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Characterization of the pleiotropic effects of the Drosophila gene mushroom body miniature B

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CHARACTERIZATION OF THE PLEIOTROPIC 
EFFECTS OF THE DROSOPHILA GENE 
MUSHROOM BODY MINIATURE B 

by 

Michael L. Ginsburg 

A thesis submitted in partial fulfillment 
of the requirements for the degree of 

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College of Sciences 

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Michael Louis Ginsburg

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Characterization of the Pleiotropic Effects
of the Drosophila Gene Mushroom Body Minature B

is approved in partial fulfillment of the requirements for the degree of

Master's of Science

Examination Committee Chair

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Graduate College Faculty Representative
The mushroom body miniature B (mbmB) mutation has documented defects in adult mushroom body anatomy, olfactory memory and female fertility. In this thesis I report that mbmB mutants also have reduced viability and growth rate. I further demonstrate, using the mushroom body cell marker dachshund, that loss of mbmB function does not affect the early development of mushroom body neuroectoderm and neuroblasts. Additionally, I found the observed delay in growth and reduced viability were not manifested during embryonic development.

Courtship behavior assays and immunohistochemical techniques were used to investigate causes of mbmB female sterility. Courtship involving mbmB females is delayed and reduced in total amount relative to that of wild type. Additional data
presented herein suggest that mechanisms required during early oogenesis are not significantly impaired by loss of \textit{mbmB} function. However, evidence is presented which demonstrates abnormalities in follicle-cell migration in \textit{mbmB} mutants.
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ABBREVIATIONS

AC    Adenylyl Cyclase
AMP   Adenosine Monophosphate
ANOVA Analysis of Variance
AP    Alkaline Phosphatase
ATP   Adenosine Triphosphate
BCIP  5-bromo-4-chloro-3-indolylphosphate
BSA   Bovine Serum Albumin
cAMP  cyclic Adenosine Monophosphate
CCD   Closed Circuit Digital
Cl    Courtship Index
CNS   Central Nervous System
CREB  cAMP Response Element Binding Protein
CS    Canton Special
Cy    Curly
CyO   Curly of Oster
DAC   Dachshund (Protein)
DAPI  4', 6-diamidino-2-phenylindole
DIC   Differential Interference Contrast
DMSO  Dimethyl Sulfoxide
dnc   dunce
EMS   Ethyl Methyl Sulfonate
GAL-4 Yeast Transcription Factor, Binds Upstream Activation Sequence
GFP   Green Fluorescent Protein
Gprk2 G-protein coupled receptor kinase-2
HEPES N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
IgG   Immunoglobulin
In    Inversion
KC    Kenyon Cell
Kr    Kruppel
L     Lobe
L     Latency
MB    Mushroom Body
mbmB  mushroom body miniature B
MBNb  Mushroom Body Neuroblast
MTOC  Microtubule Organizing Center
Nb    Neuroblast
NBT   Nitro-Blue Tetrazoleum
NGS  Normal Goat Serum
PBT  Phosphate Buffered Saline, Tween
PBS  Phosphate Buffered Saline
PBTS Phosphate Buffered Saline, Tween, Bovine Serum Albumin
PKA  Protein Kinase A
rut  rutabaga
w    white
YO-PRO-1 Quinolinium Methyl Benzoazolylidene Trimethylammoniopropyl Diiodide
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GENERAL INTRODUCTION

Ode to *Drosophila*

During the last century, the fruit fly, *Drosophila melanogaster*, has been utilized extensively as a biological model organism. The fly has aided researchers in studies on the theory of inheritance, such as those performed by Thomas H. Morgan in 1910 (Morgan 1910). More recently, the fly has been used in studies determining complex genetic interactions through the use of microarray technology (De Gregorio et al. 2002). Since the early experiments of Morgan, an immense wealth of information related to the field of biology has been generated with the *Drosophila* model. In addition to the historical significance, there are numerous practical advantages to using *Drosophila*. The fly has an inexpensive upkeep, a rapid generation time of about 10-12 days, and highly fecund females capable of producing hundreds of offspring. Furthermore, and perhaps most importantly, the fly has a relatively small genome that has recently been sequenced and there exist mutants of many of its genes (Adams et al. 2000; Lindsley and Zimm 1992).

The *Drosophila* model system has been the center of major scientific advances in nearly all fields of biology. For example, the fly was used by developmental biologists in deciphering genetic control of early development, earning its investigators: Ed Lewis, Christiane Nusslein-Volhard, and Eric Wieschaus, the Nobel Prize for medicine in 1995. Researchers in neurobiology
used the fly model to map complex neuronal signaling pathways such as those involved in olfaction (Carlson 1996; Carlson 2001). Biochemists and cell biologists have been able to determine protein as well as cell-signaling pathway homologies between *Drosophila* and other organisms, including humans. These homologies include the *Drosophila eyeless* gene product, which shares a great deal of sequence and functional similarity with the human paired box, Pax6 protein. Both proteins are involved in eye development in their respective organism (Quiring et al. 1994; Ton et al. 1991; for review, see Macdonald and Wilson 1996). Also, the hedgehog-signaling pathway, originally described in *Drosophila*, has been implicated in various aspects of development both in flies and in humans (Nusslein-Volhard and Wieschaus 1980; Wicking et al. 1999; for review, see Peifer and Bejsovec 1992). Advances in evolutionary biology have also occurred due to the vast amount of genetic homology such as those found in homeotic genes throughout the animal kingdom (Levine et al 1984; McGinnis et al 1984).

Today, cell ablation, mosaic analysis, and transgenic *Drosophila* construction permit researchers to make rapid advances in fields of biology, such as the study of behavior, previously thought to be too complex for analysis using the *Drosophila* model. Now, many aspects of behavior, including learning and memory can be studied at a molecular genetic level in the fly (Greenspan 1995; Dubnau and Tully 1998). *Drosophila* genes influencing the biochemistry of learning and memory (e.g., *rutabaga*, *dunce*, and *Drosophila cyclic-AMP response element binding protein*) have already been characterized (Yin et al. 1994; Yin et al. 1995).
The products of these genes are involved in the cyclic adenosine monophosphate (cAMP) pathway and are crucial for short and long-term memory in *Drosophila*. Other genes, such as *linotte* and *mushroom body defect*, play significant roles in the development of brain structures called mushroom bodies (MBs), described below, which are necessary for learning and memory (Prokop and Technau 1994; Simon et al. 1998; Heisenberg 1998; de Belle and Kanzaki 1999).

*Drosophila* Mushroom Bodies

*Drosophila*, with its relatively 'small brain' and complex, yet well-characterized stereotypic behavior, is particularly suited for the study of behavior and development. Compared to other organisms, *Drosophila* is easy to manipulate both physically and genetically. The *Drosophila* brain contains approximately 100-150 thousand neurons, whereas higher mammals such as primates possess 10-50 billion neurons in the brain (Strausfeld 1976; Delcomyn 1998). The *Drosophila* neuropil is organized into well-defined modular substructures such as the MBs, antennal lobes, central complex, and optic lobes. The MBs are found in nearly every insect species and have been described in annelids and all arthropod groups other than crustaceans (Strausfeld 1998). Studies have shown the MBs of flies, bees, and locusts are required for such behaviors as olfactory learning and memory, walking, and courtship (de Belle and Heisenberg 1994; de Belle and Kanzaki 1999; O'Dell et al. 1995; Erber 1980; Stopfer et al. 1997). The studies described herein began as an investigation into the embryonic development of the *Drosophila* MBs.
Until very recently, little was known about the origin, development, and substructural organization of the MBs. It is now known that the MBs are developmentally continuous structures of embryonic origin (Tettamanti et al. 1997). The MBs arise from 4 MB neuroblasts (MBNbs) of embryonic origin, with each Nb contributing equally to the entire adult MB structure (Ito et al. 1997; Armstrong et al. 1998). It has also been shown using mosaic analysis with a repressible cell marker that each Nb is capable of generating three distinct types of neurons in the MBs based upon temporally-differing axonal projection patterns corresponding to the embryonic, larval, and pupal stages of organismal development (Lee et al. 1999). The result of this temporal switching is a subdivision of the adult MB structure into 5 MB lobes: the α and β lobes, the α' and β' lobes, and the γ lobe (see figure 1). These lobes correspond to the axonal projections arising from neurons with a particular temporal birth. Neurons born during embryonic development project their axons into the γ lobe, larval-born neurons project into the α' and β' lobes, and neurons born after pupation project into the α and β lobes (Lee et al. 1999).

While much has been elucidated concerning the development of the MBs, what remains unresolved is whether genetic or functional subdivision (i.e., substructural specialization) exists among the different MB lobes. The GAL4 enhancer detector system has proved quite useful in addressing part of this question. The GAL4 system is a method in which genetic enhancer expression patterns can be visualized, revealing the enhancer's normal regulatory properties.
Figure 1. Schematic representation of the *Drosophila* adult mushroom body structure. Mushroom body lobes are shown: $\alpha$, $\beta$, $\alpha'$, $\beta'$, and $\gamma$. (MBC) mushroom body cells, or Kenyon cell bodies. (C) Calyx. (ACT) antennocerebral tract. The antennal lobe (AL) and antennal nerve (AN) are not part of the mushroom bodies. Armstrong et al. 1998.
(Brand and Perrimon 1993). This system was invaluable in demonstrating previously unknown gene expression patterns in the MBs. Approximately twenty characterized MB GAL4 enhancer lines show differing patterns of expression within the adult MB substructure (Yang et al. 1995; Armstrong et al. 1998). Several well-studied genes such as rutabaga, dunce, and the catalytic subunit of protein kinase A are also known to have varying patterns of expression in the adult MBs (Han et al. 1992; Nighorn et al. 1991; Skoulakis et al. 1993). These studies suggest it is possible there might be not only functional subdivision but also subdivision of genetic control over the development of the MBs.

