Genes involved in mushroom body development and behavior in Drosophila

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GENES INVOLVED IN MUSHROOM BODY DEVELOPMENT AND BEHAVIOR

IN DROSOPHILA

by

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Bachelor of Arts
University of Colorado, Boulder, Boulder, CO
2001

A dissertation submitted in partial fulfillment
of the requirements for the

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Christine Nicole Serway

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be accepted in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in Biological Sciences**

School of Life Science

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Mushroom bodies (MBs) are the site of multi modal sensory integration critical for associative conditioning in *Drosophila*. They have been central to research on the structure function relationship in the brain for over one hundred years due to their unique shape and readily accessible physiology. This dissertation incorporates three different approaches to further elucidate the genetic and molecular nature of this structure function relationship.

First, the suite of genetic and molecular tools available in *Drosophila melanogaster*, facilitated the molecular mapping of a 25-year old MB structural mutant called *mushroom body miniature B (mbmB)* to the gene *Pendulin (Pen)*, also known as *importin-α2 (imp-α2)*. Anatomical rescue, protein expression in the brain and functional domain analysis in *mbmB* mutants have shown that Imp-α2 is necessary for MB development, which likely gives rise to its learning, long term memory and amnesia resistant memory defects. Imp-α2 is a central component of nuclear cytoplasmic trafficking, mitotic spindle orientation, and injury response in the nervous system. The work described in this dissertation provides the first evidence that Imp-α2 also has a critical role in MB development and associative conditioning.
Second, MB specific Gal4 lines were used to identify novel genes associated with MB development through the identification of their flanking sequence. Ten Gal4 inserts were localized to introns, exons, and some intragenic regions of eight genes, likely to have interesting and testable roles in MB development and/or function. These candidate genes include: $\beta$FTZ-F1, Odorant receptor 42a, no extended memory, TAK1-associated binding protein, frizzled, Ecdysone-induced protein 75B, Casein Kinase 1$\gamma$ and eyeless. Overall, the inserts themselves had minimal effects on MB development, likely due to their positions in non-coding regions. Protein levels in three homozygous MB Gal4 inserts, all found upstream of the frizzled gene, appeared reduced, indicating that these inserts can in fact disrupt protein levels independent of any effects they may or may not have on MB gross morphology. New evidence that genetic background influences MB anatomy is also provided through the analysis of two Gal4 lines in different genetic backgrounds. This work brings to light novel signaling pathways, likely associated with MB anatomy and development, that upon further investigation will aid in our understanding of the molecular nature of how the MBs form.

Finally, the influence of MBs on walking was investigated using mutant alleles of several genes with severe MB disruptions and a chemical method for MB ablation. Over the course of fifteen minutes (the initial stages of walking), flies with disrupted MBs showed a decrease in the frequency of walking indicating a role for MBs in the up-regulation of motor coordination during its initial stages. Slight differences in orientation to landmark and velocity were also
observed, but attributed to pleiotropy rather than the MB disruptions. These findings were in contrast to conclusions made in previous work demonstrating MB’s involvement in the termination of walking bouts over longer time courses (i.e. MBs down-regulate locomotion). Both sets of data taken together implicate MBs in regulation of motor behaviors in a time dependent fashion, up regulating activity during the initial stages of walking, but suppressing activity thereafter. Therefore, MBs deliver appropriate contextual information to motor output centers in the brain by modifying the quantity of walking (activity) rather than the quality (velocity and orientation).
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ABBREVIATIONS

