Studies on the Neuroendocrine System that Controls the Mating-Induced Germline Stem Cell Proliferation

January 2018

Tomotsune AMEKU

Studies on the Neuroendocrine System that Controls the Mating-Induced Germline Stem Cell Proliferation

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy of Science
(Doctoral Program in Biological Sciences)

Tomotsune AMEKU

Table of contents

Abstract

Maintenance and regeneration of adult tissue requires a robust stem cell system that balances self-renewal with differentiation. Stem cells reside in specialized microenvironment called niche that provides short-range signals required for their maintenance and division. A number of studies have demonstrated the importance of local niche signals in regulating stem cell identity. On the other hand, stem cell activity must be modulated by systemic factor by responding to the environmental cues and changes in physiological status. But, this mechanism is poorly understood.

Here, I show that stem cell activity relies on the systemic signals via the different tissues in the fruit fly *Drosophila melanogaster*. In chapter 1, I report that female germline stem cells are activated by mating to sustain reproductive output. The mating-induced germline stem cell proliferation (both increase in number and proliferation rate of germline stem cells) is controlled by the neuroendocrine system.

In chapter 2, I report that germline stem cell is also modulated by the interorgan signaling. I identify the gut hormone that positively controls germline stem cell activity in response to mating. Importantly, the gut hormone affects niche signaling levels to control stem cell activity in a mating- and nutrition- dependent manner.

My study provides a new insight into stem cell biology to explain how systemic factors couple stem cell behavior to physiological status such as mating and diet through the signaling in the different tissues.

Abbreviations

20E: 20-Hydroxyecdysone
7dC: 7-dehydrocholesterol
BMP: Bone Morphogenetic Protein
Bm: *Bombyx mori*
CpCs: Cap Cells
Dib: Disembodied
Df: Deficiency
Dpp: Decapentaplegic
EBs: Enteroblasts
ECs: Enterocytes
EECs: Enteroendocrine cells
EcR: Ecdysone Receptor
EtOH: Ethanol
GnRH: Gonadotropin-Releasing Hormone
GSCs: Germline Stem Cells
ISCs: Intestinal Stem Cells
LH: Luteinizing Hormone
mSP: Membrane-bounded Sex Peptide
Nobo: Noppera-bo
NPF: Neuropeptide F
NPFR: Neuropeptide F Receptor
Nvd: Neverland
OSCs: Ovarian Somatic Cells
PBS: Phosphate-Buffered Saline
Phm: Phantom
pMad: phosphorylated Mad
Put: Punt
RNAi: RNA interference
RT: Room Temperature
Sad: Shadow
Sax: Saxophone
Shd: Shade
Shi: Shibire
sNPF: short Neuropeptide F
SP: Sex Peptide
SPR: Sex Peptide Receptor
Spo: Spook
Sro: Shroud
Tkv: Thickveins
TNT: Tetanus Toxin
TRF assay: Time-Resolved Fluorescence assay

General Introduction

Maintenance and regeneration of adult tissue requires a robust stem cell system that balances self-renewal with differentiation (Spradling et al., 2011). Stem cells reside in a specialized microenvironment, called niche, where they are exposed to local signals required for maintenance of stem cell function (Kirilly and Xie, 2007; Spradling et al., 2001). This robust stem cell system must be precisely modulated by the local and systemic signals since an abnormality of stem cell regulation could cause loss of tissue integrity or tumorigenesis (Drummond-Barbosa, 2008). While a number of studies have demonstrated the importance of local niche signals, it is poorly understood how systemic signals may modulate stem cell activity in response to environmental cues and changes in physiological status.

The ovary of fruit fly, *Drosophila melanogaster* is one of the most powerful tools to study adult stem cell behavior *in vivo* (Kirilly and Xie, 2007; Wong et al., 2005). *Drosophila* ovary consists many strings of egg chamber called ovariole (Spradling., 1993). In the most anterior region of ovariole called germarium, germline stem cells (GSCs) are located and give rise to the mature eggs. GSCs can divide symmetrically to produce generative cell population and also asymmetrically to produce daughter cells called cystoblasts. Cystoblast undergoes differentiation into 15 nurse cells and one oocyte in each egg chamber that is surrounded by somatic follicle cells. Therefore, the balance between self-renewal and differentiation of GSCs has pivotal role in regulating oogenesis (Spradling et al., 2011).

Despite the importance of niche signals is unquestionable, systemic signals are also required for GSC regulation, which sustains reproductive output. But, it is poorly understood whether and how crucial systemic signals influence GSC behavior in *Drosophila*.

In many animals, mating is a one of the process that is essential for activation of reproduction. What is the role of mating to achieve prosperity of the species? One of the answers is to success fertilization. On the other hand, mating also has other functions such as inducing many changes in mated females. For example, mating promotes ovulation in a cat, maintains pregnancy in a mouse and strengthens of the bonds to the partner in many animals including humans (Robertson, 2007). These are adaptive changes to achieve

effective prosperity of the species. However, little is known about the molecular mechanisms of these signaling pathways, which connect the mating input to post-mating responses. In *Drosophila*, the mating causes many behavioral changes in females such as increased egg laying and decreased receptivity to maximize the reproductive output (Kubli, 2003). Given that all eggs are fueled by GSCs, mating may affect GSC activity in response to mating. But it is still unclear whether mating input activates reproduction at cellular levels not only behavioral and physiological levels.

Chapter 1

Neuroendocrine Control of the Mating-Induced Germline Stem Cell Proliferation

Abstract

Mating and gametogenesis are two essential components of animal reproduction. Gametogenesis must be modulated by the need for gametes, but it is poorly understood how mating, a process that utilizes gametes, may modulate the process of gametogenesis. Here, I report that mating promotes female germline stem cell (GSC) proliferation in the fruit fly *Drosophila melanogaster*. Mating-induced GSC increase is stimulated by a male-derived Sex Peptide (SP) and its receptor SPR, the components of a canonical neuronal pathway that induces a post-mating behavioral shift in mated female flies. In addition, I show that ecdysteroid, the major insect steroid hormone, regulates mating-induced GSC proliferation. Ovarian ecdysteroid level increases after mating and transmits its signal through the ecdysone receptor expressed in the ovarian somatic cells to induce GSC proliferation. Importantly, neuronal SP signaling positively controls ovarian ecdysteroid biosynthesis to induce the mating-induced GSC proliferation. My study illustrates how stem cell activity is coordinately regulated by the neuroendocrine system to sustain reproductive output in response to mating.

Introduction

Gametogenesis is modulated by stimuli from environments surrounding individual organisms. One such example is mating stimulus, which accelerates egg production (Wolfner, 2009). Because mating is a process that utilizes many eggs, it is possible that mating may modulate the process of gametogenesis. However, it remains largely unknown how mating affects gametogenesis at a cellular level.

Gametes originate from a critical cell population called germline stem cells (GSCs) in most animals including fruit fly *Drosophila melanogaster*. *Drosophila* GSCs reside in a specialized microenvironment, or niche, where they are exposed to local signals required for stem cell function **(Fig 1-1A)** (Spradling et al., 2001, 2011). A number of studies have reported that the niche has a major role in regulating GSC proliferation and maintenance. However, little is known about whether and how GSC number is regulated by the signals from the external environment, such as mating.

In this study, I tackle this question and show that mating stimulates GSC proliferation in female *Drosophila*. I find that the mating-induced GSC increase is regulated by the Sex Peptide (SP) signaling pathway, a canonical pathway stimulated by seminal fluid from males. I also identify ecdysteroid as a mediator of mating-induced increase in GSC number under the control of neuronal SP signaling. My study provides evidence that there is an essential link between neuronal SP signaling and ovarian ecdysteroid signaling to control GSC activity that may sustain reproductive output.