*mushroom body miniature B*

Genetic dissection (Benzer 1973) is one method to determine how MB architecture is controlled with respect to different substructures. Once a mutation affecting the structure is obtained then the extent of the defects can be determined through comparisons to a wild-type control strain. This would bring us closer to understanding the functional role or roles of that gene. The mutation selected for this study, *mushroom body miniature B* (*mbmB*), was originally isolated during an EMS mutagenesis screen for brain structural mutations in hopes of isolating genes to study behavior (Heisenberg and Bohl 1979; Heisenberg et al. 1985). *mbmB* was then isolated and placed into the genetic background of the control strain using standard genetic crosses (Heisenberg et al. 1985). *mbmB* was mapped by recombination to region 2-31 (de Belle and Heisenberg 1996).
mbmB was chosen because of its consistent MB phenotype. Unlike other MB mutants, mbmB exhibits high expressivity and penetrance with regard to the MBs (de Belle and Heisenberg 1996). Also, unlike other brain structural mutants, the defects in the brains of mbmB are restricted to the MBs (de Belle and Heisenberg 1996). However, loss of mbmB function results in several pleiotropic effects. During the original outcrossing, it was noted that females were sterile; however, it was not known whether this was physiological sterility or an inability to mate due to behavioral defects (de Belle and Heisenberg 1996). Additional phenotypes such as a reduction in viability and a delay in growth were also detected during preliminary investigations into the effects of mbmB on MB development. A systematic approach to the characterization of these pleiotropic effects was undertaken: the results of these studies are presented herein.

This project focused on the effects of mbmB on mushroom body development, and the characterization of defects in reproduction, viability, and growth. To resolve the developmental question concerning the role of mbmB during MB organogenesis, immunohistological techniques were used to observe the proliferation of MBNbs during embryogenesis. Timed developmental assays were performed to examine the decrease in viability and the delay in growth. Questions concerning female reproduction were addressed in two ways. Courtship behavior was examined to determine the extent to which behavioral defects might affect reproductive success, and histochemical techniques were used to determine the basis of physiological female sterility.
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CHAPTER 1

THE ROLE OF \textit{mbmB} ON EMBRYONIC MUSHROOM BODY DEVELOPMENT, GROWTH RATE, AND VIABILITY

Abstract

The \textit{Drosophila} mushroom bodies are paired neuropil centers of the brain. The mushroom bodies have been implicated in various aspects of behavior, including learning and memory, courtship, and motor behavior. The mushroom body primordium is of embryonic origin, showing developmental continuity. The \textit{mushroom body miniature B (mbmB)} mutation has defects in adult mushroom body anatomy and defects in olfactory memory. Furthermore, it has been observed that \textit{mbmB} mutants have a slower growth rate and reduced viability. In this study, we demonstrate, using the mushroom body cell marker \textit{dachshund}, that loss of \textit{mbmB} does not affect the early development of mushroom body neuroectoderm and neuroblasts. Additionally, I found that the observed delay in growth and reduced viability were not manifested during embryonic development.

Introduction

The \textit{Drosophila} mushroom bodies (MB) are brain structures that have been
the focus of numerous behavioral investigations (e.g., courtship, odor-associative learning and memory, walking, and other motor behaviors) (Heisenberg 1998; de Belle and Heisenberg 1994; O'Dell et al. 1995; Martin et al. 1999). Within these studies, correlations have been drawn between the MB structure and behavioral phenotypes. Recent studies have identified and characterized the developmental roles of genes affecting MB structure. For example, the gene *dachshund* (*dac*) is required for cell differentiation and is expressed in the embryonic MB neuroblasts (MBNbs) (Martini et al. 2000). Another gene, *castor*, is required for axonal pathfinding in the MB neurons (Preat et al. 2001). Only a few genes affecting MB substructures have been characterized. For example, the *alpha-lobes-absent* gene lacks both vertically projecting lobes. Further characterization of genes affecting MB substructures would aid in understanding the complete neurogenesis of the MB. This study addresses the role of the *Drosophila* gene, *mushroom body miniature B* (*mbmB*) in the embryonic development of the MBs and in embryonic viability and growth.

The MBs of *D. melanogaster* are bilaterally symmetrical, densely packed neuropil consisting of approximately 2500 intrinsic neurons, or Kenyon cells (KC) per hemisphere (Heisenberg 1980). The KCs of the adult MBs reside in the dorsal posterior region of the protocerebrum. The dendrites of the adult MBs form the calyces, which are cup-shaped structures located beneath the cell bodies. The axonal projections of the KCs form the peduncle, or main stalk, which projects anteroventrally and branches into the MB lobes. The peduncle branches into five
Figure 1. A. Schematic representation of the Drosophila adult mushroom body structure. Mushroom body lobes are shown: α, β, α', β', and γ. (MBC) mushroom body cells, or Kenyon cell bodies. (C) calyx. (ACT) antenocerebral tract. The antennal lobe (AL) and antennal nerve (AN) are not part of the mushroom bodies. B. MB Neuroblast (Nb) proliferation. Each Nb divides asymmetrically to regenerate the Nb and to give rise to a ganglion mother cell (GMC). The GMC then divides to give rise to two Kenyon cells (KCs).
axonal arrays, or lobes: the α, α', β, β', and the γ lobe (Figure 1A). The α and α' lobes project dorsally; the β, β', and γ lobes project medially (Crittenden et al. 1998).

It has been shown that the MBs arise from 4 Nbs of embryonic origin (Ito and Hotta 1992). These Nbs are part of the protocerebral neuroectoderm, and segregate as a group of cells expressing the eyeless and dachshund proteins (Younossi and Hartenstein 1996; Noveen et al. 2000). The MBNbs delaminate from this group of cells, now called the MB neuroectoderm, during stage 9 of embryonic development (this and all subsequent staging are those of Campos-Ortega and Hartenstein, 1985; Noveen et al. 2000). Using the enhancer detector system of Brand and Perrimon, several lines of flies were developed in which GAL4-directed β-galactosidase expression can be detected in the MBs (Brand and Perrimon 1993; Yang et al. 1995). The β-galactosidase expression in the MBs of these lines shows extensive proliferation of the MBNbs beginning at embryonic stage 13 (Yang et al. 1995; Tettamanti et al. 1997). This proliferation continues throughout the remainder of development (Yang et al. 1995; Tettamanti et al. 1997).

Each MBNb proliferates asymmetrically, regenerating the Nb while giving rise to a ganglion mother cell. Each ganglion mother cell divides, giving rise to two MB neurons, or KCs (Figure 1B). Axonal outgrowth from the KCs is first observed during embryonic stage 14 and a prominent KC fiber tract contacting the main protocerebral neuropil can be detected by late stage 17 (Tettamanti et al. 1997).
Mosaic analysis with a repressible cell marker has shown that the relationship between the KCs and axonal projections into specific lobes is determined by the developmental stage at which the KC is born. The axonal projections of the γ lobe are exclusively those of KCs born during embryonic development (Armstrong et al. 1998). At the time of larval hatching, the MBs consist of about 300 KCs, with the calyx just beginning to form (Technau and Heisenberg 1982; Tettamanti et al. 1997). During early larval development, axonal projections into the γ lobe continue to arise from KCs born during embryonic development (Lee et al. 1999). As larval development ensues, an increase in the number of KCs occurs and is accompanied by further axonal projections, generating MB structures similar in size to those of the adult (Tettamanti et al. 1997). By third-instar larval development, axonal projections begin to contribute to the α' and β' lobes (Lee et al. 1999). These α' and β' projections arise from KCs born during larval development (Lee et al. 1999). KCs that develop after pupariation contribute axonal projections only to the adult-specific α and β lobes (Lee et al. 1999).

During metamorphosis nearly all neurons of the CNS undergo axonal degeneration and subsequent regeneration of their processes. The MB lobes are unique in that they experience developmental continuity; the α' and β' lobes are preserved during metamorphosis (Technau and Heisenberg 1982; Lee et al. 1999). However, the γ lobe undergoes only limited degeneration of the axons to the point of the peduncle and is subsequently re-grown. This degree of developmental
continuity allows for a unique investigative opportunity. Because the MBs are preserved during metamorphosis, defects attributed to genes functioning during embryonic and larval development can be observed in the adult.

There are genes required throughout development to ensure proper formation of the MB structure. For example, the MBs depend on enoki, a histone acetyltransferase required for neuroblast proliferation, throughout development (Scott et al. 2001). Some genes, such as the transcription factor castor, are involved only in postembryonic processes; others are necessary for MB organogenesis during two stages of development (Hitier 2001). Such is the case with fasciclin II, an adhesion molecule, which serves MB axonal pathfinding functions during embryonic and larval development (Kurusu 2002). One can begin assessment of temporal roles for particular genes involved in MB development by first examining the mutant adult substructures. Defects in the substructures of adult MBs can then be correlated to specific stages of developmental. For example, a mutation in which the α' and β' lobes were either absent or abnormally formed can be investigated by examination of the events that occur during larval development.

In the mhmB mutant, the underlying basis for the reduction in MB structure is not known. Only the overall reduction in adult MB neuropil of mhmB mutants has been described. The MB calyces of mhmB are one-quarter to one-half the size of wild-type MB calyces, and both the peduncle and lobes have been described as "thin" (Figure 2) (de Belle, J. S., and Heisenberg, M. 1996). It is not known whether these defects are attributable to the loss of a specific temporal Nb
Figure 2. 7um serial section of wild-type (CS) adults, and mbmB mutant adults. 3-5 day old adult flies are fixed and embedded in paraffin. Reductions can be seen in the size of the calyx (arrows) in mbmB relative to CS.
lineage(s), an overall lack of MB KCs due to defects during Nb proliferation, or defects occurring in γ lobe regeneration during metamorphosis.

