The Pennsylvania State University
The Graduate School
The Huck Institute Genetics Graduate Program

ROLES OF THE OCTOPAMINE RECEPTOR OAMB
IN ASSOCIATIVE LEARNING, MEMORY,
AND REPRODUCTION IN DROSOPHILA MELANOGASTER

A Dissertation in
Genetics
by
Hyun-Gwan Lee

© 2008 Hyun-Gwan Lee

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2008
The dissertation of Hyun-Gwan Lee was reviewed and approved* by the following:

Kyung-An Han  
Associate Professor of Biology  
Dissertation Advisor

Zhi-Chun Lai  
Associate Professor of Biology, Biochemistry and Molecular Biology  
Chair of Committee

Graham H. Thomas  
Associate Professor of Biology, Biochemistry and Molecular Biology

Richard W. Ordway  
Associate Professor of Biology  
Head of Intercollege Graduate Degree Program in Genetics

David S. Gilmour  
Professor of Molecular and Cell Biology

*Signatures are on file in the Graduate School
ABSTRACT

Octopamine, a biogenic monoamine, is a major neuromodulator in invertebrates. It plays a crucial role in various adaptive behaviors and in female reproduction. To mediate these diverse effects, octopamine binds to specific receptors and activates distinct signal transduction pathways. The underlying intracellular mechanism, however, remains unclear. Two octopamine receptors, OAMB-K3 and OAMB-AS, are produced by alternative splicing of \textit{oamb} transcripts and were distinctly characterized to activate for cAMP and intracellular calcium increases. Remarkably, both receptors are highly enriched in the mushroom bodies and the central complex of the brain, where both locations have been found to be key neural substrates for associative learning and memory. Furthermore, OAMB is expressed in the thoracico-abdominal ganglion, the female reproductive system, and mature eggs in the ovary. To explore OAMB’s role in conditioned courtship as a natural form of associative learning and female reproduction, null and various hypomorphic \textit{oamb} mutants were generated by P-element mediated dysgenesis. \textit{oamb} null mutants were found to be viable without gross anatomical defects and \textit{oamb} females displayed normal courtship and copulation behaviors. \textit{oamb} null mutant males were also normal in acquisition, but were impaired in short-term memory retention for conditioned courtship behaviors. Transgenic OAMB-AS expression, driven by pan neuronal \textit{elav}-GAL4, rescues the memory deficit of \textit{oamb} in short-term memory retention for conditioned courtship, suggesting that neuronal OAMB-AS is crucial for this behavior.
null mutant females have been found to be sterile. To investigate the tissue type(s) and intracellular effectors that OAMB mediates ovulation, the GAL4/UAS binary system was employed. Transgenic oamb females, with adults ubiquitously expressing either OAMB-K3 or -AS driven by heat shock-GAL4, were fecund, indicating that OAMB plays a physiological, and not a developmental, role in ovulation. Neural OAMB expression, however, is deficient for inducing ovulation in oamb null mutant females. To identify OAMB’s functional site, the enhancer GAL4 line oamb-RS-GAL4 was generated so that GAL4 could be specifically expressed in the reproductive system. Remarkably, strong oamb-RS-GAL4 expression was detectable in the oviduct epithelial cells, where endogenous OAMB is found. oamb females, with transgenic OAMB-K3 or -AS expression driven by oamb-RS-GAL4, were fully fertile, suggesting that OAMB is crucial in oviduct epithelial cells for regulating ovulation. To identify for downstream OAMB signaling molecules involved with ovulation, transgenes expressing constitutively active Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII\(^{CA}\)), CaMKII inhibitor peptide ala, and dNOS-IR were expressed by oamb-RS-GAL4 in adult oviduct epithelial cells. Ovulation defects of oamb females were rescued by transgenic CaMKII\(^{CA}\) expression in adult oviduct epithelium. CaMKII inhibitor peptides ala and dNOS-IR driven by oamb-RS-GAL4, however, reduced female fecundity in a heterozygous background. Therefore, CaMKII and possibly nitric oxide are major downstream signaling molecules of OAMB for regulating ovulation. These studies provide significant insight into the physiological and cellular mechanisms involved with octopamine function in associative learning and memory as well as for the female reproductive system.
TABLE OF CONTENTS

LIST OF FIGURES ..................................................................................................... viii

LIST OF TABLES ....................................................................................................... xi

ACKNOWLEDGEMENTS ......................................................................................... xii

Chapter 1  Literature Review ....................................................................................... 1

1.1 Drosophila as a model organism ............................................................. 4
1.2 Octopamine .............................................................................................. 6
1.3 octopamine receptors .............................................................................. 10
1.4 Conditioned courtship for studying associative learning and memory... 11
1.5 Female reproduction ............................................................................... 14

Chapter 2  Materials and Methods ............................................................................... 18

1.1 Drosophila culture and strains ................................................................. 18
2.2 OAMB expression .................................................................................... 19
2.3 Generation and characterization of oamb deletion lines ....................... 25
2.4 UAS-OAMB-K3, -AS and oamb-RS-GAL4 constructs ................. 28
2.5 Courtship behavior assay ........................................................................ 29
2.6 Fertility and ovulation assay ................................................................. 31
2.7 Data Analysis ........................................................................................... 33
Appendix A  Supplementary data................................................................................ 117

Appendix B  Published works......................................................................................119
LIST OF FIGURES

Figure 1-1. The TARGET (Temporal And Regional Gene Expression Targeting) system. ................................................................. 6

Figure 1-2. Biosynthesis pathway of octopamine in Drosophila ......................... 7

Figure 1-3. Effects of OA in invertebrates......................................................... 9

Figure 3-1. A schemetic presentation of the fly brain structure and OAMB-K3 immunoreactivity ............................................................................. 35

Figure 3-2. OAMB expression ............................................................................. 36

Figure 3-3. The characterization of oamb mutant lines. ..................................... 38

Figure 3-4. RT-PCR analysis of oamb alleles. ..................................................... 42

Figure 3-5. RT-PCR analysis of oamb309 ......................................................... 43

Figure 3-6. Immunohistochemical analysis of oamb null mutants. .................... 44

Figure 3-7. Immunohistochemical analysis of hypomorphic oamb mutants........ 45

Figure 3-8. Immunohistochemical analysis of hypomorphic oamb309 mutant. ..... 46

Figure 3-9. Baseline courtship behavior of oamb null mutant males toward CS virgin females. ........................................................................................................ 48
Figure 3-10. Conditioned courtship of oamb males ..................................................... 49

Figure 3-11. Restoration of short term memory retention of oamb$^{286}$ null males
using the pan neuronal driver, elav-GAL4. .......................................................... 51

Figure 3-12. Restoration of short term memory retention of oamb$^{286}$ males using
C747 and MB247-GAL4 ...................................................................................... 53

Figure 3-13. oamb alleles fecundity and egg hatching ............................................. 56

Figure 3-14. Dissected ovaries stained with DAPI ...................................................... 57

Figure 3-15. Normal courtship behavior of oamb females ........................................ 58

Figure 3-16. GFP labeled sperm in female sperm-storing organs ............................. 59

Figure 3-17. Ovulation levels in oamb null mutants .............................................. 60

Figure 3-18. oamb-RS-GAL4 construct .................................................................. 63

Figure 3-19. oamb-RS-GAL4 expression patterns and OAMB expression with
C380 or dTdc2-GAL4 /UAS- mCD8-GFP in the female reproductive system.... 65

Figure 3-20. Rescue of the oamb female’s ovulation defect with transgenic
OAMB expression using heat shock-GAL4 in adults ........................................... 67

Figure 3-21. Rescue of the oamb female’s ovulation defect with transgenic
OAMB expression by pan-neuronal (elav)-GAL4 ............................................... 69
Figure 3-22. Immunohistochemistry of transgenic OAMB-K3 or OAMB-AS expression driven by oamb-RS-GAL4 in the oviduct. .......................................... 71

Figure 3-23. Rescue of the oamb female’s ovulation defect with transgenic OAMB expression by oamb-RS-GAL4. ............................................................... 74

Figure 3-24. Ovulation test with possible downstream effectors activation by oamb-RS-GAL4 in the oviduct epithelium......................................................... 78

Figure 3-25. Ovulation test with possible downstream effector, dNOS-IR expression by oamb-RS-GAL4 in the oviduct epithelia. ........................................ 80

Figure 4-1. A schematic model of the signal transduction cascade triggered by OAMB during conditioned courtship. ................................................................. 90

Figure 4-2. A schematic model of the signal transduction cascade triggered by OAMB for ovulation in the oviduct epithelium. ............................................ 98
LIST OF TABLES

Table 1-1. OA and NE system.................................................................4

Table 2-1. Primers for RT-PCR.................................................................21

Table 2-2. Primers for PCR screening of oamb mutants .........................26

Primers for initial screening for imprecise excision events .....................26

Primers for mapping breakpoints in oamb mutants ...............................26

Table 3-1. Summary of dysgenesis in oamb imprecise deletion event ..........39

Table 3-2. Summary of oamb alleles based on OAMB-K3 expression in the brain ...47

Table 3-3. Sterility test of oamb mutant lines.............................................55
ACKNOWLEDGEMENTS

I am grateful to my adviser, Dr. K. Han for her constant support and encouragement. Her detailed, perceptive comments contributed to the shape and clarity of the finished thesis. I have indicated my debt to her endurance in supporting, but a great many minor points, her instructions and cheers must be acknowledged here to keep the works.

I want to express my gratitude to Drs. Ordway, Thomas, Lai, and Gilmour for thoughtful suggestions and reviewing my thesis. Also, I would like to thank the Huck Institute Genetics Graduate Program in Pennsylvania State University for resources and opportunities in Ph D. studying, Dr. D. Bai, Chonnam National University for introducing me to molecular biology, Dr. N. Paek, Seoul National University, and Dr, B. Lee Dong-Guk University in Korea for having opportunities to continue study, my Lab colleagues for plentiful helps, and David Moore and Dr. Han for reviewing my English writing. My gratitude goes towards my brothers, Dr. Han-Joon Jung, and Chungoo Park and Sunghee Kim couple for their sympathy and cooperation.

My wife, Tae-Yeon Kim has patiently allowed me to study at the expense of household chores, holidays and countless other little things. All of which only love could endure. This milestone of my career would not have been possible without her supports in taking care of my physical and mental health. I would like to thank parents, brothers and sister for provided assistance in numerous ways.
I dedicate my thesis to the family; my parents (Kum-Jae Lee, Myeong-Ja Kang, Byeong-Lim Kim, and Young-Sim Yoon), brothers (Byeong-Hun, Byeong-Gwan, and Hyun-Doo), sisters (Hyun-Ju Lee, Sun-Young Park and So-Yeon Kim), my lovely wife, son (Seung-Bai) and daughter (Da-Young), nephew (Sang-Wuk) and niece (Yeon-Ju). I owe all this work to my family. I cannot express my acknowledgment with any words for their supports, because I had lots of things from them. I’d like to thank them again.

Finally, I would like to thank NIH and NSF for the financial supports of these studies, and *Drosophila* community for lots of resources.
Chapter 1

Literature Review

Biogenic amines, which are defined as constituents or substances derived from plants or animals and containing an amine group, are known to function as regulators of various physiological processes. Two examples of biogenic amines include the catecholamine neurotransmitters norepinephrine (NE) and epinephrine (E), which are known to regulate a variety of physiological functions and biochemical processes across a wide range of systems, including the cardiovascular system, smooth muscle, white and brown fat. Norepinephrine (NE) / Epinephrine (E) are known to regulate a wide range of physiological responses such as anxiety, learning, memory, sexual arousal and responses of aggression (Milligan et al., 1994; Ressler and Nemeroff, 2001; Matthews et al., 2002; Volavka et al., 2004; Marino et al., 2005; Olson et al., 2006).

NE/E are synthesized from tyrosine and their actions are mediated through three subfamilies of receptors, α1-, α2- and β- adrenergic receptors (Hieble et al., 1995). These receptors are grouped together as subfamilies based on shared pharmacological and biochemical criteria (Hieble et al., 1995). α1A-, α1B-, and α1D-ARs are characterized for phospholipase C and IP3 activation resulting in increased calcium levels. α2A-, α2B-, and α2C-ARs are characterized for adenylate cyclase inactivation resulting in decreased cAMP levels. β1-, β2-, and β3-ARs are characterized for adenylate cyclase activation and increased cAMP levels. All three receptor subfamilies are members of the G protein-coupled receptor family (GPCR) characterized by having seven transmembrane domains.
with three extracellular and three intracellular loops (Hieble et al., 1995). Interestingly, each receptor subfamily can either have distinct expression patterns, so that they are expressed separately in different tissue and/or cell types, or they can share expression patterns where different receptor subfamilies can be expressed in the same tissue types.

NE controls and modulates various functions such as stress, depression, motivation, aggression, sexual behavior, learning and memory in the central nervous system (CNS) (Roeder, 1999; Ressler and Nemeroff, 2001; Matthews et al., 2002; Volavka et al., 2004; Marino et al., 2005; Olson et al., 2006). Experiments with pharmacological agents have been reported to demonstrate that the NE system regulates male sexual behavior in rodents (Crowley et al., 1989; Pfaus, 1999). The administration of α1 and β receptor antagonists decreases male sexual behavior, but the α2 antagonist yohimbine stimulates copulatory behavior in rodents, dogs, and humans. In females, NE facilitates sexual behavior (Crowley et al., 1989). In rats and guinea-pigs, administration of NE in the ventromedial hypothalamus (VMH) stimulates lordosis responses. In addition, studies of NE-deficient mice show that NE is required for retrieval of hippocampus-dependent memory through β1-AR in the mouse brain but not for memory acquisition and consolidation (Michael et al., 2005). Interestingly, pharmacological studies on human subjects suggest that NE plays a crucial role in the consolidation of emotional memory (Roozendaal, 2002; Roozendaal et al., 2006).

NE is also implicated in diverse physiological processes of female reproduction. In the human fallopian tube, NE levels are highest in the isthmus and in the fimbriated
end at the time of ovulation (Helm et al., 1982). Ectopic pregnancy is associated with the abnormal oviductal NE release in the cannabinoid receptor CB1 knockout mouse (Wang et al., 2004). In addition to the oviduct muscle, \( \alpha_2 \)-AR is found in epithelial cells of the bovine oviductal epithelium and induces cAMP increases (Einspanier et al., 1999). The level of the \( \alpha_2 \)-AR expression depends on an estrous cycle and significantly increased binding capacity is seen during the luteal phase (Einspanier et al., 1999). In the rabbit and human oviducts, NE enhances the secretory activity for the formation of oviduct fluid through stimulated AC activities (Tanaka et al., 1993; Dickens and Leese, 1994). These results collectively suggest that NE may modulate smooth muscle contraction in a regional and cyclic dependent manner and the oviduct fluid formation in the oviduct epithelium to regulate ovulation. These diverse functions for NE can likely be explained by a wide receptor expression pattern among many different tissue types where some of the receptors may have overlapping or compensatory functions. Consequently, the inactivation of only a single receptor, in a mouse model for example, through genetic manipulation may not necessarily unravel all, if not some, of the physiological functions that can be mediated by each receptor subtype.

NE and E have not been identified in the *Drosophila* model, but Octopamine has been reported to be functionally similar to NE (Table 1) (Roeder, 1999). It appears that the noradrenergic/adrenergic system of vertebrates is substituted by the tyraminergic/octopaminergic system in invertebrates (Monastirioti et al., 1996; Roeder, 1999, 2005). Moreover, the OA receptors share structural and pharmacological
characteristics with ARs. Thus, the *Drosophila* model system can be advantageous for studying the cellular and molecular mechanisms of the adrenergic system.

**Table 1-1. OA and NE system** (from review, Roeder, 1999).

<table>
<thead>
<tr>
<th></th>
<th>OA</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Transmitter</td>
<td>octopamine</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>2. Receptor</td>
<td>G-protein coupled receptors</td>
<td></td>
</tr>
<tr>
<td>3. Biological functions</td>
<td>stress hormone</td>
<td>immune response</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fight-or-flight response</td>
</tr>
<tr>
<td></td>
<td></td>
<td>learning and memory</td>
</tr>
<tr>
<td>4. Found in</td>
<td>invertebrates</td>
<td>vertebrates</td>
</tr>
</tbody>
</table>

1.1 *Drosophila as a model organism*

*Drosophila* can be advantageous for use as an experimental organism due to its small size, ease of culturing, short life cycle of about 10 to 12 days at 25 °C, a small genome size with four pairs of chromosomes that are composed of $1.4 \times 10^8$ base pairs of DNA, and less functional redundancy of its gene products than those of mammalian genomes.
One of great advantage in using *Drosophila* in genetic manipulations is the availability of a variety of transposable elements, such as the mobile P element. The P element can be used as an extremely useful mutagen that is helpful for the discovery and identification of novel functions for various genes. The P element can also be utilized as a carrier of exogenous genes useful for transgene expression and analysis. A transposon technique known as the enhancer trap, is also useful for the spatial and temporal expression of the tagged genes. The transgenic tool utilizing the binary system (GAL4 and UAS) (Brand and Perrimon, 1993) with the newly developed temperature sensitive GAL80 (GAL80\textsuperscript{ts}), which inhibits GAL4 binding to UAS only at room temperature, but not at 30\degree C, allows spatially and temporally controlled gene expression (McGuire et al., 2004) (Fig. 1-1). The availability of the complete sequence of the *Drosophila* genome helps facilitate genetic and transgenic manipulations. Recently, to selectively knockdown gene expression, RNA-mediated gene interference (*RNAi*) has been applied on a genome-wide scale and is a shared resource for investigators where tissue-specific *RNAi* lines are readily available to the public (http://www.vdrc.at) (Fire et al., 1998; Boutros et al., 2004; Dietzl et al., 2007). However about 40\% of identities are in the overall nucleotides of orthologs and orthologous proteins, specific regions that include catalytic domains and other features have more than 80\% similarities between human and *Drosophila*. Interestingly, more than 60\% of human genes have functional similarities in *Drosophila* (Bernards and Hariharan, 2001). Thus, the data obtained from the studies with *Drosophila* may be very useful for understanding similar physiological processes occurring in mammals.
OA was discovered in the salivary glands of the octopus, *Octopus vulgaris*, over 50 year ago (Erspamer and Boretti, 1951) and found in neuronal as well as in non-neuronal tissues of most invertebrates (Roeder, 1999). OA is a major neurotransmitter, neuromodulator, and neurohormone in invertebrates (Roeder, 1999, 2005). Tyrosine decarboxylase (Tdc) is the first step in the OA synthesis pathway that produces tyramine.
(TA) from tyrosine (Cole et al., 2005). Tyramine-β-hydroxylase (Tβh) then converts TA to OA by adding a hydroxyl group in the β-position that is a rate limiting factor for synthesizing OA (Monastirioti et al., 1996) (Fig. 1-2).

