expression patterns overlap with that of 13 Gal4 drivers expressed in neurons, that influence sperm storage, some of which directly innervate the female reproductive tract. Phenotypic analysis of mutant alleles or RNAi knock-downs of these genes indicate that they function within the neurons identified in our screen to influence this process. Overall, we find that sperm storage in the two different SSOs is supported by shared and unique genes and neuronal populations. Interestingly, we propose that storage in the spermathecae is influenced by the sex peptide signaling pathway, a major regulator of the female post-mating response. Our results add to the growing body of knowledge uncovering the role of the female nervous system in determining the fate of sperm after copulation.

INTRODUCTION

In organisms with internal fertilization, mating functions to transfer the male-produced ejaculate to the female reproductive tract. This ejaculate not only contains sperm but also seminal fluid composed of a suite of peptides important for male prolificacy and female fecundity. Equally critical to the fusion of the gametes, reproduction also involves the interaction of the seminal fluid peptides and the female-produced glandular secretions and cellular substrate that support sperm management: sperm storage, maintenance, and usage within the female reproductive tract (Adams and Wolfner, 2007; Lodi and Koene, 2016; Parada-Bustamante et al., 2016; for a review see Chen, 1984; Neubaum and Wolfner, 1999; Schnakenberg et al., 2012). The mechanisms of sperm usage and egg laying are well understood in many insect species (Avila et al., 2012; Bloch Qazi et al., 1998; Middleton et al., 2006; Rezával et al., 2014), as well as the identification of several mechanisms in the female reproductive tract that aid in sperm maintenance in insects as well as birds and mammals (Iida and Cavener, 2004; Prokupek et al., 2008; for a review see Degner and Harrington, 2016; Holt and Fazeli, 2016). Comparatively, the process of sperm storage is much less understood.

Sperm storage by females is a widespread phenomenon, occurring in a range of animals such as insects, reptiles, birds and mammals (Birkhead and Møller, 1998; Holt and Lloyd, 2010; Neubaum and Wolfner, 1999; Simmons, 2001). Sperm can be stored for various durations (Holt and Fazeli, 2016), and in a variety of different ways including simple adhesion to the female reproductive tract (Talevi and Gualtieri, 2010) or accumulation and confinement to specialized closed reservoirs (Heifetz and Rivlin, 2010). Investigations on sperm dynamics have indirectly indicated that sperm storage is likely mediated by biochemical and/or morphological engagements of the female reproductive tract as sperm motility itself is insufficient to explain the speed and efficiency of sperm amassment into SSOs (reviewed by Linley and Simmons, 1981). More direct evidence from studies that impair females during this process suggests the active involvement of muscle contractions (Bloch Qazi et al., 1998; Hellriegel and Bernasconi, 2000; LaMunyon and Eisner, 1993). Moreover, earlier studies indicated that an intact female nervous system was necessary for sperm storage (Arthur et al., 1998), implying that females may exert direct control over sperm in their reproductive tract. However, no direct mechanisms that allow for such female control have been identified.

Understanding the process of female-mediated sperm storage not only sheds light on mechanisms of female fecundity, but also on mechanisms of post-copulatory

female choice. When a female re-mates, the ejaculates of multiple males interact within her reproductive tract and compete for a place in storage, and ultimately the chance to sire offspring. As there are various costs associated with copulation, females should maximize the benefits of polyandry by increasing the genetic diversity and/or genetic quality of offspring, which involves controlling sperm's fate (Lüpold et al., 2013; Manier et al., 2010; 2013). Indeed, there is evidence that multiply-mated females can influence sperm usage as female mating behaviour (Chapter 4) and female genetic variation have been associated with altered patterns of paternity (Chow et al., 2010; 2012; Giardina et al., 2011). Moreover, some of these genes are expressed in neurons that innervate the reproductive tract (Chow et al., 2012). Identification of female-mediated mechanisms supporting sperm storage would complete our knowledge of the processes of sperm manipulation by females and how female fecundity is maximized.

The use of the model organism, *Drosophila melanogaster*, with its wealth of molecular tools and high similarity of gene coding sequences with mammals, is expected to generate much needed knowledge on sperm storage (for a review see Heifetz and Rivlin 2010). The movement of sperm into storage in this species is a very controlled process. Upon reception of the ejaculate, the female reproductive tract undergoes stereotypical and classifiable changes (Adams and Wolfner, 2007), requiring both male and female derived molecules (for a review see Schnakenberg et al., 2012; Wolfner, 2009). The process of sperm storage starts after the first minutes of mating, and continues until the female ejects all unstored sperm, unused portions of the ejaculate, and a gelatinous mating plug from her uterus (Adams and Wolfner, 2007; Manier et al., 2010). *D. melanogaster* females, like other insects, store sperm in specialized sperm-storage organs (SSOs) in the reproductive tract. These SSOs consist of the seminal receptacle (SR), a long blind-ended tube that tapers at the distal end, and the two mushroom-shaped spermathecae (Sp). Although both SSOs accumulate and hold sperm, each of these organs has a unique function (Wolfner, 2011). The SR is the primary storage organ, holding ~400 sperm at maximum (Manier et al., 2010). The Sp, on the other hand can store ~130 sperm, but also produces molecules in the spermathecal secretory cells which help recruit sperm and maintain their health in both SSOs (Schnakenberg et al., 2011; for a review see Heifetz and Rivlin 2010 and Schnakenberg et al., 2012). These molecules, along with male-derived components (Avila et al., 2011; Wolfner, 1997) and unidentified female cellular substrate most likely interact to accomplish sperm accumulation into the SSOs. Yet difficulties in directly observing the fate of sperm within the female reproductive tract have limited our understanding of the sperm storage process.

The genetic toolkit available in the model organism *D. melanogaster* provides an opportunity to fill this gap. The tools that have been developed in this species allow for the access of specific tissues, quickly identifying populations of neurons and cellular components that support sperm manipulation (Avila et al., 2012; 2015; Chow et al., 2012; Lee et al., 2015) and fluorescently tagged sperm that allows for visualization within the female reproductive tract (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Manier et al., 2010). Here we made use of these tools to identify genes and populations of neurons expressing those genes in *D. melanogaster* females that participate in the process of sperm storage. We reasoned that a genetic network acting with a hypothetical neuronal circuitry that controls sperm storage would likely be shared with other post-mating behaviours as a large pleiotropy has been found in the genes and neurons that control other aspects of the post-mating response (PMR) including female sexual receptivity and egg laying behaviour (Hasemeyer et al., 2009; Rezával et al., 2012; Soller et al., 2006; Yang et al., 2009; Yapici et al., 2008; reviewed in Chapter 2). Indeed, after mating *D. melanogaster* females show reduced sexual receptivity, increased ovulation and egg laying, and increased food intake (Carvalho et al., 2006) with a specific shift in food preference from sugar to yeast (Ribeiro and Dickson 2010). The initiation of the PMR is elicited by male-derived seminal fluid proteins (SFPs; for a review see Avila et al., 2011; Schnakenberg et al., 2012): the main actor there being sex peptide, which has been associated with all known aspects of female post-copulatory behaviour (for a review see Chapter 2). We functionally tested the involvement of subsets of neurons that express genes associated with female reproductive behaviours, especially those involved in the outcome of sperm competition (Chow et al., 2012). Functional testing was achieved by driving the expression of a temperature sensitive cation channel, dTrpA1, that can activate neuronal firing, as well as a temperature sensitive dynamin, Shibire, that can block synaptic transmission to manipulate the activity of specific neuronal population. In order to avoid the laborious and time consuming task of counting sperm in storage, we developed a relatively high-throughput sperm storage scoring system to assess differences in storage patterns of manipulated females compared to controls, and validated this scoring system with effects on fecundity. We identified 13 Gal4 lines expressed in neuronal populations that can influence sperm storage when artificially activated or silenced. We further explored their relationship to SSOs by visualizing their innervation of the female reproductive tract. Finally, we tested the function of candidate genes in these neuronal populations by targeted gene silencing. We discovered that the SR and the Sp have both shared and unique gene expression and neuronal circuitry influencing the sperm storage. Our results add to the growing body of knowledge uncovering the active role of females in reproduction.

MATERIAL AND METHODS

Drosophila stocks and rearing

Flies were reared on medium containing agar (10g/L), glucose (167mM), sucrose (44mM), yeast (35g/L), cornmeal (15g/L), wheat germ (10g/L), soya flour (10 g/L), molasses (30 g/L), propionic acid and Tegosept; and is referred to as fly food in this chapter. Flies were raised in a 12:12 hr light/dark cycle (LD 12:12) at 25°C, unless explicitly stated. Virgins were collected 0-8 hrs after eclosion using CO_2 anesthesia and were aged in same-sex groups of 20 in vials for 5-7 days prior to testing. All wild-type flies were from the *Canton-S* strain. Other strains are described below.

A series of Gal4 lines driven by fragments or full promoter region of genes implicated in female reproduction were used. These lines and the rational for their use in this study are described in Table 1. The Gal4 lines with a Bloomington Stock Center order number (Table 1) were obtained from Bloomington Stock Center. *5-HT$_7$*-gal4 (*w^-*;+;p{*5-HT$_7$Dro*-gal4}) was a gift from D. Nässel, *ppk*-gal4 (+;p{*ppk*-gal4};+), *Tdc2*-gal4 (*w^-*;+;p{*Tdc2*-gal4}), *ddc*-gal4 (*w^{1118}*;p{*ddc*-gal4};+), *fru*-gal4 (+;+;*fru*^{gal4}/*TM3*,*Sb*,*e*), *cha*-gal4 (+;+;*cha*-gal4), and *dsx*-gal4 (+;+;*dsx*^{gal4}/*TM6b*) were gifts from S.F. Goodwin.

To temporarily activate specific neuronal populations in females, we drove the expression of the temperature gated calcium ion channel *dTrpA1* with different Gal4 drivers. We crossed females with the genotype *"w^{1118};+;UAS-dTrpA1/TM6b"* (Hamada et al., 2008, obtained from Bloomington Stock Center #26264) to males from various Gal4 lines (Table 1). UAS control females were generated by crossing "*w^{1118};+;UAS-dTrpA1/TM6b*" females to "*w^{1118}*;+;+" males. For one Gal4 line (*fru^{16}*-gal4), Gal4 control females were generated by crossing +;+;*fru^{16}*-gal4 to wild-type *Canton-S* females as this line was originally back-crossed to a *Canton-S* stock.

To temporarily silence specific neuronal populations in females, we drove the expression of the temperature sensitive blocker of synaptic transmission *Shibire^{ts1}* by crossing females with the genotype *"w^{1118};+;UAS-shi^{ts1}"* (Kitamoto 2001; obtained from Bloomington Stock Center #44222) to males from various Gal4 lines (Table 1). UAS control females were generated by crossing "*w^{1118};+;UAS-shi^{ts1}*" females to "*w^{1118}*;+;+" males. All offspring from crosses involving *dTrpA1* and *Shibire^{ts1}* developed and were maintained at 18°C.

Candidate gene	Gal4 Line	BSC#	Reason for use	Gene function	Reference (Gal4)
corazonin (*crz*)	Crz-gal4	51976	coordination of ejaculate transfer[1]	neuropeptide hormone activity	Tayler *et al.* 2012
5-HT$_7$-Dro	5-HT7-gal4		effect on female mating behaviour[2]	serotonin GPCR	Becnel *et al.* 2011
apterous (*ap*)	Ap-gal4	3041	effect on female receptivity[3]	transcription factor	Lundgren *et al.* 1995.
choline acetyltransferase (*cha*)	Cha-gal4		involvement of neurotransmitter	synthesis of acetylcholine	Salvaterra and Kimoto 2001
teashirt (*tsh*)	Tsh-gal4	3040	labels all neurons in thorax	transcription repressor	Calleja et al. 1996
vesicular glutamate transporter (*vglut*)	Vglut-gal4	1102022	labels glutamatergic cells	neurotransmitter transport	Daniels et al. 2008
dopa decarboxylase (*ddc*)	Ddc-gal4		production of female behavior[4]	synthesis of dopamine and serotonin	Li et al. 2000
dNPF	dNPF-gal4	25682	sexually dimorphic expression	Neuropeptide F protein product	Krashes *et al.* 2009
doublesex (*dsx*)	dsx-gal4		labels neurons involved in PMR[5]	transcriptional activator	Rideout et al. 2010
fruitless (*fru*)	Fru-gal4		labels neurons involved in PMR[6,7]	transcription factor	Stockinger et al. 2005
	fru^{16}-gal4				Billeter and Goodwin 2004
pickpocket (*ppk*)	ppk-gal4		labels neurons involved in PMR[6,7]	channel activity	Grueber et al. 2003
tyrosine decarboxylase 2 (*dTdc2*)	dTdc2-gal4		egg-laying behavior[8]	neural tyramine and octopamine production	Cole et al. 2005
caupolican (*caup*)	GMR80B06-gal4	40068	effect on sperm competition[9]	transcription factor	Jenett *et al.* 2012
	GMR80C01-gal4	40072			
	GMR80C03-gal4	47056			
CG15765	GMR49D10-gal4	38683	effect on sperm competition[9]	carbohydrate receptor	Jenett *et al.* 2012
	GMR47E07-gal4	49568			
	GMR47D09-gal4	50306			
	GMR49B06-gal4	50409			
CG32532	GMR35A12-gal4	49814	effect on sperm competition[9]	transcription factor	Jenett *et al.* 2012
	GMR35D10-gal4	49910			
5-HT$_{2b}$-Dro	GMR74A09-gal4	39838	effect on sperm competition[9]	serotonin GPCR 2b	Jenett *et al.* 2012
	GMR74A11-gal4	39839			
	GMR83A12-gal4	40348			
	GMR83A10-gal4	48371			
	GMR83B06-gal4	48372			
dunce (*dnc*)	GMR12F01-gal4	48512	female receptivity and egg-laying[10]	cAMP phosphodiesterase	Jenett *et al.* 2012
	GMR12F06-gal4	48516			
	GMR12H08-gal4	48532			
dissatisfaction (*dsf*)	GMR29G10-gal4	49501	female receptivity[11]	hormone receptor	Jenett *et al.* 2012
	GMR30C11-gal4	49530			
lozenge (*lz*)	GMR25F04-gal4	49128	female fertility and RT morphology[12]	transcription factor	Jenett *et al.* 2012
	GMR25H03-gal4	49143			
paralytic (*para*)	GMR14H04-gal4	48665	effect on sperm competition[9]	sodium channel	Jenett *et al.* 2012
	GMR14H05-gal4	48666			
	GMR16H03-gal4	48744			
	GMR18C12-gal4	48809			