To begin to address some of these questions, I examined MB development during embryogenesis. I examined early \textit{dac} expression in the embryonic MB neuroectoderm to determine whether \textit{mbmB} has a role in MBNb formation. The MBNbs and KCs were observed following Nb delamination to assess whether \textit{mbmB} has a role during early MBNb proliferation. A reduction in the number of \textit{dac}-expressing cells during this period might be correlated to a reduction or absence of the γ lobe in the adult MB.

In working with \textit{mbmB}, it was noted that homozygous mutant progeny occurred at a much lower frequency than expected, and that these homozygotes eclosed in the stock culture later in time than did their balanced, heterozygous counterparts. These phenotypes were examined during this study to determine if a correlation exists between the MB defects and the uncharacterized phenotypes observed.

Within this study, 3 questions were addressed to assess the developmental roles of \textit{mbmB}: (1) Is early embryonic MB development affected by \textit{mbmB}? (2) What is the extent of the reduction in viability of \textit{mbmB} homozygotes, and is this reduction stage or sex specific? (3) To what extent is there a developmental delay, and is this delay stage specific?
Materials and Methods

Drosophila stocks

Wild-type stocks consisted of Canton Special (CS), mhmB In(2LR)SM5(Cy; al. l. cn. sp. ds) was constructed from a mutant strain originally obtained from Heisenberg (de Belle and Heisenberg 1996). w; L. P.CyO P[Kr-GFP^{50} w^-] was obtained from the Broadie lab (Lindsley and Zimm 1992; Castelli-Gair et al. 1994). Using standard genetic crosses, the mhmB mutation was placed in a w^- background and balanced with CyO P[Kr-GFP^{50} w^-] to allow for embryonic and larval genotype identification (see figure 3). All fly stocks were maintained on semi-defined medium originally described by Backhaus et al. (1984): 10g/L agar, 80g/L brewer’s yeast, 20g/L yeast extract, 20g/L peptone, 30g/L sucrose, 60g/L glucose, 0.5g/L MgSO_4 \cdot 6(H_2O), 0.5g/L CaCl_2 \cdot 2(H_2O), 0.6% propionic acid, 1.0% tegosept (10% p-hydroxybenzoic acid, methyl ester in 95% ethanol). During experiments, flies were reared at 24°C, 50% humidity, on a 12:12 light:dark cycle.

Analysis Of Embryonic Mushroom Body Phenotypes

Approximately 60 w^- mbmB/mbmB males and w^- mbmB/CyO, P[Kr-GFP^{50} w^-] females each were housed in ‘fly condos’, which were constructed from disposable 100mL plastic tri-pour beakers (VWR) with a hole cut in the top and plugged with a foam cap. Embryo collection plates consisting of a 60mm x 15mm polystyrene culture dish (Corning) containing grape juice agar: 30% v/v grape juice...
Figure 3. Cross performed to obtain theoretically equal ratios of \textit{mbmB} homozygotes (A) to balanced heterozygotes (B). A shows an \textit{mbmB} larva, B shows the balanced heterozygote expressing \textit{Kr-GFP} in cells associated with the gut (arrow).
concentrate (Welch's), 3% w/v agar (Sigma), 10% w/v sugar, and 1% v/v \( p \)-hydroxymethylbenzoate (Sigma) dissolved in 95% ethanol were fastened over the opening of the beaker. Freshly made yeast paste was placed on the surface of the agar. The same strategy was utilized for collection of wild-type embryos.

For embryo collections and fixations, the agar plates were removed after a period of 24 hours and replaced with a fresh plate. The embryos were loosened from the surface of the agar using a small nylon paintbrush and a solution of embryo wash (1.7M NaCl, 0.025% v/v Triton X-100), and rinsed into a nylon cell strainer (Falcon). For removal of the chorion, the cell strainer was immersed into a 50% solution of household bleach for 3 minutes, and rinsed with \( H_2O \) to remove the bleach. For fixation of the embryos, a 20 mL scintillation vial with a screw cap was filled with equal volumes of heptane and fixative (0.05M EGTA pH 7.0; phosphate buffered saline (PBS): 0.13 M NaCl, 7.0mM \( Na_2HPO_4 \) 2(\( H_2O \)), 3.0mM \( NaH_2PO_4 \) (\( H_2O \)), pH 7.0; 4.0% paraformaldehyde pH 7.0). The embryos were transferred from the cell strainer to the vial using a nylon paintbrush, the lid was secured to the vial, and the vial shaken vigorously for several seconds. The vial was then placed on a rocker for approximately 15 minutes at room temperature, or until the embryos changed from a white color to a cream color. The bottom, aqueous layer was removed using a Pasteur pipette. To remove the vitelline membrane from the embryos, an equal volume of methanol was added to vial, the lid placed securely on, and the vial shaken vigorously for 30 seconds. The vial was then placed on a rocker at room temperature for approximately 5 minutes. The embryos
were allowed to settle to the bottom. The upper, heptane layer was removed along with the vitelline membranes at the interface. The embryos were then rinsed 3 times with methanol to remove residual heptane and fixative. The embryos were then rinsed 3 times with 95% ethanol, transferred to a microcentrifuge tube, and stored at -20°C.

Fixed embryos were prepared for immunohistochemistry by removing the 95% ethanol and rinsing 3 times with PBT (PBS, 0.1% Tween-20), then rinsing 3 times with PBTS (PBS, 0.1% Tween-20, and 0.2% bovine serum albumin (Sigma) or Normal Goat Serum (Gibco)). Embryos were then incubated for 20 minutes in PBTS. The primary antibodies were diluted to the appropriate concentration with PBTS and added to the microcentrifuge tube with embryos. Mouse monoclonal anti-c/i/c antibody (generated by G. Rubin and obtained from the Developmental Studies Hybridoma Bank) was diluted 1:1000; mouse anti-GFP antibody (Jackson Immunolabs or Molecular Probes) was diluted 1:100. The embryos were either placed on a rocker for 4 hours at room temperature, or overnight at 4°C. After primary antibody incubation, the embryos were rinsed 3 times with PBT, once with PBT for 10 minutes at room temperature, and 3 times with PBTS for 5 minutes each at room temperature. For secondary antibody incubation, alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson Immuno-Research labs) was diluted 1:2000 in PBTS. The embryos were then placed on a rocker at room temperature for 4 hours. The embryos were then rinsed 3 times in PBT, then 3 times in PBTS (see above). To prepare for the alkaline phosphatase (AP) reaction,
the embryos were rinsed 3 times for ten minutes each at room temperature with AP staining buffer (0.1M NaCl, 0.05M MgCl₂, 0.1M Tris-HCL pH 9.5, 0.1% Tween-20). The staining buffer was removed and replaced with a 1mL solution of AP staining buffer containing 0.4% nitro-blue tetrazoleum (NBT) (Sigma) and 0.3% 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma). The embryos were then rocked at room temperature in the dark for a period ranging from 5 to 20 minutes, depending on the progression of the precipitation reaction as monitored under a dissecting microscope. To terminate the AP reaction, the embryos were rinsed 3 times with PBT, then dehydrated in a PBT:ethanol series of 20%, 40%, 60%, 80%, 100%, and a second 100% ethanol rinse. Embryos were cleared removing as much ethanol as possible and adding 500μL of methyl salicylate (Sigma). After the embryos settled to the bottom of the tube, the methyl salicylate was removed and replaced with another 500μL methyl salicylate.

For examination of the stained embryos, bridged microscope slides were prepared by adhering with Permount gel (Biomega Corporation) 2-18mm² glass coverslips 20mm apart on a standard microscope slide. The embryos were pipetted between the 2 coverslips and covered with a 22 x 40mm coverslip, which allowed the embryos to be moved during examination. The embryos were examined using Differential Interference Contrast (DIC) on a Zeiss Axioplan-2 microscope and photographed with Zeiss Axiovision software. Photographs were prepared using Adobe Photoshop.
**Viability Assay: Developmental Delay**

To assess the overall differences in viable adult progeny, $w': mbmB mbmB$ males were crossed to $w': mbmB CyO P[Kr-GFP]^{30} w^-'$ females (as $mbmB mbmB$ females are sterile). 20 virgin females and 20 males were placed in a vial containing Backhaus medium, under conditions stated above, for 24 hours. After 24 hours, the flies were transferred to a new egg-laying vial for 2 hours to minimize differences in developmental time. The original vial was discarded. The new vial was maintained throughout the experiment under environmental conditions described above. When adults began to eclose, the flies were collected every eight hours, and the sex and genotype of each fly was recorded. For controls, $CS \times CS$, and $CS \times CyO P[Kr-GFP]^{30} w^-'$ crosses and reciprocal crosses were carried out.

**Embryonic Lethality Developmental Delay Study**

To study the viability of $mbmB$ embryos, and to study the duration of embryonic development, $w: mbmB mbmB$ males and $w: mbmB CyO P[Kr-GFP]^{30} w^-'$ females were crossed. Matings were carried out in fly condos as described above. After a 22-hour acclimation period, the flies were allowed to oviposit on a fresh plate for the second and third hours of the light cycle to minimize differences due to time of egg laying. Embryos were obtained over a period of 4 days.

Beginning at 20 hours after egg laying, and repeated at 22, 24, and 26 hours, the plates were examined using the Zeiss Axioplan2, filter set 09 (450-490 nm excitation and emission at 515 nm). This enabled heterozygous larvae to be identified based on $Kr$-GFP expression. All larvae, presumably first instar, were
removed from the agar plate using a glass capillary tube. The number of larvae collected from each genotype was recorded and used to statistically analyze both embryonic lethality and embryonic developmental time delay.