Two TDC genes, dTdc1 and dTdc2 were identified in Drosophila. dTdc1 is mainly expressed in nonneural abdominal organs, whereas dTdc2 is found in the CNS (Cole et al., 2005). dTdc2

, a point mutation in dTdc2, causes defect in neural tyramine and octopamine synthesis and leads to female sterility (Cole et al., 2005). Drosophila females with mutations in dTdc2 are sterile by defective egg-laying (Monastirioti, 2003; Cole et al., 2005). tβh mutants show impaired development of ethanol tolerance (Scholz et al., 2000), reduced aggressive behavior (Baier et al., 2002), alterations in stress reactivity (Grunenko et al., 2000), defective appetitive learning (Schwaerzel et al., 2003), and

Figure 1-2. Biosynthesis pathway of octopamine in Drosophila (Drawn by ChemDraw Ultra 9.0).
female sterility with retaining matured eggs in their ovaries (Monastirioti et al., 1996; Monastirioti, 2003). When applied to the dissected reproductive system, octopamine modulates muscle activities in a tissue specific manner: it enhances muscle contractions in the ovary but inhibits them in the oviduct (Monastirioti, 2003; Middleton et al., 2006). OA has an important role in learning and memory processes in other insects. In the honey bee, injection of octopaminergic agonists enhances memory formation and retrieval (Mercer and Menzel, 1982). In male gypsy moths, OA modulates sensitivity to pheromone and attenuates a bimodal rhythm of locomotor and pheromone response (Linn et al., 1992). The Drosophila inactive (iav) mutant has reduced amounts of both TA and OA – about 15% of normal (O'Dell, 1993). The iav mutant is hypoactive and displays abnormal sexual behavior and sensitization to cocaine, suggesting the potential functions of OA and TA in these behaviors (O'Dell, 1993, 1994; McClung and Hirsh, 1999).

The distribution of OA in the CNS has been identified using polyclonal antiserum against OA. In the bee brain, over 100 OA-positive somata were identified and they project to the central complex, the antennal lobes, the calyces and a small part of the α-lobes of the MB, the protocerebrum, and all three optic ganglia (Kreissl et al., 1994). Especially, the optic lobes and the MB show a dense innervation by OA-containing neurons (Roeder and Nathanson, 1993). In bees, the ventral unpaired median neuron in the maxillary neuromere 1 (VUMmx1), also displays OA immunoreactivity and innervates the MB and mediates the unconditioned stimulation (sucrose) for associative olfactory learning (Hammer, 1993). In the thoraco-abdominal nervous system of insects, a group of unpaired median neurons located either on the dorsal or ventral midline called
DUM and VUM, respectively, are important sources for OA. DUM-neurons supply most of the hemolymph OA to the flight muscles. Also octopaminergic DUM-neurons of the thoracic and abdominal ganglia densely innervate other tissues, such as the leg muscle or the oviduct (Evans and O'Shea, 1977). In addition, octopamine neurons in the TAG project to the reproductive track, the ovaries, lateral and common oviducts, spermathecae, seminal receptacles, and uterus (Monastirioti, 2003; Cole et al., 2005). This implies the role of OA in the modulation of the oviduct muscle (Monastirioti et al., 1996; Monastirioti, 2003).

**Figure 1-3. Effects of OA in invertebrates.** OA acts as a neurotransmitter, neuromodulator, and neurohormone in various tissues and modulates for numerous physiological processes (a modified image from http://www.innovations-report.de/bilder_neu/52743_drosophila.jpg, Roeder, 1999).
1.3 octopamine receptors

The physiological functions of OA are mediated by OA receptors (OARs). There are at least four classes of membrane bound OARs (Roeder, 1999) belonging to the family of G-protein coupled receptors (GPCRs). OARs, activated by octopamine, lead to a signal transduction cascade involving different second messenger systems including Ca$^{2+}$, cAMP, inositol-1,4,5-trisphosphate (IP$_3$), and diacylglycerol (DAG) (Roeder, 1999, 2005).

The discovery of OARs has come from a diverse array of biological systems. OAR (Ap oa1), from the nervous system of the marine snail *Aplysia*, was cloned (Li et al., 1994) and found to function by coupling to adenylate cyclase and increasing cAMP-levels (Kaang and Li, 1995). OARs (HvOAR, and BmOAR) from the lepidopteran species *Heliothis* and *Bombyx* inhibit adenylate cyclase activity in a dose-dependent manner (von Nickisch-Rosenegk et al., 1996). In honeybee *A. mellifera*, OAR expression was detected at high levels in the mushroom bodies and optic lobes, but was observed at lower levels in the suboesophageal ganglion, thoracic ganglia, antennal lobes and the remainder of the brain suggesting its involvement with olfactory and visual processing (Degen et al., 2000; Grohmann et al., 2003).

Five OARs were characterized from the CNS of *Drosophila* (Arakawa et al., 1990; Han et al., 1998; Evans and Maqueira, 2005). Based on its receptor affinity to OA or TA,
TA/OA receptors were identified and characterized to inhibit adenylate cyclase activity, similar to the α₂-ARs (Arakawa et al., 1990). Another identified OA receptor, OAMB, has been found to increase cAMP and intracellular Ca²⁺ levels. Two isoforms of OAMB, OAMB-K3 and OAMB-AS, are produced by alternative splicing, differing after the third internal loop, but with similarities to β-ARs and α₁-ARs. These two isoforms are preferentially expressed in the MB and central complex of the brain (Han et al., 1998; Lee et al., 2003) in addition to the epithelial cells of the oviducts in the reproductive system (Lee et al., 2003). Three other OA receptors have recently been cloned, but their expression patterns and physiological functions remain unknown (Evans and Maqueira, 2005).

1.4 Conditioned courtship for studying associative learning and memory

In *Drosophila*, as in other animals, courtship is an innate behavior comprising a stereotypic pattern displayed by a male responding to multiple sensory signals. The typical courtship ritual of *Drosophila* males may be described in the following sequence: orientation to and following the female, tapping her abdomen with his foreleg, extending and vibrating one of his wings to produce a courtship song, licking her genitalia and then attempting copulation (Bastock and Manning, 1955; Greenspan and Ferveur, 2000). Male courtship behaviors in *Drosophila* may be initiated in response to various sensory inputs. Vision is the most dominant sensory input to trigger courtship behavior in males. Once a male finds a female, he immediately starts courting (Spieth, 1974). However, in the dark,
where visual cues are not available, the male is still able to initiate courting with females using other cues over short distances, such as olfaction and mechanosensation. (Spieth, 1974; Mehren et al., 2004; Ejima et al., 2005).

One attractive feature of sexual behavior in \textit{Drosophila} is the conditioned suppression of courtship in males. Siegel and Hall have established the use of male courtship behavior as a powerful assay for studying associative learning and memory (Siegel and Hall, 1979). Compared to Pavlovian olfactory conditioning for assaying associative learning, conditioned courtship provides many advantages. Conditioned courtship is derived from natural sexual behavior and stimuli so that no complex equipment and relatively small numbers of flies are needed requiring no special manipulation (Kamyshev et al., 1999). \textit{Drosophila} male courtship responses to virgin females can be suppressed upon prior exposure or experience with mated females, which actively reject approaching males. Known as conditioned courtship, these behaviors may be assayed in two stages. At first, a male is placed with a mated female for training and then paired with a virgin female for memory retention testing. The male actively courts the mated female at first, but the amount of time he spends courting her decreases over time. During the training period, the male senses the mated female that produces aversive chemical substances as unconditioned stimuli (US) and extrudes her ovipositor to block copulation (Tompkins et al., 1983; Ackerman and Siegel, 1986). When placed with a virgin female, the trained male shows a reduced amount of courtship compared with control males without prior courtship experience (Siegel and Hall, 1979). The trained male continues to suppress courtship for up to 2-3 hours (short-term memory) or up to 9
days (long-term memory) depending on the duration of training (McBride et al., 1999). Interestingly, flies with mutations in ion channels (ether-a-go-go, Shaker, nap	extsuperscript{as}), cAMP pathway (rutabaga, dunce, amnesiac, PKA), CaMKII, PKC, homer or the genes identified as crucial for odor–shock learning (latheo, turnip, cabbage) have been implicated in the formation of short-term memory in conditioned courtship (Mehren et al., 2004).

Neuroanatomical studies using several mutants of Drosophila, that are defective in learning and memory, have shown that the MB and the central complex are important for various conditioned behaviors, with cAMP signal transduction pathways being crucial (Davis and Han, 1996; Heisenberg, 1998; Sitnik et al., 2003). The MB consists of the calyx (dendritic structure), pedunculus (axonal projection), and three lobes, $\alpha/\beta$, $\alpha'/\beta'$ and $\gamma$ (axonal terminals) (Heisenberg, 1998) and receives multiple inputs from mechanosensory, olfactory, gustatory and visual sensory systems (Schwaerzel et al., 2003). The intrinsic Kenyon cells, with projections within the MB, retain short-term memory trace for olfactory conditioning and are also required for courtship conditioning memory (Joiner and Griffith, 1999). Remarkably, genes critical for learning and memory in flies- amnesiac (similar to neuropeptide PACAP), dunce (cAMP phosphodiesterase that degrades cAMP), rutabaga (adenyl cyclase that converts ATP into cAMP), DCO (catalytic subunit of protein kinase A that is activated by cAMP), and dCREB2 (cAMP-response element binding protein) - are predominantly expressed in the MB (Nighorn et al., 1991; Han et al., 1992; Davis, 1996). Notably, OAMB is also highly enriched in the MB and the central complex, and may activate the cAMP or Ca$^{2+}$ signaling cascade.
pathway to mediate physiological processes. These expression patterns of OAMB in the brain and its capacity to activate \([\text{cAMP}]\) and \([\text{Ca}^{2+}]\), make it a potential receptor for associative learning and memory of conditioned courtship (Fig. 4-1 working model).

1.5 Female reproduction

Reproduction is a crucial process and a fundamental feature of all life. In successful sexual reproduction, mating activates highly coordinated physiological processes in the female and causes modification of female physiology and reproductive behavior. Comparing virgin females to those that have mated, virgin females are found to lay fewer eggs, but accept more males, whereas females that have mated invest more resources in laying eggs and rejecting courting males. During mating, sperm and accessory gland proteins (Acps) delivered by males, induce various physiological and behavioral changes in females (Wolfner, 2007). Moreover, mating changes the sets of genes that are expressed in females by sperm, Acps, and nonsperm/non-Acp cues (McGraw et al., 2004).

While all of these mating components affect post-mating responses, seminal proteins serve as major mating signals in the *Drosophila* female (Wolfner, 2007). Five Acps have been identified to be involved in post-mating responses as CG33943, CG1652, CG1656, CG17575, and CG9997. These Acps can stimulate egg production on the first day and modulate long-term responses in maintaining post-mating behavior and physiological
changes (Ram and Wolfner, 2007). Seminal sex peptides, such as Acp70A and DUP99B, can induce rejection behavior, such as running away, kicking and ovipositor extrusion (Aigaki et al., 1991). In addition, the seminal protein Acp26A, along with ovulin, can be found at the base of the ovary right after mating and entering the female’s circulatory system (Lung and Wolfner, 1999; Heifetz et al., 2005). It stimulates egg-laying after mating (Herndon and Wolfner, 1995). The brain, the thoracico-abdominal ganglion (TAG), endocrine glands, and reproductive tissues are major binding sites of the sex peptides (Ottiger et al., 2000). However, the precise target molecules that are activated in the mated female by the seminal proteins are unknown.

From studies with genetic or transgenic mutants, several female components for sexual receptivity and ovulation have been identified. Proper levels of Sarah expression, a regulator of the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase calcineurin or Down syndrome critical region 1 homolog, are critical in ovulation. Interestingly, females ectopically expressing Sarah in the nervous system, display enhanced ovulation without mating (Ejima et al., 2004). Other components important for ovulation include dNox, which is a catalytic subunit of NADPH oxidase (Ritsick et al., 2007). Reduction of dNox in muscles causes retention of mature eggs within the ovaries and leads to decreased calcium flux and muscular contractions induced by proctolin (Ritsick et al., 2007). In addition, Ecdysone, a Drosophila steroid hormone, is crucial for ovulation since certain ecdysone receptor mutants display a mature egg-retention phenotype (Carney and Bender, 2000; Kozlova and Thummel, 2000).
While it remains to be determined whether these molecules have physiological, if not developmental, functions for ovulation, they are potential downstream targets of seminal proteins in the nervous, reproductive and/or endocrine systems. A neurotransmitter that can mediate the ovulation signal from the CNS to reproductive tissues is octopamine, which is a major monoamine in insects and is functionally similar to norepinephrine in mammals. Females with mutations in \textit{dTdc2}, encoding neuronal \textit{dTdc} and \textit{t\beta h}, are sterile due to defective egg-laying (Monastirioti et al., 1996; Monastirioti, 2003; Cole et al., 2005). Interestingly, OAMB is also expressed in TAG and reproductive tissues. However, while \textit{oamb} mutant females display normal mating and sperm retention, they are sterile due to impaired ovulation (Lee et al., 2003). Moreover, OA deficient \textit{t\beta h} females also display sterility due to defective ovulation (Monastirioti, 2003). Thus, it appears that OA controls ovulation in the oviduct epithelium of \textit{Drosophila} females through OAMB, similar to NE function in the mammalian oviduct through \(\beta\)- or \(\alpha\)-ARs. ARs are major targets for asthma, hypertension, and depression; however, their effects on the reproductive system have received less attention (Fig. 4-2 working model).
To elucidate the cellular mechanisms through which OAMB can regulate conditioned courtship and ovulation, the target tissues and critical downstream effectors of OAMB were studied. Here, I defined that both OAMB isoforms are expressed in the mushroom bodies and the central complex in the brain and the female reproductive system, especially the oviduct epithelium. By expressing transgenic OAMB-K3 or -AS using the GAL4/UAS binary system, deficits in short-term memory retention during conditioned courtship in oamb males and defective ovulation in oamb females were restored physiologically. Additionally, calcium/calmodulin dependent protein kinase II and nitric oxide were discovered to be crucial downstream effectors of OAMB in oviduct epithelial cells for regulating ovulation.
Chapter 2

Materials and Methods

1.1 Drosophila culture and strains

All fly stocks were reared on standard cornmeal/sugar/yeast agar-medium at 25 °C with a 12 h light/12 h dark cycle under approximately 50 % relative humidity, unless otherwise stated.

The wild-type Drosophila strains used in these studies were Canton Special (CS) or rosy506 (ry). The transposase line B1808 (w1118;;Dr/Δ2-3,Tm3,Sb), Heat Shock (HS)-GAL4, UAS-PKCi, and UAS-mCD:GFP (membrane-bound GFP) were obtained from the Bloomington stock center and UAS-dNOS RNAi from the Vienna Drosophila RNAi Center (Dietzl et al., 2007). oambP5391 was obtained and has been previously characterized as a mobilized P-element insertion mutant of P[1ArB] in P551, created through a transposase-mediated dysgenesis, which had placed it in the first intron, separated by 18 bp, from the first exon. oamb alleles, such as oamb96, oamb584, and oamb286 were used that were also generated from imprecise excision events of the P-element in oambP5391 by transposase-mediated dysgenesis (Lee et al., 2003). In addition, the transgenic lines C380-GAL4, elav-GAL4, MB247, C747, dTdc2-GAL4, tubP-GAL80*, UAS-PKA*, UAS-CaMKII6A (a constitutive form of CaMKII), and UAS-ala
(an inhibitor peptide of CaMKII) were kindly provided by Drs. S. Weddle (Massachusetts Institute of Technology, USA), M. Ramaswami (University of Arizona, USA), J. Hirsh (University of Virginia, USA), M. Heisenberg (University of Würzburg, Germany), R. Davis (Baylor College of Medicine, USA), K. Kiger, and L. Griffith (Brandies University, USA), respectively.

2.2 OAMB expression

In addition to the use of X-Gal staining, OAMB expression was examined for by in situ hybridization, RT-PCR and immunohistochemistry using anti-OAMB-K3 antibodies.