Candidate gene	Gal4 Line	BSC#	Reason for use	Gene function	Reference (Gal4)
sex peptide receptor (*SPR*)	GMR78E11-gal4	40001	sex peptide dependent PMR[13]	GPCR for sex-peptide	Jenett *et al.* 2012
	GMR78E12-gal4	40002			
	GMR78F01-gal4	40003			
	GMR78F03-gal4	40004			
	GMR78F08-gal4	40008			
	GMR78F09-gal4	40006			
	GMR78F10-gal4	40007			
	GMR78G01-gal4	40009			
	GMR78G02-gal4	40010			
	GMR78G03-gal4	40011			
	GMR78G04-gal4	40012			
	GMR78G06-gal4	40013			
	GMR78G08-gal4	40014			
	GMR78G09-gal4	40015			
	GMR78G05-gal4	41308			
	GMR78F02-gal4	47020			
	GMR78F07-gal4	47409			
	GMR78F06-gal4	48346			
	GMR78F12-gal4	48622			

Table 1. Description of Gal4 lines used to identify neurons supporting sperm storage. This collection of Gal4 lines were used in combination with UAS-dTrpA1 and UAS-shibire to temporary activate or silence, respectively, specific subpopulations of neurons and assess their involvement in active sperm storage by females. Gene name, function, and motivation behind its involvement are listed. All gene functions were derived from description of genes on flybase.org. Gal4 lines either drove the expression of the desired genetic construct in a full or partial expression pattern. BSC# = Bloomington Stock Center order number; GPCR = G-protein coupled receptor; PMR = post-mating response; cAMP = 3'-5'-cyclic adenosine monophosphate; RT = reproductive tract. Full references can be found in Bibliography: 1) Tayler et al. 2012; 2) Becnel et al. 2011; 3) Ringo et al. 1991; 4) Wicker-Thomas and Hamann 2008; 5) Rezaval et al. 2012; 6) Hasemeyer et al. 2009; 7) Yang et al. 2009; 8) Cole et al. 2005; 9) Chow et al. 2012; 10) Bellen et al. 1987; 11) Finley et al. 1997; 12) Anderson 1945; 13) Yapici et al. 2008.

All females were mated to males with fluorescently tagged sperm tails with green fluorescent protein (*dj*GFP; Santel et al., 1997; obtained from Blooming Stock Center #5417).

To visualize the innervation of the female reproductive tract of the selected Gal4 lines from our screen, males from Gal4 lines with a significant impact on female sperm storage were crossed to females from the line UAS-*mcd8::GFP* (w^- ;p{10xUAS-IVA-*mCD8*::GFP};+; Lee and Luo, 1999; from Bloomington stock center #32186), which encodes a membrane-bound GFP that allows visualization of cellular processes, such as neuronal projections.

To test for the functionality of candidate genes expressed in neurons influencing sperm storage, we assessed sperm storage in the following mutant females obtained from Bloomington Stock Center: dnc^1 (dnc^1;+;+; Davis and Kiger, 1981; #6020); $para^{st76}$ ($para^{st76}$;+;+; Siddiqi and Benzer, 1976; #26701); spr^- (w^-, *Df(1R)Exel6234*;+;+; Yapici et al. 2008; #7708); and egh^7 (egh^7/*FM7*/*Dp(1;2;Y)*w^+;+;+; Wandall et al., 2005; #3902). To specifically test the function of the gene of interest in the identified neuronal population, we knocked-down the gene product with RNA interference. Females from the various UAS-RNAi lines were crossed to males from the Gal4 lines with a significant impact on female sperm storage. Controls were generated by crossing UAS-RNAi females to "w^{1118};+;+" males; and males from the Gal4 lines to "w^{1118};+;+" females. *spr* RNAi (Dietzl et al., 2007; Yapici et al., 2008) was a gift from M. Soller, all others were obtained from Bloomington Stock Center from the Transgenic RNAi Project (TRiP; Perkins et al., 2015), *para*-RNAi y^1v^1;+;P{y^+v^+=TRiP.HMS00868} (#33923); *5-HT*$_{2b}$-RNAi y^1v^1;P{y^+v^+=TRiP.HMJS22882;+} (#60488); *ap*-RNAi (#41673) y^1v^1;+;P{y^+v^+=TRiP.HMS02207}, *dnc*-RNAi y^1v^1;P{y^+v^+=TRiP.HMC03573;+} (#53344), and *Tdc2*-RNAi y^1v^1;+;P{y^+v^+=TRiP.JF01910 (#25871).

Gal4 screen for neurons involved in sperm storage

Individual experimental and control females of various genotypes were paired with a single male that produced green fluorescently labeled sperm (*dj*GFP) in a petri dish 55 x 8mm with fly food. Transferring of all flies in all experiments was done by gentle aspiration. The time that copulation began was noted. In order to activate or silence the specific subpopulation of neurons in temporally regulated manner, *dTrpA1* or *Shibire*^{ts1} was expressed with use of various Gal4 driver lines. For these experiments, immediately following the start of copulation, the dish containing the mating pair was transferred to either an incubator set at 29°C, or to the lab bench

beside the incubator and remained at 22°C. For all other experiments, all dishes containing the mating pair remained on the bench at 22°C. Regardless of experiment and heat condition, 1 hour after the start of mating (1 hr ASM) females were removed from the dish, placed into a 0.5 ml centrifuge tube, and flash frozen in liquid nitrogen. 1 hr ASM was chosen as it reflects the time that maximum sperm storage is achieved (Manier et al., 2010). Samples were then stored at -20°C until dissections of the female reproductive tracts (RTs) were performed.

To score sperm storage, females were placed into PBS and the entire RT was dissected out. RTs were first checked for the presence of a mating plug that naturally auto-fluoresces (Lung and Wolfner, 2001) using a MZ10F stereomicroscope equipped with filters for UV light and the presence of a sperm mass in their uterus with use of a same MZ10F stereomicroscope equipped with a filter for GFP. If both plug and sperm mass were missing from the RT, it was noted that the female had ejected them. It was also noted if females had ovulated (had an egg in either the oviduct or the uterus) or if the sperm was not contained in the uterus but had moved to the oviducts. The proportion of females that ejected (ej), had ovulated (ov) or had sperm in their oviducts (ovi) was calculated for each genotype in each temperature condition. Additionally, the conformation of the RT may influence sperm storage as the uterus must fully unfold to permit sperm and seminal fluids to enter (Adams and Wolfner, 2007). Therefore, a "bending" score (b) was also given to each RT, which ranged between 0 and 3 (0 for completely unfolded, 1 for a small curve, 2 a slightly more pronounced curved, and 3 for severe bend greater than or equal to 45 degrees).

The amount of sperm in the two types of storage organs was assessed with a novel sperm storage scoring system. Scores ranged between 0 and 3 (0 for a complete lack of sperm stored to 3 for a full organ, see Figure 1) with use of the same MZ10F stereomicroscope equipped with a filter for GFP. A score for the seminal receptacle, and a score for each the two spermathecae (averaged) was generated for each female, and averaged for each genotype within a specific temperature condition and compared within each genotype across temperature conditions. All scoring was done blind so that scorer was unaware of both genotype and temperature condition of the female.

Fecundity assay with Gal4/UAS-dTrpA1 females

Individual Gal4/UAS-*dTrpA1* and UAS-*dTrpA1* control females were paired with a single wild-type male in a petri dish 55 x 8mm with food. The time copulation began was noted and females were immediately transferred to an incubator set at

29°C or to a different lab bench and remained at 22°C. 6 hrs ASM dishes were removed from the temperature condition and males were discarded. Any females that re-mated during this time were also discarded. To investigate the influence of the timing of this manipulation, some females (*fru*^{16}-gal4/UAS-*dTrpA1* and the controls *fru*^{16}-gal4/+ and +/UAS-*dTrpA1*) experienced the heat treatment for only 1 hr. Similar to the previous treatment, females were placed either 29°C or to a different lab bench at 22°C immediately at the time of copulation, however, pairs were removed 1 hr ASM. Regardless of the end time, immediately after the dishes were removed from the temperature condition females were transferred to a fresh vial containing fly food and placed at 18°C with a 12 hr light-dark cycle to prevent temperature-induced transgene activation and allowed to oviposit for ~48 hrs (vial 1). 48 hrs ASM, females were transferred into fresh vials for another 48 hrs (vial 2). After this, females were transferred again where they remained for 15 days (vial 3) and then discarded. The offspring developed under the same standard conditions. 18 days after the female was placed in the vial (1, 2, or 3), the offspring began to eclose. The adult offspring was counted. Due to variation in eclosion time, each vial was counted three times with at least a two-day between counts, in order to ensure all offspring was accounted for.

Egg laying assay with Gal4/UAS-dTrpA1 females

Individual Gal4/UAS-*dTrpA1* and UAS-*dTrpA1* control females were paired with a single wild-type male in a petri dish 55 x 8mm with fly food. After a successful copulation, males were removed and females were transferred to a fresh vial containing fly food and placed either in an incubator set at 29°C or to a different lab bench at 22°C and left for 24 hrs. After this time, females were discarded and number of eggs that were present were counted and compared between groups.

Re-mating assay with Gal4/UAS-dTrpA1 females

Individual Gal4/UAS-*dTrpA1* and UAS-*dTrpA1* control females were paired with a single wild-type male in a petri dish 55 x 8mm with fly food. After copulation, females were transferred singly to a fresh vial where they were kept overnight. 24 hrs ASM, the females were transferred to a fresh small petri dish 55 x 8mm with fly food, and a single virgin wild-type male was aspirated into each dish, and dish was immediately placed either into a 29°C incubator or on the bench next to the incubator at 22°C. The dishes were checked every 10 minutes for re-mating for a total of 2 hrs, and each re-mating event was noted. The proportion of females that re-mated for each genotype was determined.

Immunohistochemistry and neuronal innervation of the female reproductive tract

Staining of the female reproductive tract was performed as described in Billeter and Goodwin (2004). The stained reproductive tract was imaged on an sp8 Leica confocal microscope equipped with a 488nm Blue laser 20mW. Images were acquired using the Leica Application Suite X software and processed using the FIJI software (NIH).

Data analysis

To determine if activating or silencing the specific population of neurons influenced sperm storage, we independently scored the seminal receptacle (SR) and spermathecae (Sp) for females placed at 22°C (control group) and females at 29°C (activated or silenced) for each indicated genotype (Gal4/UAS-*dTrpA1* or Gal4/UAS-shi^{ts1}, respectively). We noticed that, in both UAS control groups, females that were exposed to the elevated temperature treatment of 29°C stored slightly more sperm compared to females of the same genotype left at 22°C (both the SR and Sp for w^{1118}*;+;UAS-dTrpA1/+* females, and only for the Sp for w^{1118}*;+;UAS-*$shibire^{ts1}$*/+* females). This indicates that temperature had a slight influence on sperm storage independent of neuronal activity during our manipulations. Therefore, we took this minor increase due to temperature into our selection process and calculated a normalized difference for each organ (SR^* and Sp^*). From these normalized differences, we created an arbitrary cut-off decision point at 0.90, so that it was determined for all lines and for each organ if females: increased, decreased, or indicated no change in sperm storage due to neuronal activity manipulation. This gave rise to 9 possible patterns of sperm storage when both organs were taken into account: SR (increase, decrease, no change) and Sp (increase, decrease, no change). We initially selected all lines in which artificial neuronal activation or silencing either lead to an increase or decrease in sperm storage relative to controls. We examined the result of this neuronal manipulation on fecundity to examine if changes seen in storage resulted in altered fecundity, and to validate our novel scoring system. From this we determined that an increase in score in the Sp did not result in a change in offspring production. Therefore, we did not include two lines that only displayed increased sperm storage in the Sp: *GMR80C01*-gal4 and *GMR12F01*-gal4. However, *GMR12H08*-gal4 was included, as it was a large increase. Furthermore, we excluded *tsh*-gal4 from this selection as the expression pattern is too broad to be informative, as well as two other gal4 lines which were not available to us at the time of experiments (*GMR78G01*-gal4 and *GMR78G06*-gal4). We decided to include $5\text{-}HT_7$-gal4 as we observed a decrease in storage in both SSOs when neurons were artificially activated, and an increase when neurons were artificially silenced as it is a pattern of interest.

Statistical analysis of the various relationships between storage scores and other screen phenotypes were assessed using GraphPad Prism 5 (GraphPad Software Inc., USA). In all tests, deviations were considered significant for $\alpha < 0.05$. The distribution of all data sets was checked with a D'Agostino & Pearson omnibus normality test. A Spearman r or a Pearson r (for non-normally and normally distributed data, respectively) was used to determine if a relationship existed between the following measurements: changes in scores due to activation and silencing of the neurons labeled by the same Gal4 line in the SR and the Sp; between the scores of the SR and Sp within the specific Gal4-UAS combination for both activation and silencing; bending scores with the scores of the SR and Sp for both activation and silencing; and percentage of females that ejected during the 1 hr neuronal manipulation with the scores of the SR and Sp for both activation and silencing.

Statistical analysis of the effect of artificial activation of neurons on fecundity, egg laying and re-mating behaviour was assessed using GraphPad Prism 5 (GraphPad Software Inc., USA). In all tests, deviations were considered significant for $\alpha < 0.05$. The distribution of the fecundity and ovipositioning data was checked with a D'Agostino & Pearson omnibus normality test. For the 12 different Gal4 lines used to validate our scoring system, the mean number of offspring produced with neurons activated during sperm storage and number of eggs laid by a female with neurons activated for the first 24 hours after mating was compared to that of the control female of the same genotype with a t-test or a Mann-Whitney U, for normally and non-normally distributed data, respectively. To compare the mean number of offspring produced by *fru*^{16}-gal4/UAS-*dTrpA1* females as well as their controls (*fru*^{16}-gal4/+ and +/UAS-*dTrpA1*) at 22°C and 29°C the distribution of the data set was first checked with a D'Agostino & Pearson omnibus normality test and mean progeny produced by the different groups of females were assessed with a two-way ANOVA with genotype and temperature as the main effects. Post hoc Bonferroni tests were used to determine differences between specific groups. To compare re-mating behaviour among the conditions of neuronal activity within each GAL4 line, we used Fisher's exact test.