Statistical Analyses

For viability, including differences attributed to gender, standard chi-square tests were performed at a .05 level of significance. For MB cell counts and developmental growth delays, two-way analysis of variance (ANOVA) was performed using genotype and developmental stage as variables and genotype and time as variables, respectively.

Results

Mushroom Body Neuroblast Proliferation Is Unaffected By mbmB During Early Embryonic Development

As discussed in the introduction, studies of the embryonic development of the Drosophila MBs revealed that the 4 MBNbs arise from the protocerebral neuroectoderm, prior to the delamination of the brain neuroblasts (Younossi-Hartenstein et al. 1996). Several genes, including eyeless, twin of eyeless, dachshund, and enoki are expressed in the embryonic neuroblasts and their progeny (Kurusu et al. 2000; Scott et al. 2001). One gene, dac, has expression that is limited to the MB neuroectoderm and to the MBNbs until after Nb delamination begins during stage 9 (figure 4A). And unlike other genes with expression in the MBs, dac is maintained in the MBNbs, ganglion mother cells, and KCs (as well as in other
Figure 4. A. Schematic (not to scale) of Dachshund expression patterns during extended germ band stage: (MBNBs) mushroom body neuroblasts, (Mbne) mushroom body neuroectoderm, (para Mbne) para-mushroom body neuroectoderm, (OL) olfactory lobe, (ED) eye disc primordia (from Noveen et. al. 2000). B. Stage 10 embryo (20X magnification, DIC) showing DAC expression, stained with alkaline phosphatase, in the MBNBs (arrow 1) and in the Mbne (arrow 2). K\textit{r}-GFP expression, also stained with AP can be seen in two cells located on either side of the ventral midline, near the stomodeal plate (arrow 3).
embryonic Nbs) throughout embryogenesis (Noveen et al. 2000). Thus, dac can be used as a cell marker to discern the MB neuroectoderm and MBNbs at the end of stage 9, just prior to Nb delamination. Stage 10 was chosen because dac expression could be examined prior to the rapid proliferation of MBNbs that begins shortly after Nb delamination. Following invagination of the MBNbs from the neuroectoderm, the Nbs are positioned on the dorsal surface of both brain hemispheres, again making them easily discernable (Younossi-Hartenstein et al. 1996). Following stage 10, dac expression is expanded to include other Nb lineages as well as other cell types. By stage 12-13, rapid proliferation of the Nbs is well underway, thereby making it difficult to distinguish between MBNbs, KCs, and other dac-expressing cells. Therefore, this period of development was chosen as the final stages during which the MBNbs and KCs could be reliably compared in mbmB and CS.

To increase the number of mutant embryos collected for examination, w; mbmB/mbmB males were crossed to w; mbmB/CyO P[Kr-GFP°] females. This allowed for not only a 1:1 ratio in expected genotypes, but also for easy identification of the heterozygous embryos (figure 4B). Using antibody detection of GFP, heterozygous embryos could be distinguished from homozygous mutants based on the Kr pattern of GFP expression. During stage 9 of embryogenesis, Kr-GFP expression can be detected in the posterior ectoderm and in the amnioserosa (Casso et al. 1999). I found that dac expression in the MB neuroectoderm and MBNbs of mutant embryos was not different from CS (figure 5A-D). In later stage
Figure 5. (A-D) Stage 10 embryos showing DAC expression in the MB neuroblasts and MB neuroectoderm, CS lateral (A) and dorsal (B) views, and mbmB lateral (C) and dorsal (D) views. (E-H) Stage 11-12 embryos showing DAC expression in MBNbs, and para-MB neuroectoderm in CS (E, F) and mbmB (G, H).
embryos examined, the MBNbs and KCs of mutant embryos also showed no difference in dac expression from CS (figure 5E-H). These results suggest that MBNb proliferation during embryogenesis is unaffected by the lack of mbmB during early to mid-embryogenesis. Counts of dac-expressing cells were performed during two different stages of embryonic development. A two-way ANOVA revealed a significant effect of developmental stage ($F_{1,56} = 1022.49, P < 0.0001$), but no influence of genotype or interaction between genotype and stage observed (Table I).

![Table 1. Mean number of DAC positive cells](image)

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<th>Stage 10</th>
<th>Stage 12</th>
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<tr>
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<td>51.13</td>
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<tr>
<td>mnmB</td>
<td>12.78</td>
<td>52.88</td>
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The mushroom body miniature B Mutation Causes a Reduction in Viability Associated with Early Larval Development in Addition to an Overall Delay in Growth.

It had been noted that the mbmB mutants exhibited a decrease in viability and a delay in developmental time. To further investigate this observation, I examined both relative viability and relative growth time of mbmB mutants. $w^+$: mbmB/mbmB males were crossed to $w^+$: mbmB/ CyO P[Kr-GFP10] females to
equalize the ratio of homozygous mutants to balanced heterozygotes. To examine the relative viability of mbmB homozygous progeny. 1686 adult flies were scored from the cross described above. 456 mutant adults were recovered compared to 1230 heterozygous adults (Table 2). Chi-square analysis revealed a highly significant difference between the numbers of homozygous and heterozygous adults recovered ($\chi^2_{0.05, 1} = 356.98, P < 0.0001, n = 1686$). A comparison of mbmB males and females recovered revealed no significant differences due to gender ($\chi^2_{0.05, 1} = 2.68, P = 0.10162, n = 1686$). I also measured the time from egg lay to eclosion (Figure 6). ANOVA revealed significant influences of genotype ($F_{13,51} = 4.80, P = 0.0051$), eclosion time ($F_{4,51} = 5.11, P = 0.0015$), and interaction between the two ($F_{12,51} = 3.29, P = 0.0014$). Effects of gender were not significant (figure 6).

Table 2. Reduction In Viable mbmB Progeny

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<tr>
<td>mbmB mbmB</td>
<td>456</td>
<td>843</td>
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<tr>
<td>mbmB CyO P[Kr-GFP]</td>
<td>1230</td>
<td>843</td>
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Next, to determine if the reduction in viability or developmental delay occurs during embryonic development, homozygous mutant and heterozygous embryos were examined for lethality and delayed growth. Mutant homozygous
Figure 6. Eclosion comparisons for males (A) and females (B). 
*mbmB* mutants exhibit a developmental delay compared to wild type. This delay was reflected in both males and females when compared to the corresponding wild-type animal.
males were again crossed to balanced heterozygous females. The females were permitted to lay eggs for 2 hours. After a 20-hour incubation period, newly hatched first-instar larvae were collected and the genotype of each animal was recorded based on larval Kr-GFP expression. Kr-GFP can be detected in the Bolwig's organ and in nerve cells associated with the gut (figure 3A) (Casso et al. 1999: Hoshizaki 1994). There was no significant difference between the number of heterozygous and homozygous mutant larvae recovered, indicating no significant reduction in viability during embryogenesis ($\chi^2_{0.05, 1} = 1.66; P=0.198, n=475$). 223 mutant larvae were recovered compared to 252 balanced heterozygotes (Table 3). The vast majority of first instar-larvae were collected within a 4-hour time frame thereby suggesting no drastic difference in developmental time between homozygotes and heterozygotes.

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<tr>
<td>$mhmB mhmB$</td>
<td>223</td>
<td>237.5</td>
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<tr>
<td>$mhmB CyO P[Kr-GFP]$</td>
<td>252</td>
<td>237.5</td>
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Discussion

Development of the central nervous system in Drosophila takes places in a very defined spatial and temporal manner (Truman and Bate 1988). Specifically,
the Nbs of the CNS during larval development are composed of three distinct populations: The optic lobe Nbs, the thoracic Nbs, and the MBNbs. All three populations begin proliferation during embryogenesis; however, the optic lobe Nbs and thoracic Nbs become quiescent just prior to larval hatching (Hartenstein and Campos-Ortega 1986; Green 1993). As discussed earlier, the MBNbs continue proliferation throughout development (Ito and Hotta 1992). There exists a brief period during the first few hours of first-instar larval development when the only proliferating Nbs are the MBNbs and one lateral neuroblast which gives rise to antennal lobe local interneurons and projection interneurons (Ito and Hotta 1992; Stocker et al. 1997). This finding is the basis for chemical ablation studies, which demonstrated the importance of the MBs in associative odor learning (de Belle and Heisenberg 1994). If mhmB functions during this period of development and is necessary for the maintenance or survival of these proliferating cells, a reduction in the adult MB calyx volume would be observed. Additionally, α' and β' lobe axonal projections should be reduced or absent. Unfortunately, an appropriate technique for marking larval MBNbs and KCs was not developed during this study. However, the examination of the MB neuroectoderm, Nbs. and KCs in mhmB mutants revealed no obvious differences in dac expression during early embryogenesis. These data suggest mhmB is not required for MBNb specification or for Nb proliferation during early embryogenesis. This does not rule out a requirement for mhmB during later developmental periods. An appropriate cell marking technique for the larval MBs is needed to properly address this issue.
The viability and developmental delay studies suggested that *mbmB* is not necessary for embryonic development. However, during the study I noticed that *mbmB* homozygous first-instar larvae were often lethargic and many were dead (Figure 7). In a preliminary study of larval lethality, the number of dead larvae was close to the overall reduction in *mbmB* adults. Furthermore, the lethargic larvae observed might explain the delay in overall growth of *mbmB* mutants. These observations suggest that *mbmB* may be crucial to larval development.