Results

Mating stimulates GSC proliferation without increased niche size

To address how gametogenesis may be stimulated at the cellular levels upon mating, I first measured the number of female GSCs in the absence or presence of mating. Virgin females were mated with males and used for assay 1 day after mating. GSC number was determined based on the morphology and position of their anteriorly anchored spherical spectrosome. I confirmed that there were germarium (tip of ovary) that contain 1, 2 or 3 GSCs in each ovariole **(Fig 1-1B)**. I found that the mated female flies had significantly more GSCs compared with the virgin females **(Fig 1-1B)**. Furthermore, mated female flies displayed an increased frequency of mitotic GSCs positive for phospho-histone H3 **(Fig 1-2A)**, suggesting that mating stimulates GSC proliferation as well. On the other hand, mating did not increase the number of cap cells **(Fig 1-2B)**, a critical GSC niche component (Spradling et al., 2001), indicating that mating-induced GSC proliferation is achieved without changing overall niche architecture.

I observed that the mating-induced GSC increase lasted for 6 days, but not 7 days after the first mating **(Fig 1-2C)**. At 7 days after the first mating, females that were the second mated with males showed an increase in GSC number after mating again **(Fig 1-2C)**. Given that the length of this increase period is consistent with the effect of male seminal fluid as most of the sperm is lost about 1 week from first mating (Manning, 1962, 1967), I speculated that the mating-induced GSC increase occurred in a seminal fluid-dependent manner.

Neuronal sex peptide signaling regulates mating-induced increase in GSC number

Several behaviors specific to post-mated female flies, such as increased egg laying and decreased receptivity, are induced by a peptide in male seminal fluid called Sex Peptide (SP) (Kubli, 2003). I therefore asked whether SP signaling is involved in the mating-induced GSC increase. I found that wild-type female flies did not show an increase in GSC number after mating with *SP* null male flies **(Fig 1-3A)**, suggesting that male-derived SP, but not physical interaction of genitalia, causes the mating-induced GSC increase.

SP is received by female neurons expressing the specific receptor gene *Sex peptide receptor* (*SPR*) (Häsemeyer et al., 2009; Yapici et al., 2008). The *SPR*-positive neurons located on the oviduct, which also co-express *pickpocket* (*ppk*) and *fruitless* (*fru*), are particularly crucial in inducing the postmating behavioral changes in female flies (Rezával et al., 2012). I therefore measured the number of GSC in transgenic RNAi animals knocked down *SPR* function in *ppk*-positive neurons, *fru*-positive neurons and almost all post-mitotic neurons using *ppk-GAL4* (Grueber et al., 2007), *NP21-GAL4* (Hayashi et al.; Kimura et al., 2008) and *elav-GAL4* (Luo et al., 1994) drivers, respectively. All of these neuronal *SPR* RNAi led to suppression of a mating-induced GSC increase **(Fig 1-3B)**. I also observed that the neuronal *SPR* RNAi disrupted an increase in mitotic GSCs after mating **(Fig 1-3C)**, suggesting that SP signaling is required for mating-induced GSC proliferation. Conversely, I also found that a forced expression of the *SP* gene in either *ppk*- or *fru*-positive female neurons caused GSC increase even in virgin female flies **(Fig 1-3B)**. Taken together, these results suggest that neuronal SP signaling is both necessary and sufficient for inducing GSC increase.

Mating-induced ovarian ecdysteroid biosynthesis mediates an increase in GSC number after mating

To explore which mechanism(s) might mediate a mating-induced GSC increase, I focused on the major insect steroid hormone, ecdysteroid. The ecdysteroid signaling in the ovary is involved in the long-lasting maintenance of GSC number throughout adult life (Ables and Drummond-Barbosa, 2010; Ables et al., 2015; König et al., 2011; Morris and Spradling, 2012; Uryu et al., 2015). However, it has never been experimentally investigated whether and how ovarian ecdysteroid biosynthesis is affected by mating, or is involved in GSC increase. To address this question, I measured the ecdysteroid level in virgin and mated female ovaries. I found that mating increased the ecdysteroid level in the ovaries of mated female flies as compared with virgin female **(Fig 1-4A)**. To investigate whether a mating-induced increase in the ecdysteroid level is owing to *de novo* biosynthesis in the ovary, I generated adult ovary-specific RNAi animals for *neverland* (*nvd*), which encodes the ecdysteroidogenic enzyme responsible for converting dietary cholesterol into 7-dehydrocholesterol (7dC)

(Yoshiyama-Yanagawa et al., 2011; Yoshiyama et al., 2006). I knocked down *nvd* expression with *c587-GAL4* driver that was active in the ovarian somatic cells (escort cells and undifferentiated follicle cells). The ovarian somatic cell-specific *nvd* RNAi females displayed reduced Nvd protein levels in the follicle cells **(Fig 1-4B)**. I confirmed that the *nvd* RNAi mated female flies did not exhibit an increase in ovarian ecdysteroid level after mating, but exhibited a significant reduction when compared with the genetic controls **(Fig 1-4A)**. Impairment of the mating-induced ovarian ecdysteroid biosynthesis was restored by co-expression of the wild-type form, but not the enzyme-dead form, of the silkworm *Bombyx mori* orthologue of *nvd* (*nvd-Bm*) **(Fig 1-4A)**, suggesting that the ovarian *nvd* RNAi phenotype is not an off-target effect.

In the ovarian *nvd* RNAi animals, I observed that neither the total number of GSCs **(Fig 1-4C)** nor the number of mitotic GSCs **(Fig 1-4D)** increased after mating. Consistent with the ecdysteroid level data, the suppression of a mating-induced GSC increase in the *nvd* RNAi animals was rescued by co-expression of the wild-type form of *nvd-Bm*, but not the enzyme-dead form in mated females **(Fig 1-4C)**. I also confirmed that the low ecdysteroid level phenotype in the *nvd* RNAi mated female flies was rescued by oral administration of 7-dehydrocholesterol, the downstream metabolite generated by Nvd **(Fig 1-5A)**. Consistent with this data, the *nvd* RNAi females fed 7dC showed a significant increase in GSC number in mated females as compared with virgin females **(Fig 1-5B)**. The phenotype of GSC number in *nvd* RNAi was also rescued by oral administration of 20-hydroxyecdysone (20E), the biologically active ecdysteroid **(Fig 1-5B)**. To further examine the role of mating-induced ecdysteroid biosynthesis in controlling ovarian GSC number, I conducted an additional experiment using the ovary-specific RNAi for *shadow* (*sad*), which is another ecdysteroid biosynthesis enzyme (Warren et al., 2002). The ovarian *sad* RNAi reduced the Sad protein level in the follicle cells **(Fig 1-4B)** and exhibited the same phenotypes as observed using the ovarian *nvd* RNAi female flies **(Fig 1-4A, 1-5B)**.

To examine whether ecdysteroids are received in the ovary, I used flies with loss of *ecdysone receptor* (*EcR*) function, which encodes a receptor component for 20E. I found that a mating-induced increase in GSC numbers was suppressed in either hypomorphic alleles or

c587-GAL4-driven RNAi of the *EcR* gene **(Fig 1-5C)**. Taken together, these data suggest that ecdysteroid biosynthesis in the ovary is activated by mating stimuli, and the ovarian ecdysteroid signaling mediates a mating-induced GSC increase.

Ovarian ecdysteroid biosynthesis is required for reproductive output

To test if the mating-induced increase in the ovarian ecdysteroid levels is required for reproductive output, not only controlling GSC increase, I counted the number of laid eggs from the mated females of the ovarian *nvd* RNAi female flies. I found that the *nvd* RNAi females showed a significant reduction in the number of laid eggs as compared with the control females **(Fig 1-6A, 1-6B)**. I also confirmed that the phenotype of the number of laid eggs in the *nvd* RNAi was restored by oral administration of 7dC or 20E **(Fig 1-6C)**. Taken together, these data suggest that ovarian ecdysteroid biosynthesis after mating is required for both an increase in egg and GSC number.