Ab     Antibody
AL    Antennal Lobe
ala    alpha lobes absent
amn    amnesiac
ANOVA  Analysis of Variance
ARM    Anesthesia Resistant Memory
βFTZ-F1 Beta FTZ-F1
BENZ   Benzaldehyde
bib    big brain
Bp     Base Pair
cAMP   cyclic Adenosine Monophosphate
CAS    cellular apoptosis susceptibility
CASB   Cellular Apoptosis Susceptibility Binding Domain
cbx    calyx bulging
CCX    Central Complex
CDs    Coding Sequences
cDNA   Cytoplasmic DNA
CG     Computed Gene
CK1γ   Casein Kinase 1 γ
CNS    Central Nervous System
CPG    Central Pattern Generator
CREB   cAMP response element binding
CS     Canton Special
CT     Control
DCO    Protein Kinase A- catalytic subunit
dFMRP  Drosophila Fragile X Mental Retardation Protein
dnc    dunce
DIM    Dimerization
DNA    Deoxyribonucleic Acid
DPM    Dorsal Paired Medial Cells
EB     Ellipsoid Body
EcR    Ecdysteroid Receptor
ELAV   Embryonic Lethal Abnormal Vision
EMS    Ethyl Methane Sulfonate
ey    eyeless
E75    Ecdysone-induced protein 75B
FB     Fan Shaped Body
fz     frizzled
FLP    Flippase Recognition Enzyme
FRT    Flippase Recognition Target
fum    fused mushroom bodies
Gal4   Yeast Transcription Factor, Binds Upstream Activation Sequence
gDNA  genomic DNA
GFP    Green Fluorescent Protein
gish  gilgamesh
Glu  Glutamate
Gln  Glutamine
hid  head involution defective
HRP  Horseradish peroxidase
HU  Hydroxyurea
imp-α2  importin-α2
KC  Kenyon Cells
IBB  importin-β binding
iPCR  Inverse Polymerase Chain Reaction
Kb  Kilobase
KC  Kenyon Cell
kDa  Kilodalton
LH  Lateral Horn
LNLSB  Large Nuclear Localization Signal Binding Domain
LTM  Long Term Memory
MB  Mushroom Body
mbd  mushroom body deranged
mbm  mushroom body miniature
mbmB  mushroom body miniature B
mbmC  mushroom body miniature C
MCH  Methyl Cyclohexanol
MTM  Middle Term Memory
mud  mushroom bodies deranged
mud  mushroom bodies deranged
Nb  Neuroblast
nemy  no extended memory
NLS  Nuclear Localization Signal
NPC  Nuclear Pore Complex
Or42a  Odorant receptor 42a
ORN  Olfactory Receptor Neuron
PBS  Phosphate Buffer Saline
PBT  Phosphate Buffer Saline, Triton-X
PBSBT  Phosphate Buffer Saline, Triton-X, BSA
PCR  Polymerase Chain Reaction
PDF  Pigment Dispersing factor
Pen  Pendulin
PFA  Paraformaldehyde
PKA-R1  Protein Kinase A Regulatory Subunit
PKA-C1  Protein Kinase A Catalytic Subunit
RNA  Ribonucleic Acid
RNAi  Ribonucleic Acid Interference
RT  Room Temperature
RT-PCR  Reverse Transcription Polymerase Chain Reaction
rut  rutabaga
smu  small mushroom bodies
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>STM</td>
<td>Short Term Memory</td>
</tr>
<tr>
<td>SNK</td>
<td>Student Newman Keuls</td>
</tr>
<tr>
<td>SNLS</td>
<td>Small Nuclear Localization Signal</td>
</tr>
<tr>
<td>SNLSB</td>
<td>Small Nuclear Localization Signal Binding Domain</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SOP</td>
<td>Sensory Organ Precursors</td>
</tr>
<tr>
<td>Tab2</td>
<td>TAk-associated binding protein</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate ethylene-diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>TIFR</td>
<td>Transient Interhemispheric Fibrous Ring</td>
</tr>
<tr>
<td>TLL</td>
<td>Tailless</td>
</tr>
<tr>
<td>TS</td>
<td>Temperature Sensitive</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activating sequence</td>
</tr>
<tr>
<td>USP</td>
<td>Ultraspiricle</td>
</tr>
<tr>
<td>w^{118}</td>
<td>white 118</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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CHAPTER 1

GENERAL INTRODUCTION

The relationship between structure and function in the brain has been well studied in many organisms spanning a variety of tissue types. It has allowed us to identify regions of the brain responsible for the generation of complex behaviors. Hippocampal lesions in rats have eliminated their conditioned fear response, with no effect on their ability to respond to other sensory stimuli (Kim & Fanselow, 1992; Phillips & LeDoux, 1992, 1994). This has implicated the hippocampus as a context-processing center in the brain. In songbirds, a highly specialized forebrain circuit is responsible for their ability to learn and recite the songs they hear during development (Nottebohm et al., 1976, 1982; Kroodsma & Konishi, 1991; Wild, 1997a, 1997b, 2004). In this circuit, the forebrain and brainstem function together allowing these birds to mimic other songs. Functional studies in the cat visual cortex have shown that structural changes occur in the receptor field during different stages of visual processing (Hirsch & Martinez, 2006), ultimately facilitating bifocal vision.