Further analyses of the role of *mbmB* during larval development should be carried out. This would entail a detailed analysis of the larval lethal phase in addition to an analysis of the length of time for each larval molt. This will allow for both an analysis of reduction in viability, and for delays in growth or molting. In addition, MB cell counts during the larval instars should be carried out to determine a possible role for *mbmB* during larval MB development.
Figure 7. CS (A) and mbmB (B) first-instar larvae. Photographed using Zeiss Axiovision 2, Darkfield image.
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metamorphosis of the corpora pedunculata in Drosophila melanogaster.”

mushroom bodies, brain centres for associative learning and memory.” Dev

in the central nervous system of Drosophila melanogaster.” Dev Biol


CHAPTER 2

*mbmB* IS NECESSARY FOR PROPER FEMALE COURTSHIP BEHAVIOR AND OOCYTE DEVELOPMENT IN *DROSOPHILA*

Abstract

Mutations in the *Drosophila* gene *mushroom body miniature B* cause an array of pleiotropic effects such as a reduction in the mushroom body adult brain neuropil, defects in behaviors such as memory and courtship, reductions in viability, a delay in growth rate, and female sterility. In this study, we used a courtship behavioral assay, in addition to histochemical techniques, in order to determine the underlying cause of *mbmB* female sterility. We have shown that *mbmB* adversely affects female courtship behavior. In courtship trials involving *mbmB* females, time to onset of wild-type courting behaviors was increased. The percentage of time that a males displayed fixed action patterns of courtship was decreased in response to the mutant female.

While courtship behavior was adversely affected, it was not abolished in *mbmB* mutants, suggesting a physiological basis for sterility. In histochemical studies, we showed that *mbmB* mutant oocytes exhibited aberrant follicle cell
migration. Additionally, mutant oocytes underwent early developmental arrest and subsequent degeneration.

Introduction

Pleiotropies of 'behavioral' genes seem to be the general rule rather than the exception, because genes involved in any number of biological processes may also have an influence on behavior of the organism (Hall 1994b). Researchers studying behavior have often ignored what they perceive to be 'secondary' characteristics of their 'behavioral' gene. For example, *Drosophila* researchers often refer to the cyclic adenosine monophosphate (cAMP) pathway as the 'learning and memory' pathway, and to mutations in this pathway as 'learning and memory' mutations (Lee and O'Dowd 2000; Kim and Wu 1996; Renger et al. 2000). The cAMP pathway actually controls myriad cell functions such as metabolism, the cell cycle, and gene expression (Beavo 1995; Conti et al. 1995; Dolmetsch et al. 1997). Unfortunately, when many of these defects are first isolated as learning mutants, the nature and extent of other defects may not be properly characterized for years. The *Drosophila* genes *rutabaga* (*rut*) and *dunce* (*dnc*) are prime examples of this problem. Both genes were initially isolated as learning and memory mutants, and have defects in cAMP metabolism (for review, see Tully 1991; Davis and Dauwalder 1991). However, the cAMP pathway is responsible for numerous functions within the organism. Although still commonly referred to as learning and memory mutants, the organismal-wide impacts of these genes are now being elucidated (Lee and O'Dowd 2000; Delgado et al. 1998). Many genes affecting the
cAMP pathway also affect larval growth and molting, as well as many aspects of oogenesis (Kiger et al. 1999; Lane and Kalderon 1993; Venkatesh et al. 2001).

Interestingly, the mushroom body miniature B mutation, originally described as a learning and memory mutant, also affects female sterility (Heisenberg et al. 1985; de Belle and Heisenberg 1986). The focus of this study will serve to characterize the nature of female sterility observed in mbmB.

*Drosophila* female sterility has been extensively studied, yet much remains to be learned about the many different factors required for proper oogenesis including the influence of nutrition, cell-cycle regulation, cell migration, biochemical signaling, and external factors from the male fly (Drummond-Barbosa and Spradling 2001; Calvi et al. 1998; de Cuevas et al. 1997; Tram and Wolfner 1999). Females homozygous for mushroom body miniature B (mbmB) produce zero progeny. Several possibilities exist to explain this phenomenon. Loss of the mbmB gene may render the female physiologically sterile or the mutation may cause a behavioral defect, interfering with mating. It is also possible that the issue of apparent female sterility may, in fact, be male sterility. I have tested whether male or female sterility exists in addition to testing whether sterility is due to a behavioral defect or physiological defect.

In addressing female physiological sterility, there exists the possibility that the mutation could be a maternal effect lethal. In this type of mutation, the maternal gene products required for survival of the embryo after fertilization are lacking in the mother. Such is the case with the bicoid gene controlling anterior development.
of the embryo (Frigerio et al. 1986; Berleth et al. 1988). In females homozygous for the \textit{bicoid} mutation, embryos fail to develop properly and lack anterior structures (Driever and Nusslein-Volhard 1988). Females mutant for a maternal effect gene produce and oviposit morphologically normal eggs, which fail to develop correctly. Female sterile mutants rarely, if ever, oviposit. The latter was found to be the case in \textit{mbmB} females, with oviposition attempts detected in less than 1% of flies examined (data not shown). In the rare event of oviposition, the eggs were smaller in size, lacked dorsal appendages, and appeared to lack a protective membrane (Figure 1). Additionally, the eggs fell apart when manipulated in any way. Thus, maternal effect lethality was not considered further. The most plausible explanations now being that of physiological sterility or behavioral sterility. still left many processes of both oogenesis and courtship behavior to examine.

The \textit{Drosophila} ovaries are paired structures residing in the abdomen. They occupy between 20\% and 50\% of the total abdomen volume in wild-type flies. Each ovary is composed of 16-20 individuals units, called ovarioles. The developing egg chambers are located within the ovariole in a sequential manner: the most mature eggs reside in the posterior tip, and progressively more immature eggs reside at the anterior tip (King 1970). This makes it rather convenient for developmental studies (Figure 2).

The developing egg chambers are composed of two distinct cell types: the germ-line derived nurse cells, germ-line derived oocyte, and the somatically derived follicle cells. The process of oocyte production can be divided into 14
Figure 1. Oviposited eggs from wild-type (CS) females (A), and mbmB mutant females (B). Note the dorsal appendage seen in wild type (A. arrow), and the aberrant, rudimentary appendage formed in the mbmB mutant egg (B. arrow). Darkfield microscopy.
Figure 2. Schematic of the Drosophila ovaries (Sullivan et al. 2000).
beginning with one or two germ-line stem cells at the anterior tip of the ovariole termed the germarium (Figure 3) (King 1970; Schupbach et al. 1978). The stem cell undergoes asymmetric division, giving rise to a cystoblast. The cystoblast then undergoes 4 successive rounds of mitosis to give rise to 16 cystocytes (King 1970; Mahowald and Kambysellis 1980). As the cystocyte undergoes mitosis, the daughter cells all remain interconnected through specialized furrows called ring canals (Mahowald and Strassheim 1970). During the 16-cell cyst stage, one of the cystocytes from the first mitotic division forms a microtubule organizing center (MTOC) by a process not well understood. The MTOC forms a network of connections extending into the remaining 15 cells. The cystocyte containing the MTOC becomes the oocyte, most likely because it is one of two cells containing the greatest number of ring canals (Mahowald and Strassheim 1970; Theurkauf et al. 1992). The remaining 15 cystocytes differentiate into nurse cells for the oocyte, becoming polytenized in the process (Mahowald 1970; Ribbert 1979; Redfern 1981). The cystoblast is enveloped by follicle cells and is now termed a stage-one egg chamber. As egg chamber development continues, the follicle cells undergo up to five rounds of mitosis in addition to becoming polytenized by undergoing up to five rounds of endoreplication (Mahowald et al. 1979; Hammond and Laird 1985; Mulligan and Rasch 1985; Bohrmann et al. 1986).

An important milestone during oogenesis occurs around stage 8. Transport of yolk proteins from the maternal fat body into the oocyte visibly distinguishes the oocyte from the nurse cells (DiMario and Mahowald 1986). By the end of stage 8,
Figure 3. Schematic of one *Drosophila* ovariole, showing relevant structures examined in this study (Spradling, 1993).
the follicle cells begin migration over the oocyte. It is also during stage 8 that
defective egg chambers degenerate in wild-type females (Giorgi and Deri 1976;
Buszczak et al. 2002; Nezis et al. 2000). By a mechanism that is not well
understood, oocytes defective in any number of developmental processes undergo
apoptosis during stage 8. For example, the *Drosophila lethal (2) giant larvae* gene
causes arrest and subsequent apoptosis during stage 8-9 due, in part, to
disorganization of the follicle cells (De Lorenzo et al. 1999).

Oocytes passing the stage 8 `checkpoint` continue along their normal
developmental trajectory, with follicle cells continuing their migration over the
oocyte. This is followed by a specialized set of follicle border cells migrating
between the oocyte and the nurse cells. By stage 11. the `dumping` of the
cytoplasmic contents of the nurse cells begins with the transport of mRNA,
ribosomes, and mitochondria into the oocyte. As the remainder of the nurse cells`
cytoplasmic contents are transferred to the oocyte, the nurse cell nuclear
membranes become permeabilized and the nurse cell cluster begins to degenerate
(Cooley et al. 1992). After cytoplasmic transport, the nurse cells undergo apoptosis
(Cavaliere et al. 1998). Once cytoplasmic transport is complete, the follicle cells
begin secretion of the chorion, or egg shell. In addition, accessory structures such
as the dorsal appendage are formed. Finally, the mature egg is transferred through
the oviduct to the uterus where it is fertilized and oviposited.