Statistical analysis of the genetic mutants and knock-downs was assessed using GraphPad Prism 5 (GraphPad Software Inc., USA). In all tests, deviations were considered significant for $\alpha < 0.05$. All data were analyzed using nonparametric tests with a Gaussian approximation. Mann-Whitney U test were used to analyze the differences in sperm storage between genetic mutants and their genetic controls

for each sperm storage organ. A Kruskall Wallis test was used to analyze the differences in sperm storage between genetic knock-downs and their controls for each sperm storage organ. If a significant effect was found, a Dunn's Multiple Comparison test was used to examine differences between knocked-down females and each control. In the case of *para* knock-down experiments, no UAS-*para* RNAi control females were available for the assay. Therefore differences between *para* knock-down females and the Gal4 controls were analyzed with a Mann-Whitney U test.

RESULTS AND DISCUSSION

Gal4 screen for neurons mediating sperm storage by females

The aim of this study was to identify neuronal populations that mediate sperm storage in the two sperm store organs (SSOs): the long tube shaped seminal receptacle (SR) and the two mushroom-shaped spermathecae (Sp). To accomplish this, we used the Gal4-UAS system to acutely manipulate the activity of defined groups of neurons and observed changes in sperm storage. We reasoned that neural circuits that control sperm storage are likely to be shared with other reproductive behaviours, and to express genes that influence female reproduction in general. To test this hypothesis, we chose 56 Gal4 lines that contain the full or partial regulatory promoter sequences of genes previously associated with female reproduction and that are expressed in the central nervous system (Table 1). We used these Gal4 drivers to target the expression of either the temperature sensitive calcium ion channel *dTrpA1* (with UAS-*dTrpA1*; Hamada et al., 2008) or the temperature sensitive synaptic blocker *Shibire*^{ts1} (UAS- *shi*^{ts1}; Kitamoto, 2001) in different neuronal populations. By placing these experimental females at 29°C immediately at the start of copulation with a male with GFP-labelled sperm, we artificially and acutely activated neurons that expressed *dTrpA1* or silenced neurons that expressed *Shibire*^{ts1}. Control females also expressed the same thermosensitive machinery in the same subset of cells but were left at permissive temperatures (22°C) and therefore have normal neuronal activity. To determine if the neuronal manipulation influenced sperm storage, the female reproductive organs were dissected and the amount of sperm stored in the SR or the Sp was scored using a relatively high-throughput scoring system that we developed here ranging from 0 to 3, with 0 indicating no sperm in storage and 3 indicating a maximum sperm in storage (Figure 1). The scores of each control and experimental line were compared to determine whether treatment resulted in an increases or decreases in sperm storage compared to controls. We manipulated neuronal activity from the start of

copulation and applied it continuously for 1 hour (Figure 2A). We found changes in sperm storage in about half of the lines tested (Table 2). The influence of our neuronal manipulation ranged from visible increases in storage compared to controls to a complete lack of storage in our manipulated females, indicating that manipulating diverse neuronal populations can lead to a modulation of sperm storage rather than a binary response (storage or no storage). Furthermore, storage was both increased and decreased with each type of neuronal manipulation (artificial neuronal activation or silencing) showing that activating neurons does not necessarily result in a decrease in sperm storage and silencing neurons in an increase in storage- or vise versa (Table 2).

Moreover, we found no general inverse relationship between activating and silencing the same neuronal population on sperm storage in the SR (Spearman r = 0.16, p = 0.26; Figure 2B) or the Sp (Pearson r = -0.06, p = 0.69; Figure 2C). In other words, if artificially activating a specific group of neurons resulted in decreased sperm storage, silencing did not result in increased sperm storage- and vice-versa. This suggests that sperm storage neurons function to either recruit or restrict sperm into the SSOs, but usually not both. Finally, we found a significant positive correlation between the scores of the SR and Sp within the specific Gal4-UAS combination for both activation (Pearson r = 0.47, p = 0.0003; Figure 2D) and silencing (Pearson r = 0.57, $p < 0.0001$; Figure 2E), meaning that changes in sperm storage in the SR was usually accompanied with changes in the Sp in the same direction. However, as this is not always the case (for example see *GMR78F06*-gal4/UAS-*dTrpA1* in Table 2), these results suggest not only that neurons are involved in sperm storage, but also that there may be both shared and SSO-specific neural circuitry.

In addition to scoring sperm storage, we also noted other phenotypes of the reproductive tract that may indicate how storage can be altered. These phenotypes included: bending of the uterus scored on a 4 point scale (see methods for explanation), the probability of sperm ejection during the 1 hr temperature treatment, ovulation, and the presence of sperm within the common or lateral oviducts (Table 2). As the uterus conformational changes were predicted to be necessary for successful sperm storage (Adams and Wolfner 2007), we compared our bending scores with the scores of the SR and Sp (Figure 2F and G).

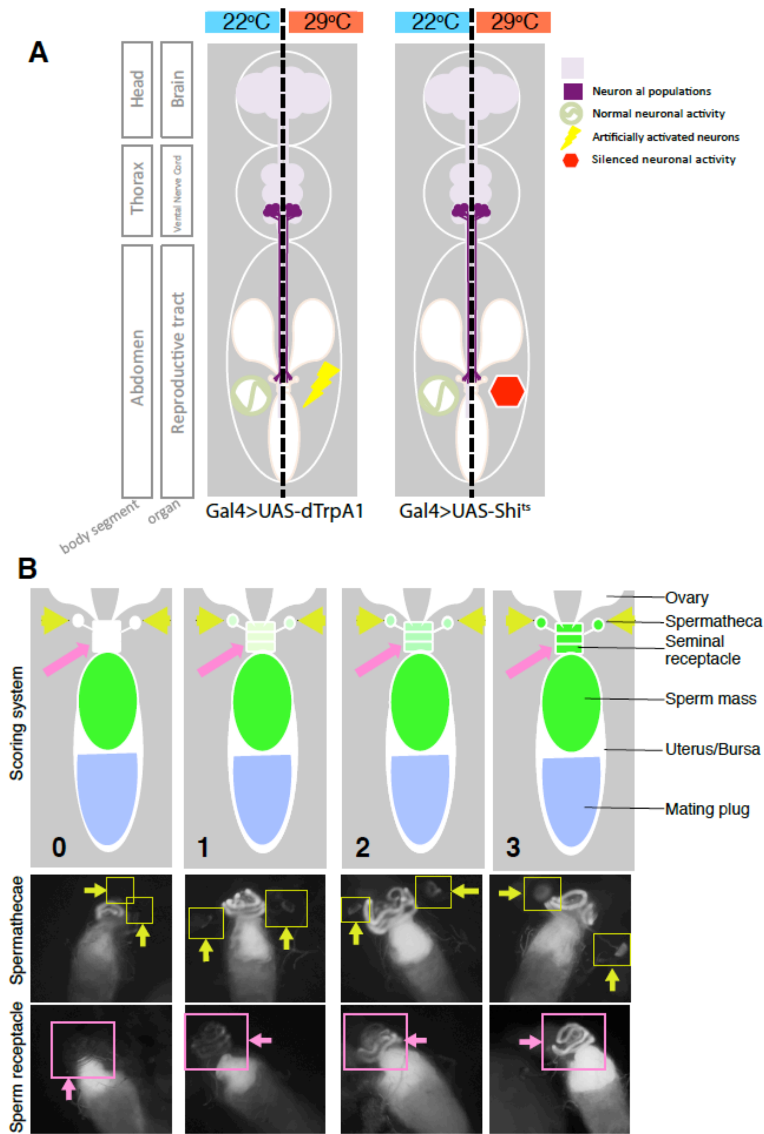


Figure 1. Sperm storage scoring system. **(a)** Experimental paradigm of neuronal activity manipulation. Schematic of the body plan of female *Drosophila* is depicted: three body segments are indicated with the relevant organ. *dTrpA1* or *Shibire^ts* was expressed in a specific population of cells (represented in dark purple) with use of the Gal4-UAS system. At temperatures below 25°C, females experience normal neuronal activity. At 29°C, neuronal activity is manipulated either via artificial activation or silencing. **(b)** Top images are representations of the scoring system used to assess GFP signal in the spermathecae (yellow arrows) and seminal receptacle (pink arrow) with scores from 0-3. Bottom images representative examples of each of the scores. The spermathecae are surrounded by a yellow box, and indicated with yellow arrows, while the seminal receptacle is surrounded by a pink box, and indicated with pink arrows.

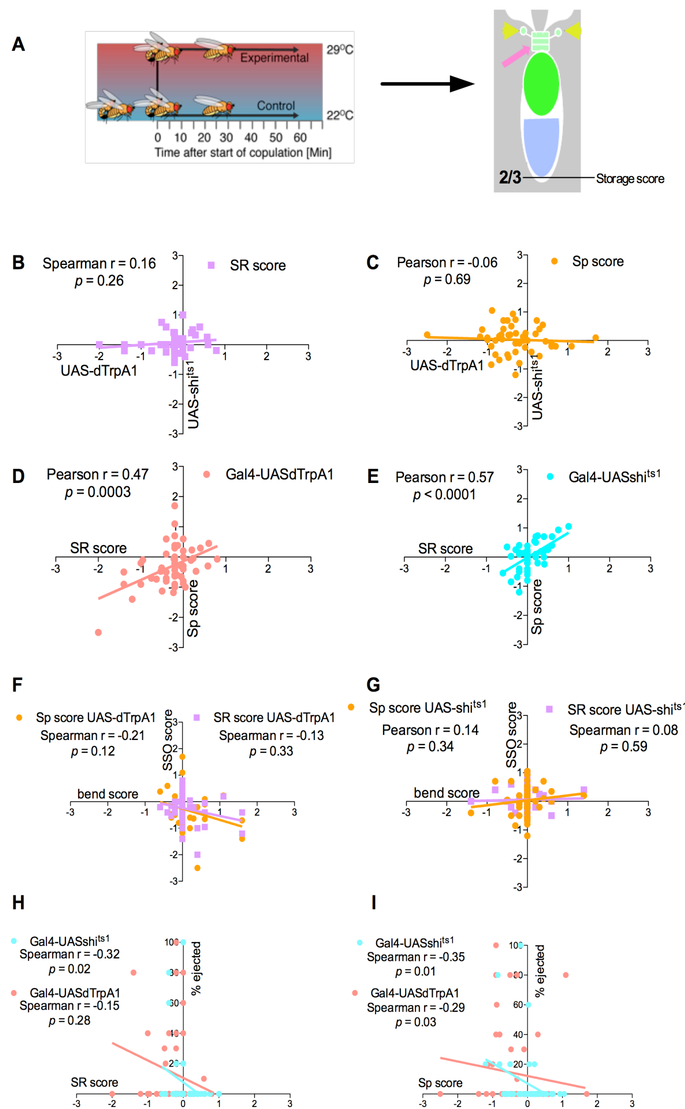


Figure 2. Sperm storage in the SR and Sp are correlated with each other and the likelihood of ejecting but not morphology. (a) Protocol of the temperature application to activate or silence neuronal activity. Arrow indicates removal of female from experiment and reproductive tract dissection. Schematic of female reproductive tract in grey box represents storage scoring process (see Figure 1). **(b-c)** Correlation between the normalized mean (mean of control minus means of manipulated females) of each Gal4 driver with UAS- *dTrpA1* and UAS-*Shibire*^{ts1} seminal receptacle (SR* in Table 2), B, and spermathecae (Sp), C. **(d-e)** Correlation between the normalized mean of the SR and the Sp of the same Gal4 line for females expressing either *dTrpA1*, D, or *Shibire*^{ts1}, E. **(f-g)** Correlation of the mean normalized bending score and mean normalized sperm score for the SR (purple) or Sp (orange) in each Gal4 line expressing *dTrpA1*, F, or *Shibire*^{ts1}, G. **(h-i)** Correlation of the percentage of experimental females that ejected and mean normalized sperm score for the SR, H, or mean normalized sperm score for the Sp, I, in each Gal4 line expressing *dTrpA1* (pink) or *Shibire*^{ts1} (blue). Outcome of the statistical tests are indicated on the top left corner of each panel. A Spearman r or a Pearson r (for non-normally and normally distributed data, respectively) was used to determine relationship between the indicated factors.

Gene	Gal4 line	Gal4/UAS-*dTrpA1* 22°C-29°C SR	Sp	Normal. SR*	Sp*	Result SR	Sp	Additional phenotypes b	ej	ovu	ovi	Gal4/UAS-*shi*^{ts1} 22°C-29°C SR	Sp	Normal. Sp*	Result SR	Sp	Additional phenotypes b	ej	ovu	ovi	V
crz	crz	0.20	0.30	0.00	0.00			0.0	0.0	0.0	0.0	0.20	0.20	0.00			0.0	0.0	0.0	0.0	
5HT7	**5HT7***	0.20	0.20	0.40	0.50			-0.2	0.0	0.0	0.0	0.60	0.90	0.70			-0.4	0.0	0.0	0.0	
ap	**ap***	0.75	0.20	0.95	0.10			0.6	0.0	0.0	0.0										
cha	cha	0.20	0.40	0.40	0.10			0.6	0.0	0.0	0.0	0.00	0.20	0.40			-1.4	0.0	0.0	0.0	
ddc	ddc	0.00	0.10	0.20	0.20			0.0	0.0	0.0	0.2	0.00	0.60	0.80			0.0	0.2	0.0	0.0	
dNPF	dNPF	0.00	1.00	0.20	0.70			0.0	0.0	0.0	0.0	0.25	0.00	0.20			0.3	0.0	0.0	0.0	
dsx	**Dsx***	0.80	0.10	1.00	0.20			0.4	0.0	0.0	0.0										
fru	**fru***	0.20	0.58	0.00	0.88			0.0	0.6	0.2	0.0	1.00	1.25	1.05			0.0	0.0	0.0	0.0	
fru16	**fru16***	1.80	2.20	2.00	2.50			0.4	0.0	0.0	0.0	0.00	0.40	0.20			0.0	0.2	0.0	0.0	
ppk	ppk	0.20	0.50	0.00	0.20			-0.2	0.0	0.0	0.0	0.00	0.43	0.23			0.0	0.0	0.0	0.0	
Tdc2	**Tdc2***	0.20	0.00	0.00	0.30			0.0	0.8	0.0	0.0	0.20	1.00	1.20			0.0	0.2	0.0	0.0	
tsh	Tsh	1.00	1.10	1.20	1.40			1.6	0.0	0.0	0.0										
vglut	Vglut	0.20	0.40	0.40	0.70			1.6	0.0	0.0	0.0	0.25	0.48	0.68			0.0	0.0	0.0	0.0	
caup	GMR80B06	0.20	0.50	0.00	0.80			-0.1	0.4	0.0	0.0	0.00	0.20	0.00			0.6	0.0	0.0	0.6	
caup	GMR80C01	0.00	1.40	0.20	1.10			0.0	0.8	0.0	0.0	0.00	0.00	0.20			0.0	0.0	0.0	0.0	
caup	GMR80C03	0.60	0.60	0.40	0.30			0.0	0.0	0.0	0.0	0.60	0.60	0.40			0.0	0.0	0.0	0.0	
CG15765	GMR49D10	0.00	0.30	0.20	0.00			0.0	0.0	0.0	0.0	0.00	0.20	0.00			0.0	0.0	0.0	0.0	
CG15765	GMR47E07	0.00	0.00	0.20	0.30			0.0	0.0	0.0	0.0	0.00	0.10	0.10			-0.2	0.0	0.0	0.0	
CG15765	GMR47D09	0.80	0.75	0.60	0.45			0.0	0.0	0.0	0.0	0.00	0.17	0.03			-0.2	0.0	0.0	0.0	
CG15765	GMR49B0	0.33	0.17	0.53	0.47			-0.3	0.3	0.0	0.0	0.00	0.40	0.60			0.0	0.0	0.0	0.0	
CG32532	GMR35A12	0.00	0.33	0.20	0.63			0.3	0.0	0.0	0.0	0.00	0.25	0.05			-0.5	0.2	0.0	0.0	
CG32532	GMR35D10	0.00	0.30	0.20	0.60			0.2	0.0	0.0	0.0	0.20	0.60	0.40			0.2	0.0	0.0	0.0	
5HT2B	GMR74A0	0.00	0.10	0.20	0.20			0.0	0.0	0.0	0.0	0.00	0.10	0.10			0.0	0.0	0.0	0.0	