2.2.1 in situ hybridization

The 5’ half of OAMB-K3 cDNA (Han et al., 1998) clone was used to make RNA probes with digoxigenin–UTP and T3 or T7 RNA polymerase (Roche Applied Science, Boehringer Mannheim, IN, USA). After DNase I treatment and ethanol precipitation, digoxigenin-labeled riboprobes were resuspended in 50% formamide/5X SSC (as recommended by the manufacturer, Roche Applied Science, Boehringer Mannheim, IN, USA). Ten-micrometer head cryosections of CS were fixed in 4% paraformaldehyde
made up in PBS (0.1M phosphate buffer with saline, pH 7.2) for 10 min. After two washes in PBS for 10 min each, the sections were treated with 0.2 N HCl for 20 min, and then washed in 2X SSC for 30 min, and treated with Pronase (117 μg/ml in 50 mM Tris-HCl buffer (pH 7.5), 5 mM EDTA) for 5 min. The sections were fixed again in 4% paraformaldehyde in PBS and acetylated in PBS containing 0.1M triethanolamine and 0.25% acetic anhydride for 10 min. After washing in 2X SSC, the sections were prehybridized in hybridization buffer (50% formamide, 5X SSC, 5% dextran sulfate, 1X Denhardt's solution, 0.65 mg/ml sonicated salmon sperm DNA, 0.1% SDS), followed by hybridization in the same buffer containing digoxigenin-labeled riboprobes at 42 °C overnight. The sections were treated with RNase A (20 μg/ml in 3X SSC, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA) at room temperature for 10 min and unbound probe was washed in 0.1X SSC at 65°C for 10 min. For the immunological detection of hybridized probe, the sections were placed in blocking solution (2% sheep serum in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl with 0.3% Triton X-100) for 1 hour, and then treated with 1:500 diluted anti-DIG antibody conjugated to alkaline phosphatase (BM) for 3 hours. After washing 5 times for 10 min in a 100 mM Tris-HCl (pH 7.5), 150 mM NaCl solution, sections were then rinsed in a 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂ solution for 5 min. For colorometric detection of the phosphatase signal, NBT/X-phosphate with Levamisol in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂ was used. The signal was developed for 1-12 hours in a humid and dark environment and the slides were washed, dried, and subsequently mounted in Permount (Nighorn et al., 1991).
2.2.2 RT-PCR

For *oamb* transcript analysis, total RNA was prepared from the heads, the bodies, the dissected oviducts, and the female reproductive system by using the High Pure RNA Tissue Kit (Roche Applied Science, Boehringer Mannheim, IN, USA). RT-PCR was performed on total RNA with a Titan One Tube RT-PCR System (Roche Applied Science, Boehringer Mannheim, IN, USA) using primer sets following the manufacturer's recommended procedures (Table 2-1). *cdc2c* was used as a positive control, insuring for the integrity of the cDNA and genomic DNA, in addition to the use of *oamb-K3* or -AS cDNA as the positive control for the RT-PCR reaction.

<table>
<thead>
<tr>
<th>Table 2-1. Primers for RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cdc2c</em>: 5'-GCACACCTGATGAAACAAAT-3'</td>
</tr>
<tr>
<td>5'-CTGGGGGCTATTGGACACGA-3'</td>
</tr>
<tr>
<td>E4-E5: 5'-CGTTCTGGTCATCGGTGG-3'</td>
</tr>
<tr>
<td>5'-CGTGGACATTATGCTTGG-3'</td>
</tr>
<tr>
<td>E6-E7: 5'-CACCGCCGCAGAACAGAC-3'</td>
</tr>
<tr>
<td>5'-CGCTCTGATTACCGCAT-3'</td>
</tr>
<tr>
<td>E6-E8: 5'-CACCGGCCGAACAGAC-3'</td>
</tr>
<tr>
<td>5'-CGCTCGTGACCCACATCC-3'</td>
</tr>
</tbody>
</table>

E, exon.
2.2.3 Immunohistochemistry

To generate OAMB-K3 or -AS specific polyclonal antibodies, a 296 bp fragment from positions 1824-2120 of \textit{oamb}-K3 (Genebank Accession no. AJ007618, (CG3856-RB)) and a 324 bp fragment from positions 1515-1839 of \textit{oamb}-AS (Genebank Accession no. AJ007617, (CG3856-RA)) were amplified by PCR and subcloned in a pGEX-4T2 vector using EcoRI – EagI (Pharmacia, NJ; New England Biolabs, MA). These amplified regions represent for the coding region of the OAMB third cytoplasmic loop. After over expression with IPTG induction, the fusion protein, with the glutathione S-transferase (GST) tag, was purified with SDS-PAGE (Han et al., 1998) and used to immunize mice for the generation of polyclonal antisera. After the third intraperitoneal injection of the fusion protein into a Swiss Webster mouse, ascites fluid was collected (Harlow and Lane, 1988). The specificity of the polyclonal antibody was determined by immunohistochemical analysis in CS brain sections. The mouse pre-immune serum was used as a control.

For affinity purification of the antibodies, one cm$^2$ of nitrocellulose membrane was incubated for 2 hrs at room temperature with 0.2 ug/μl of the gel-purified GST-OAMB-K3 or -AS fusion protein in PBS. After washing in PBS for one min, the membrane was incubated with 3% normal goat/horse serum in PBS with 0.02% sodium azide for 2 hrs followed by overnight incubation 4 °C with 1:100 diluted OAMB-K3 or -AS antibody ascites fluid in PBS containing 0.2% triton X-100, 3% normal goat serum,
and 0.02% sodium azide. The antigen specific purified antibody was eluted with 0.2M Glycine (pH 2.5) buffer with 0.1 % BSA and neutralized with 1M Tris-HCl (pH 7.9). To increase the concentration and for desalting, the solution was concentrated by Centricon (Millipore Co, MA). The affinity-purified antibodies were used to stain adult head sections and the body sagittal sections.

For detecting OAMB expression by immunoreactivity in cryosections, the flies were fixed for 3 hours at 4 °C in a freshly made 4% paraformaldehyde in PBS solution containing 40 mM lysine and then placed overnight in 25% sucrose in PBS after two 10 min washes in PBS. 10 μm cryosections were prepared on gelatin-coated slides and then fixed in a fixative solution for 10min. After washing in PBS and PBHT (20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 0.2% Triton X-100), the sections were preincubated with PBHT containing 5% normal goat or horse serum for 3 hr for blocking and incubated overnight at room temperature with the affinity-purified anti-OAMB antibody (1:200 for anti OAMB-K3 and 1:50 for anti OAMB-AS antibody). For colorometric detection, the sections were then incubated for 3 hr at room temperature with biotinylated horse anti-mouse IgG antibody (1:1000; Vector, Burlingame, CA) and washed in PBHT for 10 min 3 times. Horseradish peroxidase-conjugated avidin (ABC kit, Vector, Burlingame, CA) was then applied according to the manufacturer’s recommended protocol. The immunoreactivity was visualized with 1 mg/ml diaminobenzidine and 0.03% hydrogen peroxide in PBHT and mounted in Glycergel. Alternatively, Alexa Fluor 568 conjugated goat anti-mouse IgG (1:1000, Invitrogen, Eugene, OR) was used as a secondary antibody to detect the immunoreactivity by fluorescence for 3 hr and washed
and mounted in Vectashield mounting media (Vector, Burlingame, CA). For β-Galatosidase immunoreactivity, the OAMB immunostaining procedure was followed substituting a rabbit anti-β-Galatosidase antibody as the primary antibody (1:1000, Cappel Lab, Cochranville, PA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Fisher Science (Fair Lawn, NJ) and VWR (West Chester, PA).

Images were taken by DMR epifluorescence (Leica, Heidelberg, Germany) or using a FluoView confocal microscope (Olympus, Melville, NY). Confocal image stacks were collected at 1 – 2 micron steps with a 20X objective and stacked by maximum-projection, analyzed with the software Image pro plus 5.1 (Cybermediatics, Betheda, USA), and Adobe Photoshop 9 (Adobe, USA).

2.2.4 X-gal staining

The female reproductive system of oambP5391 was dissected in PBS and fixed in 2% paraformaldehyde in PBS containing 40 mM lysine for 20 min. After washing in PBS twice, the samples were stained with 4% X-Gal in DMF (N, N Dimethylformamide) for 3 to 24 hr at 37 °C and then counterstained with 0.2% Nuclear Fast Red (Sigma, St. Louis, MI) in 10% of Aluminum Sulfate for 10 min. Ten μm whole body cryosections of oambP5391 females were fixed in 4% paraformaldehyde in PBS containing 40 mM lysine for 10 min and stained. The X-Gal stained specimens were mounted in 70% glycerol
(Sullivan et al., 2000). The digital images were taken by DMR epifluorescence (Leica, Heidelberg, Germany).

2.3 Generation and characterization of oamb deletion lines

Local hop and imprecise excision experiments were performed as described (Tower et al., 1993; Dalby et al., 1995). The oamb gene is located on the third chromosome at cytological position 92F (Han et al., 1998). To generate the deletion in oamb, the enhancer trap line P551 carrying a P[1ArB] element was initially identified that has an insertion approximately 50 kb upstream of the oamb gene (Cooley et al., 1988; Bellen et al., 1989). Mobilizing P[1ArB] in P551, transposase-mediated dysgenesis was done and new insertion lines obtained as Df(3R)P6679, oambP213, and oambP5391 in the oamb locus (Tower et al., 1993; Dalby et al., 1995; Sullivan et al., 2000). oambP5391 has P-element insertions in the first intron that is separated by 18 bp. oambP5391 is homozygous viable with a normal OAMB immunoreactivity and no external abnormalities. Using oambP5391, P-element-mediated dysgenesis was performed (Voelker et al., 1984) for the imprecise excision of oamb. To collect the deletion lines, F1 progeny with ry eye color were screened by PCR using multiple primer sets (Table 2-2).
Table 2-2. Primers for PCR screening of oamb mutants

Primers for initial screening for imprecise excision events:

P primer : 5'-CGACGGGACCACCTTATGTTATTTCATCATG-3'
291U : 5'-TGGCTAACATCCTTTTGCTTCG-3'
245C : 5'-AGAAAGCGTCGCAAATCAAAGGG-3'

Primers for mapping breakpoints in oamb mutants:

Next CG31205U : 5'-ATTGCAGGTGCTGGATTCTTC-3'
Next CG31205D : 5'-AGCGAGGACTACCCACTGTGT-3'
14KUU : 5'-ACTACGGACATCAACGGACTA-3'
14KUD : 5'-TTGCCCCGTCATCATCTTACTA-3'
5KUU : 5'-GCCCTAACATGGTGCCTCC-3'
5KUD : 5'-CCTCACCCGCTCCCTCGGATT-3'
1KUU : 5'-GTTGCAGGTACATTATCTACCT-3'
1KUD : 5'-GTCAGGCCTATCCGTCTTC-3'
E2-U : 5'-CCAACCAAGCACTGAAAAC-3'
E2-D : 5'-GAGATTAGCGAGGAGCCCGTC-3'
I2-1U : 5'-CCAGGCGGTGAAGATTTCAGTAT-3'
I2-1D : 5'-GCCCAATACACCGGACAGACT-3'
I2-2U : 5'-TCAGAGGTGGTGGAGGTG-3'
I2-2D : 5'-GCTTAGGTGGGTAGCAATAAAGAAT-3'
I2-3U : 5'-GCGACAAGGCGAAATCCTC-3'
I2-3D : 5'-TGGATGAGGATTGGGTCTGC-3'
I2-4U : 5'-GCGGCAACTGTGAAAAGATG-3'
I2-4D : 5'-AAACGACGAATGCTGAATGT-3'
E3-U : 5'-TCAGTGATGTGCCCCTAAT-3'
E3-D : 5'-CCTGCCAGCCAATCAGTAAA-3'
I3-1U : 5'-ATTAGCAGAGCCCCCACTTT-3'
I3-1D : 5'-TTGCCAGCGACTCTATTTACC-3'
I3-2U : 5'-GCAGCAGTTTCTCTCTACGATT-3'
I3-2D : 5'-GTCGCTGTGATCGCCTGTGA-3'
I3-3U : 5'-GTGGAGGAATGAATGAAGGA-3'
I3-3D : 5'-TCCCAAGTTTCTGCCCAATG-3'
CG4000-U : 5'-AAGGCAATGGGCACAAAG-3'
CG4000-D : 5'-CGGGAGGAGGAGCTTGAG-3'
CG5474-U : 5'-CAGCAGGGCTCCTACTC-3'
CG5474-D : 5'-TCAACGGGCACACCTC-3'

E, exon; I, intron; U, upstream; D, downstream; K, kilobasepairs.
To investigate the abnormality of egg development in oamb alleles, oamb<sup>96</sup>, oamb<sup>286</sup>, or oamb<sup>192</sup> along with ry females were subjected to DAPI staining. Females were kept with CS males for 3 days in regular cornmeal-food vial containing yeast paste. The ovaries were dissected in PBS and fixed in 2% paraformaldehyde in PBS for 20 min. After washing in PBS twice, they were incubated with 1 μg/ml of DAPI (Molecular Probes, Eugene, OR) in PBS containing 0.1% of Triton X-100 for 10 min, and washed in PBS twice before mounting in 70% glycerol. Moreover, immunohistiochemistry were performed to address the expression of OAMB in oamb alleles.

2.4 UAS-OAMB-K3, -AS and oamb-RS-GAL4 constructs

To generate transgenic lines expressing OAMB, OAMB-K3 (Han et al., 1998) or OAMB-AS cDNA (CG3856-RC) were cloned in a pPBretU vector (Roman et al., 1999). Four independent transgenic lines, UAS-OAMB-K3I (X chromosome), UAS-OAMB-K3III (3<sup>rd</sup> chromosome), UAS-OAMB-ASII (2<sup>nd</sup> chromosome), and UAS-OAMB-ASIII (3<sup>rd</sup> chromosome) were obtained by germ line transformation in rosy<sup>506</sup> (Spradling and Rubin, 1982). The transgene insertion sites were identified by inverse PCR and sequencing (Sullivan et al., 2000; FlyBase-Consortium, 2003). To obtain transgenic lines expressing GAL4 in the oviduct driving transgenic OAMB-K3 and/or -AS, the oamb region (-1484 to +2880) was amplified by PCR with the primer set 5’- CACTAGTCCACGTCAGCCCATTC-3’ and 5’-GAAGATCTCCCTGCTGCTTGGC-3’. The PCR segment was cloned in the pPTGAL vector containing the promoter-less GAL4.
gene (Sharma et al., 2002) (Fig. 3-18) and germ-line transformation was performed in the isogenic \( w^{1118} \) (Spradling and Rubin, 1982). The chromosomes with \( RS\)-GAL4 insertions were mapped using the balancer line \( (w; +/CyO; +/TM6B, Tb) \) and GAL4 expression patterns were visualized with UAS-\( mCD8:GFP \). One line, out of six independent lines, was used for the rescue experiments after backcrossing with the isogenic \( w^{1118} \) for six generations. For the rescue experiments, the transgenic lines, \( heat\text{-}shock\text{-}GAL4 \), \( elav\text{-}GAL4 \), \( RS\text{-}GAL4 \), UAS-\( OAMBs \), and UAS-\( CaMKII^{fC4} \), were used with the \( oamb^{286} \) or \( oamb^{96} \) mutant.

### 2.5 Courtship behavior assay

Because behavioral assays are extremely sensitive to the genetic background of the tested animal, individual alleles have been backcrossed to the parental line \( ry \) or \( CS \) for six generations to minimize for any potential problems caused by genetics. In addition, because courtship behavior is sensitive to circadian rhythms, all courtship behavior assays were done between 9 am to 4 pm, timed so that the flies are actively moving around.

Courtship behaviors were tested in the courtship chamber (9mm diameter x 5 mm height) with a damp paper disk in over 70% humidity under two 13W fluorescent lights (Tompkins et al., 1983). All flies were transferred to the chamber by aspiration, without
CO\textsubscript{2} anesthetization, and with minimized shocks to avoid for mechanical stress. The courtship behaviors were recorded by a CCD camera and monitored for 20 minutes. Six pairs in different chambers were simultaneously assayed at once and the recorded video clips were scored to calculate courtship index. A courtship index (CI) was calculated as the percentage of time that the male spent courting a female, including the steps taken towards females to attempt copulation, but excluding a complete copulation. A complete copulation was found to be rare in either the training period with mated females or in the memory testing with decapitated virgin females as a partner. A performance Index (PI) was calculated as the percent reduction of the CIs with training vs. without training. Naïve male or female flies as experimental groups were collected within 8 hr after eclosion, and were aged individually. The partners were collected under the same conditions as the experimental group, with the exception of keeping 20-30 as a group per vial.

For measuring a baseline in the male courtship assay, 5 day old naïve males were paired with decapitated virgin CS females in the courtship chamber and videotaped for scoring (Fig. 3-9) for 20 min. For the conditioned courtship assay, 5 day old naïve males were trained by placing them with mated CS females for 1 hr and then tested with decapitated virgin CS females for memory retention for three time points: right after, 1 hour, and 3 hours after training (Fig 3-8). To examine for any role OAMB would have in conditioned courtship behaviors, the targeted expression of oamb transgenes in oamb null mutants was achieved by utilizing the GAL4/UAS system. The pan neuronal GAL4 line (elav-GAL4) and two transgenes, UAS-OAMB-K3 or -AS, in the oamb null mutants was
first tested for whether conditioned courtship is mediated by neuronal OAMB activity. Mushroom body specific GAL4 lines (C747 and MB247) were also used for rescue experiments to address that OAMB expression in mushroom bodies was required in conditioned courtship.

To quantify courtship and copulation activities in oamb null mutants, 4-day-old naïve females were individually paired with naïve CS males in a courtship chamber and videotaped. The percentage of time that CS males spent courting females in the first 20 min within the chamber was calculated. Once they engaged in copulation, the duration of their copulation was recorded (Fig. 3-15).

2.6 Fertility and ovulation assay

For fecundity tests, 2- to 3-day-old individual females were placed with three CS males on grape juice medium where eggs and larvae were counted 8 days after pairing. The same experiment was performed on cornmeal medium to count pupae and adult progeny 14 days after pairing (Table 3-3 and Fig. 3-13 A).

For the hatching assay, five females were placed with 15 CS males on a regular cornmeal medium for 1 day, and transferred to a new food vial for egg collection for 24 h.
The hatched eggs from each vial were counted 48 h later (Lannutti and Schneider, 2001) (Fig. 3-13 B).

To measure ovulation rates, 4-day-old virgin females were individually paired with CS males in a courtship chamber for 1 h and then moved on standard cornmeal media. At various time intervals after mating, the mated females were tested on ice to determine the presence of an egg in their uteri by gently pushing the tip of their abdomens or in the tip of her ovipositor (Fig. 3-17) (Aigaki et al., 1991).

Ovulation tests with increased precision are described in Figures 3-20 through 3-25 where virgin females were collected within 12 h after eclosion and aged to 5 to 7 days. Ten females were paired with 30 wild-type CS males in a regular cornmeal food vial for 3 h for mating and then the female reproductive system was dissected to check for the presence of an egg in the common oviduct or the uterus on ice. The percentage of females having an egg per vial was counted as one data point.