*Drosophila melanogaster* is capable of a multitude of complex behaviors and offers unique techniques that can be used to investigate the genetic, molecular and cellular basis of behavior and its underlying neuronal circuitry in the brain. The function and interactions of many different types of genes as well as their respective regulatory mechanisms give rise to anatomy and behavior. Here, I will focus on a specific structure in the insect brain called mushroom bodies (MBs), which are directly correlated with many complex behaviors, most notably
associative olfactory conditioning (review: Davis, 2005). Without these densely packed neuropilar structures, flies are unable to learn in a Pavlovian associative olfactory conditioning paradigm (de Belle & Heisenberg, 1994). Although a great deal of detailed information has been compiled regarding MB development and function, we are far from understanding the complete picture of how this interplay generates complex and adaptive behaviors. The work outlined in this dissertation begins to fill in the gaps providing unique insight to each avenue of work using three very different approaches.

The *Drosophila* olfactory system is an ideal circuit to investigate the relationship between structure and function because its physiology is well characterized and it is known to be the entry point for critical environmental stimuli. The highly organized developmental patterning of ~1,200 olfactory receptor neurons (ORNs) on the antenna project their axons to less than 50 target glomeruli in the antennal lobe (AL) (Laissue et al., 1999; Stocker, 1994). Projection neurons extend from the AL glomeruli to the mushroom bodies (MBs) or lateral horn (LH) (Laisseu et al., 1999; Stocker, 1994).

MBs serve as the information processing centers in the *Drosophila* olfactory system. They are paired neuropil composed of thousands of densely packed kenyon cells in the protocerebrum, separated from the rest of the brain by a thin layer of glial cells. Each Kenyon cell body sends out dendritic projections, collectively called the calyx. The calyx receives olfactory information from the antennal lobe via the antennal-cerebral tract (Heisenberg, 1998). The Kenyon cell axons project rostrally below the calyx as a structure called the pedunculus,
which then bifurcates and gives rise to a series of lobes. These include two
dorsally projecting (α, α′,) and three medially projecting (β, β¹ and γ) lobes (Yang
et al, 1995; Crittenden et al., 1998) that all arise from only four progenitor
neuroblasts (Ito et al., 1997; Lee et al., 1999).

Groundbreaking evidence linking MB’s to learning and memory was
accomplished with the selective ablation of the four mushroom body progenitor
neuroblasts. The cytostatic chemical hydroxyl urea (HU) was used to inhibit
protein synthesis by killing dividing MB cells through inhibition of ribonucleotide
reductase (Timson, 1975). In this experiment, flies were fed HU 8-12 hrs after
larval hatching, when there are only 5 neuroblasts (Nb’s) proliferating in each
hemisphere of the brain (Truman & Bate, 1998), four of which give rise the
mushroom bodies, while the 5th gives rise to local and projection neurons within
the antennal lobe (Stocker et al., 1997; Ito & Hotta, 1992). This selectively
ablated the MB’s of adult flies (de Belle & Heisenberg, 1994). These MB-less
flies were then unable to perform olfactory associative learning tasks, providing a
strong correlation between mushroom bodies and learning and memory (de Belle
& Heisenberg 1994).