SP signaling positively controls ecdysteroid biosynthesis to induce mating-induced increase in GSC number

The important question to be addressed is whether SP signaling regulates the mating-induced GSC increase via ovarin ecdysteroid signaling. I found that a mating-induced increase in the ovarian ecdysteroid level was significantly suppressed in the mated female flies of neuronal *SPR* RNAi **(Fig 1-7A)**. Conversely, the ovarian ecdysteroid level increased in the neuronal forced expression of *SP* in virgin female flies **(Fig 1-7A)**. These results suggest that SP signaling is both necessary and sufficient for activating ovarian ecdysteroid biosynthesis.

I also examined if SP-SPR signaling affects transcription levels of ecdysteroid biosynthesis enzyme genes. The expression levels of most of ecdysteroidogenic enzyme genes (*nvd*, *sro*, *spo*, *phm*, *dib*, *sad* and *shd*) in the mated female ovaries were upregulated after mating **(Fig 1-7B)**. The transcriptional increase in *nvd* and *phm* were significantly impaired in neuronal *SPR* RNAi adult female flies **(Fig 1-7B)**. These data imply that mating-

dependent transcriptional up-regulation of ecdysteroidogenic enzyme genes is partially, but not fully, regulated by SP-SPR signaling.

Importantly, the GSC number phenotype of the *ppk-GAL4-* or *NP21-GAL4*-driven *SPR* RNAi mated female flies is owing to ecdysteroid loss, since the GSC phenotype was rescued by oral administration of 20E **(Fig 1-7C)**. In contrast, curiously, the impairment of GSC increase in female flies mated with *SP* null males was not rescued by oral administration of 20E **(Fig 1-7D)**. Taken together, my data strongly suggest that the mating-induced GSC increase is regulated by ovarian ecdysteroid whose biosynthesis is positively controlled by neuronal SP signaling in mated female flies **(Fig 1-8A)**, while there must be an unknown pathway dependent on SP but independent of the SPR-ecdysteroid cascade **(Fig 1-8B)**.

Discussion

In *Drosophila*, the behavioral shift, known as the post-mating response, is activated by the seminal peptide SP via binding to SPR that causes silencing of *SPR*-positive neurons (Feng et al., 2014; Häsemeyer et al., 2009). Although *SPR*-positive neurons are detected in the female reproductive tract, ventral nerve chord, and brain (Hussain et al., 2016; Yapici et al., 2008), a restricted subset of *SPR*-positive neurons located on the reproductive tract, which is also *ppk*- and *fru*-positive, is necessary and sufficient for inducing many aspects of the post-mating response (Rezával et al., 2012; Yapici et al., 2008).

I here propose that SP-SPR signaling in the shared reproductive tract neurons also play a crucial role in regulating GSC proliferation. Previous study has reported that *SPR*-positive neurons on the reproductive tract innervate the downstream SP abdominal ganglion neurons, which further project and signal into the central brain to modulate post-mating switch (Feng et al., 2014). It is interesting to examine how shared these downstream neurons are to control mating-induced GSC proliferation and the canonical post-mating response, whereas the precise neuronal circuit transmitting the SP signal has not yet fully elucidated.

My results show that ovarian ecdysteroids are required for post-mating GSC proliferation. Previous studies have shown that EcR and the downstream signaling molecules are required for maintaining female GSCs during the aging process in *Drosophila* without a change of cap cell number (Ables and Drummond-Barbosa, 2010; König et al., 2011; Morris and Spradling, 2012). My study, in conjunction with these previous results, suggests that ovarian ecdysteroid signaling is essential for not only long-lasting maintenance, but also the mating-induced acute proliferation.

In contrast to the necessity of the ecdysteroid signaling for GSC proliferation, my results suggest that ecdysteroids appear not to be sufficient for the phenomena, as oral 20E administration itself does not increase GSC number in virgin female flies. Thus, the ovarian ecdysteroid appears to be a permissive, but not instructive, factor for mating-induced GSC increase. Related to the insufficiency of ecdysteroids, my data also suggest that SP-dependent induction of GSC increase appears not to be solely due to the SPR-ecdysteroid

cascade. First, *SP* overexpression is sufficient to induce ovarian ecdysteroid biosynthesis and GSC proliferation. Second, the GSC phenotype in *ppk/fru*-promoter-driven *SPR* RNAi animals is rescued by oral administration of 20E. Third, nevertheless, the impairment of GSC increase in female flies mated with *SP* null males is not rescued by oral administration of 20E. These results imply that there must be an unknown pathway that acts downstream of SP and is independent of the SPR-ecdysteroid cascade. In facts, a recent study has proposed that there must be additional receptor(s) required for inducing SP-dependent post-mating switch (Haussmann et al., 2013). Elucidating such additional receptor(s) and its/their signaling pathway would be important to comprehensively understand the mating-induced GSC proliferation in future.

The important question to be addressed in the future is which factor(s) directly stimulate ovarian ecdysteroid biosynthesis downstream of SP signaling. In the larval stages, several peptidergic and monoaminergic factors play pivotal roles in stimulating ecdysteroid biosynthesis in the endocrine organ called the prothoracic gland (Niwa and Niwa, 2014; Ohhara et al., 2015; Shimada-Niwa and Niwa, 2014). However, molecular mechanisms regulating ovarian ecdysteroid biosynthesis are still poorly understood in *Drosophila*. It can be assumed that *SPR* is expressed in ovarian somatic cells, and SP might be directly received by SPR in the ovaries, as SPR suppresses ecdysteroid biosynthesis by receiving a neuropeptide during larval development of the silkworm *Bombyx mori* (Yamanaka et al., 2010). However, based on immunohistochemistry using anti-SPR antibody, it is unlikely that SPR is present in the ovary (Yapici et al., 2008), implying that an existence of indispensable neuronal and/or humoral factors that connect SP-SPR signaling in the reproductive tract neurons with ovarian ecdysteroid biosynthesis for mating-induced GSC increase.

A possible candidate of such a factor is ovary ecdysteroidogenic hormone (OEH) in the mosquito *A. aegypti*, the neuropeptide which is known to induce ovarian ecdysteroid biosynthesis and regulate egg maturation (Brown et al., 1998; Vogel et al., 2015), while a role of OEH in GSC proliferation has not yet been examined. Somehow it seems that orthologues of *OEH* genes have been lost from the *D. melanogaster* subgroup (Veenstra, 2010; Vogel et al., 2015). It would therefore be intriguing to identify and characterize

14

specific humoral factor(s) controlling ovarian ecdysteroid biosynthesis in response to SP signaling in *D. melanogaster*.
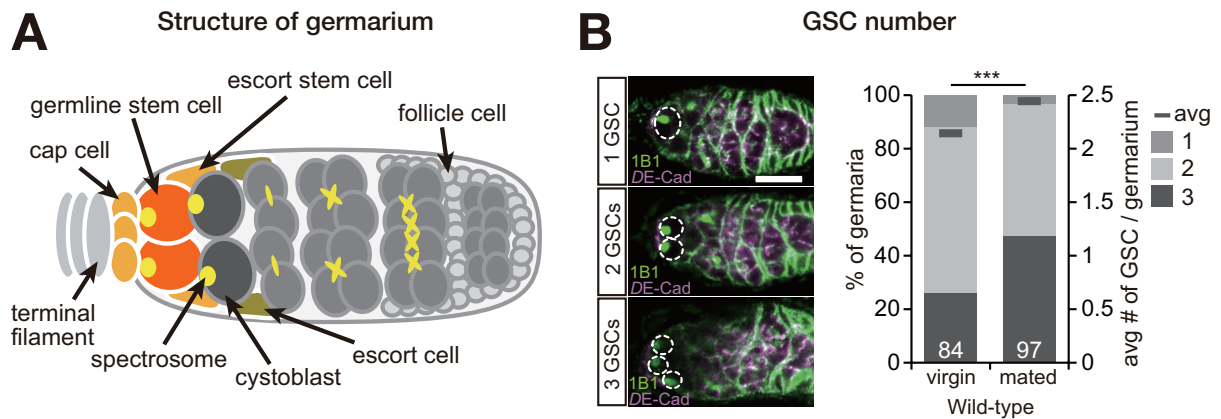
Figures

Figure 1-1. Mating increases GSC number

(A) *Drosophila* germarium (The tip of ovariole). GSCs reside in a niche, comprising somatic cells such as cap cells, terminal filament, and escort stem cells. GSCs are identifiable by their typical spectrosome morphology and their location (adjacent to the cap cells). GSC division produces one self-renewing daughter and one cystoblast that differentiates into a germline cyst.