To induce transgenic OAMB-K3 and/or -AS expression in adults, the oamb mutant females carrying heat-shock-GAL4 reared at 18° to 20 °C were heat shocked at 37 °C for 1 h twice a day for 3 days before testing for ovulation. For the ovulation test experiments with tubP-GAL80°, 2 to 3 day-old females were reared at 30 °C for 3 days and subjected to mating and ovulation tests. All other females were kept at 20 °C as uninduced controls along with CS and oamb°.
2.7 Data Analysis

Minitab Release 14.1 (Minitab Inc., State College, PA, USA) was used for statistical analyses. Before statistical analysis was applied, normality tests were done for determining which statistical analysis method to utilize. The student’s t-test, for comparing the means of two groups with normally distributed data, the analysis of variance (ANOVA) test, and a general linear model followed by the post hoc Tukey-Kramer test, for more than two groups, was used. All data are presented as mean ± standard error of the mean.
Chapter 3

Results

3.1 OAMB expression in Drosophila

To study OAMB’s physiological function, OAMB expression patterns were first examined by in situ hybridization, RT-PCR, immunohistochemistry with anti-OAMB-K3 antibodies, and X-Gal staining. LacZ reporter expression in oamb\textsuperscript{P5391}, which contains an enhancer trap P[I\text{ArB}] element in the first intron of the oamb gene, reveals an endogenous enhancer activity for oamb expression (Lee et al., 2003). OAMB is highly enriched in the brain, particularly in the mushroom bodies (MB) and the ellipsoid body (EB), as visualized by immunohistochemistry with an anti-OAMB-K3 antibody (Fig. 3-1) (Rein et al., 2002). X-Gal staining in adult oamb\textsuperscript{P5391} females displayed β-gal activities in the thoracic and abdominal ganglia (TAG), mature eggs, and the reproductive system (Fig. 3-2 A-C, and F). The dissected female reproductive system with X-Gal staining showed OAMB expression in the epithelial tissues of lateral and common oviducts, follicle cells of the mature egg in each ovariole, seminal receptacles, spermathecae, and accessory glands (Fig. 3-2) (Lee et al., 2003). To determine whether the β-gal activity in the body reflected authentic OAMB expression, in situ hybridization was performed on sagittal sections of CS female bodies using riboprobes representing the 5’ half of oamb cDNA. As shown in Figure 3-2. D and E, OAMB transcripts were detectable in cells
scattered in the TAG cellular cortex and the oviduct in a similar pattern with the X-gal stain. This suggests that β-gal expression reflects endogenous enhancer activities for OAMB expression in the CNS and the oviduct.

**Figure 3-1. A schematic presentation of the fly brain structure and OAMB-K3 immunoreactivity.** Ten μm cryosections, from the anterior (A) to posterior (C) frontal brain were immunostained with a anti-OAMB-K3 mouse antibody and chromatic signal detected (ABC kit, Vector). Mushroom body lobes (α, β, γ), ellipsoid bodies (eb), and pedunculus(P) are indicated. Bar, 100 μm. (the brain structure, modified from http://www.flybrain.org/Flybrain/html/contrib/2000/rein/index.html, Rein et al., 2002)
Figure 3-2. OAMB expression. X-gal staining (A, B, C, F) and in-situ hybridization (D, E). The oamb$^{P5391}$ line contains the enhancer P-element P[lArB] in the first oamb intron. P[lArB] has a LacZ reporter gene, thus its expression likely parallels the expression pattern of oamb. The sagittal sections of oamb$^{P5391}$ were stained with X-gal and counterstained with Nuclear Fast Red. (A), Thoraco-abdominal ganglion (B), Abdomen (C), the dissected female reproduction system (F) (D), thoracico-abdominal ganglion labeled with antisense probes made from the oamb common exon (E), Abdominal section. AcGl, accessory gland; Od, oviduct; AG, abdominal ganglion, Spt, spermatheca; SmRcp, seminal receptacle; T, thoracic ganglion. Bars in A B D E, 100 μm. Bars in C and F, 50 μm.
3.2 Generation of oamb mutants

\( oamb^{p5391} \), with a P-element insertion in the first intron, was generated by mobilizing the P-element in P551, the enhancer trap line carrying a P[lArB] insertion, approximately 50 Kb upstream of the oamb gene on the third chromosome (92F). Using P-element mediated dysgenesis, serial excision lines of the oamb locus in \( oamb^{p5391} \) were generated (Voelker et al., 1984). 1357 independent excision lines (F1) were initially selected that showed ry eye color and performed PCR to identify the deletion region. Out of 1357 independent excision lines, 16 excision lines were selected for various deletion events in the oamb locus as summarized in Figure 3-3 and Table 1. Two deletion lines (\( oamb^{236, 345} \)) have the deletion upstream of the P-element insertion site in oamb and 11 lines (\( oamb^{192, 45, 309, 341, 390, 96, 126, 218, 432, 584, 286} \)) are downstream. Three lines (\( oamb^{174, 311, 368} \)) are deleted in both directions (Figure 3-3).
Figure 3-3. The characterization of oamb mutant lines. To generate deletions in oamb, P-element mediated dysgenesis was used. PCR was performed to screen and define the deleted regions in the lines with excision events. Boxes show OAMB exons and the boxes filled with lines for the alternatively spliced exons for OAMB-K3 (E7) and OAMB-AS (E8). P-element in oambP5391 is in the first intron (I1). The arrow represents the mobilized P-element of P551 and dotted lines delineate deleted genomic regions of oamb alleles.
To examine for OAMB expression in the excision lines, RT-PCR and immunostaining were conducted (Fig. 3-4, 3-5, 3-6, 3-7, and 3-8) (Lee et al., 2003). Two isoforms, OAMB-K3 and OAMB-AS, are produced by alternative splicing (Fig. 3-3). RT-PCR was performed, targeting the common region for both isoforms, using a unique sequence in OAMB-K3 or OAMB-AS, and cdc2c as a control (Fig. 3-4). While no expression of \textit{oamb} was detected in \textit{oamb}^{286}, \textit{oamb}^{96, 126, 309} had OAMB-K3 transcripts in the head, but with deletions up to I2 or I3 and no OAMB-AS was detectable. \textit{oamb}^{236}, with an upstream deletion, contained both transcripts in all tissue types (Fig. 3-4 and 3-5).

**Table 3-1. Summary of dysgenesis in \textit{oamb} imprecise deletion event.**

<table>
<thead>
<tr>
<th>Excision events</th>
<th>Frequency</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="deletion" /></td>
<td>5.6 % (98)</td>
<td>Deletion of downstream</td>
</tr>
<tr>
<td><img src="image" alt="deletion" /></td>
<td>48.7 % (674)</td>
<td>Deletion of upstream</td>
</tr>
<tr>
<td><img src="image" alt="deletion" /></td>
<td>31.8 % (398)</td>
<td>Internal deletion of P-element</td>
</tr>
<tr>
<td><img src="image" alt="deletion" /></td>
<td>14 % (187)</td>
<td>Precise deletion or large deletion</td>
</tr>
</tbody>
</table>

Using P-element mediated dysgenesis, serial excision lines of \textit{oamb} locus in the \textit{oamb}^{P5391} were generated and 1357 independent excision lines were screened.
RT-PCR analysis was done on heads, dissected oviducts, and bodies that include the TAG (Fig. 3-4 and 3-5). The oamb locus encodes for two isoforms, OAMB-K3 and OAMB-AS. They are produced by alternative splicing of the seventh exon (Fig. 3-3) to yield divergent sequences from the putative third cytoplasmic loop. RT-PCR was performed for the common region for both isoforms, the unique sequence in OAMB-K3 or OAMB-AS, along with cdc2c as a RNA control. OAMB transcripts were not detectable in oamb286 containing a deletion of the OAMB coding sequence. Similarly, both OAMB transcripts were absent in the oviduct, the reproductive system, and the body of oamb96 with a breakpoint in I3. Interestingly, the head of oamb96 contained OAMB-K3, but not OAMB-AS, transcripts. Similarly, the bodies of oamb309 females with a breakpoint in I2 had no OAMB transcripts, while their heads expressed both transcripts (Fig. 3-5). In contrast, the fertile lines oamb192 and oamb236 contained both transcripts in all tissue types examined (Fig. 3-4). Thus, the RT-PCR analysis revealed that both OAMB isoforms were normally expressed in all examined tissues.

OAMB-K3 was highly enriched in the MB and the EB in ry, a control line as shown in the serial frontal sections stained with the anti-OAMB-K3 antibody (Fig 3-1, 3-6, and 3-7 A-D). No immunoreactivity was detected in oamb286, oamb96 or oamb584 (data not shown) and they represent null mutants on serial frontal sections (Fig. 3-6 E-L). OAMB immunoreactivity was detectable in several lines and their expression levels varied depending on the extent of deletions in oamb. While oamb126 had normal
expression of OAMB-K3 in the EB (Fig. 3-7 G), \textit{oamb}^{545} \text{ and } \textit{oamb}^{126} \text{ had reduced OAMB-K3 expression in the MB (Fig. 3-7 F and G). On the other hand, } \textit{oamb}^{236} \text{ had overall reduced OAMB expression in the MB and EB. Thus, these lines represent hypomorphic alleles. OAMB-K3 was detectable in the brain of } \textit{oamb}^{309} \text{, indicating that OAMB-K3 expression in these structures was not required for ovulation (Fig. 3-8). Likewise, the ovulation process may not depend on expression of OAMB-AS, whose transcripts were detectable in the head of the sterile allele } \textit{oamb}^{309} \text{ by RT-PCR in the brain (Table 3-2).}
Figure 3-4. RT-PCR analysis. Total RNA was prepared by using a High Pure RNA Tissue Kit (Roche Applied Science, Boehringer Mannheim, IN, USA). RT-PCR was performed with primer sets specific for common (E4-E5), OAMB-K3 (E6-E7), and OAMB-AS (E6-E8) (see Fig. 3-3). The genotypes assayed are shown in the top panel. The areas amplified in OAMB transcripts are indicated in the right panel. OAMB cDNA clones of both isoforms (cDNA) and ry genomic DNA (gDNA) were used as controls for PCR, and cdc2c a control for RNA presence; H, male and female heads; B, female bodies; O, oviducts. (Lee et al., 2003)
Figure 3-5. RT-PCR analysis. Total RNA was prepared by using a High Pure RNA Tissue Kit (Roche Applied Science, Boehringer Mannheim, IN, USA). RT-PCR was performed with primer sets specific for common (E4-E5), OAMB-K3 (E6-E7), and OAMB-AS (E6-E8) (see Fig. 3-3). The genotypes assayed are shown in the top panel. The areas amplified in OAMB transcripts are indicated in the right panel. OAMB cDNA clones of both isoforms (cDNA) and ry genomic DNA (gDNA) were used as controls for PCR, and cdc2 a control for RNA presence; H, male and female heads; B, female bodies.
Figure 3-6. Immunohistochemical analysis of oamb null mutants. Polyclonal antibodies were made against the third cytoplasmic region of OAMB-K3 and used for immunostaining after affinity purification. A-D, frontal head sections of ry, E-H, frontal head sections of \textit{oamb}^{286}, I-L, frontal head sections of \textit{oamb}^{96}. Mushroom body lobes (\(\alpha\), \(\beta\), \(\gamma\)), ellipsoid bodies (eb), pedunculus(P), and noduli(no) are indicated.
Figure 3-7. Immunohistochemical analysis of hypomorphic oamb mutants.
Polyclonal antibodies were made against the third cytoplasmic region of OAMB-K3 and used for immunostaining after affinity purification. A-D, frontal head sections of ry; E-H, frontal head sections of oamb
$^{126}$; I-L, frontal head sections of oamb$^{345}$. Mushroom body lobes (α, β, γ), ellipsoid bodies (eb), pedunculus(P), and noduli(no) are indicated.
Figure 3-8. Immunohistochemical analysis of hypomorphic oamb mutant. Polyclonal antibodies were made against the third cytoplasmic region of OAMB-K3 and used for immunostaining after affinity purification. A-C, frontal head sections of ry, D-F, frontal head sections of oamb<sup>309</sup>. Mushroom body lobes (α, β, γ), ellipsoid bodies (eb), and pedunculus(P) are indicated.
Behavioral assays are extremely sensitive to the genetic background of the tested animal. To minimize for any potential problems caused by genetics, individual alleles have been backcrossed with a parental line ry for six generations for courtship behavior. Male courtship is triggered by various sensory cues, including visual and chemical stimuli. Therefore, the baseline courtship behavior of the oamb null mutants was checked before testing whether the oamb mutants had the phenotype in conditioned courtship. The oamb mutant males displayed normal courtship toward CS virgin females (Fig 3-9). The conditioned courtship assay was subsequently conducted to investigate the roles of
OAMB in associative learning and memory. Compared with the *ry* control, *oamb*\textsuperscript{206} and *oamb*\textsuperscript{584} males showed a significant defect in short term memory retention right after and 1 hour after training in conditioned courtship, but learning during training was not affected (Fig. 3-10). This suggests that short-term memory (STM) retention in conditioned courtship may be mediated by OAMB.

![Figure 3-9. Baseline courtship behavior of *oamb* null mutant males toward CS virgin females.](image)

Four day old naïve *oamb* null mutant males were paired with decapitated virgin CS females in the courtship chamber. The recorded video clips were analyzed for 20 min for scoring courtship behavior. There were no significant differences (P > 0.05, ANOVA). CI, the percentage of time that the male spent courting the female.
Figure 3-10. Conditioned courtship of oamb males. (A) Five day old males were paired with mated females for 1 hour training ($P > 0.05$, ANOVA) and (B) then tested with 5 day old decapitated virgin females right after training for acquisition (5 min), or 1 hour and 3 hours after training for short term memory retention (*, $P < 0.05$, ANOVA). PI, performance index, percent reduction in CI without and with conditioned CI. Mock-conditioned males were used as controls.
3.4 Rescue short-term memory defect of conditioned courtship in oamb males

To analyze for OAMB function in conditioned courtship, the targeted expression of oamb transgenes in oamb null mutant oamb$^{286}$ was achieved by using the GAL4/UAS system. There are two isoforms of OAMB, OAMB-KS and OAMB-AS, which are produced by alternative splicing (Fig. 3-3). They have differences after the 5th transmembrane domain in the C-terminal region (Lee et al., 2003). To investigate the role of OAMB in conditioned courtship, UAS lines carrying OAMB-K3 or OAMB-AS cDNAs were generated by germ line transformation (Spradling and Rubin, 1982). Two independent lines were established in each construct, OAMB-K3 (UAS-K3I, X chromosome, and -K3III, 3rd chromosome) and OAMB-AS (UAS-ASII, 2nd chromosome and -ASIII, 3rd chromosome) and recombined with the oamb$^{286}$ mutant background.

The pan neuronal GAL4 line (elav-GAL4) and two transgenes – OAMB-K3 (UAS-K3) or OAMB-AS (UAS-AS) – in the oamb null mutants were first used to test whether conditioned courtship is mediated by OAMB activity in the neuronal tissues. OAMB-AS driven by elav-GAL4 rescued the short-term memory defect of oamb mutant males 1 h after training (Fig. 3-11), suggesting that OAMB-AS, but not OAMB-K3, in the CNS is important for short-term memory retention in conditioned courtship.
Figure 3-11. Restoration of short term memory retention of oamb<sup>286</sup> null males using the pan neuronal driver, elav-GAL4. Performance index (PI) was calculated at 1 hour after training. CS and oamb<sup>286</sup> were used as controls. OAMB-AS expression in neuronal tissues restores the defect of STM retention of conditioned courtship (ANOVA $F_{[5,83]} = 5.15$, * $P < 0.05$, with post hoc Tukey–Kramer simultaneous comparison to wild type). $n = 6$-22.
To investigate which regions of OAMB expression could function as a mediator of conditioned courtship, the mushroom body specific GAL4 drivers, MB247- or C747-GAL4, were employed to express the OAMB transgenes in oamb male. The expression of two transgenes, OAMB-K3 (UAS-K3) or OAMB-AS (UAS-AS), in the mushroom body driven by MB247- or C747-GAL4 increase their performance (Fig. 3-12). Interestingly, the level of PI, 1 h after training, was in the middle range between CS and oamb mutants in the transgene expression of either OAMB-K3 or -AS by MB specific GAL4 (MB247- or C747-GAL4). The 1h STM defect of conditioned courtship in oamb was not fully restored by the expression of the transgenes in MB only. This suggests that other possible means exist by which OAMB can mediate conditioned courtship, such as a requirement for OAMB outside of the mushroom bodies or the presence of more than one OAMB isoform.
Figure 3-12. Restoration of short term memory retention of \textit{oamb}^{286} males using \textit{C747} and \textit{MB247-GAL4}. Performance index (PI) 1 hour after training. CS and \textit{oamb}^{286} were used as controls. There was no significant difference among different genotypes (ANOVA $F_{[5,83]} = 5.15$, $P < 0.05$, with \textit{post hoc} Tukey–Kramer simultaneous comparison to wild type). $n = 6-22$. 
3.5 Female sterility in oamb mutants