Perturbations to individual components of the olfactory circuit have provided
further support for the MBs in associative conditioning. Dunkelberger (2008) has
shown through analysis of a suite of MB structural mutants that there is a tight
correlation between reductions in MB cell number and poor olfactory learning. A
similar result was observed in wildtype heat shocked flies, whose MBs were
reduced along with their learning scores (Wang et al., 2007).
Axonal projections are also important for the regulation of associative conditioning. Work on a mutant missing α lobes (known as *alpha lobes absent* or *ala*) has shown defects in long term memory (Pascual & Préat, 2001). β-lobe fusion across the midline in adults has been associated with reductions in olfactory learning and memory in a handful of genes including *mushroom body miniature B (mbmB)* (Dunkelberger, 2008), *Fragile X mental retardation protein (dFMRP)* (Bolduc et al., 2008; Michel & Restifo, 2004; McBride et al., 2005), *linotte* (Moreau-Fauvarque et al., 1998; Moreau-Fauvarque et al., 2002) and *fused mushroom bodies (fum)* (de Belle & Kanzaki, 1999).

In addition to cell number and patterning, communication between cells is another necessary component of a functional neuronal circuit. When communication from the dorsal paired medial (DPM) neurons to the MBs is disrupted in *amnesiac (amn)* mutants (a known 30 minute memory mutant), short term memory is blocked (Waddell et al., 2000). Disruption of MB synaptic function can alter MB cell integrity as well. Blocked neurotransmission of MB signaling has been accomplished through the use of a temperature-sensitive *dynamin* transgene (*shibire*⁶⁸) whose induction can be regulated in a matter of minutes (Kitamoto, 2002). This system has been used to show that MB neurotransmission is necessary for memory retrieval, yet has no effect on its storage (Dubnau et al., 2001; McGuire et al., 2001). In contrast to the short lived changes initiated by *shibirie*⁶⁸, long lasting synaptic plasticity can give rise to both structural and functional changes at the synapse, through the storage of mRNAs and initiation of LTM, which is related to CREB-mediated transcription of
downstream genes (Dubnau et al., 2003). It has also been shown that LTM requires cAMP signaling as well as protein synthesis (Tully et al., 1994). This work implicates both the quantity (cell number) and quality (projection patterning, NT release, and translational regulation) of MB circuitry as necessary components for processing signals required for associative conditioning.

In Chapter 2 of this dissertation, anatomical, behavioral and molecular characterization of mbd provided a unique opportunity to directly correlate the cellular requirements for MB development with its function. Our lab has shown that mbd mutants exhibit axonal guidance defects, reduced MB cell numbers and associative conditioning defects (Dunkelberger, 2008). My work has shown that intact Imp-α2 is necessary for accurate MB development at the axonal and cell body level. This is the first evidence that nuclear cytoplasmic transport is critical for MB development.

Candidate genes identified in Chapter 3 brought to light new cellular pathways and biochemical processes that have yet to be associated with MB development. My work offers a preliminary investigation of their role in shaping the MB and provides promising avenues for future work on genes not previously thought to be required for MB development.

Taken together, my work on mbd and the Gal4 lines provides the opportunity to further dissect MB lobes functionally, determining “where” memories are stored in the brain. The MB lobe specific expression exhibited by the Gal4 lines I analyzed in Chapter 3 as well as the genes whose expression pattern they mimic offer a powerful dataset because of the different expression
levels in each lobe as well as the various lobe combinations from line to line. I have provided a cellular explanation for the β lobe fusion exhibited in mbmB as well, which can now be correlated to its behavioral defects. Additionally protein expression patterns of both mbmB and the candidate genes from the Gal4 screen have shown that expression outside the MBs in the rest of the brain can have a significant influence on MB development and associative behavior.

MB’s physically link the external olfactory world to behaviors initiated by the brain, with the Kenyon cells being a mere two synapses away from the olfactory receptors of the AL. Although it has been clearly demonstrated that the MBs are necessary for learning (described above), they have no effect on general sensory or motor skills (de Belle & Heisenberg, 1994). MBs have also been implicated in regulations of different aspects of courtship memory (Sakai & Kitamoto, 2006; Joiner & Griffith, 2000), aggression (Edwards et al., 2009; Baier et al., 2000), sleep (Seugnet et al., 2008; Joiner et al., 2006; Pitman et al., 2006), centrophobism and thigmotaxis (Besson & Martin, 2005) and down-regulation of motor activity over long periods of time (Martin et al., 1998; Helfrich-Förster et al., 2002). Initially, this was somewhat surprising because these behaviors require little to no olfactory input, rather they utilize visual and tactile stimuli for their initiation. These behaviors do require the integration of sensory information however, which is likely facilitated by the MBs.