(B) Left: Representative images of the germaria of wild-type flies containing one, two, or three GSCs in each germaria. Samples were stained with 1B1 (green) and anti-*D*E-cadherin (magenta) antibodies, which visualized GSCs (dotted circles) and overall cell membranes, respectively. Right: Frequencies of germaria containing one, two, and three GSCs (left y axis), and average number of GSCs per germarium (right y axis) in virgin and mated females in wild-type flies. Mated females showed increased GSC numbers as compared with virgin females.

Notes: The number of germaria analyzed is shown inside bars in **B**. For statistical analysis, a Wilcoxon rank sum test was used in **B**. ***$P \leq 0.001$.
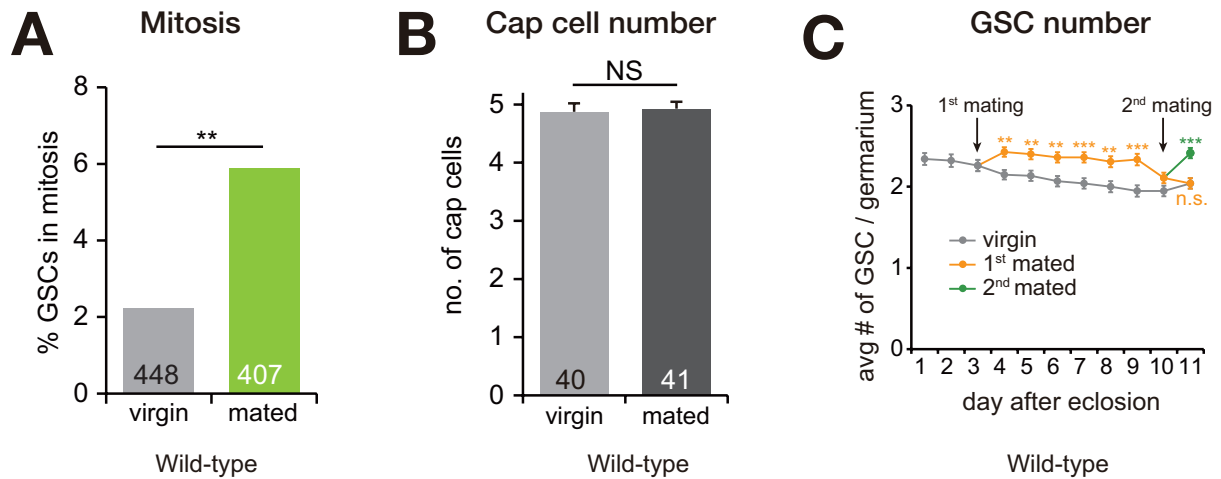
Figure 1-2. Mating stimulates GSC proliferation without changing in niche cell number

(A) Frequency of mitotic GSCs was counted by staining with anti-phospho-histone H3, which is a marker for mitotic cell division. Mated females showed an increased rate of mitotic GSCs as compared with virgin females.

(B) The number of cap cells was counted by staining with anti-Lamin-C antibody, which is a marker for the cap cells. Mating did not change the number of cap cells.

(C) Temporal changes in GSC number in virgin and mated females. Females were mated with males for the first time 3 days after eclosion (1st mating) and in the second time 7 days after 1st mating (2nd mating).

Notes: The number of germaria analyzed is shown inside bars in **A** and **B**. Values are presented as the mean with standard error of the mean in **B** and **C**. For statistical analysis, Chi-square analysis was performed for **A**. A Student's t-test was used for **B**. A Wilcoxon rank sum test was used for **C**. ***$P \leq 0.001$, **$P \leq 0.01$, NS, non-significant ($P > 0.05$).
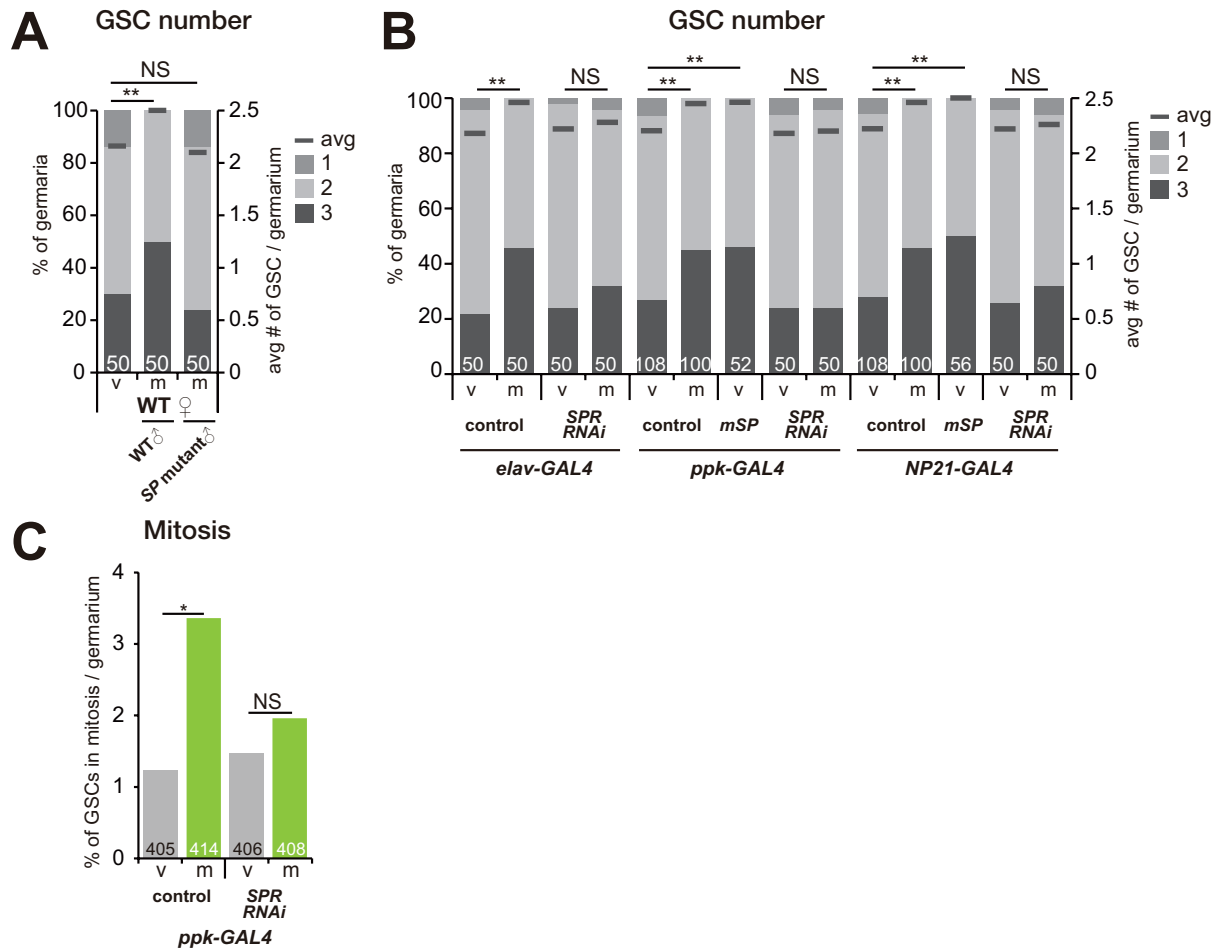
Figure 1-3. Neuronal SP signaling regulates the mating-induced GSC proliferation

(A, B) Frequencies of germaria containing one, two, and three GSCs (left y axis), and average number of GSCs per germarium (right y axis) in virgin (v) and mated (m) female flies. **(A)** Wild-type females were mated with wild-type and *SP* trans-heterozygous mutant adult male flies ($SP^0/SP^{\Delta 41}$). **(B)** Adult female flies overexpressing the membrane bound form of *SP* (*mSP*) or transgenic *SPR* RNAi were mated with wild-type male flies under the control of several neuronal GAL4 drivers (*elav-GAL4*, *ppk-GAL4* and *fru (NP21)-GAL4*).