16 oamb alleles were viable with normal external morphology; but homozygous female alleles that had the deletion within I2 were sterile. To characterize this further, individual homozygous females from each excision line were placed with three wild-type CS males on grape juice food for counting eggs. Females of ry, oamb$^{192}$, oamb$^{236}$, and oamb$^{345}$ produced many progeny, but females of the other 13 lines produced no or very few eggs (Table 3-3) (Lee et al., 2003). In Table 3-3, all CS females were in the “High” category with two upstream excision lines, oamb$^{236}$ and oamb$^{345}$, that had a significant number of females scoring “High”. This suggests that the excision lines, with genomic deletions in only upstream regions, have normal fecundity that is comparable to the genetic control lines. oamb$^{192}$ females containing a relatively small deletion in the first intron region, also scored in the “High” category. The lines with breakpoints downstream of E2 were in the “Low” group. Females trans-heterozygous in oamb$^{96}$ or oamb$^{286}$ with Df(3R)$^{P6679}$ were fully fertile, indicating that the sterility of oamb$^{96}$ and oamb$^{286}$ females was complemented by Df(3R)$^{P6679}$, which contains a deletion in its upstream regulatory sequence. Taken together, these results suggest that the enhancer(s) for egg laying is likely placed in I2 between the downstream breakpoints of oamb$^{192}$ and oamb$^{109}$. 
Table 3-3. Sterility test of oamb mutant lines. Individual females of each line were placed with three wild-type CS males in grape-juice agar food vials and eggs were counted eight days after mating.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Deletion</th>
<th>Percentage of &quot;High&quot; females</th>
<th>Average number of progeny from &quot;Low&quot; females</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>control</td>
<td>100 (11)</td>
<td>N/A</td>
</tr>
<tr>
<td>ry</td>
<td>85.4 (41)</td>
<td>7.5 ± 0.6 (6)</td>
<td></td>
</tr>
<tr>
<td>236</td>
<td>upstream</td>
<td>67.7 (31)</td>
<td>4.9 ± 1 (10)</td>
</tr>
<tr>
<td>345</td>
<td>upstream</td>
<td>90.9 (33)</td>
<td>2.7 ± 2.7 (3)</td>
</tr>
<tr>
<td>192</td>
<td></td>
<td>100 (18)</td>
<td>N/A</td>
</tr>
<tr>
<td>309</td>
<td></td>
<td>0 (13)</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>45</td>
<td></td>
<td>0 (11)</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>341</td>
<td></td>
<td>0 (12)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>390</td>
<td></td>
<td>0 (16)</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>126</td>
<td>downstream</td>
<td>0 (26)</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>96</td>
<td></td>
<td>0 (67)</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>218</td>
<td></td>
<td>0 (28)</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>432</td>
<td></td>
<td>0 (27)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>584</td>
<td></td>
<td>0 (27)</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>286</td>
<td></td>
<td>0 (63)</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>174</td>
<td>both side</td>
<td>0 (39)</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>311</td>
<td>both side</td>
<td>0 (24)</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>368</td>
<td>both side</td>
<td>0 (22)</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>96/P6679</td>
<td>Transheter-</td>
<td>100 (15)</td>
<td>N/A</td>
</tr>
<tr>
<td>286/P6679</td>
<td>ozygous</td>
<td>100 (14)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

oamb deletion lines are presented by allelic numbers in the left column; CS and ry served as controls. In the middle column, “High” represents females that produced at least 10 progeny; numbers in parentheses represent the total number of females tested. The right column shows the average progeny number from “Low” females ± standard error of means. Females with less than 10 progenies for each genotype belong to the “Low” group. Total numbers of “Low” females are in parentheses for ry, oamb^{236}, and oamb^{345}. None of CS, oamb^{192}, oamb^{96}/Df(3R)^{P6679}, and oamb^{286}/Df(3R)^{P6679} females belong to the “Low” group (N/A). For the remainder, all of the tested females belong to the “Low” group, and thus were used for calculating the average progeny number. Both developing and adult progeny were counted for this analysis.
To examine whether eggs laid by oamb females develop normally, females of oamb^{584}, oamb^{96}, oamb^{286}, or ry were mated with CS males, and their eggs were examined for hatching (Fig. 3-13 B). The percentages of viable eggs laid by oamb^{96} and oamb^{286} females, however, were highly variable due to the small number of eggs. The percentages of hatched eggs from the oamb alleles oamb^{584}, oamb^{96}, and oamb^{286}, were similar to that from the control ry, suggesting that maternal OAMB is not needed in normal embryonic development.

**Figure 3-13. oamb alleles fecundity and egg hatching.** (A) Female fecundity of a subset of oamb alleles and the control ry. The percentages of “Low” females that produced less than 4 eggs/larvae (E/L) and pupae/adults (P/A) are shown by black solid bars; those that produced equal or more than 4 by bars filled with hatched lines; and “High” females that produced more than 10 E/L or P/A by bars filled with dots. (B) However most of sterile oamb females did not lay eggs, some laid 1-2 eggs. The percentage of hatched eggs was calculated from the vials that contained eggs (mean ± SEM). There was no significant difference among different genotypes ($P > 0.05$, one-way ANOVA). $N = 12$ (ry), 8 (oamb^{584}), 3 (oamb^{96}), and 3 (oamb^{286}).
To investigate abnormality on egg development in oamb females, DAPI staining was performed in the ovaries of oamb\textsuperscript{96}, oamb\textsuperscript{286}, oamb\textsuperscript{192}, or ry females mated with CS males. ry and oamb\textsuperscript{192} have continuously developing oocytes with approximately one mature egg per ovariole but oamb\textsuperscript{96} and oamb\textsuperscript{286} reveal more than two mature eggs per ovariole that were similar to unmated 10 day-old ry female ovaries (Fig. 3-14). No abnormalities in nurse cells, egg chambers, ring canals and overall morphology was detectable in one-day-old ovaries of the sterile females (Fig. 3-14 D and E). This suggests that the sterility of the oamb mutant females may not be due to defective oogenesis (Lee et al., 2003).

Figure 3-14. Dissected ovaries stained with DAPI. (A) and (B) ry, (C) oamb\textsuperscript{192}, (D) oamb\textsuperscript{96}, and (E) oamb\textsuperscript{286}. (A) and (C–E) are from 3- to 4-day-old mated females, and (B) is from a 10-day-old virgin ry female. Mature eggs do not have nurse cells with large nuclei, and have dorsal appendages; many of them are visible in the ovaries of oamb\textsuperscript{96} and oamb\textsuperscript{286} as well as aged virgin ry females. Bars, 50μm.
Because unmated 10-day-old *ry* females had a high number of mature eggs as seen with *oamb* females (Fig. 3-14 B), the *oamb* females’ courtship behaviors – receptivity and the duration of the copulation – were tested and they were comparable to those of *ry* (Fig. 3-15). This indicated that the *oamb* sterility was not related to courtship or copulation failure.

Figure 3-15. Normal courtship behavior of *oamb* females. Individual females were paired with CS males in the courtship chamber and time that the male spent courting the female was measured for 20 minutes (A) (ANOVA $F_{[2,32]} = 3.16, P > 0.05$). $n = 10-16$: CI (courtship index), the percentage time that the male spent courting. The copulation duration was also measured (B). *oamb* represents comparable basal courtship behavior to CS control. (ANOVA $F_{[2,42]} = 3.03, P > 0.05$). $n = 15$ each.
Egg laying is enhanced by mating and is known to be mediated by sperm and seminal fluids (Wolfner, 2007). To investigate whether the sterility of the oamb mutant females is due to an inability to retain the sperm from males, the sterile oamb females were mated with males that carry a chimeric don-juan (dj)-GFP fusion gene (Santel et al., 1997) and they showed no differences with the control ry females (Fig. 3-16). These results indicate that the observed sterility was not due to issues with sperm receptivity or storage in the reproductive system of oamb mutant females.

Figure 3-16. GFP labeled sperm in female sperm-storing organs. Females were mated with males that carrying GFP labeled sperm (dj-GFP) for 3 hours and their reproductive organs were dissected. Odi, oviduct; Spt, spermatheca; SmRcp, seminal receptacle; Utrs, uterus. Bars, 50μm.
Whether the sterility of oamb females was caused by an ovulation defect was tested. Individual females of \textit{oamb}^{96}, \textit{oamb}^{286}, or \textit{ry} were mated with CS males and then an egg in their uteri was monitored during 3 hour intervals. For up to 12 hours after mating, an increased number of \textit{ry} females with an egg was observed while \textit{oamb}^{96} and \textit{oamb}^{286} did not have an egg in their uteri at all (Fig. 3-17). Thus, \textit{oamb} females had a defect in releasing mature eggs to the uterus which is defined as ovulation. As a result, the ovulation deficit in \textit{oamb} mutants may cause their infecundity.

\textbf{Figure 3-17. Ovulation rate.} Females were mated with CS males for 3 hours and then separated. Females with eggs in the uterus and the abdominal tip were counted at various time intervals. \textit{ry} females showed eggs with increasing level upto 12 h while \textit{oamb}^{96} and \textit{oamb}^{286} did not have an egg in their uteri at all.
Therefore, the oamb alleles with downstream deletions, except for oamb$^{192}$, were defective in ovulation and an enhancer for ovulation is likely located in I2 between the deletions of oamb$^{192}$ and oamb$^{309}$. Considering the OAMB expression levels data measured by RT-PCR (Fig. 3-4 and 3-5) and the immunoreactivity experiments (Fig. 3-6, 3-7, and 3-8) described earlier, OAMB-K3 and/or -AS in the body appears to be required for normal female ovulation, which is independent of oamb expression in the brain.

3.6 Reproduction system specific GAL4 driver and OAMB localization in the oviduct epithelium

To investigate whether the major site of OAMB’s function for ovulation is in the reproductive system, GAL4 drivers with specific expression targeting female reproductive tissue were screened. 67 enhancer lines, which were selected based on the reported expression of GAL4 in an epithelial cell type or in reproductive tissue, were obtained from the Bloomington stock center (26 lines) and other labs (9 lines from Dr. Lai (Penn state Univ.) and 32 lines from Dr. Bourbon (France)). A previous study of female sterility in oamb mutants suggested that an ovulation enhancer element may be located in a region of the second intron of the oamb locus (Lee et al., 2003). Thus, efforts were focused on examining the oamb genomic region for any reproductive tissue specific enhancer elements. A PCR fragment from this region containing the endogenous oamb promotor (-1484 to +2880) was cloned into an upstream region of GAL4 within the
pPTGAL vector (Sharma et al., 2002) (Fig. 3-18). Six independent transformed flies 
(oamb-RS-GAL4, named RS for Reproductive System) were identified using the white+ 
marker in pPTGAL and the chromosomal positions of the insertions are mapped using a 
w; +/CyO; +/TM6B,Tb balancer stock. To verify oamb-RS-GAL4 expression in the 
female reproductive system, UAS-mCD8-GFP was utilized. oamb-RS-GAL4 was 
specifically expressed in the reproduction system but not in the central nervous system 
(Fig. 3-19 A-C). oamb-RS-GAL4 was highly enriched in the epithelial cells of the 
oviduct, as shown by the endogenous OAMB expression pattern which was weakly 
expressed in subsets of spermathecae, seminal receptacles, and the accessory gland (Fig. 
3-2 B and Fig. 3-19 A, B, D). The oamb-RS-GAL4 line, that has an insertion in the 
second chromosome, was moved into an oamb286 background. mCD8-GFP driven by 
motor neuronal-GAL4 C380 clearly showed that motor neurons innervated nuclear-
localized β-Gal reporter gene expression, which indicates that OAMB expressing cells 
are in the oviduct epithelium (Fig. 3-19 E). Moverover, the octopamine neurons labeled 
by dTdc2 (neuronal Drosophila tyrosine decarboxylase)-GAL4 driver was visualized 
with UAS-mCD8-GFP showing that abdominal ganglion neurons were projected 
between the epithelial layer and muscle layer in the oviduct where endogenous OAMB is 
expressed (Fig. 3-19 F). It is a useful GAL4 expressing line that can be used for oviduct 
specific expression driving trangenes in rescue experiments.
Figure 3-18. *oamb-RS-GAL4* construct. To get the reproductive system specific GAL4 driver, the *oamb* locus was used. *oamb-RS-Gal4* was generated by cloning a ~4.4Kb (from -1484 to +2880) PCR fragment from the *oamb* genomic segment region in *pPTGAL* (Sharma et al., 2002).
3.7 Rescue of oamb female sterility

3.7.1 OAMB expression in the adult stage by HS-GAL4

To rescue female sterility in oamb, the GAL4/UAS binary system was employed. To investigate whether the female sterility in the oamb mutant was due to an absence of OAMB, heat shock (HS)-GAL4 along with OAMB-K3 (UAS-K3III) and/or OAMB-AS (UAS-ASII and UAS-ASIII) transgenic lines were utilized for ubiquitous expression in adult oamb96 and oamb286 null mutants. The transgenic oamb females carrying HS-GAL4
and UAS-OAMB-K3 or/and OAMB-AS along with control lines (oamb mutants carrying only HS-GAL4 or UAS-OAMB, oamb mutants, and CS controls) were reared at 18 °C prior to heat shock treatments. Ubiquitous expression of transgenic OAMB-K3 and/or -AS driven by HS-GAL4 in oamb females was induced by heat shocks at 37 °C for an hour twice a day in adult stage only. The females were mated with 5-7 day-old CS males at room temperature and the reproductive systems were dissected to count ovulating females which present eggs in the common oviduct or uterus. The oamb females with heat-induced OAMB-K3 or OAMB-AS expression showed similar ovulation rates to those of CS (Fig. 3-20). While the oamb females carrying HS-GAL4 and UAS-OAMB-K3I presented heat-shock dependent rescue to the wild-type ovulation rate, the oamb females carrying HS-GAL4 and UAS-OAMB-ASII or -ASIII showed increased levels of ovulation without heat treatments, which could be due to leaky transgene expression. The control lines with or without heat treatments did not rescue female sterility, clarifying that neither transgene insertions or heat treatments alone induced the increased ovulation levels. Thus, both OAMB-K3 or/and OAMB-AS play a crucial role in ovulation. Moreover, the rescue of the sterility phenotype with transgene expression in adults suggests that OAMB is involved in physiological processes, not in developmental processes, for ovulation.
Figure 3-20. Rescue of the *oamb* female’s ovulation defect with transgenic OAMB expression using heat shock-GAL4 in adults. For the ubiquitous expression of UAS-OAMB-K3 or OAMB-AS (UAS-K3I, -ASII, and -ASIII), HS-GAL4 was used in the *oamb* background. Virgin females were reared at 18 °C prior to heat treatments. Ten 5 to 7 day-old females were crossed with 30 CS males at 25 °C in each cornmeal/yeast/agar food vial for 3 hours and the reproductive systems were dissected to count eggs in the uteri and oviduct for ovulation rate. For inducing HS-GAL4, the females were incubated at 37 °C for 1 h twice a day, 3 days before testing. All transgenic OAMBs in *oamb* females rescued the female sterility with heat shock treatments and UAS-ASII, and -ASIII with HS-GAL4 has fecundity without heat-shocks. CS females were used as a wild type control. (ANOVA $F_{[15,115]} = 20.93$, $P > 0.3$, with post hoc Tukey–Kramer simultaneous comparison to wild type). $n = 3-12$. ns, no significance.
3.7.2 OAMB expression in the neuronal tissues by elav-, Cha-, and C747-GAL4

OAMB is present in the central nervous system and the reproductive system (Lee et al., 2003). However, OAMB is not required in the brain for ovulation based on the expression of OAMB in oamb alleles-oamb<sup>309</sup> or oamb<sup>126</sup>, suggesting that ovulation might be regulated by OAMB in the body through thoracicoabdominal ganglia and/or reproductive tissues (Lee et al., 2003). To address where OAMB is required for controlling ovulation, the elav-GAL4 line, in which GAL4 is expressed in all neurons (Robinow and White, 1988, 1991), was initially used to drive transgenic OAMB expression. Transgenic oamb females carrying UAS-OAMB-K3 and/or UAS-OAMB-AS did not rescue oamb female sterility with elav-GAL4 and had similar ovulation levels to those of oamb females without any transgene, elav-GAL4 or UAS-OAMB alone (Fig. 3-21). oamb females, however, carrying both UAS-OAMB isoforms slightly increased ovulation. The OAMB transgenic, whose expression was driven by elav-GAL4, did not display significant changes in the ovulation rate (Fig. 3-21). The other neuronal driver, cha-GAL4, which is expressed in cholinergic neurons of the central and peripheral nervous systems (Appendix A, A-1), and C747, a MB specific expression driver with transgenic OAMB-K3 and -AS, did not rescue the ovulation phenotype of oamb females (data not shown). Therefore, OAMB expressed in the nervous system is likely insufficient for modulating the ovulation process.
Figure 3-21. Rescue of the oamb female’s ovulation defect with transgenic OAMB expression by pan-neuronal (elav)-GAL4. To dissect the target tissues of OAMB in ovulation, pan neuronal GAL4 (elav-GAL4) was used for driving UAS-OAMB-K3 or -AS expression in the oamb background. Females were crossed same as the previous described condition at room temperature and their reproductive systems were dissected for ovulation tests. No significant changes in the female sterility were detected with transgenic OAMB expression with or without elav-GAL4. CS females were used as a wild type control (ANOVA $F_{[8,46]} = 23.42$, $P < 0.0005$, with post hoc Tukey–Kramer simultaneous comparison). $n = 5-8$. ns, no significance.
3.7.3 OAMB expression by reproductive system specific GAL4 (RS-GAL4)