Several phenotypic classes of female sterile mutants have been described.
Agametic, or tumorous, mutations cause very early arrest of the oocyte; mature egg
chambers are rarely, if ever, detected. The ovaries of these females are also extremely small and, in some cases, not detected at all (King and Storto 1988). For example, the ovaries of ovarian tumor mutants contain a disorganized mass of cells and recognizable egg chambers are generally absent (Steinhauer et al. 1989). Early arrest and degenerative mutants make up another class of female sterile phenotypes. The egg chambers of these mutants generally degenerate around mid-stage in oogenesis, very likely failing the ‘checkpoint’ described earlier. The ovaries of these mutants are roughly half the size of wild type. The egg chambers of early arrest or degenerative mutants appear normal usually until stages 7 to 9, during which time the egg chamber undergoes apoptosis or necrosis and begins degeneration. The similar size of the developing egg chambers allows the ovarioles of these mutants to be described by one mutant gene name: heads on a string (Cooley et al. 1988) (Dorn et al. 1986). Another phenotypic class is comprised of mutants where nurse cell cytoplasmic transport is disrupted. These egg chambers generally develop part of the chorion, but usually lack accessory structures due to disruption by the persistent nurse cell cluster that remains attached to the egg (Schupbach and Weischaus 1991) (Cooley et al. 1992). More recent studies such as those performed on follicle cell migrations, intercellular bridges/ring canals, and egg shell, or chorion synthesis focused on more specific developmental processes during oogenesis, allowing for the expansion of phenotypic classifications (Lane and Kalderon 1995; Sokol and Cooley 1999; Peri and Roth 2000). Oogenesis mutants can now be described more accurately based on specific defects.
In this study, I investigated different aspects of oogenesis to determine the nature of sterility in \textit{mbmB} mutants. Initially, the structure of the entire ovary was examined for relative size, presence or absence of ovaries, and stages of oogenesis missing. I also examined ovarioles and individual egg chambers for defects or alterations in the number of nurse cell and follicle cell nuclei and intercellular bridges/ring canals. The results of this detailed analysis are described herein.

To determine if a behavioral component to \textit{mbmB} female sterility exists, courtship trials were performed. Defects in courtship behavior might cause the female to appear sterile. This is similar to defects seen when harsh environmental conditions exist (Drummond-Barbosa and Spradling 2001). Failure of the female to mate due either to a defect in courtship behavior in either sex, or absence of males can result in oocyte ‘backlog’. This backlog of egg chambers generally leads to degeneration of the late-stage egg chambers.

Courtship for \textit{Drosophila} consists of a series of fixed action patterns described primarily in males (Figure 4) (for review, see Hall 1994a and Greenspan 1995). Male courtship usually begins with the recognition of the female. The first recognized display is orienting, where the male places itself in close proximity to the female target. The male will then begin tapping its foreleg on the female’s abdomen. Next, the male will vibrate its wing closest to the head of the target female. The frequency of this wing vibration is known as the courtship song, which is species specific (Shorey 1962). Generally, the male will perform these first three behaviors in quick succession. After displaying the courtship song, the male will
Figure 4. Species-specific fixed action patterns of *Drosophila* courtship. The courtship "singing", or wing vibration shown in 3 represents the onset of courtship for all trials conducted in this study (Greenspan and Ferveur 2000).
extend its proboscis and lick the genitalia of the female, followed by curling its abdomen towards the female genitalia. Finally, if successful, the male will then copulate with the female. The role of the female in the courtship process appears to lie in whether to accept or reject the advances by the courting male. The target female may slow movements such as walking or assume a receptive posture in response to an acceptable male. Conversely, the female may show displays of rejection by kicking towards the male, flicking its wings, curling its abdomen, or extruding its ovipositor. Unfortunately, none of these behaviors have been well studied (Connolly and Cook 1973; Nakano et al. 2001). Defects in female courtship behavior can be detected either by direct assessment of female behavior, or by assessment of male courtship behavior in response to test females. This study uses male courtship fixed action patterns to assess courtship in behavior in both male and female mhmB mutants.

Materials and Methods

Drosophila Stocks For Courtship

Wild-type stocks consisted of Canton Special (CS). mhmB/In(2LR)SM5(C); al. l. cn. sp. ds) were constructed from a mutant strain initially obtained from Heisenberg (de Belle and Heisenberg 1996). Through extensive outcrossing and recombination with CS, the mhmB SM5 strain is expected to be approximately 99% isogenic to the CS control strain (de Belle and Heisenberg 1996). Both mhmB and
CS male flies were isolated 0-6 hours after eclosion and placed in individual vials containing 1 mL of Backhaus medium (Backhaus et al. 1984, see Chapter 1). Wild-type CS females and females homozygous mutant for mhbB were isolated 0-6 hours after eclosion. Approximately 20 females were placed in vials containing 10 mL Backhaus medium. All flies were kept at 24°C, 50% humidity, on a 12:12 light:dark cycle for 3-5 days.

Courtship Trials

Courtship trials were conducted 3-4 hours after the onset of the light cycle to ensure active courtship behavior. Flies were placed into a courtship wheel (Figure 5). Females underwent cold anesthesia for placement into the mating chambers and males were directly aspirated into the chambers. When placed into the closed position, the courtship wheel contains 4 individual chambers. The individual chamber pairings were as follows: (1) CS males and CS females, (2) CS males and mhbB females, (3) mhbB males and CS females, (4) mhbB males and mhbB females. The courtship wheel remained in the open position while the flies were allowed to acclimate to the wheel for approximately 15-20 minutes. The courtship trials were all conducted under dark red light at 24°C and 50% humidity. At the start of each trial, the courtship wheels were placed into the closed position and the trials were recorded by CCD digital video cameras (World Precision Instruments) through Zoom-7000 macro lenses (Navitar) mounted approximately 33 cm from the courtship wheel. Twenty-six trials were recorded for a period of 10
Figure 5. Courtship wheel. Males and females, first separated, are permitted to acclimate (A). When a courtship trial begins, the pins (arrow) are brought together, which lines up the chambers and brings males and females into contact.
Courtship Scoring and Statistical Analyses

Two parameters of courtship behavior were scored. The first, courtship latency (L), was defined in this study as the time elapsed between initiation of the courtship trial and the first display of wing vibration by the male. The wing vibration was chosen in order to eliminate the scoring of non-courtship-specific behavior expected to occur in the small confines of the courtship chamber. The second measure of courtship behavior, courtship index (Cl), was defined in this study as the proportion (% x 100) of the observation period spent by the male displaying any of the courtship behaviors from wing vibration to copulation (Siegel and Hall 1979). Data were collected by scoring the videotaped trials for Cl and initiation of wing vibration. Each trial was scored without prior knowledge of pairing. Statistical analyses were performed on all measured behaviors using two-way analyses of variance (ANOVA) to test for significant influences of genotype. Comparisons between the means were examined using the Student-Newman-Keuls test (Zar 1974).

Drosophila Stocks For Ovary Studies

Using standard genetic crosses, the mhmB mutation was placed in a w− background and balanced with Cy O P[Kr-GFP^{20} w−]. CS and mhmB homozygous females were collected 3-5 days post eclosion. To stimulate egg production and to
avoid defects attributed to environmental or nutritional defects, the females were and placed into vials containing Backhaus medium, fresh yeast paste, and several 3-5 day old CS males. The vials were kept at 24°C and 50% humidity under 12:12 light:dark cycles for 48 hours.

*Ovary Dissections*

Ovary dissections were performed with the use of a dissecting microscope. Flies were cold anesthetized and placed onto a depression slide containing approximately 200 µL of Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 10 mM HEPES, pH 6.9, filter sterilized). Flies were held with the ventral side of the abdomen exposed and dissected using Dumont #5 forceps. One set of forceps was used to remove the posterior tip of the abdomen and the genitalia. The abdomen was then gently squeezed, exposing the oviduct and posterior portion of the ovaries. The oviduct was then grasped with the forceps and removed from the abdomen along with the ovaries. The oviduct and any accessory tissue still connected to the ovaries were removed with the forceps. Depending upon the experiment, the ovaries were either left intact, or the individual ovarioles were gently separated. The ovaries/ovarioles were then placed into a microcentrifuge tube containing 1.0 mL of Ringer's solution and kept on ice.

*Histochemistry*

Ovaries stained with either DAPI (4', 6-diamidino-2-phenylindole) or
rhodamine-conjugated phalloidin. were first fixed by removing the Ringer's solution and replacing it with 100µL devitellinizing buffer (1/6 buffer B: 100mM KH$_2$HPO$_4$, pH 6.8: 1/6 formaldehyde 36%: 2/3 H$_2$O) and 600µL heptane. The tubes were vortexed for 30 seconds and placed on a rotator platform for 10 minutes at room temperature. The tubes were then pulse-centrifuged for 10 seconds, the devitellinizing buffer removed and rinsed twice with PBS. For DAPI staining, 1µg/mL in PBS was added for 5 minutes, removed, and the ovaries were rinsed once in PBS. The ovaries were mounted using Fluoromont gel mounting solution (Biomeda Corporation) and DAPI staining was detected using a Zeiss Axioplan 2, filter set 05 (395-440nm excitation, 470nm emission). Ovaries stained with rhodamine-conjugated phalloidin were dissected and fixed as described above. Rhodamine-conjugated phalloidin was added to the 1mL tube at a final concentration of .15µM. Rhodamine-stained ovaries were examined using the Zeiss Axioplan 2, filter set 15 (546nm excitation, 590nm emission).

Ovaries stained with propidium iodide and YO-PRO-1 (Molecular probes. Vybrant® kit #4), were dissected as described above. The ovaries were rinsed once with Ringer's solution. Propidium iodide and YO-PRO-1 (.01µM and 1µg/mL, in H$_2$O and DMSO, respectively) were then added to the tube. The cells were incubated for 20 minutes on ice, rinsed once in PBS, then mounted with Fluoromont gel mounting solution and examined immediately using a Zeiss Axioplan 2, filter set 09 (450-490nm excitation, 515nm emission). All images were photographed using Zeiss Axiovision and formatted using Adobe Photoshop.