		Gal4/UAS-*dTrpA1*										Gal4/UAS-*shi*^{ts1}									
		22°C-29°C		Normal.		Result		Additional phenotypes				22°C-29°C		Normal.	Result		Additional phenotypes				
Gene	Gal4 line	SR	Sp	SR*	Sp*	SR	Sp	b	ej	ovu	ovi	SR	Sp	Sp*	SR	Sp	b	ej	ovu	ovi	V
5HT2B	GMR74A11	0.20	0.90	0.00	0.60			0.0	0.0	0.0	0.0	0.00	0.10	0.10			0.0	0.0	0.0	0.0	
5HT2B	**GMR83A12***	0.80	0.60	1.00	0.90			0.0	0.4	0.0	0.2	0.00	0.00	0.20			0.0	0.0	0.0	0.0	
5HT2B	**GMR83A10***	1.20	0.20	1.40	0.50			0.0	0.8	0.6	0.4	0.20	0.30	0.50			-0.4	0.2	0.0	0.0	
5HT2B	**GMR83B06***	1.20	0.60	1.40	0.90			0.0	0.8	0.6	0.6	0.00	0.00	0.20			0.0	1.0	0.0	0.0	
dnc	GMR12F01	0.31	0.06	0.51	0.36			0.0	0.0	0.0	0.0	0.75	1.13	0.93			0.0	0.0	0.0	0.0	
dnc	GMR12F06	0.43	0.25	0.23	0.05			0.0	0.0	0.0	0.0	0.50	0.46	0.26			0.0	0.0	0.0	0.0	
dnc	**GMR12H08***	0.00	2.00	0.20	1.70			0.0	0.0	0.0	0.0	0.20	0.30	0.10			0.0	0.0	0.0	0.0	
dsf	GMR29G10	0.00	0.60	0.20	0.30			0.0	0.4	0.0	0.0	0.60	0.35	0.55			0.0	0.0	0.0	0.0	
dsf	GMR30C11	0.00	0.20	0.20	0.10			-0.2	0.3	0.0	0.0	0.40	0.20	0.00			0.2	0.0	0.0	0.0	
lz	GMR25F04	0.00	0.00	0.20	0.30			0.0	0.0	0.0	0.0	0.20	0.20	0.40			0.0	0.0	0.0	0.0	
lz	GMR25H03	0.00	0.68	0.20	0.38			-0.6	0.0	0.0	0.0										
para	**GMR14H04***	0.00	0.70	0.20	1.00			0.0	0.2	0.0	0.0	0.20	0.30	0.10			0.4	0.0	0.0	0.0	
para	GMR14H05	0.05	0.30	0.15	0.60			-0.3	0.0	0.0	0.0	0.20	0.90	0.70			0.4	0.0	0.0	0.0	
para	**GMR16H03***	0.25	0.70	0.05	1.00			0.2	0.0	0.0	0.0	0.40	0.23	0.03			0.0	0.6	0.0	0.2	
para	GMR18C12	0.15	0.35	0.05	0.65			0.6	0.0	0.0	0.0	0.00	0.40	0.20			-0.2	0.0	0.0	0.0	
SPR	GMR78E11	0.20	0.50	0.40	0.80			0.0	0.4	0.0	0.0	0.00	0.30	0.50			-0.2	0.0	0.0	0.0	
SPR	GMR78E12	1.00	0.23	0.80	0.07			0.0	0.0	0.0	0.0	0.19	0.33	0.13			0.0	0.0	0.0	0.0	
SPR	GMR78F01	0.02	0.24	0.22	0.06			0.0	0.0	0.0	0.0	0.41	0.04	0.24			0.0	0.0	0.0	0.0	
SPR	GMR78F03	0.00	0.13	0.20	0.43			0.0	0.0	0.0	0.0	0.40	0.40	0.20			1.4	0.0	0.0	0.0	
SPR	GMR78F08	0.13	0.01	0.07	0.29			0.0	0.0	0.0	0.0	0.29	0.45	0.25			0.0	0.0	0.0	0.0	
SPR	GMR78F09	0.20	0.10	0.00	0.40			0.0	0.0	0.0	0.0	0.20	0.70	0.50			0.0	0.0	0.0	0.0	
SPR	GMR78F10	0.43	0.43	0.63	0.73			0.0	0.0	0.0	0.0										
SPR	GMR78G01	0.30	0.83	0.50	1.13			0.0	0.2	0.0	0.0	0.00	0.58	0.38			0.0	0.0	0.0	0.0	

Gal4/UAS-*dTrpA1*

Gene	Gal4 line	22°C-29°C		Normal.		Result		Additional phenotypes			
		SR	Sp	SR*	Sp*	SR	Sp	b	ej	ovu	ovi
SPR	GMR78G03	0.07	0.68	0.13	0.38			0.0	0.0	0.0	0.0
SPR	GMR78G04	0.50	0.17	0.30	0.47			0.0	0.0	0.0	0.0
SPR	GMR78G06	0.03	0.88	0.23	1.18			0.0	0.0	0.0	0.0
SPR	GMR78G08	0.38	0.19	0.18	0.11			0.0	0.0	0.0	0.0
SPR	GMR78G09	0.40	0.53	0.20	0.23			1.1	0.0	0.0	0.0
SPR	GMR78G05	0.78	0.00	0.58	0.30			0.0	0.1	0.0	0.0
SPR	**GMR78F02***	0.00	0.60	0.20	0.90			0.0	1.0	0.3	0.0
SPR	GMR78F07	0.55	0.40	0.75	0.70			0.0	0.0	0.0	0.0
SPR	GMR78F06	0.20	0.90	0.40	0.60			-0.4	0.0	0.0	0.0
SPR	GMR78F12										
-	UAS controls	0.2	0.3	0	0			0.0	0.0	0.0	0.0

Gal4/UAS-*shi*^{ts1}

Gene	Gal4 line	22°C-29°C		Normal.	Result		Additional phenotypes				V
		SR	Sp	Sp*	SR	Sp	b	ej	ovu	ovi	
SPR	GMR78G03	0.17	0.75	0.55			0.0	0.0	0.0	0.0	
SPR	GMR78G04	0.33	0.39	0.19			0.0	0.0	0.0	0.0	
SPR	GMR78G06	0.00	0.33	0.13			0.0	0.0	0.0	0.0	
SPR	GMR78G08	0.10	0.33	0.13			0.0	0.0	0.0	0.0	
SPR	GMR78G09	0.40	0.90	0.70			-0.8	0.0	0.0	0.0	
SPR	GMR78G05	0.25	0.93	0.73			0.0	0.0	0.0	0.0	
SPR	**GMR78F02***	0.40	0.65	0.85			-0.3	0.8	0.2	0.0	
SPR	GMR78F07	0.20	0.35	0.15			0.0	0.0	0.0	0.0	
SPR	GMR78F06	0.00	0.00	0.20			-0.2	0.0	0.0	0.0	
SPR	GMR78F12	0.50	0.55	0.35			0.6	0.0	0.0	0.3	
-	UAS controls	0	0.2				0.0	0.0	0.0	0.0	

Table 2. Gal4 screen for neurons involved in sperm storage. Each indicated Gal4 line was crossed separately to UAS-*dTrpA1* and UAS-*shib*^{ts1}, female offspring were mated to males expressing green fluorescent protein on tails of sperm, and sperm storage was assessed using a 4 point storage system (0-3, see Figure 1) for each sperm storage organ (SR = seminal receptacle; Sp = spermathecae). The first 2 column heat map (22°C-29°C) represents difference in average sperm storage scores for females of the same genotype in different temperature conditions. The number in the cell represents the numerical difference in average score, the colour represents the direction so that a red cell indicates less sperm in storage. and a green cell indicates more sperm in storage in females placed at 29°C and experienced neuronal manipulation relative to females that remained at 22°C and experienced normal neuronal activity. The normalized (Normal.) column heat map represents the differences altered to take into account that UAS control females placed at 29°C were found to store slightly more sperm compared to UAS control females that remained at 22°C. We made an arbitrary decision point at 0.90, meaning any difference greater than 0.90 was considered a considerable change in sperm storage amount. A subset of these Gal4 lines were selected to validate the sperm storage scoring system (indicated with a grey cell in column "V"; see Figure 2). Based on the patterns of this screen and the validation results 13 lines were selected to investigate further (indicated an asterisks. see methods). Additional phenotypes were also scored: beding of the reproductive tract (b); proportion of females that ejected (ej), were ovulating (ovu), and had sperm present in their oviducts (ovi). Sample size for each group ranged from 5-12.

We did not find a relationship between this morphological phenotype and sperm storage changes for either activation (SR: Spearman r = -0.13, p = 0.33; Sp: Spearman r = -0.21, p = 0.12; Figure 2H) or silencing (SR: Spearman r = 0.08, p = 0.59; Sp: Pearson r = 0.14, p = 0.34; Figure 2I). This suggests that females can progress through the steps of uterine conformation and still show deficiencies in sperm storage. Therefore, we conclude that the neurons identified in our study that decrease sperm storage may directly act on the SSOs rather than more broadly influencing the morphology of the reproductive tract, indirectly affecting sperm storage.

Another post-mating behaviour that influences sperm storage is sperm ejection. Sperm ejection happens on average 3 to 6 hrs after mating (Duménil et al., 2016; Laturney and Billeter, 2016; Chapter 3; Lee et al., 2015; Manier et al., 2010). Previous studies have found that decreased sperm ejection latency can reduce the amount of sperm stored from a single mating (Lee et al., 2015) and can reduce the number of offspring sired by the second male in a twice mated female (Lupold et al., 2013). We therefore anticipated that females that ejected within the first hour ASM, during which the temperature treatment is applied, would have less sperm in storage. Interestingly, different Gal4 drivers triggered sperm ejection during this timeframe (Figure 2H and I). We compared the percentage of females that ejected with the changes in storage in the SR and Sp due to neuronal activation (Figure 2H) and silencing (Figure 2I). We found a negative relationship between this post-mating behaviour and storage changes in SR (via activation with dTrpA1: Spearman r = -0.15, p = 0.28; and silencing via Shibire^{ts1}: Spearman r = -0.32, p = 0.02; Figure 2H) and Sp storage (via activation with dTrpA1: Spearman r = -0.29, p = 0.03; and silencing via Shibire^{ts1}: Spearman r = -0.35, p = 0.01; Figure 2I) suggesting that females that were likely to eject during the manipulation also tended to store less sperm. This could indicate that either by manipulating the activity of neurons that support ejection, females were forced to eject, which indirectly resulted in a decrease in sperm storage; or that neuronal populations influence both behaviours independently. However, earlier ejection (defined at more than half the females ejecting within the hour) did not always result in decreased sperm storage (equal to or more than 0.5 point decrease in storage)- some even showing increased sperm storage (*Tdc2*-gal4/UAS-*dTrpA1*; *GMR80C01*-gal4/UAS-*dTrpA1*; *GMR83B06*-gal4/UAS-*shi*^{ts1}, *GMR16H03*-gal4/UAS-*shi*^{ts1}, *GMR78G05*-gal4/UAS-*dTrpA1*; Table 2). Due to these exceptions, our findings suggest that sperm ejection may function to modulate sperm storage, but sperm storage can also be influenced through ejection-independent mechanisms.

Finally, as the location of sperm in the female reproductive tract is tightly controlled (Adams and Wolfner 2007), we reasoned that finding sperm outside of the uterus could indicate a lack of control over the ejaculate. Although rare, we did find that activation of a few populations of neurons did lead to disorganized sperm management (indicated by the presence of sperm within the common or lateral oviduct). We determined that 5 specific Gal4-UAS combinations resulted in at least one of the females showing sperm within the oviducts (*GMR83A12*-gal4/UAS-*dTrpA1*; *GMR83A10*-gal4/UAS-*dTrpA1*; *GMR83B06*-gal4/UAS-*dTrpA1*; *GMR16H03*-gal4/UAS-shi^{ts1}; *GMR78F12*-gal4/UAS-shi^{ts1}). Interesting, this was consistently associated with reductions in storage of the SR as all of the mentioned genotypes showed at least mild decreases (equal to or greater than 0.40 decrease) compared to controls (Table 2).

Validation of scoring system via fecundity assessment

Due to the range of quantitative differences in sperm storage scores between Gal4 lines, we created an arbitrary cut off point to qualify the action of each Gal4 line in terms of increased, decreased, or no change in sperm storage for each SSO. We determined that a score of 0.90 or above for a given SSO indicates increased sperm storage, and -0.90 and below a decrease, anything in between being considered as no change. We chose this arbitrary cut-off as it indicates an observable change according to our scoring scale (Figure 1).