OAMB-K3 and/or OAMB-AS transgenes driven by elav-GAL4 in oamb females remained infertile (Fig. 3-21). The CNS, or neural OAMB expression, is not sufficient or is not required for ovulation. Thus the major site of OAMB’s function for ovulation may be the reproductive system. To address whether OAMB regulates ovulation in the female reproductive system, the oviduct epithelium specific oamb-RS-GAL4 was used. The over-expression of OAMB-K3 and/or -AS by oamb-RS-GAL4 in oamb null mutants is depicted in Figure 3-20. To visualize transgenic OAMB expression by oamb-RS -GAL4, the sagittal sections of female bodies were immunostained with anti-OAMB-K3 (Fig, 3-20 A, C, E, and G) and -AS (Fig, 3-20 B, D, F, and H) anti-serum. CS as a wild type and oamb286 null mutant were used for controls of UAS-OAMB-K3 and/or UAS-OAMB-AS expression in oamb mutants (Fig. 3-22). Like endogenous OAMB expression in the female reproductive system (Fig. 3-22 A and B), oamb-RS-GAL4 was well localized in the oviducts expressing transgenic OAMB-K3 and/or -AS. No cross immunoactivity was observed with the OAMB-K3 or -AS antibody to transgenic OAMB-AS or OAMB-K3, respectively (Fig. 3-22 F and G). Thus, oamb-RS-GAL4 was utilized to drive transgenic OAMB-K3 and -AS for rescuing the oamb female ovulation defect, elucidating the role of OAMB in ovulation in oviduct epithelium cells. The ovulation rates of transgenic OAMB-K3 or -AS expressed by oamb-RS-GAL4 in oamb females were similar to those of CS wild type control (Fig. 3-23 A). These results indicate that OAMB-K3 or -AS plays an essential role in ovulation in the oviduct epithelium.
Figure 3-22. Immunohistochemistry of transgenic OAMB-K3 or OAMB-AS expression driven by oamb-RS-GAL4 in the oviduct. The transgenic oamb lines carrying UAS-K3 or UAS-AS in oamb background were crossed with oamb-RS-GAL4; oamb and the female progeny containing transgenes in the oamb background were examined by immunohistochemical analysis using anti-OAMB-K3 (1:200) and anti-OAMB-AS (1:50) antibodies along with control lines (CS and oamb^{286}) in ten um sagittal sections. CS (A, B); oamb^{286} (C, D); UAS-OAMB-K3/+; oamb-RS-GAL4/+; oamb^{286} (E, F); UAS-OAMB-AS/oamb-RS-GAL4; oamb^{286} (G, H). A, C, E, G against anti OAMB-K3, and B, D, F, H against anti OAMB-AS. Scale bar, 50μm.
The transgenic oamb females carrying oamb-RS-GAL4 and UAS-OAMB-K3 or UAS-OAMB-AS were examined for ovulation, which were rescued to similar levels to those of CS females (Fig. 3-23 A). Both transgenic OAMB expression levels did not further increase ovulation rates in the oamb female oviduct. These results suggest that OAMB in the oviduct epithelium is adequate to rescue the ovulation defects in oamb females and that the oviduct epithelium is the major site for OAMB regulation of ovulation. Either OAMB isoform has similar capacities in ovulation. The rescue of female sterility in oamb mutants by HS-GAL4 (Fig. 3-20) suggests that OAMB has a physiological role in ovulation. To probe that OAMB is required in the physiological process for ovulation, the TARGET (GAL80ts/GAL4/UAS) system was employed. TARGET allows for control of spatial and temporal transgene expression (McGuire et al., 2004). At lower than 20 °C, GAL80ts binds to GAL4 to inactivate UAS transcription. However, the temperature sensitive GAL80ts is released from GAL4 at 30 °C, which acts on UAS to induce downstream gene expression. Thus, oamb females carrying tubP-GAL80ts (GAL80ts under tubulin promoter for ubiquitous expression), oamb-RS-GAL4 and UAS-OAMB-K3 or -AS are incubated for 3 days at 30 °C to induce transgenic OAMB expression in only adult oviduct epithelial cells. Their ovulation levels were similar to those of CS females after induction, whereas ovulation levels of the oamb females with the same transgenes remained low without temperature shift at 20 °C (Fig. 3-23 B). This indicates that OAMB is required during a physiological, but not developmental, process for ovulation in the oviduct epithelium.
Figure 3-23. Rescue of the *oamb* female’s ovulation defect with transgenic OAMB expression by *oamb-RS-GAL4*. To address whether OAMB regulates ovulation in the female reproductive system, transgenic OAMB expression was targeted by *oamb-RS-GAL4* in *oamb*286. (A) Transgenic *oamb* carrying *oamb-RS-GAL4* and UAS-OAMB-K3 and/or OAMB-AS along with control lines; *oamb*286, CS, and transgenic *oamb* mutants carrying only *oamb-RS-GAL4* or UAS-OAMB were reared at 25 °C before the ovulation test. Ten 5 to 7 day-old females were mated with 30 CS males in each cornmeal/yeast/agar food vial for 3 hours and their reproductive systems were dissected to count eggs in the uteri and oviduct for ovulation. The ovulation rates of transgenic OAMB expression driven by *oamb-RS-GAL4* in *oamb* females were no different in a CS wild type control (P > 0.9). (ANOVA $F_{[6,89]} = 58.40$, ** P < 0.0005, with post hoc Tukey–Kramer simultaneous comparison). n = 9–15. (B) Oviduct specific enhancer *oamb-RS-GAL4* was employed for dissecting the role of OAMB in ovulation. By using *oamb-RS-GAL4* with the TARGET system (McGuire et al., 2004), transgenic OAMB-K3 or OAMB-AS was able to control their expression in a temporal and spatial manner to clarify whether OAMB is required developmentally or physiologically in ovulation in the oviduct epithelium. To inactivate GAL80ts, females were incubated for 3 days at 30 °C. Expression of either OAMB isoform driven by *oamb-RS-GAL4* in *oamb*286 females at 30 °C had no differences when compared to CS wild type control (P > 0.9). (ANOVA $F_{[5,99]} = 45.24$, ** P < 0.0001, with post hoc Tukey–Kramer simultaneous comparison). n = over 20 each. ns, no significance.
3.8 Ca²⁺/calmodulin-dependent protein kinase II in the downstream signaling molecules of OAMB for ovulation.

To dissect the downstream signaling effectors of OAMB in ovulation, the oviduct specific enhancer oamb-RS-GAL4 was used with the TARGET (Temporal And Regional Gene Expression Targeting) system. TARGET is a modified GAL4/UAS system for the temporal and spatial regulation of transgene expression (McGuire, et al., 2004) and can be used to address whether OAMB is required physiologically or during development for ovulation. Because OAMB activates for cAMP and intracellular Ca⁺² increases when assayed with in vitro cell lines (Han et al., 1998; Balfanz et al., 2005), the most reasonable downstream candidate protein kinases were initially screened. Potential cellular targets for these signaling molecules are protein kinase A (PKA) for cAMP, protein kinase C (PKC) and CaMKII for intracellular Ca²⁺. PKAr*, a mutated regulatory subunit that inhibits protein kinase A, PKCi, a peptide inhibitor of protein kinase C which is a dominant negative effector (Broughton et al., 1996; Kiger et al., 1999), and ala, a Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) inhibitor peptide (Joiner and Griffith, 1997) were driven by oamb-RS-GAL4. PKAr* or PKCi expression did not cause sterility in the oviducts by oamb-RS-GAL4. ala, however, in the oamb heterozygous background, induced a defect in female fecundity (Fig. 3-24 B). To investigate their physiological roles in ovulation, the TARGET (GAL80ts/GAL4/UAS) system was employed (McGuire et al., 2004). When the oamb heterozygous females carrying tupP-GAL80ts and oamb-RS-GAL4 along with UAS-PKAr*, UAS-PKCi or UAS-ala were reared at 20 °C, they showed normal ovulation similar to the oamb heterozygous females
Conversely, when the females of the same genotypes were raised at 30 °C for 3 days to inhibit targeted protein kinase activities, the females carrying UAS-ala had reduced levels of ovulation (Fig. 3-24 A). These results suggest that CaMKII is involved in physiological processes in ovulation and may function as a downstream signaling molecule of OAMB. If CaMKII is a downstream signaling molecule of OAMB in ovulation, the constitutive activation of CaMKII would bypass the requirement of OAMB to rescue the ovulation defect of oamb females. To test this, constitutively active CaMKII (CaMKII\textsuperscript{CA}) whose activity is independent on the upstream signals Ca\textsuperscript{2+} and calmodulin (Jin et al., 1998; Mehren and Griffith, 2004) was expressed in adult oviduct epithelium cells in oamb females by oamb-RS-GAL4. The ovulation level of oamb females carrying tupP-GAL80\textsuperscript{ts}, RS-GAL4 and UAS-CaMKII\textsuperscript{CA} reared at 20 °C was comparable to oamb females. However, after temperature shift to 30 °C for 3 days, the ovulation level was increased up to that of the control CS females (Fig. 3-24 C). Thus, CaMKII is a downstream effector of OAMB in the oviduct epithelium for regulating ovulation. Moreover, CaMKII\textsuperscript{CA}-mediated ovulation required mating since the virgin oamb females expressing CaMKII\textsuperscript{CA} did not show ovulation (Appendix A, A-2), suggesting that mating activates OAMB-dependent as well as OAMB-independent processes to trigger ovulation.
Figure 3-24. Ovulation test with possible downstream effectors activation by oamb-RS-GAL4 in the oviduct epithelium. To modulate the possible downstream protein kinases of cAMP or Ca\(^{2+}\), (A) PKAr*, mutated regulatory subunit that constitutively inhibits protein kinase A (Kiger et al., 1999) and PKCi, peptide inhibitor of protein kinase C as a dominant negative effector (Broughton et al., 1996) driven by oamb-RS-GAL4 were induced in the oviducts at 30°C incubation for 3 days before dissecting, but no sterility was caused in oamb heterozygous females. However, ala, Ca\(^{2+}\)/calmodulin-dependent protein kinase II inhibitor peptide (Joiner and Griffith, 1997) in the oamb heterozygous background induceing the defect of female fecundity (ANOVA \(F_{[9,160]} = 64.35, \** P < 0.0005\), with post hoc Tukey–Kramer simultaneous comparison). \(n = 10-20\)

(B) Instead of PKAr*, PKCi, and ala induction in the adults, dominant negative effectors were expressed in whole stages of fly from developmental to adult stage. The ovulation reduction was affected only in the ala expressing line (ANOVA \(F_{[4,98]} = 53.46, \** P < 0.0005\), with post hoc Tukey Simultaneous comparison). \(n = 20-21\)

(C) To confirm the effect of UAS-ala driven by oamb-RS-GAL4 in ovulation, constitutively active CaMKII (Jin et al., 1998) was expressed by oamb-RS-GAL4 in the adult oviduct epithelial cells in oamb females. To activate the expression of constitutively active CaMKII in the adult stage, virgin females were incubated in 30°C for 3 days before examining their ovulation. In addition CaMKII inhibitor peptide ala caused sterility in the oamb heterozygous background, the ovulation defect of oamb females was rescued with activating CaMKII\(^{CA}\) in the adults. (ANOVA \(F_{[7,143]} = 55.94, \** P < 0.0005\), with post hoc Tukey Simultaneous comparison). \(n = 10-26\)
3.9 Nitric oxide in the oviduct epithelium for ovulation

Ovulation is an active transport process of mature eggs from the ovary to the uterus that highly coordinates contraction and relaxation of the oviduct muscle. This process in insects is primarily regulated by the neuromodulators, OA and glutamate (Rodriguez-Valentin et al., 2006). OA may modulate ovulation through OAMB in the oviduct epithelium. Possible mechanisms that epithelial OAMB uses to mediate ovulation would be epithelial fluid secretion or by indirectly modulating oviduct muscle activities. One candidate is nitric oxide (NO). NO is a gaseous paracrine signaling molecule which is well known as an endothelium-derived relaxing factor for smooth muscles (Schneider et al., 2003). Furthermore, NO plays a secretory function in the epithelium (MacPherson et al., 2001; Ulbrich et al., 2006). Nitric oxide synthase (NOS) is activated by Ca\(^{2+}\) (Regulski and Tully, 1995; Davies et al., 1997) and is one of the known downstream molecules of CaMKII (Schneider et al., 2003; Jones et al., 2007; Song et al., 2008). To address whether NO is a downstream molecule of OAMB in the oviduct epithelium, a NOS double-stranded RNA (VDRC stock #, 27725 (Dietzl et al., 2007)) was utilized with oamb-RS-GAL4 to perform a tissue-specific knockdown of dNOS expression in Drosophila.

oamb-RS-GAL4 induced the expression of dNOS-IR (inverted-repeat Drosophila nitric oxide synthase fragment) during the whole life (RS-GAL4/UAS-dNOS-IR; oamb\(^{286/+}\)) or only at the adult stage (RS-GAL4/UAS-dNOS-IR;GAL80\(^{ts}\), oamb\(^{286/+}\)) by
incubating at 30°C for 3 days before ovulation tests showing significantly reduced ovulation levels compared to those of CS females, the females without dNOS-IR induction (reared at 20 °C) or with dNOS-IR only (Fig. 3-25).

Figure 3-25. Ovulation test with possible downstream effector, dNOS-IR expression by oamb-RS-GAL4 in the oviduct epithelia. To address whether the OAMB regulates ovulation in the female reproductive system through NO, dNOS-IR was targeted by oamb-RS-GAL4. oamb heterozygous background carrying oamb-RS-GAL4 and UAS-dNOS-IR along with control line; CS and only UAS-dNOS-IR line were reared at 27 °C before ovulation test, and followed previously described methods for ovulation rates. The ovulation level for dNOS-IR expressed by females driven by oamb-RS-GAL4 showed significant differences to CS wild type control (ANOVA $F_{[2,64]} = 40.46$, **P < 0.0005,
These results reveal a crucial role of NO in ovulation and support the hypothesis that NO generated in the oviduct epithelium may serve as a paracrine signal to regulate the oviduct muscle activities or luminal fluid secretion for successful ovulation.

In conclusion, 16 oamb alleles containing various deletions in the oamb locus were generated and used to study the roles of OAMB in behavioral and reproductive physiological processes. The expression of OAMB-K3 or -AS in the epithelial cells of oviducts was crucial for ovulation whereas the neuronal expression of OAMB-AS was important in conditioned courtship. Moreover, CaMKII and NO as the downstream signaling molecules of OAMB-K3 or -AS are crucial to modulate ovulation in the oviduct epithelium.
Chapter 4

Discussion

4.1 OAMB expression and the characterization of oamb mutants

OA is a major biogenic amine in invertebrates. To investigate OA mediated physiological function and/or associated molecular mechanisms, identifying its receptors is essential. The first OA specific receptor, OAMB, was identified in the brain in *Drosophila* (Han et al., 1998). Activated OAMB is highly enriched in the mushroom bodies of the brain and can increase cAMP and intracellular Ca$^{2+}$ levels (Han et al., 1998). Balfanz et al. has suggested that OAMB is likely to mediate its physiological effects through intracellular Ca$^{2+}$ increases (Balfanz et al., 2005). OAMB has also been categorized as OAR1, which is similar to the vertebrate $\alpha$-adrenergic receptor (Evans and Maqueira, 2005). Recently, three more octopamine receptors have been identified in *Drosophila* (Maqueira et al., 2005) in addition to octopamine receptors cloned in the honeybee and other insects (Blenau and Baumann, 2001; Huang et al., 2007). The cellular mechanisms mediating their physiological function, however, have not been examined. In the experiments designed and described in this study, additional details of OAMB expression in the brain, the TAG, and the female reproductive system are described using immunohistochemistry, *in situ* hybridization, RT-PCR, and X-gal staining. The expression of two OAMB isoforms, derived from alternative splicing of the
last exon leading to differences on the third intracellular loop, have also been examined (Fig.3-1, 3-2, 3-4, and 3-6, Lee et al., 2003). The observed OAMB expression pattern implicate that OAMB has a wide-ranging function in the brain and other tissues. To further investigate the roles of OAMB, null and various hypomorphic oamb mutants containing various deletions in the oamb locus were generated by P-element mediated dysgenesis (Fig. 3-3). These mutants were observed to possess defective phenotypes in conditioned courtship and female reproductive behavior, especially in ovulation.

Studies using OA deficient mutants have reported impaired development of ethanol tolerance (Scholz et al., 2000), reduced aggressive behavior (Baier et al., 2002), alterations in stress reactivity (Grunenko et al., 2000), and defective appetitive learning (Schwaerzel et al., 2003). Several Drosophila mutants for the cAMP pathway (rutabaga, dunce, amnesiac, PKA), in addition to mutants of CaMKII, PKC, homer, latheo, turnip, and cabbage, are defective in learning and memory. Neuroanatomical studies have shown that the MB and the central complex are important for various conditioned behaviors (Nighorn et al., 1991; Davis and Han, 1996; Heisenberg, 1998). Intrinsic Kenyon cells, with projections within the MB, retain short-term memory traces for olfactory conditioning and are required for courtship conditioned memory (Joiner and Griffith, 1999). cAMP and Ca^{2+} signal transduction pathways are crucial for associative learning (Sitnik et al., 2003; Mehren and Griffith, 2004). Interestingly, OAMB is highly expressed in the MB, pedunculus and three lobes, α/β, α’/β’ and γ, and the central complex. cAMP and intracellular Ca^{2+} levels were also observed to be increased by OAMB activation (Han et al., 1998). In the experiments designed in this study, the oamb null mutants,
oamb\textsuperscript{99}, oamb\textsuperscript{286} and oamb\textsuperscript{584}, which were generated by P-element mediate dysgenesis, were tested in a conditioned courtship assay. These mutants were found to present a defect in short-term memory retention in conditioned courtship (Fig. 3-10). As a result, the role of OAMB may be mediating OA through the cAMP and Ca\textsuperscript{2+} signaling pathways in the MB or the central complex for associative behavior.

The deletion between oamb\textsuperscript{192} and oamb\textsuperscript{309}, where the second exon and the second intron exist, are referred to as oamb imprecise excision lines and associated with female sterility (Fig. 3-3 and 3-13). After mating, the female’s reproductive physiology and behavior are modified by the sperm and seminal fluid proteins that the female received during copulation (Chapman et al., 2003). The sex peptides, Acp70A, and Ductus ejaculatorius, DUP99B are crucial for changing the receptivity of mated females (Aigaki et al., 1991). The Drosophila seminal fluid protein ovulin (Acp26Aa) stimulates the release of mature eggs from the ovary after mating (Heifetz et al., 2000). The sex peptides SP and DUP99B, through the use of radiolabeling, have been shown to have high affinity binding sites on peripheral nerves, the subesophageal ganglion, the cervical connective, parts of the thoracic ganglion, and to the genital tract in adult females (Ottiger et al., 2000). The expression of a membrane-bound form of SP has also been reported to influence post-mating behavior in the brain (Nakayama et al., 1997). Taken together, these studies suggest that seminal proteins like ovulin and the sex peptides, which are transferred from a male to a female during copulation, may travel to the brain where post-mating behavior can be modulated. The mating signals received in the brain may then be delivered to the TAG to activate rejection behavior (running away, kicking,
and ovipositor extrusion) and/or to stimulate ovulation. These reports collectively suggest the observation that OAMB in the body is crucial for female fecundity.