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Results

Female Courtship Behavior is Abnormal in mbmB Mutants

The courtship behavior was observed in 26 trials each of CS paired with CS, mbmB males paired with CS females, mbmB females paired with CS males, and mbmB paired with mbmB. Both the courtship index (Cl) and courtship latency (L) were adversely affected in trials involving mhmB females. The Cl was decreased in trials involving mhmB females (Figure 6). A two-way ANOVA revealed a significant difference from wild type Cl in trials where CS males were paired with mhmB females and mhmB males were paired with mhmB females, although there was no significant difference between them ($F_{3, 10} = 23.30; P = 0.0001$).

When courtship latency was assessed for the trials indicated above, similar results were obtained. However, because L is a measure of time elapsed prior to the initiation of courtship, L is expected to increase. Again, in trials involving mhmB females, two-way ANOVA revealed a significant increase in latency ($F_{3, 110} = 6.68; P = 0.0004$) (Figure 7).

mushroom body miniature B Causes Early Arrest and Subsequent Degeneration of Drosophila Oocytes

DAPI staining was performed to better visualize the ovaries of both CS and mhmB flies. This allowed for better microscopic examination of the ovaries as a whole as well as the nuclei of individual follicle and nurse cells. The ovaries of mhmB females were found extremely reduced in size usually occupying less than
Figure 6. Male display of courtship behavior is significantly decreased in trials involving \textit{mbmB} females (see text for details).
Figure 7. Male courtship latency is significantly increased in trials involving *mbmB* females (see text for details).
half the normal volume occupied by wild type ovaries (Figure 8A-B). Ovaries, however, were present in nearly all mutant flies examined; less than 2% of the 186 mhmB flies were lacking one or both ovaries. In CS flies examined, absence of one or both ovaries was not observed (Figure 8C).

Examination of individual ovarioles in mhmB mutants revealed that most egg chambers had not progressed beyond stage 9 of oocyte development (Figure 9). Moreover, numerous degenerating egg chambers were detected. The degenerating egg chambers were present in the developmentally linear ovariole beyond stage 8 chambers. This suggests that the mhmB egg chambers had somehow failed to pass the 'checkpoint' during stage 8. Similar egg chambers were rarely detected in the CS ovaries examined (Figure 9). To determine the possible underlying cause of premature degeneration, I began a systematic examination of the different components of the oocyte. The egg chamber nurse cells were examined and counted to ensure that the proper numbers were present, and that no obvious morphological differences occurred between CS and mhmB (Figure 10).

Multinucleated nurse cells are often detected in nurse cell transport mutants such as female sterile(1)217 (Swan 2001). In these mutants, fewer numbers of nurse cells are generally detected. DAPI staining revealed that mhmB mutant egg chambers contained 15 nurse cells, as normally occurs in wild type. DAPI staining also revealed degeneration of the egg chamber as well as irregularities during follicle cell migration (Figures 10-11). However, the egg chamber degeneration in mhmB was not completely penetrant. As discussed earlier, an extremely small number of
Figure 8. *mbmB* mutants (B) show a drastic reduction in ovary size as compared to wild type (A). This difference appears to be caused by the lack of later stage egg chambers in *mbmB* mutants. Accessory structures, such as the common oviduct (arrows, A and B) do not appear to differ. Ovaries stained with DAPI. (C) Total numbers of flies missing one or more ovaries.
Figure 9. DIC images of wild-type (A-B) and mutant ovarioles (C) show normal progression of CS egg chamber development, and degeneration of mbmB egg chamber. No difference in development until after stage 8 (the checkpoint).
Figure 10. Ovarioles stained with DAPI. (A) CS egg chamber progression through stage 10. (B) mbmB progression to stage 9, and subsequent degeneration of the egg chamber (arrow in B).
Figure 11. Follicle cell migration begins to cover presumptive oocyte in CS (A), while in mbmB, follicle cells appear irregularly distributed, particularly over the anterior portion of the egg chamber (B). Oocytes stained with DAPI.
mutant egg chambers did progress beyond stage 8-9, with some chambers
beginning to form rudimentary accessory structures such as the dorsal appendages
(Figure 12). These accessory structures were never found complete in mhmB
mutant egg chambers. The nurse cells of these advanced stage egg chambers could
still be detected, not ruling out a disruption during nurse cell transport.

Often, in mutations disrupting ring canal structure, the nurse cells will
become multinucleated, resulting in several clusters of nurse cells instead of 15
distinct nuclei. Cytoplasmic transport is also severely disrupted since the structures
enabling the transport are defective. I already determined that the numbers of nurse
cell nuclei in mhmB egg chambers were not different from wild type. However, I
could not rule out disruptions in cytoplasmic transport. For this reason, I examined
the ring canal structures with the use of rhodamine-conjugated phalloidin.
Phalloidin binds specifically to F-actin, a component of the nurse cell intercellular
bridges/ring canals. The ring canals in mhmB egg chambers were examined for the
correct number, 15; no differences in numbers were detected between mhmB and
CS flies (Figure 13).

Nurse cell apoptosis is normally complete by stage 14 in wild-type egg
chambers. Some persistent nurse cell nuclei were detected in the rare, aberrant late
stage mhmB egg chambers (Figure 12). For this reason, I examined the general
ability of mhmB egg chambers to undergo apoptosis. Using the Vybrant apoptosis
kit, apoptosis was detected in CS egg chambers following cytoplasmic transport,
during normal nurse cell breakdown beginning at stage 13 (Figure 14 A). In mhmB
Figure 12. Advanced mutant egg chamber showing the formation of the dorsal appendage in CS (A, arrow), and aberrant dorsal appendage formation in mbmB mutants (B, bracket). Egg chambers stained with DAPI.

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Figure 13. CS (A) and \textit{mbmB} (B) egg chambers stained with rhodamine-conjugated phalloidin. The rings canals do not differ morphologically or numerically. The different planes of focus between A and B allow visualization of the oocyte and cytoskeletal components in B, but not in A.
Figure 14. Apoptosis in CS (A) and mbmB (B) egg chambers visualized through the Vybrant apoptosis assay. Propidium iodide (red) enters necrotic cells, while YO-PRO-1 (green) enters apoptotic cells. In A, cells undergoing apoptosis during late stage oogenesis can be seen. In B, apoptosis is expanded throughout a presumably stage 9 egg chamber. Necrosis is detected in the next egg chamber.
mutants, apoptosis was most commonly detected in egg chambers beyond stage 8. This was followed by necrosis throughout the egg chamber (Figure 14 B). In the aberrant late stage mutant egg chambers, both apoptosis and necrosis were detected.

Discussion

The results of the courtship behavioral trials revealed abnormal courtship behavior displayed by wild type males when mhmB females were used as the targets of courtship. The extent of these defects, however, was not so severe as to render mhmB females completely behaviorally sterile: mhmB females were observed copulating. However, no eggs were detected from mhmB females during preliminary studies. These data might explain a reduction in reproductive success for mhmB females, but they do not suggest behavioral sterility. These results are consistent with courtship trial experiments in which the MBs were chemically ablated (Grizzle 2000. unpublished thesis). In those trials, females that underwent chemical ablation of the MBs elicited an increase in courtship latency and a decrease in the courtship index from wild-type males. The ablation studies, taken with the findings presented here, suggest the defects in courtship behavior are a result of the reduction in MB neuropil and not the cause for female sterility in mhmB.

Data presented here suggest that the morphology of the ring canals and nurse cells are not responsible for the degeneration of the developing egg
chambers. Additionally, an inability to undergo apoptosis was also ruled out. The ring canals were detected in both *mhmB* and *CS* egg chambers beginning with the earliest stages of egg chamber formation. I also was able to detect the ring canals in both *CS* and in *mhmB* during all stages observed (data not shown). In *CS*, the ring canals were observed until such time as nurse cell apoptosis was well underway. In *mhmB*, the ring canals were typically observed until such time as the egg chamber began degeneration.

Next, nurse cell and follicle cell morphology were examined. The DAPI-stained egg chambers revealed that there were in fact 15 nurse cells in each chamber, as in wild-type females. Also, the majority of *mhmB* ovarioles exhibited egg chamber degeneration after stage 9. Because nurse cell cytoplasmic 'dumping' begins at the end of stage 10, it is likely that the ring canal structures are not the primary cause of premature degeneration of mutant egg chambers. However, I noted that there might be secondary effects on the efficiency of follicle-cell migration.

The data presented suggest that mechanisms required during the early stages of egg chamber development are not significantly impaired in the *mhmB* mutant. There exists numerous, complex activities during the later stages of oocyte development. These activities include chorion and vitelline membrane synthesis, and construction of the accessory structures such as the micropyle and dorsal appendages. However, in studies performed by Montell et al., Calvi et al., and Peri and Roth mutations in these late stage processes occurring after stage 10 allowed
oogenesis to progress rather than causing a breakdown of the egg chamber as observed in the majority of *mbmB* mutants (Montell et al. 1992; Calvi et al. 1998; Peri and Roth 2000). This suggests that *mbmB* is necessary prior to the oogenesis ‘checkpoint’. Attention was now turned to those processes occurring around stage 10 or earlier.