As our scoring system evaluated visible differences in GFP-labeled sperm signal intensity, we reasoned that these deviations could represent profound differences in the amount of stored sperm and consequently influence the number of offspring produced. In order to investigate this possibility and validate our scoring system, we selected 12 Gal4 lines in combination with *dTrpA1* at 29°C that were scored either as resulting in decrease, increase or no change in sperm storage, compared to their controls (indicated in Table 2 and referred to as the "validation subset" of Gal4 lines). We again expressed *dTrpA1* under the control of the subset of these Gal4 drivers and artificially activated these neurons by placing mating pairs at either 29°C, or allowed for normal neuronal activity at 22°C, during sperm transfer and lasting for 6 hrs ASM to include the entire sperm storage process (Figure 3A). After this period, females were placed in vials with fly food and produced offspring, which were then counted and compared between groups (Figure 3B).

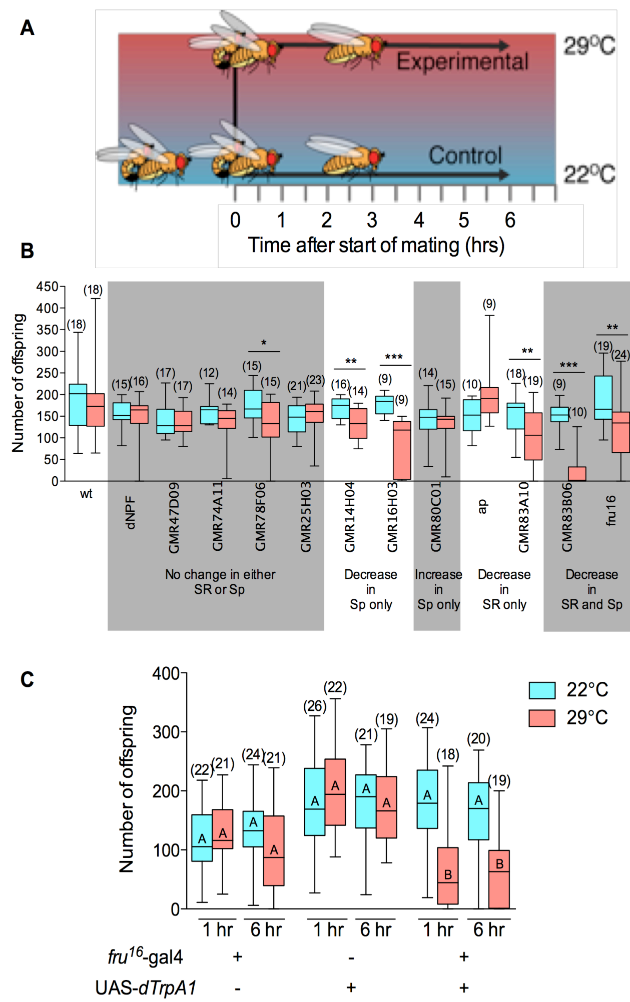


Figure 3. Activation of neurons during sperm storage reduced offspring production for Gal4 lines that inhibited sperm storage. (a) Protocol of the temperature application to activate or silence neuronal activity. **(b)** Box plots displaying mean number of progeny produced by females expressing UAS-*dTrpA1* with the indicated driver that experienced artificial activation (placed at 29°C, shown in pink) or with normal neuronal activity (remained at 22°C, shown in blue) during sperm storage. Wild-type (wt) females are shown as temperature controls. Differences within genotype across temperature treatments were determined by an unpaired t-test or a Mann-Whitney U test for normally and non-normally distributed data, respectively. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Sample sizes are indicated in brackets. Whiskers indicate min and max. **(c)** Box plot indicating number of offspring produced by different groups: females expressing UAS-*dTrpA1* under the control of the *fru^{16}*-gal driver were placed at either 29°C (shown in pink) to artificially force activation of these neurons, or remained at 22°C (shown in blue) for normal neuronal activity for 1 or 6 hours starting at the beginning of copulation. Sample sizes are indicated in brackets. Groups were compared with Two-way ANOVA followed by Bonferonni post-hoc. Significant differences are indicated by different letters. Whiskers indicate min and max.

We found that, in general, the scores generated during our screen and ultimately the category of sperm storage highly reflected the patterns in offspring production (Figure 3B; Supplementary Table 1). Of the 12 lines we validated, we predicted that 5 would have no effect on offspring production as we found no difference in sperm storage scored between activated and control females in either SR or Sp. We indeed found no significant difference in 4 of the 5 lines, however, the activation of the neurons labeled by *GMR78F06*-gal4 caused female to produce significantly less offspring compared to controls (Figure 3B; Supplementary Table 1). Interestingly, of the 5 Gal4 lines in that group, *GMR78F06*-gal4/UAS-*dTrpA1* females did demonstrate the largest decrease in SR storage (SR^{*} = -0.40; Table 2). This suggests that a reduction in sperm storage scored as 0.40 in the SR can lead to a decrease in

sperm storage resulting in a significantly reduced offspring production. This implies that our cut-off of 0.90 is conservative and that we mostly expect to call false-negatives. In line with this conclusion, we found that all but one of the lines that we categorized as causing a decrease in SR or decrease in SR and Sp produced significantly less offspring (Figure 3B). This was especially true for the two lines we found a decrease in both SR and Sp (decrease in both SR and Sp *GMR83B06*-gal4 and *fru*^{16}-gal4). We conclude that the sperm storage scoring system for the SR reflects the amount of sperm in storage as shown indirectly with changes to offspring production.

The relationship between our category of sperm storage in Sp and consequential offspring production is less clear. Similar to SR, we found that the lines that we categorized as causing a decrease in Sp produced significantly less offspring (Figure 3B) suggesting that restricting sperm storage into this organ can influence total number of offspring produced even when SR storage was not altered. However, an increase in Sp storage did not lead to an increase in offspring production (see *GMR80C01*-gal4/UAS-*dTrpA1*; Figure 3B). A possible explanation is that by manipulating neuronal activity and recruiting more sperm into storage, it may have resulted in a volume too large for the females to efficiently use the sperm. In this way, increases in sperm storage to the Sp would not lead to increases in progeny production. Overall, as these two SSOs have very different storage capacities, with SR holding much more sperm than the Sp (Manier et al., 2010), it is likely that observed deviations in SR scores may indeed have a larger influence on progeny production. However, the number of sperm in both SSOs can influence the number of offspring produced as we see that decreases in one accompanied with no change in the other still results in decreased offspring production. Overall, we validated our scoring system: we reliably showed that when we activated specific subpopulations of neurons during sperm storage we find females store perceptibly less sperm and also go on to produce less offspring.

Sperm storage: plastic or fixed?

It is clear that manipulating neuronal activity during the entire sperm storage process, ie for 6 hr ASM (Figure 3B) spanning from the start of copulation and including sperm ejection, the end of the sperm storage process (Manier et al., 2010), can lead to changes in offspring production, particularly for those manipulations that lead to less storage. We wondered if we removed the neuronal manipulation in neurons that restrict sperm into storage before the end of this process, ie before sperm ejection, can females recover? Is the process of sperm

storage plastic enough to go on, recruit more sperm into storage, and consequently produce more offspring? We chose to investigate this in *fru*^{16}-gal4/UAS-*dTrpA1* females as activation of these neurons during sperm storage led to the largest reduction in storage in our screen, and our results suggest that these neurons function exclusively as restrictors as silencing with UAS-*shi*^{ts1} did not increase sperm storage. To test the plasticity of sperm storage, we activated these neurons for either the first hour of sperm storage or for the entire process of sperm storage.

We again drove the expression of UAS-*dTrpA1* in this sub-set of *fruitless* labeled cells (*fru*^{16}-gal4 driver) and placed the mating pair at 29°C to activate these neurons, or remained at 22°C for normal neuronal activity, during sperm transfer and storage. We applied the same heat treatments to the genetic controls (*fru*^{16}-gal4/+ and UAS-*dTrpA1*/+). Since the process of sperm storage is formally ended by the female with the ejection of unused portions of the ejaculate and the mating plug (Manier et al., 2010; Lee et al., 2015), to ensure that females in the 1 hr treatment had not ended sperm storage, and females in the 6 hr treatment had ended this process, all females were checked for ejections. Females were then placed in vials with fly food and produced offspring, which were then counted and compared between groups.

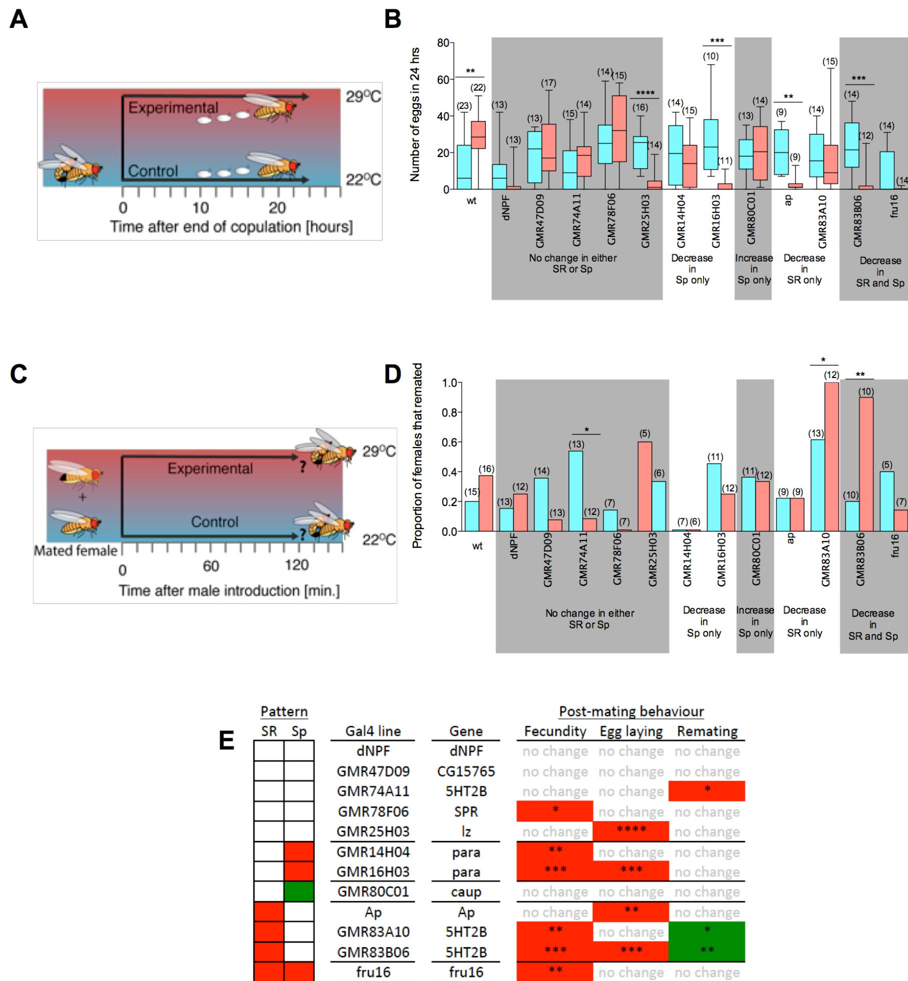
We compared the mean number of offspring produced by females of each group with a Two-way ANOVA and found a significant interaction of genotype and temperature, $F(6, 245) = 6.78$, $p < 0.001$. With Bonferroni post hoc tests we found that females that experienced the artificial activation of *fru*^{16}+ neurons produced significantly less offspring compared to controls (Figure 3C). Interestingly, females that experienced neuronal manipulation for only a portion of the sperm storage process (were removed 1 hr ASM and had not ejected) still produced significantly less offspring compared to controls (Figure 3C) and not significantly different from females that experienced neuronal manipulation for the entire process (were removed 6 hr ASM and had ejected). This indicates that females were unable to recover, did not go on to store normal amount of sperm and increase offspring production after the temperature condition was removed and normal neuronal activity was restored. This suggests that there is window of opportunity for storage, and the neuronal activity within the first hour of storage can profoundly impact the outcome hours later. Interestingly, Manier et al. (2010) showed that although maximum amount of sperm storage is achieved 1 hour ASM, exchange between uterus/bursa continues until ejection, which suggests that entry of sperm into the SSOs at later time points is at least possible. However, our results may indicate that a neural-mediated event in the early stage of the sperm storage process may be required for full storage potential. Alternatively, full storage may require a male-

derived component to interact with the nervous system. Indeed, multiple male-derived seminal fluid proteins present in the ejaculate have been found to be required for sperm to enter into storage (Avila and Wolfner, 2009; Bloch Qazi and Wolfner, 2003; Neubaum and Wolfner, 1999) and this male signal may be degraded over time and therefore is not able to encourage sperm accumulation into the SSOs 1 hr ASM.

Neurons that influence sperm storage represent both shared and unique circuitry

Previous studies have revealed that the female neuronal circuitry underlying the PMR has a common frame that supports many PMRs and branches that control individual PMRs. The common frame is constituted by the neurons that sense sex peptide, the main trigger of the PMR, in the bursa and relay this information to the brain (Avila et al., 2012; Carvalho et al., 2006; Fleischmann et al., 2001; Hasemeyer et al., 2009; Isaac et al., 2010; Nakayama et al., 1997; Rezával et al., 2012; 2014; Ribeiro and Dickson, 2010; Rideout et al., 2010; Schmidt et al., 1993; Yang et al., 2009; Yapici et al., 2008; reviewed in Chapter 2). Neuronal circuits unique to each individual PMR often diverge down-stream of sex peptide sensing, although some represent a completely sex peptide-independent circuit (Haussmann et al., 2013; Rexhepaj et al., 2003; Xue and Noll, 2000; Yang et al., 2009). As sperm storage occurs after mating, we wondered if the neuronal populations we identified in our sperm storage screen are shared with other PMRS, or whether they are unique to sperm storage. To investigate this, we expressed *dTrpA1* under the control of the 12 validation Gal4 drivers. We mated these females to wild-type males and artificially activated these different neuronal populations during egg laying (neuronal activation started at the end of copulation and continued for 24 hrs; Figure 4A) and re-mating (neuronal activation started when mated female was presented with a new virgin male 24 hrs after virginal mating Figure 4C).