Interestingly, OAMB has been found to be expressed in the oviduct epithelium, which was not expected as octopamine has been hypothesized to modulate muscle activity. Putative octopamine receptors on the muscle have been thought to mediate muscle activity. Efforts to clone these putative octopamine receptors, however, have not yet been successful, implying that octopamine receptors may not be directly involved with muscle contraction. Additionally, OAMB expression was detected only in the follicle cells of fully mature eggs in the ovariole. These cells undergo apoptosis when the egg exits the ovary (Nezis et al., 2002). The expression of OAMB in the follicle cells is not crucial for the viability and development of the eggs as oamb females were observed to occasionally lay eggs while undergoing normal development. As a result, instead of being involved with muscle activity, octopamine is likely involved with apoptosis or ovulation.

Although a large body of research has been conducted on oocyte and embryonic development, there is very limited information on the molecular and cellular components or on the mechanisms involved with ovulation and oviposition. Understanding the unique genetics and expression of OAMB will help advance our understanding of octopamine, through which we hope to gain knowledge about the underlying mechanisms regulating physiological function.
4.2 Role of OAMB in conditioned courtship

OA plays a crucial role in behavioral plasticity in *Drosophila*. *oamb* null mutant males, *oamb*[^2^6] and *oamb*[^5^8^4], have a significant short term memory retention defect right after and 1 hour after training in conditioned courtship compared with the *ry* control. The acquisition during training, however, was not affected (Fig. 3-10). Studies with chlordimeform, a octopaminergic insecticide, showed that OA could mediate classical olfactory learning and memory (Dudai, 1987) and that OA is crucial for the formation of appetitive olfactory memory (Schwaerzel et al., 2003). The role of OA in experience-dependent courtship modification toward mature males has also been described (O'Dell, 1994). These reports support the hypothesis that short-term memory (STM) retention in conditioned courtship may be mediated by OA through OAMB. To address the role of OAMB in conditioned courtship in neuronal tissues, pan-neuronal GAL4 driver *elav*-GAL4 was utilized. The rescue of *oamb* mutant male’s defect, in 1 h STM of conditioned courtship by *elav*-GAL4, reveals that the expression of OAMB-AS in the CNS is important for mediating conditioned courtship behavior (Fig. 3-11).

To study for the sites in the brain responsible for conditioned courtship, chemical ablation of the mushroom bodies with hydroxyurea were reported suggesting that MB is crucial for the consolidation of short-term and long-term associative memories (McBride et al., 1999). Moreover, the expression of the CaMKII inhibitor in the mushroom bodies was examined and reported to affect only initial conditioning, not retention, while
conversely, expression in the central complex was found to affect only retention, not initial conditioning (Joiner and Griffith, 1999). Odor information received during olfactory conditioning is conveyed to the mushroom body calyces (Davis, 2004). In the mushroom bodies, conditioned stimulus (CS) and unconditioned stimulus (US) inputs converge through distinct receptors and activate signal transduction cascades to modulate synaptic output (Han et al., 1998, Fig. 4-1 model).

OAMB is highly enriched in the mushroom body, which is a principal neuroanatomical location mediating normal olfactory conditioning and conditioned courtship (O'Dell et al., 1995; Davis, 1996; Davis and Han, 1996). Initially, to dissect the brain structure(s) in which OAMB mediates short-term memory for conditioned courtship, the GAL4 drivers $C747$ and $MB247$ that express GAL4 in the MB, were used (Fig. 3-12). The level of PI, 1 h after training, with the expression of two transgenes – OAMB-K3 or -AS in the mushroom body driven by $MB247$- or $C747$-GAL4 was in the middle range between CS and oamb mutants (Fig. 3-12). The 1h STM defect of conditioned courtship in oamb males was not fully restored by the expression of the transgenes in MB only. The dominant region for olfactory memory, however, is the mushroom body. R4m neurons, in the ellipsoid body, are important for long-term memory consolidation (Wu et al., 2007), suggesting that a much broader and more complex neuronal circuitry is required (Joiner and Griffith, 1999). It also indicates that other possible means by which OAMB mediates conditioned courtship exist. Perhaps there may be a requirement for OAMB outside of the mushroom bodies or perhaps more than one OAMB isoform is present for normal conditioned courtship behavior. Therefore, to clarify whether OAMB expression is
required in other brain regions for conditioned courtship, the \textit{C232}- or \textit{C819-GAL4} lines that specifically expresses GAL4 in the EB need to be studied (Renn et al., 1999). Also, the transgenic \textit{oamb}\textsuperscript{286} lines that carrying OAMB-K3 and -AS together driven by \textit{C747-}
or \textit{MB247-GAL4} will be studied for the ability to restore the \textit{oamb} male’s defect of STM in conditioned courtship. Moreover, \textit{oamb} alleles that have hypomorphic expressions in the brain are useful resources to clarify the crucial structures of the brain where mediate conditioned courtship by OAMB. These results will help in elucidating what role that OAMB has in modulating the physiology of the mushroom bodies and the central complex underlying conditioned courtship learning.

OAMB activates cAMP and intracellular Ca\textsuperscript{2+} increases (Han et al., 1998). Thus, OAMB may be activated by modulatory neurons delivering US information for activating cAMP or intracellular Ca\textsuperscript{2+} increases. Mutants in \textit{dunce}, \textit{rutabaga}, and \textit{amnesiac} display disrupted associative conditioning in a classical aversive olfactory conditioning (Tully and Quinn, 1985) and suppressed conditioned courtship (Siegel and Hall, 1979; Gailey et al., 1984). The cAMP signaling pathway was disrupted in these mutants. Formamidine treatment significantly reduces the learning of \textit{rut} flies (Dudai, 1987) and other ACs (Levin et al., 1992) distinct from the \textit{rut} AC may be coupled to OAMB. In addition, calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) has been reported to be affected in conditioned courtship behavior (Griffith et al., 1993; Griffith et al., 1994; Kane et al., 1997).
cAMP and Ca\textsuperscript{2+} activated by OAMB may directly and indirectly modulate the intracellular signaling cascades involving PKA, PKC, CaMKII, and MAPK for conditioned courtship (Fig. 4-1 model). Therefore, cAMP, PKA, CaMKII, and PKC are the putative downstream mediators of OAMB. However, other biogenic amine receptors or various regions of the brain are likely to be involved in associative learning behavior, because dopamine receptors DAMB and dDA1 are preferentially expressed in the mushroom body lobes and pedunculi (Han et al., 1996; Kim et al., 2003; Kim et al., 2007). The constitutively active form CaMKII, driven by either 30Y-GAL4 or cha-GAL4 in cholinergic inputs into the antennal lobes, improved trainer-dependent courtship suppression (Mehren and Griffith, 2006). The mutations in the potassium channels encoded by Shaker and eag cause the suppression in conditioned courtship and the courtship suppression was mediated by modulation of Eag function as a substrate of calcium/calmodulin-dependent protein kinase II (Cowan and Siegel, 1984; Griffith et al., 1994). Octopamine mediating US information has been demonstrated using the proboscis extension reflex conditioning of the honeybee, in which octopamine injection into the calyces during training substitutes for a sugar reward given to the proboscis (Hammer and Menzel, 1998).

Given the diversity of neuromodulatory systems, it is also conceivable that OAMB may process different sensory inputs. OAMB with distinct biochemical and anatomical properties should help dissect the elaborate neuromodulatory systems underlying behavioral plasticity.
Figure 4-1. A schematic model of the signal transduction cascade triggered by OAMB during conditioned courtship. Unconditioned stimuli (US), such as aversive pheromones from mated females, can activate neurons to trigger a signal transduction cascade, mediated by OAMB in the MB and/or EB. Increases in intracellular cAMP or Ca^{2+} from OAMB activation may be responsible for modifying or processing the attractive pheromones (as a conditioned stimuli, CS) from virgin females. Mechanisms for neuronal regulation through OAMB induced cAMP and Ca^{2+} increases have been described to act through cyclic nucleotide-sensitive potassium channels, protein kinase A/C (PKA/C), and CaMKII (modified from Han et al., 1998).
4.3 Role of OAMB in ovulation

Ovulation is the process where matured ova are released from the ovaries to the uterus. For reproduction in *Drosophila*, ovulation is a critical process of rhythmical contraction and relaxation of smooth muscle cells in the oviduct. Neuronal expression of OAMB, however, was not enough to rescue female sterility. *oamb* null mutant ovulation defects in females was rescued with the expression of either OAMB-K3 or -AS transgenes driven by the oviduct specific *oamb-RS-GAL4*, clearly showing that OAMB expression in the oviduct, not neuronal tissues, regulates for ovulation. To control expression of either OAMB-K3 or -AS transgene spatiotemporally, *GAL80ts* was employed. The rescue experiments of *oamb-RS-GAL4* with *GAL80ts*, to drive transgenic OAMB-K3 or -AS expression at the adult stage, reveal the physiological role of OAMB in ovulation. Furthermore, OAMB was found to modulate ovulation through CaMKII and NO in oviduct epithelium cells.

OA is the invertebrate counterpart of the vertebrate noradrenergic transmitters (Roeder, 1999; Roeder et al., 2003). Previous studies showed that OA relaxes oviduct muscles to allow for the release of mature eggs into the oviducts in locusts (Nykamp and Lange, 2000; Donini and Lange, 2004). To study the cellular and molecular mechanisms of epinephrine/norepinephrine in female reproduction, especially in regulating ovulation, the *Drosophila* model system was applied, as they have similar physiological roles.
There are two key enzymes to generate OA from tyrosine; tyrosine decarboxylase (TDC) and tyramine β-hydroxylase (TβH) (Roeder, 1999). In Drosophila, dTdc2, is expressed in the CNS and innervates the female reproductive tract. tβh mutant tβhnM18 displays a female sterility phenotype that has no octopamine, but female sterility was reversed by the expression of dTDC2 or TβH in the neuronal projection from the ventral ganglia in the female oviduct system by GAL4/UAS (Monastirioti et al., 1996; Monastirioti, 2003; Cole et al., 2005), implying that octopamine is crucial for female reproduction. dTdc2 neurons in the ventral ganglion innervate the posterior ovariole and oviduct, suggesting OA from neuronal supply activate OAMB to control ovulation in oviduct (Fig. 3-19 F, and Fig. 4-2 model, Cole et al., 2005). In addition, OAMB is highly expressed in the female reproductive system and Drosophila oamb mutants, containing deletions over the second intron of the oamb locus have impaired female reproduction (Fig. 3-2 and 3-13) (Han et al., 1998; Lee et al., 2003). The underlying mechanisms, however, are poorly understood. Thus, the Drosophila OA receptor, OAMB, was chosen for study to investigate the molecular mechanisms in female reproduction.

To investigate the requirement of OAMB in ovulation, HS-GAL4 for ubiquitous expression of transgenic OAMB in adults, was utilized for rescuing female sterility in oamb mutant females. The over/ectopic-expression of either OAMB-K3 or -AS in Drosophila driven by HS-GAL4, was induced in the adult stage by heat shock given for one hour at 37 °C, twice a day for 3 days, before the ovulation test and restoration of female fecundity (Fig. 3-20). OAMB-ASs with HS-GAL4 has normal ovulation even
without heat-shock induction, which is possibly due to leaky expression of the UAS-ASII or -ASIII. This clearly suggests that either OAMB-K3 or -AS is crucial in ovulation.

To dissect the tissue type(s) that requires OAMB in ovulation, pan-neuronal elav-GAL4 was initially employed for expressing transgenic OAMB in neurons because OAMB is highly expressed in the CNS (Lee et al., 2003). Transgenic OAMB expression driven by elav-GAL4 did not rescue the sterility in oamb females (Fig. 3-21), suggesting that neural OAMB expression is inadequate for regulating ovulation. To address whether OAMB’s functional site is the reproductive system, as suggested by the reported OAMB expression levels in the reproductive system (Lee et al., 2003), enhancer GAL4 lines from public fly stock centers were screened. These flies have specific OAMB expression in the female reproductive system or show similar patterns of endogenous OAMB where it is specifically expressed in oviduct epithelium cells. However, no GAL4 line is available that limits expression to the female reproductive system, but not for neuronal expression. Therefore, the enhancer GAL4 line oamb-RS-GAL4 was generated where GAL4 was specifically expressed in the oviduct epithelium using the oamb locus in pPTGAL (Fig. 3-18 and 3-19 A-C) (Sharma et al., 2002). From the female fecundity of the serial deletions of oamb alleles, the enhancing element for the expression of the reproductive system is located in the 2nd intron. Thus, the region which encompassed the possible enhancing element and the promoter region in the oamb locus was cloned in pPTGAL vector (Sharma et al., 2002). The strong GAL4 expression of oamb-RS-GAL4 with UAS-mCD8-GFP was revealed in the oviduct epithelium, not in the CNS (Fig. 3-19 A - C), where endogenous OAMB is expressed (Fig. 3-19. D) and drove transgenic
OAMB expression in the oviduct (Fig. 3-22). The expression of transgenic OAMB-K3 or -AS driven by oamb-RS-GAL4 rescued oamb female sterility (Fig. 3-23 A) and also transgenic expression only in the adult stage was sufficient to restore oamb female fecundity (Fig. 3-23 B). This suggests that OAMB is required in oviduct epithelium cells for modulating ovulation physiologically.

Octopamine neurons in the abdominal ganglion innervate the female reproductive system and play a crucial role in female reproduction (Monastirioti, 2003; Rodriguez-Valentin et al., 2006; Hardie et al., 2007). Interestingly, activated OAMB induces cAMP and intracellular Ca\(^{2+}\) increases (Han et al., 1998; Evans and Maqueira, 2005). The role of cAMP in gonad development and fertility are well studied in *Drosophila* (Lannutti and Schneider, 2001), as well as for Ca\(^{2+}\) and cGMP in ovarian steroidogenesis in the blowfly (Maniere et al., 2002, 2003). Proctolin has been reported to act as an antagonist of octopamine which induces insect visceral muscle contractions by increasing cytosolic calcium concentrations (Ritsick et al., 2007) in addition to octopamine relief oviduct contractions with cAMP increases in the oviduct muscle (Rodriguez-Valentin et al., 2006). These observations support the potential for downstream molecules of OAMB in the oviduct to mediate for ovulation through the effectors related to cAMP and/or Ca\(^{2+}\). Therefore, to dissect the cellular mechanisms of OAMB in ovulation, the known secondary signaling effectors of OAMB were studied. Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is activated by intracellular Ca\(^{2+}\) increases and is involved in various cellular mechanisms. It is preferentially expressed in the brain, thoracic ganglion, and gut of the adult (Takamatsu et al., 2002). To investigate whether CaMKII is the downstream
effector of OAMB in ovulation, CaMKII inhibitor peptide ala or constitutively active forms of CaMKII was expressed in the adult oviduct epithelial cells by oamb-RS-GAL4. CaMKII\textsuperscript{CA} rescued the ovulation defect of oamb females (Fig. 3-24 C), but ala induced sterility in the oamb heterozygous background (Fig. 3-24 A and B), suggesting that CaMKII is a major downstream signaling molecule of OAMB for regulating ovulation.

Another potential effectors for increased intracellular Ca\textsuperscript{2+} is nitric oxide (NO) synthase (NOS). NO is a polyvalent molecule which plays a critical role in regulating multiple functions, such as a paracrine/autocrine mediator for multifunctional signaling in the reproductive process, fertility, ovulatory process, labor, pregnancy, sexual behavior, oviduct function, steroidogenesis, tissue remodeling, placenta, penile erection, sperm motility, and sperm motility (Griffith and Stuehr, 1995; Rosselli et al., 1998). In smooth muscle, NO triggers for relaxation by activating soluble guanylate cyclase and increasing cGMP levels, and PKG and CaMKII are involved in the relaxation on gastric fundus smooth muscles (Arnold et al., 1977; Abraham et al., 1996; Cohen et al., 1999; Kim and Perrino, 2007). Moreover, CaMKII regulates endothelium-dependent relaxation and NO synthesis in endothelium-intact aortic rings of normotensive rats and CaMK II inhibitor KN-93 decreased NO synthesis in a porcine aortic endothelial cell line (Schneider et al., 2003). NO in the female reproductive tissues has been shown to be a important modulator in the physiology of the oviduct such as ovulation, fertility, and pathological situations (Rosselli et al., 1998). These observations all implicate CaMKII as an important molecule for NO synthesis.
Endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), in bovine oviduct epithelium, were induced by the oestrous cycle, and iNOS was localised in secretory epithelial cells as well as in the lamina muscular (Ulbrich et al., 2006). Moreover, NOSs, neuronal nitric oxide synthase (nNOS), eNOS, and iNOS, were expressed by hormonal regulation and differential expression along the oviduct throughout the estrous cycle (Lapointe et al., 2006). These observations suggest that NO production by temporal-spatial NOSs is crucial for reproductive events in the oviduct.

Furthermore, NO is involved with smooth muscle relaxation through the cGMP pathway in the human fallopian tube (Ekerhovd and Norstrom, 2004). The NO-cGMP signaling process in *Drosophila* has been primarily studied for neuronal activity. However, the secretory cells in malpighian tubules expressed dNOS, increases in calcium and intracellular cGMP, and also amplified fluid secretion rates depending on the activation of a soluble guanylate cyclase (Davies et al., 1997; MacPherson et al., 2001; Kean et al., 2002; Broderick et al., 2003). These studies suggested that NO has possible secretory roles and indirectly oviduct muscle relaxation for modulating oviduct functions through physiological mechanisms in conjunction with the possibility that OAMB modulates ovulation (Fig. 4-2 model).

In summary, octopaminergic neurons from abdominal ganglion innervate the female reproductive system and activate OAMB to modulate ovulation in oviduct epithelial cells after mating. By OAMB activation, increased intracellular Ca\(^{2+}\) activates calcium/calmodulin dependent kinase II and NO as the downstream effect molecules of OAMB in oviduct basal epithelium cells for regulating ovulation. Here, I describe that
OA receptor OAMB in the epithelium cell layer may modulates luminal fluid secretion or oviduct smooth muscle through CaMKII and NO.