(C) Frequency of mitotic GSCs was counted by staining with anti-phospho-histone H3 in *SPR* RNAi female flies. *SPR* RNAi suppressed promoted cell division after mating.

Notes: The numbers of germaria analyzed are shown inside the bars in **A**, **B** and **C**. For statistical analysis, a Wilcoxon rank sum test was used for **A** and **B**. Chi-square analysis was performed for **C**. **$*P \leq 0.01$, *$P \leq 0.05$, NS, non-significant ($P > 0.05$).
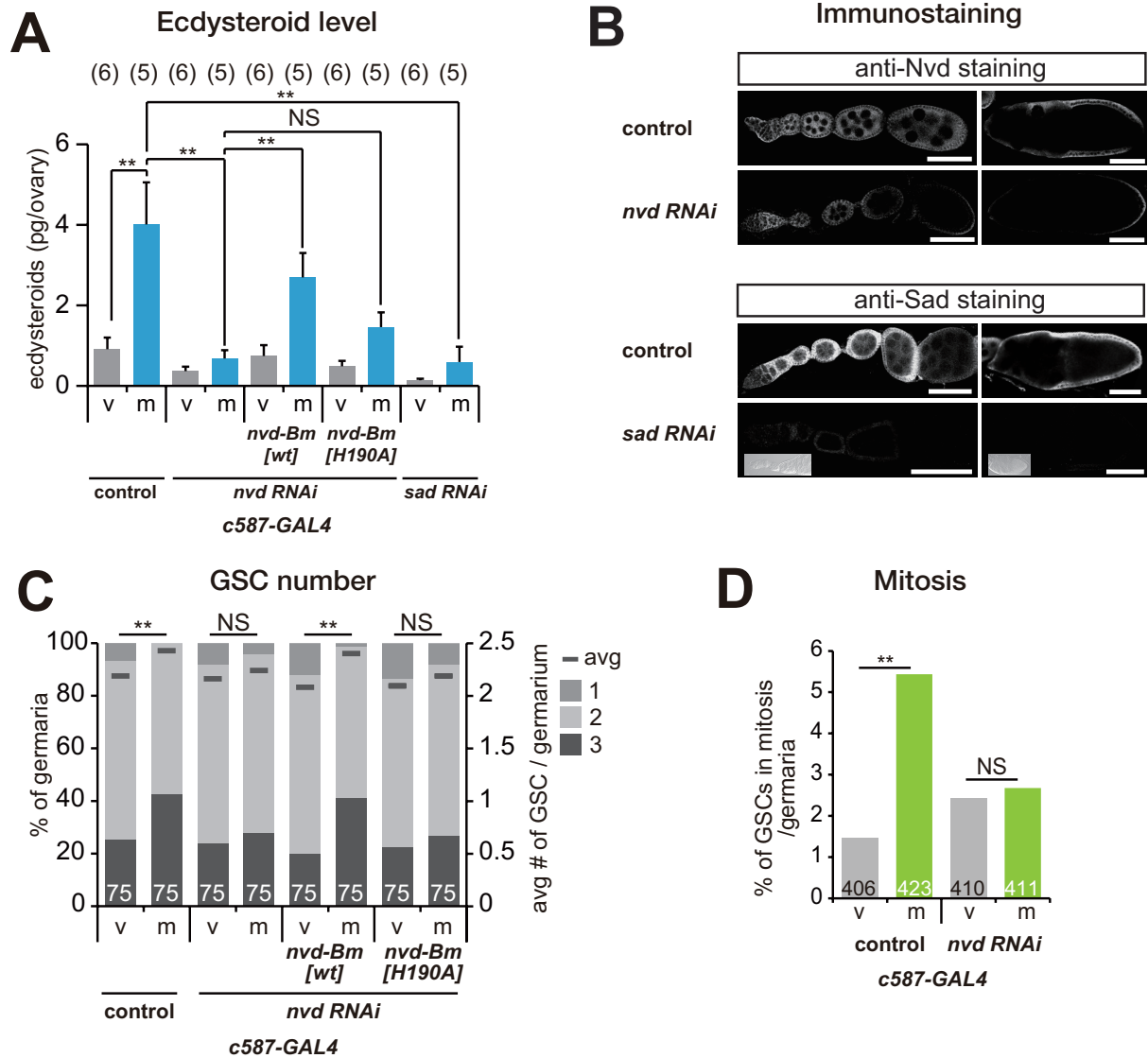
Figure 1-4. Ovarian ecdysteroid biosynthesis regulates GSC proliferation after mating

(A) Ecdysteroid levels in virgin (v) and mated (m) females in the ovarian somatic cell-specific (escort cells and follicle cells) *nvd* RNAi female flies. *c587-GAL4* driver was crossed with control or *UAS* transgene strains as indicated. *UAS-nvd-Bm [wt]* and *UAS-nvd-Bm [H190A]* overexpressed the wild-type form and enzymatic inactive form of *Bombyx mori nvd* transgenes, respectively.

(B) Anti-Nvd and anti-Sad immunostaining in ovarioles in somatic follicle cell-specific RNAi for *nvd* and *sad*, respectively. In *nvd* RNAi ovarioles, the anti-Nvd signal was particularly reduced in stages 2–6 (left column) and stage 10 (right column) follicle cells. Anti-sad signal was reduced in stages 2–6 and stage 10 follicle cells of *sad* RNAi female flies. Inset of

antiSad immunostaining image is a light-field image of the same specimen. Scale bar represents 50 μm.

(C) Frequencies of germaria containing one, two, and three GSCs (left y axis), and average number of GSCs per germarium (right y axis) of follicle cell-specific *nvd* RNAi animals with or without the *B. mori nvd* transgene.

(D) Frequency of mitotic GSCs was counted by staining with anti-phospho-histone H3 in *nvd* RNAi female flies. Knocking down *nvd* suppressed promoted cell division after mating.