In the work I present in Chapter 4, I used multiple measurements of simple locomotion (velocity, activity and orientation) to dissect which components of walking require intact MBs during the initial stages of locomotion. My results
contradict the conclusions of previous work on the role of MBs in locomotion (Martin et al., 1998; Helfrich-Förster et al., 2002), showing that MBs up-regulate activity during the initial stages of locomotion. These results have allowed me to develop a more accurate time-dependent model for MB function throughout the course of locomotion. Their role as up-regulators during the initial stages of locomotion switches to down-regulation over time. Our data provides further evidence that MBs function as sensory integration centers modulating the frequency of behaviors by regulating their termination in a context dependent fashion.

Conclusion

The diversity of genes associated with MB development likely reflects the complexity required to modulate multi-sensory based functions. To further investigate MB anatomy and function, I used MB mutants generated in several different ways (EMS mutagenesis, P-element mutations, and chemical ablation) each causing different levels of disruption to their respective genes. This unbiased approach brought to light novel cellular and molecular pathways, and answered some very detailed questions regarding MB function.
References


CHAPTER 2
IDENTIFICATION OF MUSHROOM BODY MINIATURE B: THE DROSOPHILA
IMPORTIN-α2 IS IMPLICATED IN MUSHROOM BODY DEVELOPMENT AND
ASSOCIATIVE CONDITIONING

Abstract

Integration of neuronal inputs in the brain that facilitate appropriate behavioral outputs requires accurate functioning of biochemical processes and molecular pathways converging in both space and time. This is no small feat even for the simplest behaviors or rudimentary neuronal networks. Learning and memory are complex behaviors that have been well studied in Drosophila melanogaster because they possess relatively simple underlying circuitry compared to higher organisms. The extensive genetic and molecular tools available and wealth of knowledge about Drosophila development make it the ideal system to study this structure-function relationship. Although the central components of learning and memory have not been shown to be a single biochemical process or molecular pathway, the mushroom bodies (MBs) stand out as a key structure in the Drosophila brain necessary for associative behaviors (de Belle & Heisenberg, 1994; review: Davis, 2005). Spatial rescue experiments with short term memory (STM) mutants like rutabaga (rut) as well as experiments at the cellular level disrupting neurotransmission using shibiriťe have implicated specific lobes of the MBs in different aspects of learning and memory (Zars et al., 2000; Krashes et al., 2007). In this study, I investigated the gene mushroom body miniature B (mbmB), which has been known for almost three decades to have severe MB
structural defects as well as significant deficits in associative odor learning and memory performance. I have further characterized its anatomical defects and molecularly mapped it to the gene Pendulin, the *Drosophila importin-α2 (imp-α2)*. I expressed an *imp-α2* cDNA in the MBs with the UAS-Gal4 system and achieved a rescue of its MB structural defects. My work provides critical and novel insight into the connection between MB development and the cell biology of learning and memory.