Taken as a whole, this study can enhance understanding of the physiological role of the α-adrenergic receptor in mediating ovulation. As aforementioned, the adrenergic system is important in female reproductive processes such as ovulation. While drugs targeting adrenergic systems for hypertension, asthma and depression are heavily used, the effects in reproductive systems are not clearly understood. Thus, it is of great interest to conduct mechanistic research of the adrenergic system on female reproduction using a Drosophila model system. Knowledge obtained from this research and follow-up studies may provide significant insight into the physiological and cellular basis of adrenergic function in reproductive systems and may help prevent potential side effects caused by adrenergic drugs on ovulation.
Figure 4-2. A schematic model of the signal transduction cascade triggered by OAMB for ovulation in the oviduct epithelium. OA neurons, which are innervated from the abdominal ganglia to the basal region of the oviduct epithelium, activate OAMB, to induce for intracellular Ca\textsuperscript{2+} level increases. The Ca\textsuperscript{2+} signaling pathway then activates CaMKII or NOS for muscle relaxation and/or fluid secretion.
Chapter 5

Future directions

In this study, OAMB-AS in neuronal tissue plays a crucial role in short-term memory retention of conditioned courtship. The \textit{oamb} phenotype in conditioned courtship using mushroom body specific GAL4 (\textit{MB247} and \textit{C747}) lines, however, were not fully restored. Therefore, hypomorphic \textit{oamb} alleles, with differential OAMB expression in the mushroom bodies and the central complex, in addition to central complex specific GAL4 lines, will be used to map which brain structures require OAMB for short term memory in conditioned courtship. Moreover, tissue specific GAL4 lines that rescue the defect of \textit{oamb} mutant males in short-term memory retention of conditioned courtship will be applied to dissect the downstream signaling effectors that OAMB mediates. For conditioned courtship, cAMP and Ca^{2+}, activated by OAMB, may directly or indirectly modulate the intracellular signaling cascades involving PKA, PKC, CaMKII, and MAPK. To investigate the cellular and molecular mechanisms mediated by OAMB for short-term memory of conditioned courtship (see Fig.4-1 for model), the GAL4/UAS system will be used with GAL80ts to drive the transgenic downstream effectors or RNA-mediated gene interference (\textit{RNAi}) (McGuire et al., 2004; Dietzl et al., 2007).

The sterility in \textit{oamb} females was rescued by the expression of transgeneic OAMB-K3 and/or -AS and CaMKII\textsuperscript{CA} with \textit{oamb-RS-GAL4}, suggesting that OAMB in the oviduct epithelium is crucial and recruits CaMKII activation for ovulation. \textit{oamb-RS-}
GAL4, with a strong expression level in the oviduct epithelium, can be utilized to drive the possible downstream signaling effectors of OAMB in ovulation. By rescuing for ovulation defects in oamb females when oamb-RS-GAL4 is expressed, downstream effectors can be identified. To identify downstream OAMB effectors with minimal bias, genetic interaction screening will be conducted to identify genetic suppressors that rescue the ovulation phenotype when overexpressed in oamb mutant females. For overexpression of candidate effectors in the oviduct, oamb-RS-GAL4, in combination with EP lines (P-element vector containing multiple UAS randomly inserted in the genome), will be obtained from a public stock center and transgenic oamb mutants overexpressing candidate effectors in the oviduct will be tested for ovulation. The dominant suppressors identified from this screen may help elucidate cellular mechanisms mediated by OAMB for ovulation.

Taken together, the knowledge obtained from these studies will elucidate the physiological and cellular mechanisms associated with octopamine function in the reproductive system, as well as for associative learning and memory in Drosophila and/or other organisms, such as humans.
Chapter 6

Summary

The biogenic amine octopamine is a major neuromodulator in invertebrates. Two octopamine receptors, OAMB-K3 and OAMB-AS, which are produced by alternative splicing of oamb transcripts, were identified and found to activate for cAMP and intracellular calcium increases. Both receptors are expressed in the central nervous system and the reproductive system. Males or females lacking both isoforms are impaired in short-term memory of conditioned courtship and ovulation.

First, expression levels of OAMB in the CNS and the female reproductive system were investigated. OAMB was found to be highly enriched in the mushroom bodies and the ellipsoid bodies of the Drosophila brain, which are known to be a center of associative learning and memory, and also observed to be expressed in the thoracico-abdominal ganglion and the female reproductive system (oviducts, seminal receptacles, spermatheca, and follicle cells in the mature eggs).

Second, the roles of OAMB in associative learning and female reproduction were studied by generating null and various hypomorphic oamb mutants using P-element mediated dysgenesis. oamb null mutant males are defective in short-term memory retention, but not for acquisition of courtship conditioning whereas oamb female alleles that have deletion beyond the second exon display female sterility.
Thirdly, to examine for the tissue(s) where OAMB mediates its function, transgenic OAMB-K3 and –AS were established and used with various tissue specific GAL4 lines, \textit{elav-}, \textit{heat-shock-}, and \textit{oamb-RS-GAL4}. The short term memory of the conditioned courtship phenotype in \textit{oamb} males was rescued with the neuronal GAL4 driver, \textit{elav-GAL4}, indicating that OAMB–AS expression in the CNS is crucial, but not in other tissues. Ovulation defects, however, were rescued by transgenic OAMB-K3 and/or OAMB-AS expression in the oviduct epithelium at the adult stage, suggesting that OAMB’s functional site is the reproductive system through a physiological regulation and not a developmental one.

Lastly, OAMB was found to activate intracellular cAMP and Ca\textsuperscript{2+}, suggesting protein kinase A (PKA), protein kinase C (PKC), MAP kinases and CaMKII are possible candidates for downstream effectors. To dissect the downstream signaling molecules that OAMB activates for ovulation, PKAr*, PKCi, CaMKII inhibitor peptide ala, constitutively active CaMKII (CaMKII\textsuperscript{CA}), and \textit{dNOS-IR} were expressed in adult oviduct epithelial cells by \textit{oamb-RS-GAL4}. CaMKII\textsuperscript{CA} rescued the ovulation defect of \textit{oamb} females after mating whereas ala and \textit{dNOS-IR} reduced ovulation levels in an \textit{oamb} heterozygous background. Thus, CaMKII and possibly NO are major downstream signaling molecules of OAMB for regulating ovulation.

In summary, these studies were conducted to better understand the reproductive behavior and physiology that can be mediated by the octopaminergic system. Through these experiments and by future experiments, the underlying cellular and molecular mechanisms involved with these processes can be elucidated.
References


Drosophila mushroom bodies by selective feminization of genetically defined
of noradrenergic signaling by the nucleus tractus solitarius in mediating opiate
156.
Drosophila Ca2+/calmodulin-dependent nitric oxide synthase. Proc Natl Acad Sci
U S A 92:9072-9076.
analysis of the Drosophila ellipsoid body neuropil: organization and development
Ressler KJ, Nemeroff CB (2001) Role of norepinephrine in the pathophysiology of
neuropsychiatric disorders. CNS Spectr 6:663-666, 670.


A-1. Rescue of the oamb female’s ovulation defect with transgenic OAMB expression by cholinergic neuronal (cha)-GAL4. To dissect the target tissues of OAMB in ovulation, cholinergic neuronal GAL4 (cha-GAL4) was used for driving UAS-OAMB-K3 or -AS expression in the oamb background. Females were crossed at room temperature for 3 h and counted the females that hold egg in the uterus or the tip of genitalia for ovulation levels. No significant changes in the female sterility were detected with transgenic OAMB expression with or without cha-GAL4. CS females were used as a wild type control, n = 3-19. ns, no significance.
A-2. Ovulation test with possible downstream effectors activation by *oamb-RS-GAL4* in the oviduct epithelium. To investigate whether the mating activates OAMB to induce ovulation through CaMKII, constitutively active CaMKII driven by *oamb-RS-GAL4* was expressed in the adult oviduct epithelial cells in *oamb* females and test ovulation without mating. To activate the expression of constitutively active in the adult stage, virgin females were incubated in 30°C for 3 days before examining their ovulation (see Fig. 3-24 C for more mated data).
Appendix B

Published works – Abstracts only


Recurring ethanol exposure induces disinhibited courtship in *Drosophila*.

**Lee HG**, Kim YC, Dunning JS, Han KA.

Alcohol has a strong causal relationship with sexual arousal and disinhibited sexual behavior in humans; however, the physiological support for this notion is largely lacking and thus a suitable animal model to address this issue is instrumental. We investigated the effect of ethanol on sexual behavior in *Drosophila*. Wild-type males typically court females but not males; however, upon daily administration of ethanol, they exhibited active intermale courtship, which represents a novel type of behavioral disinhibition. The ethanol-treated males also developed behavioral sensitization, a form of plasticity associated with addiction, since their intermale courtship activity was progressively increased with additional ethanol experience. We identified three components crucial for the ethanol-induced courtship disinhibition: the transcription factor regulating male sex behavior Fruitless, the ABC guanine/tryptophan transporter White and the neuromodulator dopamine.

fruitless mutant males normally display conspicuous intermale courtship; however, their courtship activity was not enhanced under ethanol. Likewise, white males showed negligible ethanol-induced intermale courtship, which was not only reinstated but also augmented by transgenic White expression. Moreover, inhibition of dopamine neurotransmission during ethanol exposure dramatically decreased ethanol-induced intermale courtship. Chronic ethanol exposure also affected a male's sexual behavior toward females: it enhanced sexual arousal but reduced
sexual performance. These findings provide novel insights into the physiological effects of ethanol on sexual behavior and behavioral plasticity.


D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*.

Kim YC, Lee HG, Han KA.

*Drosophila* has robust behavioral plasticity to avoid or prefer the odor that predicts punishment or food reward, respectively. Both types of plasticity are mediated by the mushroom body (MB) neurons in the brain, in which various signaling molecules play crucial roles. However, important yet unresolved molecules are the receptors that initiate aversive or appetitive learning cascades in the MB. We have shown previously that D1 dopamine receptor dDA1 is highly enriched in the MB neuropil. Here, we demonstrate that dDA1 is a key receptor that mediates both aversive and appetitive learning in pavlovian olfactory conditioning. We identified two mutants, dumb1 and dumb2, with abnormal dDA1 expression. When trained with the same conditioned stimuli, both dumb alleles showed negligible learning in electric shock-mediated conditioning while they exhibited moderately impaired learning in sugar-mediated conditioning. These phenotypes were not attributable to anomalous sensory modalities of dumb mutants because their olfactory acuity, shock reactivity, and sugar preference were comparable to those of control lines. Remarkably, the dumb mutant's impaired performance in both paradigms was fully rescued by reinstating dDA1 expression in the same subset of MB neurons, indicating the critical roles of the MB dDA1 in aversive as well as appetitive learning. Previous studies using dopamine receptor antagonists implicate the involvement of D1/D5 receptors in various pavlovian conditioning tasks in mammals; however, these have not been supported by the studies of D1- or D5-
deficient animals. The findings described here unambiguously clarify the critical roles of D1 dopamine receptor in aversive and appetitive pavlovian conditioning.


Classical reward conditioning in *Drosophila melanogaster*.

Kim YC, Lee HG, Han KA.

Negatively reinforced olfactory conditioning has been widely employed to identify learning and memory genes, signal transduction pathways and neural circuitry in Drosophila. To delineate the molecular and cellular processes underlying reward-mediated learning and memory, we developed a novel assay system for positively reinforced olfactory conditioning. In this assay, flies were involuntarily exposed to the appetitive unconditioned stimulus sucrose along with a conditioned stimulus odor during training and their preference for the odor previously associated with sucrose was measured to assess learning and memory capacities. After one training session, wild-type Canton S flies displayed reliable performance, which was enhanced after two training cycles with 1-min or 15-min inter-training intervals. Higher performance scores were also obtained with increasing sucrose concentration. Memory in Canton S flies decayed slowly when measured at 30 min, 1 h and 3 h after training; whereas, it had declined significantly at 6 h and 12 h post-training. When learning mutant t beta h flies, which are deficient in octopamine, were challenged, they exhibited poor performance, validating the utility of this assay. As the Drosophila model offers vast genetic and transgenic resources, the new appetitive conditioning described here provides a useful tool with which to elucidate the molecular and cellular underpinnings of reward learning and memory. Similar to negatively reinforced conditioning, this reward conditioning represents classical olfactory conditioning. Thus, comparative analyses of learning and memory mutants in two assays may help identify the molecular and cellular components that are specific to the unconditioned stimulus
information used in conditioning.


Microcolumn separation of amine metabolites in the fruit fly.

Paxon TL, Powell PR, Lee HG, Han KA, Ewing AG.

Electrophoretic resolution of 14 biogenic amines and metabolites with similar mobilities is addressed by employing micellar electrokinetic capillary chromatography coupled to amperometric electrochemical detection. The present study describes the optimization of separation conditions to achieve resolution of analytes of biological significance within 20 min in a single separation. They include dopamine, epinephrine, norepinephrine, octopamine (Einspanier et al.), L-3, 4-dihydroxyphenylalanine, tyramine (TA), and serotonin as well as metabolites 5-hydroxyindolacetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 3-methoxytyramine in addition to N-acetylated metabolites including N-acetyldopamine, N-acetyloctopamine (naOA), and N-acetylserotonin. The optimized conditions used result in excellent reproducibility and predictable peak shifting, thus enabling identification of several metabolites along with their biogenic amine precursors in biological samples, specifically from the fruit fly Drosophila melanogaster. The separation method is sensitive, selective, and quantitative as demonstrated by its capacity to detect changes in TA, OA, and naOA present in the head homogenates of the Canton-S and mutant inactive(1) Drosophila lines. Quantitative analysis of metabolites in conjunction with their biogenic amine precursors in a single separation offers tremendous potential to understand the physiological processes and underlying mechanisms mediated by various biogenic amines in Drosophila and other animals.

Octopamine receptor OAMB is required for ovulation in *Drosophila melanogaster*.

**Lee HG**, Seong CS, Kim YC, Davis RL, Han KA.

Octopamine is a major monoamine in invertebrates and affects many physiological processes ranging from energy metabolism to complex behaviors. Octopamine binds to receptors located on various cell types and activates distinct signal transduction pathways to produce these diverse effects. We previously identified one of the Drosophila octopamine receptors named OAMB that produces increases in cAMP and intracellular Ca	p+ upon ligand binding. It is expressed at high levels in the brain. To explore OAMB's physiological roles, we generated deletions in the OAMB locus. The resultant oamb mutants were viable without gross anatomical defects. The oamb females displayed normal courtship and copulation; however, they were impaired in ovulation with many mature eggs retained in their ovaries. RT-PCR, in situ hybridization, and expression of a reporter gene revealed that OAMB was also expressed in the thoracicoabdominal ganglion, the female reproductive system, and mature eggs in the ovary. Moreover, analysis of various alleles pinpointed the requirement for OAMB in the body, but not in the brain, for female fecundity. The novel expression pattern of OAMB and its genetic resource described in this study will help advance our understanding on how the neuromodulatory or endocrine system controls reproductive physiology and behavior.


Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of *Drosophila melanogaster*.

Kim YC, **Lee HG**, Seong CS, Han KA.
The diverse physiological effects of dopamine are mediated by multiple receptor systems. The dDA1 represents one of the Drosophila dopamine receptors that activate the cAMP cascade. To gain insight into the role of dDA1, we generated a polyclonal antibody against the unique sequence in dDA1 and investigated dDA1 distribution in the central nervous system (CNS) of Drosophila melanogaster. In both larval and adult CNS pronounced dDA1 immunoreactivity was present in the neuropil of the mushroom bodies, a brain structure crucial for learning and memory in insects, and four unpaired neurons in each thoracic segment. In addition, the larval abdominal ganglion contained two dDA1 cells in each segment. This expression pattern appeared to be maintained in the condensed adult abdominal ganglion although the precise number and the intensity of staining were somewhat variable. The adult CNS also exhibited intense dDA1 immunoreactivity in the central complex, a structure controlling higher-order motor function, moderate expression in several neurosecretory cells, and weak staining in two unpaired neurons in the mesothoracic neuromere. The dDA1 expression in these areas was only detected in adult, but not in third instar larval CNS.
VITA

Hyun-Gwan Lee

E-mail: hil1@psu.edu, hglee602@gmail.com

Education:
Feb. 1995 : Bachelor degree in Science, Department of Biology, Chonnam National University
Feb. 1997 : Master degree in Science, Department of Biology, Chonnam National University, Korea
Thesis Title: Purification and Characterization of the Lipid Body Membrane Proteins Specifically Expressed during Germination Period of Maize Seed
Aug. 2008 : PhD degree in Genetics, Penn State University, USA
Thesis Title: Roles of the octopamine receptor OAMB in associative learning, memory, and reproduction in Drosophila melanogaster

Research Experience:
Mar. 1995-Aug. 1997: RA in Hormone Research Center, Chonnam National University, Korea
Mentor: Dr. Dong-Gyu Bai
Research Project: Mechanism of Gene Expression Controlled by Plant Hormones in Maize

Mentor: Dr. Nam-Chon Paek
Research Project: Genes Identification and Expression by Photo-Signal in Arabidopsis

Sep. 1999-Mar. 2000: RA in Plant Molecular Genetic Breeding Center, Seoul National University, Korea
Mentor: Dr. Nam-Chon Paek
Research Project: Genes regulated by phytohormone in Arabidopsis

Apr. 2000-Dec.2002: RA in Department of Biobehavioral Health, Pennsylvania State University, USA
Mentor: Dr. Kyung-An Han
Research Project: Identification of expression patterns of octopamine receptor isoforms in Drosophila melanogaster nervous system, and generation and characterization of the octopamine receptor mutants using various behavioral paradigms.

Teaching Experience:
Sep.1998-Aug.1999 : TA in Department of Biology, Chonnam National University
Teaching undergraduate students in general biology laboratory

Jan.2005-Dec.2005 : TA in Department of Biology, Penn State University
Teaching undergraduate students in Bio230W and Bio240W laboratory

Jan. 2008-May.2008 : TA in Department of Biology, Penn State University
Teaching undergraduate students in Bio240W laboratory

Research Paper Presentation (see Appendix B):