Follicle cell migration, one of the landmark events of oogenesis, begins during stage 9. The follicle cells migrate towards the posterior portion of the egg chamber to cover the entire oocyte. This leaves the nurse cells sparsely covered by about 50 follicle cells. Shortly after this initial follicle cell migration begins, a subset of follicle cells begins centripetal migration along the border between the oocyte and nurse cells. These cells are known as the border cells (King 1970). Failure of the follicle cells to migrate properly, such as in *cup* or other follicle-cell mutants, causes degeneration of the oocyte (Schupbach and Weischaus 1991). Failure of the follicle border cells to undergo centripetal migration usually results in progression of oocyte development. However, this loss of border cell migration results in malformation or absence of the chorion and accessory appendages. When border cells are removed by laser ablation, only defects in the chorion accessory structures were detected (Montell et al. 1992). Unfortunately, little is known about the mechanisms controlling migration of the follicle cells. Consequences of incomplete or disrupted follicle-cell migration could lead to a gradient in the severity of defects, depending upon the relative progression of migration. This could explain the differences in severity in *mbmB* mutants, from degeneration of
stage 8-9 egg chambers, to more mature eggs lacking dorsal appendages and complete chorion. To determine the nature of possible follicle cell migration defects in \textit{mbmB} mutants, further studies addressing specific events such as border cell migration are necessary.

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GENERAL CONCLUSIONS AND DISCUSSION

Data presented in this thesis contributes to the understanding of pleiotropic effects of the Drosophila gene, mushroom body miniature B (mbmB). These mutants exhibit decreased viability and growth, and defects in oogenesis and courtship behavior. The data presented here also suggests that mbmB is not necessary for embryonic mushroom body neuroblast specification or proliferation. As discussed earlier, this seemingly normal development of the MB primordia in the embryo indicates that the adult MB γ lobe should be unaffected in mbmB mutants. A closer examination of the adult γ lobes would allow for the distinction between a reduction in adult MBs either due to a defect in proliferation during late embryogenesis or a defect due to some later stage process such as axonal pathfinding during metamorphosis. Additionally, the preliminary data from larval lethality experiments suggests that mbmB may have a role in early larval growth. It is possible that mbmB is also required for MBNb proliferation during early larval development. To properly address these issues, larval growth and larval viability must be examined, so must larval MB development.

With a more complete characterization of the phenotypes associated with mbmB, a search for Drosophila mutants exhibiting similar phenotypes was performed. The search yielded several interesting genes involved in the cyclic
adenosine monophosphate (cAMP) signaling pathway. Mutations affecting \textit{cAMP-dependent protein kinase 1 (pka-c1)} show defects in learning and memory, abnormal courtship behavior, larval lethality and delayed larval molting, and defects in oogenesis (Skoulakis et al. 1993; O'Dell et al. 1999; Lane and Kalderon 1993; Lane and Kalderon 1995). Mutations in \textit{dunce (dnc)} exhibit reductions in learning and memory, defects during oogenesis including degenerating egg chambers, and aberrant neuronal arborization (Quinn et al. 1974; Swan et al. 2001; Lee and O'dowd 1999; Yao 2000). Similarly, mutations in \textit{rutabaga (rut)} also affect learning and memory as well as viability (Folkers 1982; Lane and Kalderon 1993).

In addition to searching for phenotypic similarities among different mutants, a search for genes in the same region of the genome was performed. Both \textit{mbmB} and \textit{pka-c1} have map positions at 30C. on the left arm of the second chromosome (de Belle and Heisenberg 1996; Foster et al. 1998; Flybase.org). The data presented here show a striking similarity in \textit{pka-c1} and \textit{mbmB} mutant phenotypic defects. This suggests that \textit{mbmB} may either be allelic to \textit{pka-c1} or involved in the cAMP pathway. Complementation tests between mutants of \textit{pka-c1} and \textit{mbmB}, in addition to P-element mediated gene disruption of \textit{mbmB} are currently underway by Christine Serway of the de Belle lab at the University of Nevada, Las Vegas.

In eukaryotes, cells are capable of responding to extracellular signals via membrane receptors. In response to this first messenger, cells can produce a
secondary messenger such as cAMP. The cAMP second messenger is used not only in signal transduction events sensed by G-protein coupled receptors, but also in other transduction events such as ion concentration changes sensed by calmodulin. These membrane receptors can be stimulatory, causing an increase in intracellular cAMP, or they may be inhibitory, causing a decrease in cAMP. Once the extracellular signal is received by the G-protein coupled receptor, the G protein is able to dissociate from the receptor and bind to adenylyl cyclase (AC). The AC enzyme is responsible for dephosphorylation of ATP, converting it to cAMP. Assuming that a stimulatory G-protein coupled receptor was activated, an increase in the concentration of intracellular cAMP occurs. The second messenger, cAMP, is now capable of activating downstream targets such as PKA. PKA activates the cAMP response element binding protein (CREB), which brings about gene transcription. The increase in cAMP levels caused by the first messenger is, however, transient. Phosphodiesterases are responsible for the hydrolysis of cAMP to AMP, decreasing the concentration of intracellular cAMP.

In mammalian systems, cAMP-dependent protein kinase A is composed of two catalytic subunits and two regulatory subunits. Initially, this holoenzyme is inactive due to the inhibitory effect of the regulatory subunits. cAMP binds these regulatory subunits, causing them to dissociate from the catalytic subunits. The catalytic subunits are now free from inhibition and capable of phosphorylating downstream targets. In Drosophila, PKA is activated when cAMP binds to the regulatory subunits and brings about a conformational change. This conformational
change releases the now active PKA catalytic subunits. In *Drosophila*, three genes for catalytic subunit isoforms (*pka-C1, pka-C2, and pka-C3*) and 2 genes for regulatory subunit isoforms have been identified (*pka-RI and pka-RII*) (Foster et al. 1988; Lane and Kalderon 1993; Goodwin et al. 1997; Muller 1997).

In *Drosophila*, *G-protein coupled receptor kinase* 2 (*Gprk2*), is thought to activate AC and increase intracellular cAMP levels (Figure 1). Mutations in *Gprk2*, which is expressed most abundantly in the MBs and in the ovaries, result in reduced egg laying, malformations of the egg chamber dorsal appendages, and incomplete oocyte nurse cell dumping (Lannutti and Schneider 2001). Another gene, *dnc*, involved in the cAMP pathway encodes a type-2 cAMP phosphodiesterase (PDEII) responsible for cAMP degradation. Surprisingly, even though *dnc* and *pka-C1* perform opposite cellular functions, mutations in each gene result in very similar phenotypes. *dnc* mutants display defects in learning and memory, courtship behavior, and oocyte development (Dudai et al. 1976; Odell 1994; Swan et al. 2001). Egg chambers of *dnc* females show dorsal appendage malformation in addition to degeneration of late-stage egg chambers (Swan et al. 2001).

It has been shown that cAMP levels are reduced in *Gprk2* mutants and cAMP levels are elevated in *dnc* mutants (Lannutti and Schneider 2001; Devay et al. 1986). This suggests that perhaps it is the concentration of cAMP that is crucial for proper signal transduction: an increase as detrimental to the system as is a decrease. Interestingly, when null alleles of *dnc* are introduced into a *Gprk2* mutant
Figure 1. cAMP pathway schematic. (PKA) Protein Kinase A, (R) PKA regulatory subunit, (C) PKA catalytic subunit.
background, the defects associated with *Gprk2* are suppressed; introduction of *Gprk2* into *dnc* mutant background has the same affect. This further supports the idea that cAMP normalization is critical (Lannutti and Schneider 2001). Another gene found to have high levels of expression in the MBs, *rut*, encodes an AC responsible for conversion of ATP to cAMP. Mutations in *rut* have been shown to cause defects in learning and memory, courtship behavior, and larval molting and viability (Folkers 1982; Gailey et al. 1985; Lane and Kalderon 1993). When mutant alleles of *rut* are introduced into a *dnc* background, there is a partial rescue of both the female sterility and the learning and memory deficit (Livingstone et al. 1984; Feany 1990). It has also been shown that reductions in cAMP levels inhibit protein synthesis during larval development (Friedrich and Gallyas 1989). A reduction in cAMP levels during larval development most likely affects PKA activity as well. Thus, further supporting the idea that defects in *mhb* are manifested during larval development.

Researchers have amassed a plethora of information regarding learning and memory processes through use of G-protein coupled receptors and the cAMP pathway in *Drosophila*. However, very little attention has been given to the role of these pathways in development. It seems quite obvious that these pathways do impact development of the organisms as is evidenced from research presented in this thesis as well as the research of others discussed here. Further investigation of the processes affected by *mhb* can only serve to aid in understanding the various aspects of development regulated by the cAMP pathway.
Studies are currently underway in the de Belle lab to gain the molecular identity and characterization of \textit{mbm}B. Once this information is obtained, a number of experiments should be performed in order to determine the exact nature of the \textit{mbm}B mutation. For example, defects in \textit{mbm}B share the most commonalities with those of \textit{dnc}. Since the \textit{dnc} gene product is involved in lowering cAMP levels and \textit{mbm}B is suspected of causing a defect in PKA, it would seem that the defects in \textit{mbm}B mutants would be more similar to those found in \textit{pka-C}I mutants. However, it is possible that the nature of the \textit{mbm}B mutation may cause a loss of PKA inhibition. This loss of inhibition would mimic the effects of chronically-elevated cAMP levels as observed in \textit{dnc} mutants. To test this, biochemical phosphorylation assays using PKA substrates could be used to determine if PKA activity is increased or decreased relative to wild-type activity. Another phenotype, which is observed in \textit{mbm}B alone, is that of the reduction in MB neuropil. Although this same reduction is not observed in the MBs of the other mutants discussed here, there are well-documented studies showing defects in synaptic plasticity and in growth of neuronal processes in cAMP pathway mutations (Sanyal et al. 2002; Renger et al. 2000; Yao et al. 2000). It is possible that the \textit{mbm}B mutation affects axonal and/or dendritic outgrowth in a specific fashion as to cause the MB neuropil reduction. A more complete study into the existence of substructural abnormalities in the MBs of \textit{mbm}B mutants would aid in making this determination. Again, once the isolation of the \textit{mbm}B gene is complete, a systematic investigation into its temporal and spatial regulation, as well as its function in learning and memory.
behavior, and development can be pursued.

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