Introduction

For over a century neuroscientists have been investigating how brain circuitry provides the framework for complex behaviors like learning and memory. Neural networks are capable of producing complex behaviors because they are able to regulate innate, predictive and adaptive circuitry in both space (anatomically) and time (developmentally). Experimental work in behavioral neurobiology using *Drosophila melanogaster* and other model organisms has shed a great deal of light on this structure-function relationship. The first olfactory classical conditioning experiments in *Drosophila* date back over 30 years (Quinn et al., 1974). Many individual genes responsible for poor olfactory based associative conditioning have now been molecularly characterized (review: Davis 2005). Interestingly, the first two learning and memory genes, *dunce (dnc)* and *rutabaga (rut)* were both part of the same pathway, the cyclic AMP signaling cascade (Dudai et al., 1976; Qui & Davis, 1993; Dauwalder & Davis, 1993; Livingston et al., 1984; Levin et al., 1992). Other members of this pathway have since been
implicated in different aspects of learning and memory including Protein kinase A - regulatory sub unit (Pka-R1), Protein Kinase A - catalytic sub unit (DCO), amnesiac (amn), and cAMP response element binding protein (CREB) (Skoulakis et al., 1993; Goodwin et al., 1997; Tully et al., 1994; Margulies et al., 2005; Yin et al., 1994). The large number of genes identified in the cAMP pathway associated with learning and memory facilitated the belief that it may be the learning and memory pathway, but cAMP signaling was just the tip of the iceberg. More recently, several other classes of genes have been shown to be critical for learning and memory. These genes span many cellular functions including cell adhesion: fasciclin II (Chang et al., 2001) and Volado (Grotewiel et al., 1998); RNA transport: oskar, staufen, e1f-5c (Dubnau et al., 2003); neurotransmitter function and synaptic plasticity: damb (Han et al., 1996), oamb (Han et al., 1998), Neurofibromin (Guo et al., 2000); and development: Latheo (Boynton et al., 1992), Linotte/derailed (Dura et al., 1993), and alpha lobes absent (Pascual & Préat, 2001). It is now obvious that a broader neurobiology approach will be necessary to more fully understand the cell and molecular basis behind learning and memory. Recent work has begun to fill in the gaps with more detailed knowledge about axon guidance, neurotransmitter functioning and transport, cell proliferation and cytoskeletal structure (review: Davis, 2005; Margulies et al., 2005). Insight into the cell biology and genetic composition underlying complex behavior is still only the first step, as complete resolution in both space and time is necessary to understand how behaviors become responsive as well as adaptive.
While progress was being made on the molecular front, the search for the structural / anatomical location of a memory engram focused on the mushroom bodies (MBs) (review: Heisenberg, 2003). Work on associative olfactory memory in flies has also implicated the involvement of dorsal paired medial neurons (DPMs) (Yu et al., 2005; Waddell et al., 2000) and the ellipsoid body, one component of the central complex (CCX) (Wu et al., 2007) in memory formation, yet their roles have not been characterized as thoroughly as the MBs.

The MBs of adult Drosophila are bilateral neuropilar structures in the protocerebrum composed of approximately 2500 intrinsic Kenyon cells (KCs) per hemisphere. They undergo a stereotyped developmental program, where four neuroblasts give rise to three spatially and morphologically unique subsets of KCs during larval and pupal development. MB $\gamma$ neurons are the first to develop, prior to the mid-third instar stage. The $\alpha'$ / $\beta'$ neurons develop next between mid third instar and puparium formation, and finally the $\alpha$ / $\beta$ neurons arise after puparium formation (Lee et al., 1999). KC dendrites arborize at the calyx, while their axons project to form a bundle of neurons called the pedunculus. Once the adult fly ecloses, three neuronal classes form two dorsal ($\alpha$ and $\alpha'$) and three medial ($\beta$, $\beta'$ and $\gamma$) lobes that bifurcate from the pedunculus (Heisenberg, 2003).

Mushroom bodies are well known for their central role in olfactory based Pavlovian conditioning (de Belle & Heisenberg, 1994; review: Margulies et al., 2005). Additionally they have more complicated roles in other behaviors often considered associative including motor activity (Serway et al., 2009; Besson & Martin, 2005; Helfrich-Förster et al., 2002), aggression (Baier et al., 2002), sleep
(Joiner et al., 2006; Pitman et al., 2006; Seugnet et al., 2008) and even some aspects of courtship memory (Joiner & Griffith, 2000; McBride et al., 1999). MBs preferentially express many protein products of genes known to be central to the generation of memories as well, giving further weight to their importance as a site of cellular memory (review: Keene & Waddell, 2007; Nighorn et al., 1991; Han et al., 1992; Skoulakis & Davis, 1996; Grotewiel et al., 1998; Cheng et al., 2001; Folkers et al., 2006; Crittenden et al., 1998). Genetic studies selectively blocking synaptic transmission within the MBs have provided additional support for MB involvement in learning and memory (Dubnau et al., 2001; McGuire et al., 2001; Krashes et al., 2007).