Notes: Values are represented as the mean with standard error of the mean in **A**. The numbers of samples examined are indicated in parentheses in **A**. The numbers of germaria analyzed are shown inside bars in **C** and **D**. For statistical analysis, Dunnett's test was used for **A**. A Wilcoxon rank sum test was used for **C**. Chi-square analysis was performed for **D**. **$P \leq 0.01$, *$P \leq 0.05$, NS, non-significant ($P > 0.05$).
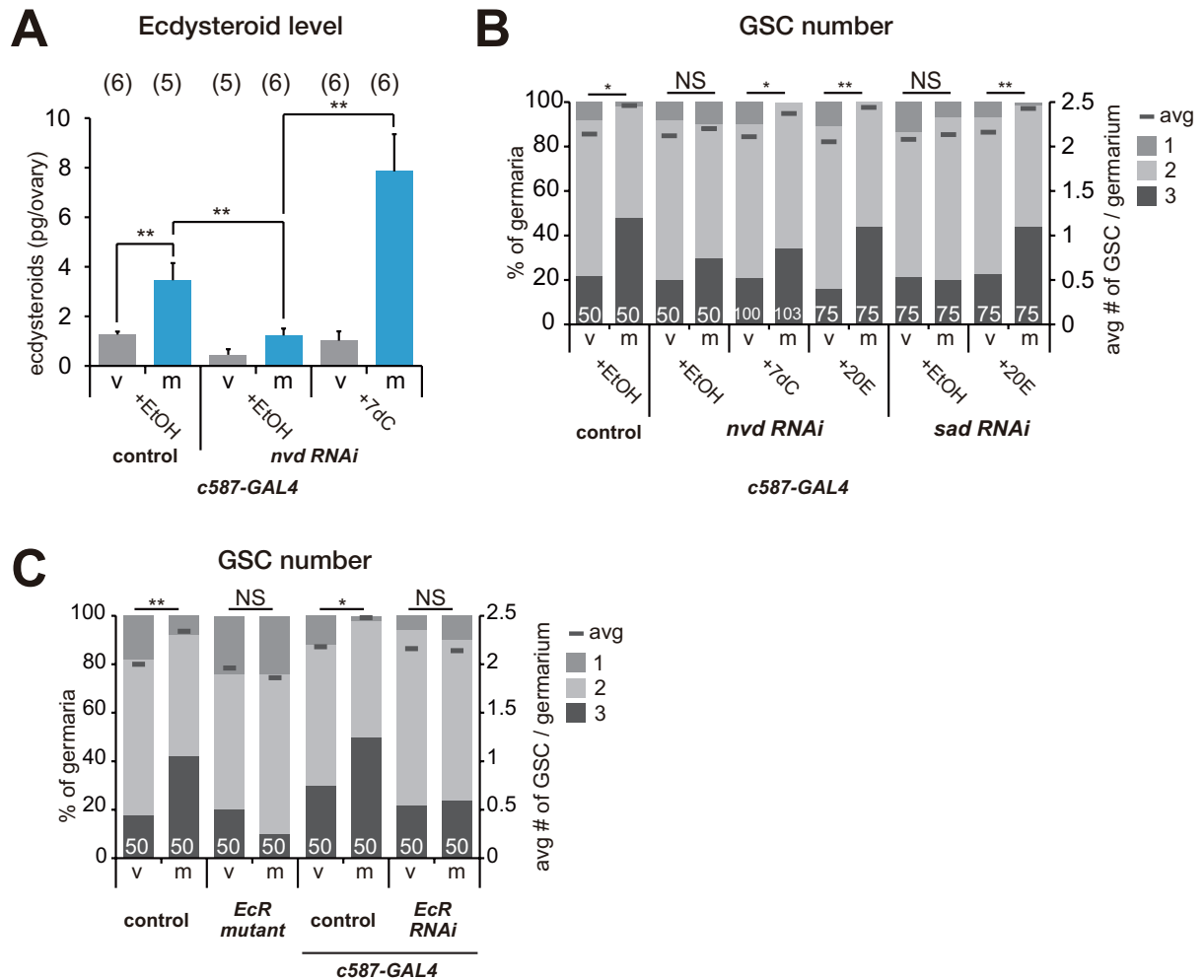
Figure 1-5. Ovarian ecdysteroid signaling regulates the mating-induced GSC proliferation

(A) Ecdysteroid levels in virgin (v) and mated (m) females in the ovarian somatic cell-specific (escort cells and follicle cells) *nvd* RNAi female flies. *nvd* RNAi female flies were fed food supplemented with ethanol (EtOH; for control) and 7-dehydrocholesterol (7dC).

(B, C) Frequencies of germaria containing one, two, and three GSCs (left y axis), and average number of GSCs per germarium (right y axis). **(B)** The ovarian somatic cell-specific *nvd* and *sad* RNAi female flies that were fed food supplemented with EtOH (for control), 7dC and 20E.

(C) The ovarian somatic cell-specific *EcR* RNAi female flies and transheterozygous mutants for *EcR* (*EcR*^A483T^ and *EcR*^M554fs^, mutants in the predicted ligand- binding domain)

Notes: Values are represented as the mean with standard error of the mean in **A**. The numbers of samples examined are indicated in parentheses in **A**. The numbers of germaria analyzed are shown inside bars in **B** and **C**. For statistical analysis, Dunnett's test was used

for **A**. A Wilcoxon rank sum test was used for **B** and **C**. **$P \leq 0.01$, *$P \leq 0.05$, NS, non-significant ($P > 0.05$).
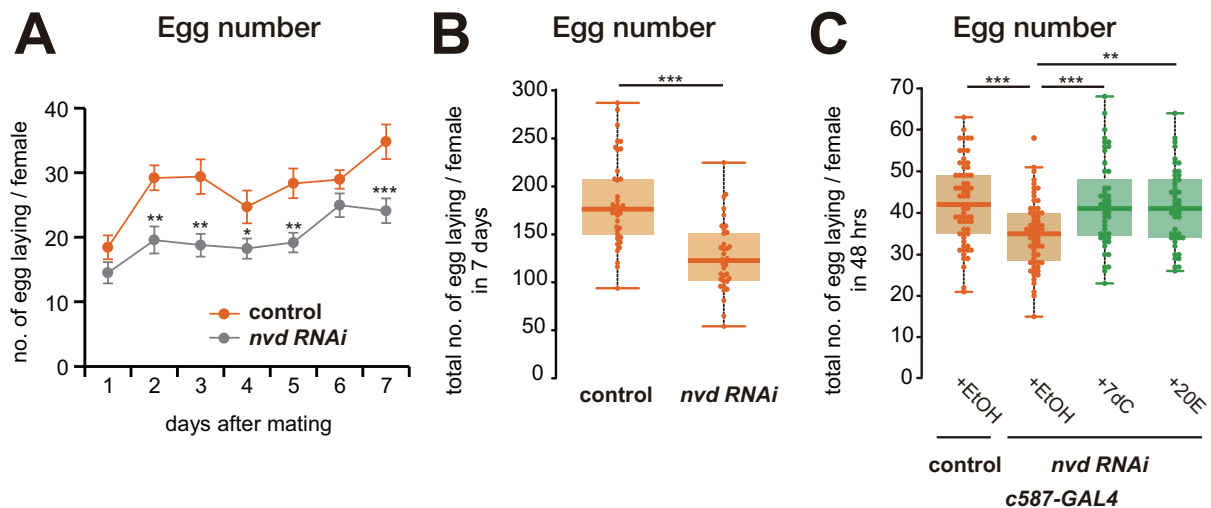
Figure 1-6. Ovarian ecdysteroid biosynthesis regulates egg laying

(A–C) The number of laid eggs was measured in ovaries of follicle cell-specific *nvd* RNAi female flies. **(A)** Temporal changes in laid eggs were measured for 7 days. **(B)** Total number of laid eggs in 7 days after mating is shown. **(C)** Total number of laid eggs in 48 hours in *nvd* RNAi female flies that were fed food supplemented with EtOH (for control), 7dC and 20E. Feeding of 7dC or 20E rescued decreased egg number phenotype in *nvd* RNAi.