We found differences in both egg laying (Figure 4B) and re-mating behaviour (Figure 4D) in both lines that did and did not render sperm storage differences (for an overview see Figure 4E). Five of these lines had altered sperm storage patterns (Table 2) and produced significantly less offspring compared to controls (Figure 3B). Interestingly, activation of the neurons in 2 of these lines (*GMR14H04*, subset of *para*; and *fru^{16}*, subset of *fru*) did not lead to a change in egg laying or sexual receptivity (Figure 4E) suggesting that these populations represent sperm storage-specific circuitry.



SR	Sp	Gal4 line	Gene	Fecundity	Egg laying	Remating
		dNPF	dNPF	no change	no change	no change
		GMR47D09	CG15765	no change	no change	no change
		GMR74A11	5HT2B	no change	no change	*
		GMR78F06	SPR	*	no change	no change
		GMR25H03	lz	no change	****	no change
		GMR14H04	para	**	no change	no change
		GMR16H03	para	***	***	no change
		GMR80C01	caup	no change	no change	no change
		Ap	Ap	no change	**	no change
		GMR83A10	5HT2B	**	no change	*
		GMR83B06	5HT2B	***	***	**
		fru16	fru16	**	no change	no change

Figure 4. Neurons that support sperm storage are both unique and shared with the neuronal circuitry that supports PMR. (a and **c)** Protocol of the temperature application to activate neuronal populations (placed at 29°C) and controls (placed at 22°C) to assess egg laying, A, and remating, C. **(b)** Box plot displaying mean number of eggs laid by females that experienced artificial activation (pink) and controls (blue) of given genotype. Differences across temperature conditions for each genotype were determined by a unpaired t-test of a Mann-Whitney U test for normally and non-normally distributed data, respectively. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Sample sizes are indicated in brackets. Whiskers indicate min and max. **(d)** Bars indicate proportion of females that experienced artificial activation (pink) and controls (blue) of given genotype that re-mated. Differences between temperature conditions for each genotype were determined by a Fisher's Exact test. * = $p < 0.05$, ** = $p < 0.01$. **(e)** Summary of the effect of activating the different population of cells defined as our validation set on sperm storage ("Pattern" see Table 2), fecundity (see Figure 3), egg laying, and remating. The direction of the change, with respect to controls, is indicated by colour (green = increase, red = decrease, white = no change) * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

The other three lines displayed reduced PMR in either one of both behaviours tested. Activation of *GMR16H03*-expressing neurons (subset of *para*) showed significantly reduced egg laying (Figure 4B) but no change in re-mating (Figure 4D). Similarly, activation of *GMR83B06*-expressing neurons (subset of *5-HT$_{2b}$*) caused an increase in re-mating (Figure 4D), with no effect on egg laying (Figure 4B). Taken together, these results indicate that there is unique and shared neuronal circuitry supporting these three PMRs.

Identification of Gal4 lines that influence sperm storage

From the results of the fecundity test, we determined: first, that a reduction of 0.90 was in most cases sufficient to produce a significant reduction in fecundity; and second, that increases in Sp was not associated with an increase in fecundity. From here, we selected all lines that displayed a change in the SR and/or a decrease in the Sp equal to or greater than 0.90 (Table 2). We excluded *tsh*-gal4 from this selection as the expression pattern in the whole ventral nerve cord is too broad to be informative, as well as two other Gal4 lines which were not available to us at the time of experiments (*GMR78G01*-gal4 and *GMR78G06*-gal4). We also included *5-HT$_7$*-gal4 as we observed a decrease in storage in both SSOs when neurons were activated, and an increase when neurons were silenced. Furthermore we also included one line that displayed an Sp only increase, *GMR12H08*-gal4, as the increase was very large (1.70). This left us with 13 lines (indicated by an asterisk in Table 2) that we further studied, out of the original 56 we screened.

Innervation of female reproductive tract of Gal4 lines that label sperm storage neurons

The similarities in the effect on sperm storage shared between the selected Gal4 lines as a result of our neuronal manipulation may reflect a small subset of neurons being labeled by multiple Gal4 drivers. Alternatively, common effects may stem from different neurons at different positions along the circuitry. As the mechanisms of sperm storage remain almost completely unknown, it is important not only to identify which neurons influence this process but also determine how they might do so. For this, we investigated the expression pattern of the 13 identified Gal4 drivers in both the central nervous system (Figure 5A) and their innervation pattern of the female reproductive tract (Figure 5B). Using the Gal4-UAS system we drove the expression of a membrane associated GFP, mCD8::GFP, and performed immunostaining to visualize the innervation pattern of the female reproductive tract by confocal microscopy.

We scored the presence or absence of neuronal innervation in individual regions of the female reproductive tract and observed innervation patterns that varied between the lines (Figure 5C). It was not the case that one specific region of the female reproductive tract was consistently innervated by all lines. This indicates that sperm storage can be influenced by different subsets of neurons innervating different regions of the female reproductive tract.

Moreover, we found no innervations of the reproductive tract in two of lines (*ap*-gal4 and *GMR78F02*-gal4, a subset of *spr*$^{+}$ cells) that do affect sperm storage, suggesting that those represent central nervous system neurons that are upstream of those innervating the reproductive tract. Some lines have GFP-positive cells in the reproductive tract that lack neurites suggesting they are non-neuronal cells. Those line include *GMR83A10*-gal4, which had a strong GFP signal in cells of the common oviduct; *GMR83A10*-gal4, which had GFP-positive cells in the distal end of the SR (Figure 5); and *GMR12H08*-gal4, which has one neuron that runs dorsally down the bursa but also has heavy non-neural staining at the pre-storage area (Figure 5B).

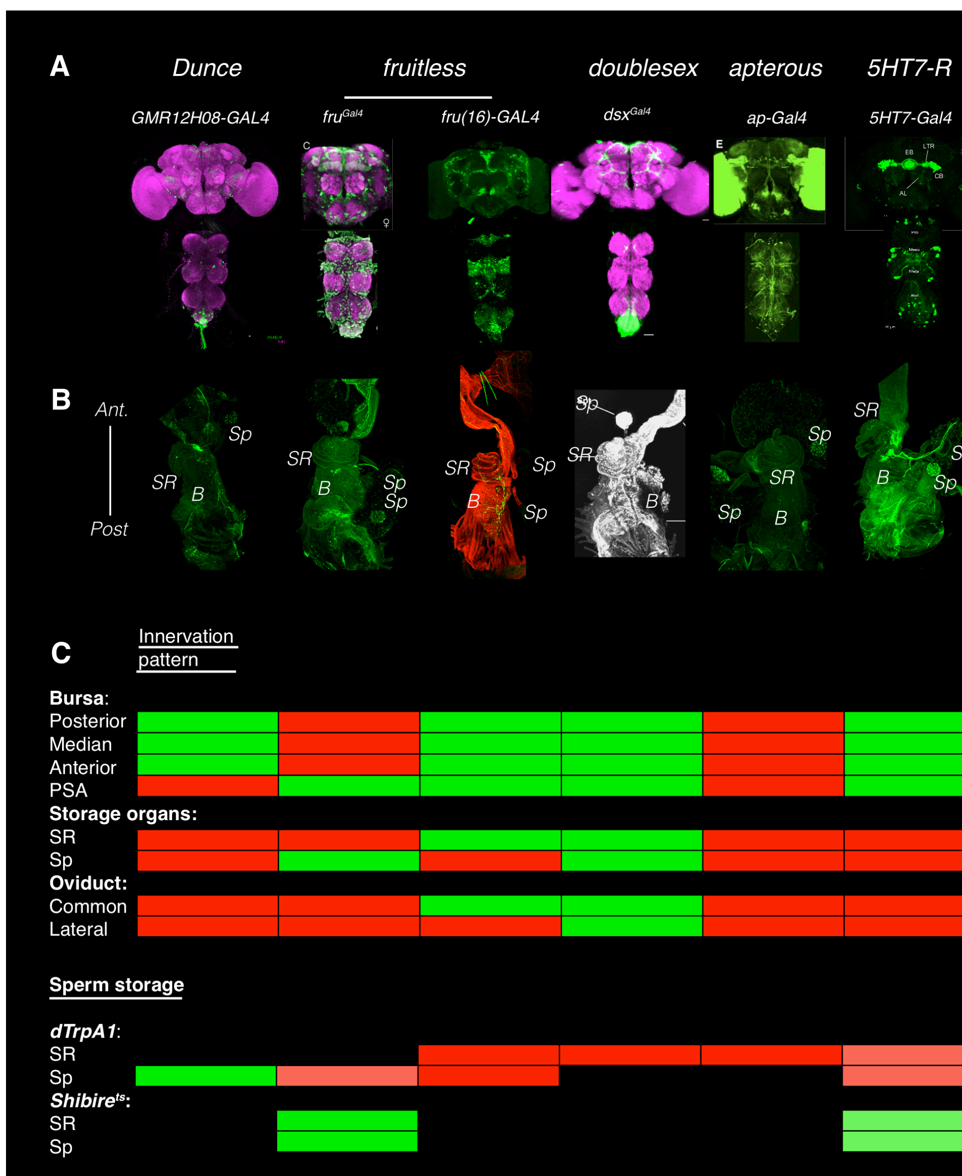
We also found both broadly and specifically expressed lines. For example, *dsx*-gal4 heavily innervates all regions of the reproductive tract (Figure 5A); and *GMR83A12*-gal4, which stains one major neurite that runs posterior but does not innervate the bursa, and also labels neurons that innervate the anterior bursa, the surface of the SR, the stalk of the Sp, pre-storage area and heavy innervation of the common oviduct (Figure 5B and C). One line, *GMR14H04*-gal4, appears to label 2 bilateral sensory neurons, which cross the bursa and innervate the pre-storage area (Figure 5B).

We found two lines that appeared to innervate the muscles of the genitalia: *5-HT*$_7$-gal4 labels a single neuron, which innervates the pre-storage area as well as the muscles of genitals; and *GMR16H03*-gal4, along with vast innervation of entire bursa and stalk of the Sp it also labels a neuron, which innervates the external genitalia (Figure 5). The most interesting was the innervation pattern of *fru*^{16}-gal4 as it is very specific, only innervating the SR, the pre-storage area and the common oviduct (Figure 5B and C).

In general, we could not directly explain the effect of artificially manipulating sperm storage patterns via a specific Gal4 driver with the patterns of neuronal innervation of the female reproductive tract. Changes in Sp storage were not related to Sp innervation, as lines that innervate this organ did not all necessarily

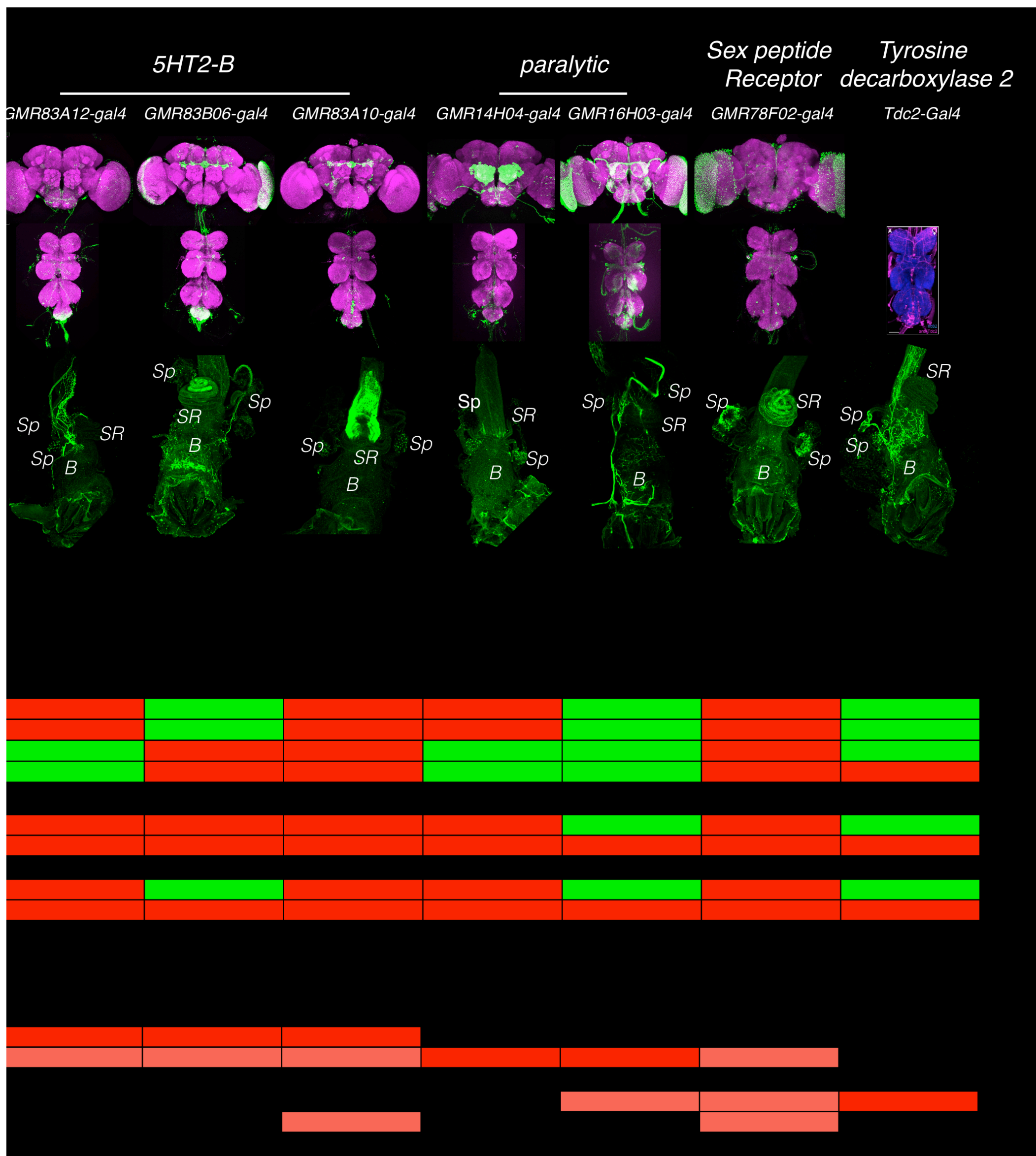
influence storage here, and all lines that showed changes in storage did not necessarily innervate the organ. Interestingly, all the lines that innervated the SR (*GMR83A12*-gal4, *dsx*-gal4, and *fru*^{16}-gal4) showed reduced sperm storage in the SR when activated with dTrpA1. However, reduced sperm storage in this organ was also achieved through gal4 drivers that showed no innervation of this organ (*GMR83A10*-gal4, *GMR83B06*-gal4, and *ap*-gal4).