Almost 30 years ago, the first EMS induced MB structural mutants were identified (courtesy of J. Nüsslein-Volhard; Heisenberg et al., 1985). In that group of early mutants was a disruption to the gene, mushroom body miniature (mbm¹), which showed significantly reduced MBs and female odor learning defects (Heisenberg et al., 1985; de Belle & Heisenberg, 1996). Cloning and characterization of mbm¹ implicated zinc-finger based nucleic acid binding as a necessary component of brain development and olfactory learning (Raabe et al., 2004). The anatomical mutant mushroom body deranged (mbd) was another early MB anatomical mutation with abnormal MB morphology and a memory acquisition defect (Heisenberg et al., 1985; de Belle & Kanzaki, 1999).

The single mutant allele of mushroom body miniature B (mbmB) was selected from a screening of 1400 ethyl methane sulfonate (EMS) treated second chromosome lines (courtesy of J. Nüsslein-Volhard). mbmB displayed a
significantly reduced MB calyces, peduncle and lobes, as well as mild CCX
defects, and female sterility (de Belle & Heisenberg, 1996). Recently our lab has
shown that mbmB exhibits reduced viability and growth rate, yet it has no effect
on the early development of MB neuroectoderm and neuroblasts through stage
13 of embryonic development (Ginsburg, 2002). I have quantified the MB defects
in mbmB seen throughout the structure, as it exhibits β lobe fusion across the
midline, as well as reduced lobe size, calyx volume and cell number. I believe
this anatomical reduction is correlated with its significant reduction in learning,
the anesthesia-resistant component of memory (ARM) and long term memory
(LTM) (Dunkerberger, 2008), yet without molecular characterization of mbmB,
the mechanism remained elusive.

Originally, mbmB was mapped by recombination to 2-31 (Heisenberg et al.,
1985; Heisenberg, 1989; Lindsley & Zimm, 1992). Because it was an EMS-
generated mutation, I expected a change (or changes) in single nucleotides.
Traditionally cytological mapping and characterization of single gene mutations,
particularly those generated by EMS have presented a big challenge, often
requiring a great effort and time (e.g., mbm was cloned nearly 20 years after it
was first described (Raabe et al., 2004). Additionally the original wildtype that
mbmB was generated from is no longer available, leaving us unable to make
comparisons. The increasing availability of P-elements and gene traps with
thorough chromosomal coverage has greatly improved the likelihood of mapping
single gene mutations. In the case of mbmB, screening phenotypes of interest
(brain anatomy and behavior) is labor intensive because it requires the testing of
hundreds of lines for complementation to the mutant. The difficulty in cloning a mutant with defects in the brain is compounded by the fact that brain anatomy is often subject to change based on genetic background (de Belle & Heisenberg, 1996). Unfortunately I did not have the parent strain that mbmB was generated in, further complicating the matter. I set out to map and identify mbmB using a suite of the new genetic and molecular tools, thereby assigning a molecular identity to its anatomical and behavioral phenotypes.

In this study, I have found that mbmB is Pendulin (Pen), also known as the Drosophila importin-α2 (referred to from here on as imp-α2), encoding a carrier protein that utilizes the nuclear pore complex for nuclear cytoplasmic trafficking of nuclear localization signal (NLS) bearing cargo proteins. My work reveals a novel role for Imp-α2 in MB development, learning and memory consolidation. I show that mbmB has a 45% reduction in MB calyx volume, as well as a 52% reduction in MB cell number. Furthermore, I succeeded in rescuing these anatomical phenotypes through the introduction of a full length UAS imp-α2 cDNA driven in the MBs with the UAS-Gal4 system (Brand & Perrimon, 1993). The brain defects in mbmB flies correspond with reduced learning scores seen in our homozygous mbmB flies. Interestingly, mbmB flies also show a decrease in both spaced (LTM) and massed (ARM) long-term memory (Dunkelberger, 2008). As it is known that long-term memory is protein synthesis dependent (Tully et al., 1994), I propose that the LTM defects our lab has observed in mbmB mutants are a function of inadequate nuclear-cytoplasmic trafficking of critical NLS
bearing transcription factors. This study provides new insight into the impact of Imp-α2 on brain development and its influence on learning and memory.