Notes: Values are presented as the mean with standard error of the mean in **A**. Each value is plotted as a dot. Box plot shows 25–75% (box), median (band inside) and minima to maxima (whiskers) in **B** and **C**. For statistical analysis, a Student's t-test was used for **A** and **B**. Dunnett's test was used for **C**. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.
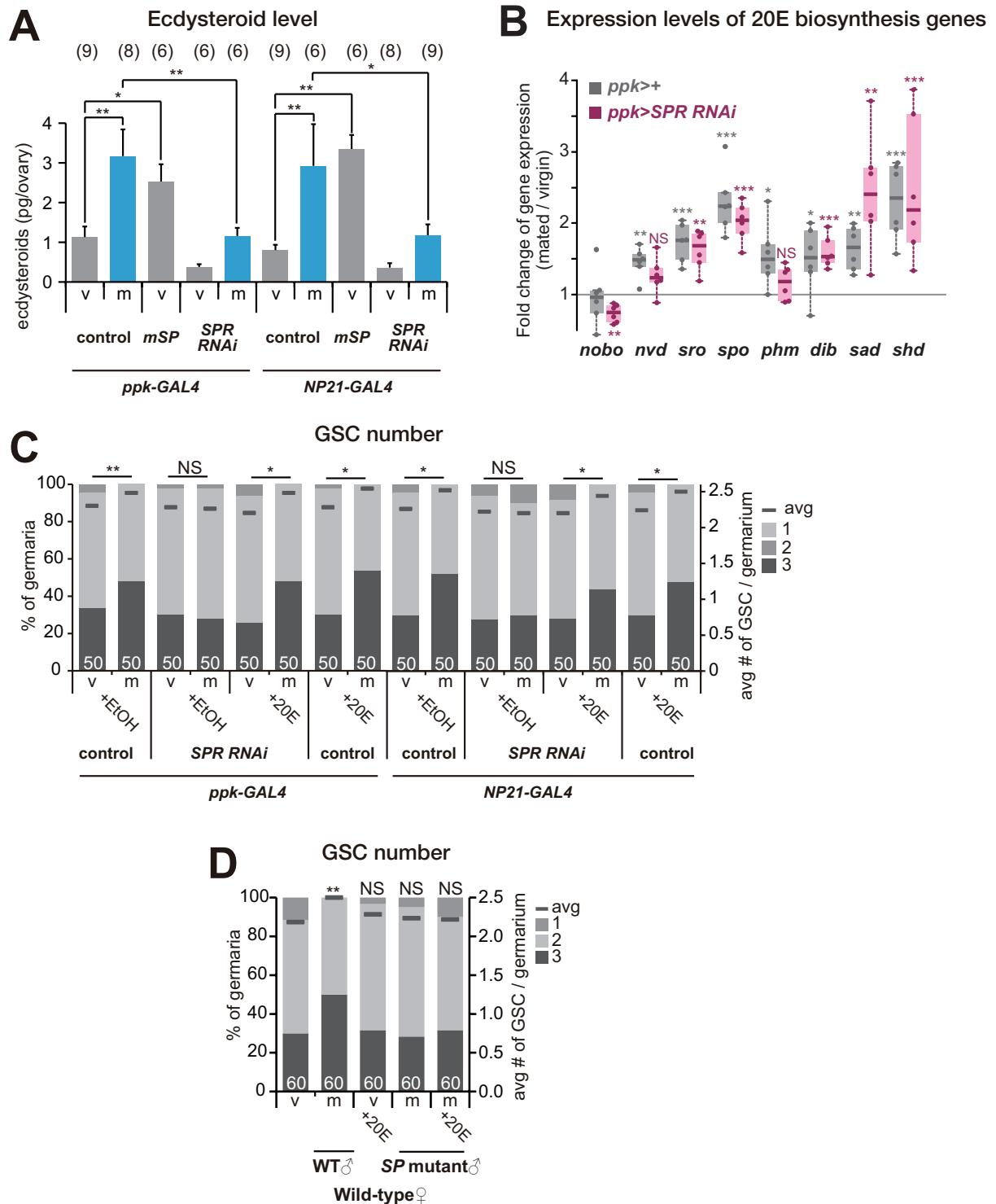
Figure 1-7. Neuronal SP-SPR regulates the mating-induced GSC proliferation through ecdysteroid biosynthesis

(A) Ecdysteroid levels in virgin (v) and mated (m) female ovaries isolated from adult female flies overexpressing the membrane bound form of *SP* (*mSP*) and transgenic *SPR* RNAi.

Transgenes were driven by *ppk-GAL4* or *fru (NP21)-GAL4*.

(B) Expression levels of ecdysteroid biosynthesis enzyme genes in the ovaries (mated/virgin). Most of the enzyme genes, except for *nobo*, transcriptionally increased by mating in control female ovaries. Increased transcript levels of *nvd* and *phm* by mating were suppressed in *SPR* RNAi females.

(C, D) Frequencies of germaria containing one, two, and three GSCs (left y axis), and average number of GSCs per germarium (right y axis) in virgin (v) and mated (m) female flies. **(C)** The neuronal *SPR* RNAi adult female flies that were fed food supplemented with EtOH (for control) and 20E. **(D)** Females mated with *SP* null mutant males did not show mating-induced increase in GSCs and this phenotype was not rescued by oral administration of 20E. Notes: Values are presented as the mean with standard error of the mean in **A**. The numbers of samples examined are indicated in parentheses in **A**, or inside bars in **C** and **D**. For statistical analysis, Dunnett's test was used for **A**. Student's t-test was used for **B**. A Wilcoxon rank sum test was used for **C** and **D**. **$P \le 0.01$, *$P \le 0.05$, NS, non-significant ($P > 0.05$).