Figure 5. Expression pattern and neuronal innervation of the female reproductive tract of neurons that support sperm storage. The expression pattern of the different Gal4 lines identified in the screen to influence sperm storage are shown. **(a)** Expression pattern in the central nervous system (brain and ventral nerve cord) of indicated Gal4 line: images were obtained from various sources: *fru*^{16} (Billeter and Goodwin, 2004); *fruitless* (Stockinger et al., 2005); *doublsex* (Rezával et al., 2012); *apterous* (Soller et al., 2006); 5HT7 (Becnel et al., 2011); and *Tdc2* (Rezával et al., 2014). See next page.



We conclude that the neuronal circuit that influences sperm storage and ultimately progeny production is composed of different neuronal populations, some of which directly innervated the reproductive tract, and others are located in the central nervous system.

note: an image of the brain *Tdc2* expression pattern was unavailable. The remaining images were obtained via Fly Light (http://flweb.janelia.org/). **(b)** Innervation of the female reproductive tract: expression pattern of the identified lines, as visualized with a *UAS-mCD8-GFP* reporter driven by the indicated Gal4 line, and anti-GFP antibody (green) staining. **(c)** Innervation pattern from analysis indicated as either absent (red) or present (green). **(d)** Summary from sperm storage (Table 2) indicated as either decreased (red), increased (green), or no change (black) when neurons either activated



GENES THAT FUNCTION TO INFLUENCE SPERM STORAGE IN LABELED NEURONS

Many of the Gal4 drivers included in the sperm storage screen were generated by transcriptionally fusing segments of the regulatory region of specific genes to the Gal4 transcription factor, leading to Gal4 expression in a subset of the full expression pattern of specific genes, and possibly ectopically in other cells. For example *GMR80B06*-gal4, *GMR80C01*-gal4, and *GMR80C03*-gal4 each represent a subset of the full regulatory region of the gene *caupolican (caup*). When these different groups of neurons were temporary activated or silenced during sperm storage, all three produced very different patterns of sperm storage compared to controls (Table 2). However, the Gal4 drivers *GMR83A12*-gal4, *GMR83A10*-gal4, and *GMR83B06*-gal4, which represent subset of the $5\text{-}HT_{2b}$ receptor gene expression, all gave very similar disrupted patterns of sperm storage. This may reflect that, unlike *caup*, $5\text{-}HT_{2b}$ not only labels neurons involved in sperm storage but also has a functional role within this neuronal circuitry with regards to sperm storage. Furthermore, it is likely that the protein products of these genes, whose regulatory sequences were used to manipulate sup-populations of neurons, are directly involved in sperm storage since they had been previously associated with other female reproductive behaviours. To test the functional role of the genes that labeled the neurons identified in our screen, we assessed female sperm storage for all available mutants as well as RNAi-mediated knock-downs of the gene product in the specific cellular population. The latter was achieved by specifically targeting RNAi constructs to the identified sub-populations of neurons via the Gal4-UAS system.

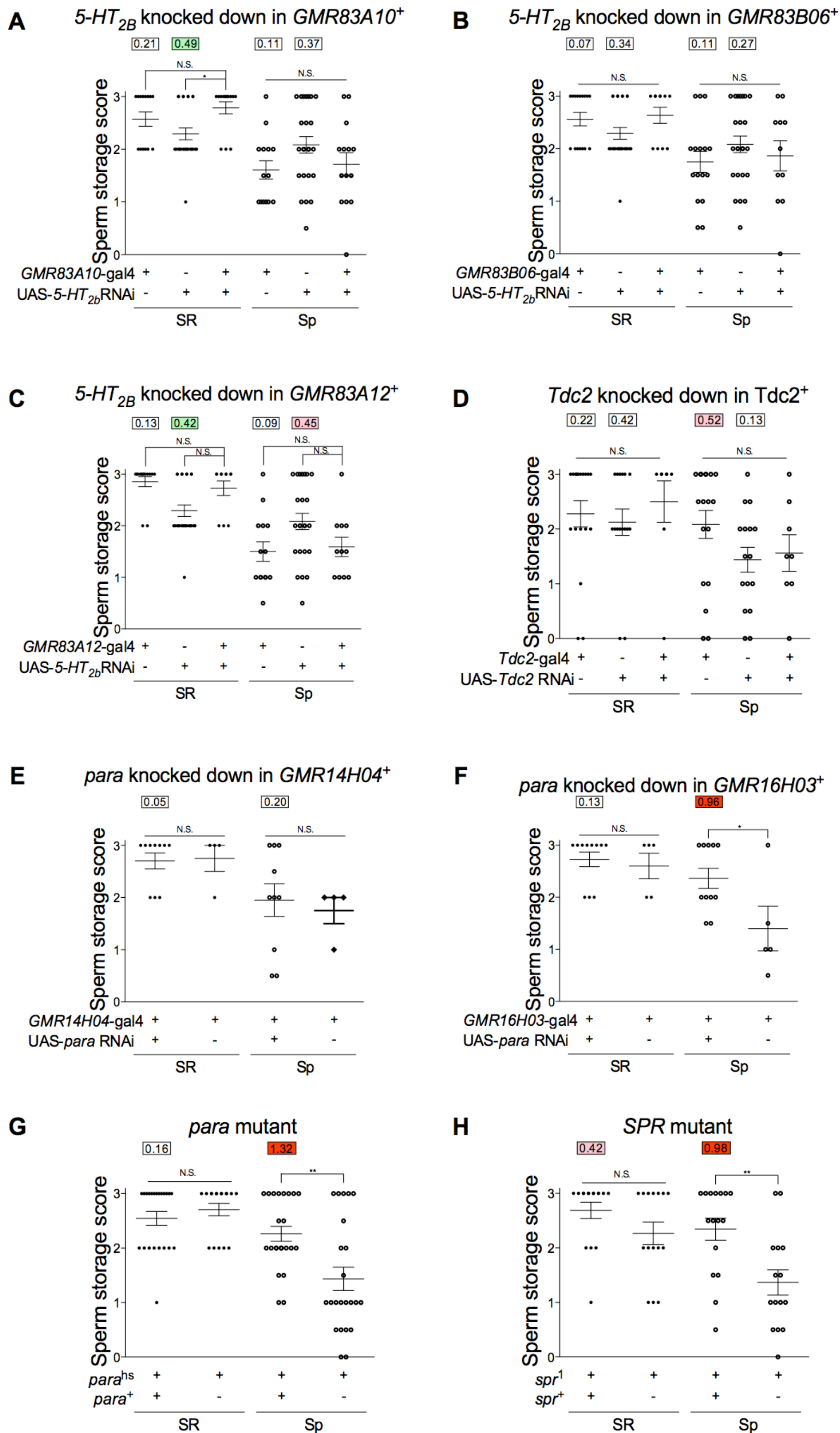
$5\text{-}HT_{2b}$ labels neurons but gene production is not involved in sperm storage

Although we found severe sperm storage restriction when we artificially activated three separate populations of $5\text{-}HT_{2b}$ receptor labeled cells, we did not find any significant decreases in sperm storage when we knocked-down this gene product in any of the populations, compared to controls (Figure 6A, B, and C; Supplementary Table 2).

This was surprising as this gene encodes a G-coupled protein serotonin receptor and serotonin has been associated with production or suppression of reproductive behaviours in many groups of animals including various species of insects (Pooryasin and Fiala, 2015; Vergoz et al., 2012; Yamane, 2014) and mammals

(Zhang et al., 2013) (for reviews see (Angoa-Pérez and Kuhn, 2015; Hull, 2011)). In *D. melanogaster*, variants in *5-HT$_{2b}$* are associated with the outcomes in sperm competition (Chow et al., 2012); and serotonin levels in the anterior bursa change as a response to mating (Heifetz et al., 2014), logistically poised to influence sperm storage. Furthermore, in this study, we found that these 3 non-overlapping neuronal populations of *5-HT$_{2b}$* derived Gal4 drivers all influenced sperm storage, and other PMRs, albeit with varying effects. We will therefore need to further characterize whether the RNAi constructs effectively reduced *5-HT$_{2b}$* to reach gene knock-down levels sufficient to give a phenotype.

One of the *5-HT$_{2b}$* drivers, *GMR83B06*-gal4, resulted in significantly decreased storage and fecundity, laid significantly less eggs, and significantly enhanced likelihood to remate when these population of neurons were artificially activated. This Gal4 driver is expressed in neurons that innervate the reproductive tract but not in areas that would necessarily indicate influence on sperm storage (posterior/mid bursa and common oviduct, Figure 5C). Similarly, another subpopulation of *5-HT$_{2b}$* expressing cells, *GMR83A10*-gal4, had very similar storage and PMR defects when artificially activated via dTrpA1 heat-shock. Interestingly, this line is not expressed in neurons that innervate the reproductive tract. The lack of innervation is not surprising, as there is very little neuronal expression in the ventral nerve cord, which is the site of neurons that send projection in the abdomen to innervate the reproductive tract (See Figure 1 for an illustration; Figure 5A and B). However, both *GMR83B06*-gal4 and *GMR83A10*-gal4 are expressed in the central complex in the brain. This structure supports male sexual behaviour, which makes this structure a candidate for a function in female sexual behaviour (Popov et al., 2005; Sakai and Ishida, 2001). Moreover, cells that express *5-HT$_7$*, another of the 5 serotonin receptors in *D. melanogaster* (Gasque et al., 2013), also showed reduction in sperm storage in both organs when artificially activated and increased sperm storage during neuronal silencing (Table 2). Interestingly, we again did not find *5-HT$_7$* expressing neurons innervating the SSOs but this gene is also expressed in the central complex. The third subpopulation of *5-HT$_{2b}$* expressing cells, *GMR83A12*-gal4, which also displayed restricted sperm storage did not show reduced fecundity. Surprisingly, neurons that project from the VNC do indeed innervate the SSOs.


A
5-HT2B knocked down in GMR83A10+
B
5-HT2B knocked down in GMR83B06+
C
5-HT2B knocked down in GMR83A12+
D
Tdc2 knocked down in Tdc2+
E
para knocked down in GMR14H04+
F
para knocked down in GMR16H03+
G
para mutant
H
SPR mutant
Sperm storage score
SR
Sp
N.S.

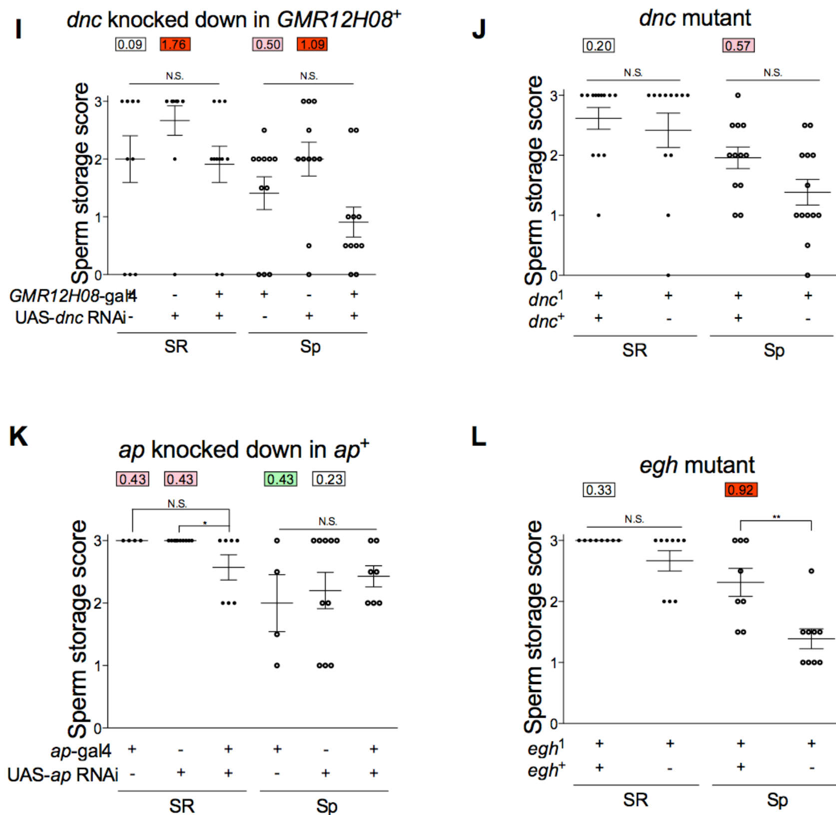


Figure 6. Identified genes both label and function within the neural circuitry that supports sperm storage. The effect of sperm storage was reduced in some females that were either had reduced gene expression via RNAi knock-down (**A**-**F**, **I**) or genetic mutation (**G**, **H**, and **J**). Scatterplot displaying individual female sperm storage scores (see Figure 1) of females of indicated genotype. Bar indicates mean and s.e.m. Colour box above controls represents the mean difference in sperm storage between control and experimental females. Direction and degree of differences indicated: white is no difference; pink is slight decrease and light green slight increase, dark red large decrease. N.S. = not significant, * = $p < 0.05$, ** = $p < 0.01$.

Taken together, it is clear that *5-HT$_{2b}$* expressing cells are involved in sperm storage, however, we do not have evidence that supports the necessity of this receptor in these cells within this process. Furthermore, we deduce that *GMR83A10*-gal4 and *GMR83B06*-gal4 expressing cells are most likely upstream within the circuitry because activation of both populations of neurons have very similar effects on female reproductive behaviour, both are expressed in the central complex, and one labels neurons that do not innervate the reproductive tract.

Tdc2 labels neurons but gene production is not involved in sperm storage

Similarly, to the serotonin receptors, knock-down of *Tdc2* failed to influence female sperm storage (Figure 6D; Supplementary Table 2). *Tdc2* is expressed in the central nervous system and regulates the production of tyramine and octopamine (Cole et al., 2005). Previous research also found that females that lacked tyramine and octopamine had normal sperm accumulation into storage (Avila et al., 2012), but abnormal sperm release (Avila et al., 2012) and egg laying behaviour (Cole et al., 2005).