A

Mating

Neuron **Sex Peptide signaling**

Ovary 20E

EcR 20E biosynthesis genes

Niche cells Late follicle cells

GSCs → Oocyte → Mature eggs

Brain

SPR+ neuron

SP Ovary

20E: 20-hydroxyecdysone
EcR: Ecdysone receptor

B

SP
↓
SPR
↓
20E
↓
ECR
↓
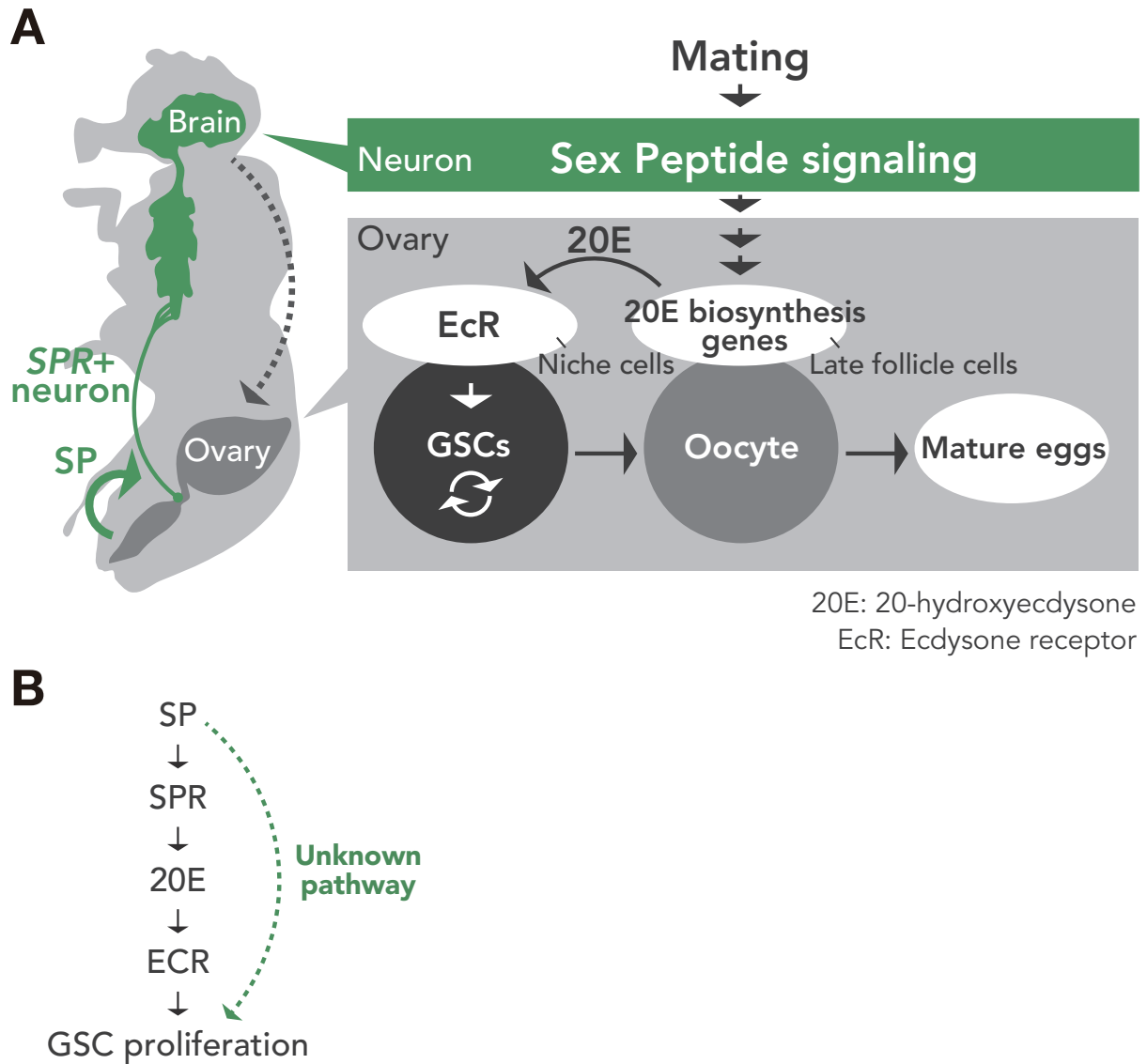GSC proliferation

Unknown pathway

Figure 1-8. Neuroendocrine control of GSCs

(A) Schematic of neuroendocrine control of a mating-induced GSC proliferation. Neuronal sex peptide signaling induced by mating increases GSC number via activated ovarian ecdysteroid signaling. Since overexpression of *SP*, but not feeding of 20E into virgin females was sufficient to induce GSC increase, there might be another pathway activated by *SP* to control GSC number.

(B) There must be an unknown pathway dependent on SP but independent of the SPR-ecdysteroid-EcR cascade.

Methods

Fly strain. Flies were raised on cornmeal-agar-yeast medium at 25°C. *yw* was used as the control strain. *elav-GAL4* (#8765) (Luo et al., 1994) was obtained from the Bloomington Drosophila Stock Center (BDSC, stock number #8765). EcR^{M554fs} (#4894) and EcR^{A483T} (#5799) (Bender et al., 1997) were also obtained from BDSC. *UAS-SPR-IR* (VDRC KK106804), *UAS-sad-IR* (VDRC KK106356) and *UAS-EcR-IR* (VDRC KK37059) were obtained from the Vienna Drosophila RNAi Center. SP^0, SP^Δ (Liu and Kubli, 2003) and *NP21-GAL4*, an enhancer trap line inserted near the *fruitless* promoter (Hayashi et al.; Kimura et al., 2008), were gifts from Nobuaki Tanaka (Hokkaido University, Japan). *c587-GAL4* (Kai and Spradling, 2004; Zhu and Xie, 2003) and *ppk-GAL4* (Grueber et al., 2007) were gifts from Hiroko Sano (Kurume University, Japan), Tadashi Uemura (Kyoto University, Japan) and Günter Korge, respectively. *UAS-SP.GGT* for overexpressing a transgene of membrane-bound form of *SP* (Nakayama et al., 1997) was a gift from Toshiro Aigaki (Tokyo Metropolitan University). Other strains used were *UAS-nvd-IR*, *UAS-nvd-Bm [wt]*, *UAS-nvd-Bm [H190A]* (Yoshiyama-Yanagawa et al., 2011).

Mating assay. Flies were reared at 25°C and aged for 3 days. About 15–30 virgin female flies were introduced into a vial with cornmeal-agar-yeast media containing more *yw* male flies than female flies for 6 hours at 25°C. The mating protocol using 3- or 4-day-old adult female flies has also been used in previous studies to assess mating-induced *Drosophila* behaviors. Individual female flies were introduced into a vial with yeast paste on grape juice-agar media for 16 hours (for GSC count) or 24 hours (for ecdysteroid measurement) at 25°C. I determined mating success by checking egg laying and hatching larvae. I transferred flies into fresh vials and counted egg-number manually after 48 hours.

Immunohistochemistry. Ovaries were dissected in Grace's Insect Medium, Supplemented (Life Technologies) and fixed in 4% paraformaldehyde in Grace's medium for 20 min at room temperature (RT). For staining of germaria, the fixed samples were washed three times in phosphate-buffered saline (PBS) supplemented with 0.1% Triton X-100. For staining of

whole ovarioles, the fixed samples were washed twice in PBS with 1% Triton X-100 and once in PBS with 2% Triton X-100. After these washing steps, the samples were blocked in the blocking solution (PBS with 0.3% Triton X-100 and 0.2% BSA) for 1 hour at RT, and then incubated with a primary antibody in the blocking solution at 4°C overnight. The primary antibodies used in this study were mouse anti-Hts 1B1 (Ding et al., 1993) (1:50; purchased from Developmental Studies Hybridoma Bank), rat anti-DE-cadherin DCAD2 (Oda et al., 1994) (1:100; purchased from Developmental Studies Hybridoma Bank), rabbit anti-phospho-histone H3 (Millipore; 1:1000), mouse anti-Lamin-C LC28.26 (Riemer et al., 1995) (1:10; provided from Satoru Kobayashi), rabbit anti-Sad (Gibbens et al., 2011) (1:200; a gift from Michael B. O'Connor), and guinea pig anti-Nvd (Ohhara et al., 2015) (1:200). Fluorescent (Alexa488 or Alexa546)-conjugated secondary antibodies (Life Technologies) were used at a 1:200 dilution and incubated for 2 hours at RT in the blocking solution. All samples were mounted in FluorSave reagent (Calbiochem). GSC number were determined based on the morphology and position of their anteriorly anchored spherical spectrosome.

Ecdysteroid level measurements. Flies were reared at 25°C and aged for 4 days. Ovaries were dissected in PBS. Samples were homogenized in 50 µL of methanol in 1.5-mL tubes using a pestle. After centrifugation at 20,913 x g for 1 min, the supernatants were transferred to new tubes and dried with a centrifugal evaporator. Samples were resuspended in 50 µL of EIA buffer (Cayman Chemicals) according to the manufacturer's protocol and incubated at 4°C overnight. Ecdysteroid levels were quantified by an enzyme-linked immunosorbent assay using anti-20E antiserum (Cayman Chemicals) and 20E-acetylcholinesterase (Cayman Chemicals) as essentially described [60]. Note that the antiserum used in this study is known to recognize not only 20-hydroxyecdysone (20E) but also ecdysone (Yamanaka et al., 2015). In this study, I used 20E (ENZO Life Sciences) as a standard, and the ecdysteroid amount was expressed in 20E equivalents. Absorbance was measured at 415 nm using a microplate reader Multiskan GO (Thermo Fisher Scientific).

Feeding experiments using 20-hydroxyecdysone and 7-dehydrocholesterol. Flies were reared at 25°C and aged for 1 day and placed on cornmeal-agar-yeast media mixed with a solution of 20E in ethanol, resulting in a final concentrations of 10^{-5} M. Flies were also fed on regular food containing 0.1% wet weight 7-dehydrocholesterol (7dC, Sigma). The experiment using 7dC was carried out under constant dark conditions because 7dC is unstable in light. The same concentration of methoprene was used in a previous study for *Drosophila* female adults (Reiff et al., 2015). GSC counts or ecdysteroid measurements were performed on mated and unmated 4-day-old female flies.

Egg laying assay. 3-day-old females were transferred to a vial with the same number of males to allow mating. Individual females were transferred to a fresh vial and allowed to lay eggs for 24 hours. We used a round chamber (23 mm diameter and 40 mm height) with cornmeal-yeast-agar media.

Statistical analysis and graphing. Statistical analysis and graphing were performed with Excel (Microsoft) and an add-in software for statistics (Excel Toukei 2011; Social Survey Research Information). The mean values were calculated with standard errors. For analyzing the values of GSC numbers, a Wilcoxon rank sum test was applied. For analyzing the values of number of cap cells, number of laid eggs, ecdysteroid levels, and weight of ovaries, Student's *t*-test or Dunnett's test (For multiple comparisons) were applied. For analyzing the values of frequency of mitotic GSCs, Chi–square analysis was applied. The *P* value is provided for comparison with the control shown as $*P \le 0.05$, $**P \le 0.01$, $***P \le 0.001$; NS, non-significant ($P > 0.05$).