It is very likely that neurons that are involved in sperm storage are also involved in egg fertilization, as the process of sperm release from storage must also be controlled for efficient sperm use. Indeed *Tdc2*$^{+}$ cells, when silenced reduced sperm storage in the spermathecae suggesting that these neurons are normally activated in a mated female and function to keep sperm in storage. This is also supported by the innervation pattern as these neurons innervate the stalk of the spermathecae Taken together, although *Tdc2* may label sperm organ control neurons, the functional role of *Tdc2* (tyramine and octopamine) in these neurons may indeed be limited to fertilization and egg related behaviour and may not extend to sperm accumulation.

Paralytic gene product is involved in spermathecae-specific sperm storage

We observed the sperm storage patterns of females either mutant for *para* or females that had *para* knocked-down in two different populations of *para*$^{+}$ cells. We found that mutant females, and females with *para* knocked-down in *GMR16H03*-gal4 labeled cells displayed significantly reduced sperm storage specific to their spermathecae, and no differences in sperm storage in females with *para* knocked-down in *GMR14H04*-gal4^{+} cells (Figure 6E, F, and G; Supplementary Table 2). The specific effect of reduction in the spermathecae does complement our data on expression as *GMR16H03*-gal4 labels neurons that innervate the stalk of the spermathecae (where *GMR14H04*-gal4 does not) and the results from our original screen that activation of these neurons reduced storage in this organ. *para* encodes the only voltage-gated sodium channel in *Drosophila* (Loughney et al., 1989) and is involved in neuronal excitability (Lilly et al., 1994). This suggests that *para* may function to influence the activity level of the *GMR16H03*-gal4 labeled neurons to directly influence the amount of sperm accumulated into storage. Alternatively, as *para* mutations also influence olfactory perception (Lilly et al., 1994), this sensory

system may be required for proper assessment of male quality and could, at least in theory, influence this process from a central location that influences cryptic female choice.

sex peptide receptor, dunce, and aperterous label SP-signal pathway and are involved in spermathecae-specific sperm storage

Neurons expressing these three genes are part of sex peptide signal transduction neuronal pathway, required for females to elicit a normal PMR (Chapman et al., 1996; Soller et al., 2006; Yapici et al., 2008). Interestingly, genetic manipulations interfering with the expression of all three genes lead to very similar effects on sperm storage. *SPR* mutant females had significantly reduced storage specific to the spermathecae (Figure 6H; Supplementary Table 2), and is identical to the sperm storage defects of artificially activating of a subset of *SPR*$^{+}$ cells with the *GMR78F02*-gal4 (Table 2). Previously, *SPR* has been shown to mediate sperm release from storage (Avila et al., 2015). Similar to our results with *Tdc2*-gal4, it seems that neurons involved in the controlled release from sperm storage are involved in the initial storage. However, the subset of *SPR*$^{+}$ cells that are labeled by our *GMR78F02*-gal4 line do not innervate the reproductive tract and are very sparse in CNS (Figure 5A). They are therefore most likely not part the same population as those labeled by *Tdc2*-gal4 (Figure 5A). And unlike *Tdc2*, *SPR* appears to function in SPR expressing neurons as SPR mutants stores less sperm in the Sp compared to controls (Figure 6H). *SPR* encodes a G-coupled protein receptor responsible for the effect of sex peptide on the PMR (Yapici et al., 2008). A hallmark of behaviour that is included in the PRM is that it not only post-mating behaviour but also that it occurs as a response to a mating-specific signal, ie a seminal fluid peptide. Although sperm storage is clearly a post-mating behaviour, the involvement of SPR suggests that sperm storage could be included in PMR.

Likewise, we assessed sperm storage in females either mutant for *dnc*, or had *dnc* knocked-down in a specific population of cells. Mutant females apparently stored less sperm compared to controls, but this was not statistically significant (Figure 6I and J; Supplementary Table 2). Similar to the sperm storage defects in *SPR* mutants, these reductions were specific to the spermathecae. *dnc* is also involved in the sex peptide response as mutant females injected with the peptide are significantly more likely to mate than controls (Chapman et al., 1996). Taken together, it appears that sex peptide may be involved in the storage process specific

to the spermathecae, and the transmission of this signal not only involves *SPR* and *dnc* expressing neurons, but also their gene products as well.

Finally, we knocked-down *ap* in *ap*$^{+}$ cells and found, similar to activation of these cells, significantly lower sperm storage scores in the SR (Figure 6K; Supplementary Table 2). As *ap*-gal4 does not label any neurons that innervate the female reproductive tract, (Figure 5B), it is likely that *ap* acts in neurons that are upstream in the circuitry. Some *ap*-expressing neurons in the ventral nerve cord are required for the sex peptide-dependent PMR (Soller et al., 2006) and these cells require normal *egh* expression and gene product during development for proper neuronal targeting (Soller et al., 2006). Therefore, we assessed *egh*$^{-}$ females to assess if this mutant also functions in sperm storage. Interestingly, *egh* mutants show sperm storage decrease in the spermathecae (Figure 6L; Supplementary Table 2), which reflects the sperm defects of *SPR* and *dnc* rather than that of *ap*. Together, these results suggest that *ap* functions in *ap*-expressing cells to influence sperm storage in the seminal receptacle. But this effect is separate from that of *egh*. The overlap between the results of *egh*, *dnc*, and *SPR* mutants does suggest the involvement of the sex peptide signal specifically for spermathecae storage. Neurons that express these three genes may represent a sex peptide signaling pathway representing neuronal circuitry supporting spermathecal-specific sperm storage.

It would be interesting to observe if variation in sperm storage could be influence by either genetic variation of sex peptide and/or SPR. Additionally, the involvement of this pathway also suggests that sperm storage may also be under conflict between males and females. It is possible that in the context of polyandry, females benefit from storing less sperm from each mating in order to maximize genetic diversity of offspring, where males would benefit from maximizing the number of sires from each copulation. It would be interesting to observe if variation in sperm storage could be influence by the amount of sex peptide males transfer as males who are exposed to rival males and therefore perceive increased sperm competition transfer for sex peptide to female during copulation compared to males that are raised in isolation (Wigby et al., 2009).

CONCLUSION

Although sperm storage by females is found across taxa, we know surprisingly little about the mechanisms that support it. The large interspecific variation in how sperm is stored, such as differences in storage organ morphology and duration of

storage (Holt and Fazeli 2016), indicates that sperm storage has evolved under many different constraints, probably related to the evolution of mating systems. The process of sperm storage is central to the reproductive success of many species, including *Drosophila melanogaster*, as any perturbations to it can have drastic consequences on fecundity. The female reproductive tract in this species is highly innervated (Avila et al., 2012; Rezaval et al., 2014; Rezaval et al., 2012; Figure 5 of this study) and these neurons most likely to control sperm during storage (Arthur et al., 1998) and usage (Avila et al. 2015; Chow et al., 2012), and finally sperm ejection (Lee et al., 2015, Chapter 6).

During copulation, the female receives the ejaculate, composed of sperm and the seminal fluid containing a variety of male derived compounds. Upon reception that virgin female reproductive tract undergoes well-defined stages of morphological changes (Adams and Wolfner 2007). Sperm eventually is transferred from the uterus, or bursa, to the two types of SSOs located at the anterior of the bursa. These two structures are very different with respect to morphology and function (see review Schnakenberg et al., 2012) and consistent with previous literature we also found that different neuronal populations can influence sperm storage in one organ and not the other. The sperm storage process is ended via sperm ejection: the removal of the unstored sperm and gelatinous mating plug (Lee et al., 2015; Manier et al., 2010). Previous experiments associated decreased ejection latency with reductions in offspring production (Lee et al., 2015; Lupold et al., 2013). Consistent with these findings, we found a relationship between probability of ejection and reduction in sperm storage in females with manipulated neuronal activity. Our findings strengthen the relationship between timing of ejection and sperm storage suggesting that females may use ejection behaviour to modify offspring production or even patterns of paternity in a polyandrous context.

Here we identified neurons for sperm storage, providing evidence that females actively control the process of sperm accumulation into storage organs. Future investigations into the neuronal circuitry of sperm storage should determine if/how these populations of neurons communicate with each other to explain patterns that we found in shared and unique genetic and cellular manipulations. In general, understanding of the female contributions to sperm storage pales in comparison to the wealth of knowledge on the male derived compounds that influence female reproductive behaviour. As the cellular substrates and female-derived gene products involved in post-copulatory female reproduction are discovered, the male-female interactions can start to be fully appreciated. As we have uncovered the influence of the sex peptide signaling pathway, it is possible that sperm storage is indeed a behaviour of the PMR and may have evolved under sexual conflict.

Furthermore, understanding neuronal circuitry supporting sperm storage in a once mated female would no doubt lead to, at the very least, the generation of specific testable hypothesis about the neuronal control of a twice mated female and the mechanisms of female cryptic choice.

ACKNOWLEDGEMENTS

We thank Ilse Jansen, Eva de Goeij, Lara van Renssen, and Nikki Spaan for help during the experiments. We are grateful to Jenke Gorter for critical comments on the manuscript. This work was supported by a grant from the Dutch organization for scientific research (NWO; reference: 821.02.020).

AUTHOR CONTRIBUTIONS

M.L. and J.-C.B. designed and interpreted the study. M.L. performed all experiments and the statistical analysis. M.L. and J.-C.B. wrote the manuscript.

SUPPLEMENTAL INFORMATION

		Fecundity						Egg-laying						Remating
		Distribution		Activated vs. natural				Distribution		Activated vs. natural				Activated vs. natural
Gal4 line	T	K2	*p* value	test	*t* or *U*	*df*	*p* value	K2	p value	test	*t* or *U*	*df*	*p* value	Fisher's exact test
Wild-type	22°C	1.24	0.5376	MW	112.00		0.1172	3.32	0.1898	t	3.83	43	**0.004**	$p = 0.433$
	29°C	22.93	**0.0001**					1.11	0.5727					
dNPF	22°C	6.10	**0.0473**	MW	112.00		0.7667	8.60	**0.0135**	MW	64.00		0.2642	$p = 0.645$
	29°C	22.05	**0.0001**					14.46	**0.0007**					
GMR47D09	22°C	3.24	0.1977	t	0.25	32	0.8065	3.30	0.1915	t	0.49	28	0.6295	$p = 0.165$
	29°C	0.79	0.6738					2.20	0.3326					
GMR74A11	22°C	2.01	0.3667	MW	55.00		0.1427	1.81	0.4044	t	1.25	27	0.2234	**$p = 0.030$**
	29°C	12.22	**0.0022**					0.79	0.672					
GMR78F06	22°C	0.67	0.7144	t	2.25	28	**0.0327**	0.73	0.695	t	0.91	27	0.3696	$p = 1.000$
	29°C	3.77	0.1515					3.69	0.1578					
GMR25H03	22°C	2.21	0.3316	MW	214.50		0.5334	1.34	0.5103	MW	7.00		**< 0.0001**	$p = 0.567$
	29°C	10.13	**0.0063**					20.41	**0.0001**					
GMR14H04	22°C	3.90	0.142	t	3.51	28	**0.0015**	3.95	0.1387	t	0.56	27	0.5775	$p = 1.000$
	29°C	3.44	0.1794					1.57	0.4561					
GMR16H03	22°C	0.76	0.6842	t	4.11	16	**0.0008**	4.82	0.0898	MW	3.50		**0.0003**	$p = 0.400$
	29°C	3.32	0.1902					9.62	**0.0081**					
GMR80C01	22°C	4.74	0.0937	MW	96.00		0.7104	0.20	0.9012	t	0.49	25	0.6298	$p = 1.000$
	29°C	16.70	**0.0002**					3.70	0.1573					
Ap	22°C	0.94	0.626	MW	21.00		0.0548	2.82	0.2446	MW	4.00		**0.0014**	$p = 1.000$
	29°C	14.50	**0.0007**					20.23	**0.0001**					
GMR83A10	22°C	3.09	0.2129	t	3.26	35	**0.0023**	2.24	0.3256	MW	89.00		0.4981	**$p = 0.039$**
	29°C	2.25	0.3249					7.89	**0.0194**					
GMR83B06	22°C	4.92	0.0854	MW	2.00		**0.0005**	0.58	0.7485	MW	16.50		**0.0005**	**$p = 0.005$**
	29°C	8.78	**0.0124**					25.62	**0.0001**					
fru^{16}	22°C	2.00	0.3684	t	3.27	41	**0.0022**	2.59	0.2735	MW	68.00		0.1022	$p = 0.569$
	29°C	0.28	0.8703					16.26	**0.0003**					

Supplementary Table 1. Summary of statistical tests to compare the effect of artificial activation of neurons on post-mating behaviour. All females expressed the temperature gated calium ion channel *dTrpA1* determined by the indicated Gal4 driver, with the exception of the wild-type controls. Within each genotype, mated females experienced 29C (artificially activation the neurons) or 22C (natural activity). The distribution of the fecundity and ovipositioning data was first analyzed for normality. To determine difference between temperature (Temp.) conditions within the same genotype, a t-test (t) was used if both groups were normally distributed, and a Mann Whitney (MW) was used if at least one was not. A Fisher's exact test was used to determine differences in remating behaviour.

	Gal4/UAS	Kruskal-Wallis		Dunn's Multiple Post hoc test		Mann Whitney	
organ	mutant	*KW*	*p*	vs. Gal4	vs. UAS	*U*	*p*
SR	GMR83A10	7.63	**0.022**	5.46, ns	**12.06, $p < 0.05$**		
Sp		4.03	0.13				
SR	GMR83B06	3.87	0.14				
Sp		1.64	0.44				
SR	GMR83A12	11.29	**0.003**	-3.12, ns	9.89, ns		
Sp		6.35	**0.042**	1.82, ns	-8.95, ns		
SR	Tdc2	2.02	0.36				
Sp		4.55	0.10				
SR	GMR14H04					19.00	0.94
Sp						16.00	0.61
SR	GMR16H03					24.00	0.73
Sp						9.50	**0.045**
SR	para-					163.00	0.50
Sp						137.00	**0.005**
SR	spr-					84.00	0.16
Sp						52.00	**0.007**
SR	ap	6.67	**0.036**	-4.50, ns	**-4.50, $p < 0.05$**		
Sp		0.49	0.78				
SR	egh						
Sp						8.50	**0.007**
SR	dnc	5.49	0.064				
Sp		5.77	0.056				
SR	dnc1	73.50	0.83				
Sp		45.00	0.069				

Supplementary Table 2. Summary of statistical analysis of the comparison of sperm storage scores (4 point scale 0-3) between genetic mutants or knock-downs and their respective genetic controls. SR = seminal receptacle, Sp = spermatheca, KW = Kruskal-Wallis statistic, ns = not significant.