Materials and Methods

Fly Strains

I used wildtype *Canton Special* derived from Würzburg stocks (CS), as well as white\textsuperscript{1118} \(w^{1118}\) (FBst0307124) backcrossed to CS for seven generations as controls in all experiments [hereafter referred to as \(w^{1118}\) (CS)] Berlin was used as an additional wild type control during sequencing. \(mbmB^1\) (CS) was used for initial anatomical characterization, and \(w;mbmB^1\) (CS) was used in the rest of the experiments. Both \(mbmB\) strains used were backcrossed to CS to control for genetic background effects (de Belle & Heisenberg, 1996). For mapping of \(mbmB\), a series of disruptions to chromosome 2L, including the 2L deficiency kit (Bloomington Stock Center), and a series of P-elements were crossed to \(mbmB\) (Table 2.1B-C; Appendix Figures A.1, A.3). Table 1.1A lists all fly strains used in each experiment, including wildtypes, mutants, Gal4 lines and transgenes. The strain \(y\ w; D14/y^+ CyO;TM6/+\) refers to \(Pen^{D14}\), an interstitial deletion of \(imp-\alpha2\) and will be referred to here as \(imp-\alpha2^0\) (Gorjánácz et al., 2006). I performed rescue experiments with \(imp-\alpha2\) cDNA and a series of MB specific Gal4 drivers both in the \(w;mbmB^1\) (CS) background. For anatomical analysis of each domain, all transgenic constructs were crossed into a CS genetic background and combined with \(mbmB^1\) (CS). To drive expression each strain was crossed to the MB specific Gal4 line in the cantonized \(mbmB\) background.
w;mbmB¹(CS):P[Gal4]c772 (for details on all crossing schemes, see Appendix B).

All flies were grown on standard cornmeal and molasses food supplemented with live baker’s yeast (Bloomington, Indiana, United States). They were maintained in either plastic bottles with 40 ml of food, or vials with 8 ml of food, sealed with cotton plugs, and kept at 24°C with 50% humidity in a constant 12:12 light dark cycle.

**Anatomical Analysis**

Paraffin mass histology was performed to analyze central brain morphology of MBs, CCX (Fan shaped body + ellipsoid body) and Antennal Lobe (AL). This method was used to initially characterize and then genetically map mbmB, to assess rescue mbmB functions and to characterize functional domains in mutant flies. Briefly, 2 to 6 day old flies were chilled on cold plates then placed in mass histology collars, fixed in Carnoy’s solution, dehydrated in ethanol and then embedded in paraffin (Heisenberg & Böhl, 1979). Heads were sliced in 7 µm serial sections and visualized using a fluorescent microscope (Zeiss, Thornwood, New York, USA). Volumes of MB calyx, CCX and AL were estimated from planimetric measurements of serial sections of brains using AXIOVISION software (Zeiss, Thornwood, New York, USA) (Serway et al., 2009; Wang et al., 2007).

Right wing area and right forelimb length were measured for mbmB homozygotes and compared to CS as well as to mbmB heterozygotes (Serway et al., 2009; Wang et al., 2007). Flies were cold anaesthetized to facilitate the
removal of their appendages, which were then mounted on glass slides, covered
with a cover slip and sealed with nail polish. A light microscope in concert with
AXIOCAM digital camera and software were used to collect and measure all
images (Zeiss, Thornwood, New York, USA).

The UAS/Gal4 binary expression system is a genetic tool that allows us to
drive expression of either reporter constructs (ex: GFP) or transgenes (ex: imp-
\( \alpha2 \) cDNA) in a tissue specific manner (Brand & Perrimon, 1993). This method
was used to investigate changes in MB cell number in the \textit{mbmB} mutant allele,
CS, and rescue flies. Whole mount fly brains were dissected in PBS, mounted
and viewed under a fluorescent confocal microscope using the far blue (FITC)
filter. Z-series were captured at 1 \( \mu \)m virtual sections spanning all of the MB cell
bodies. GFP-labeled KC nuclei were counted manually every 7th section using
IMAGE-J software (Abramoff et al., 2004) in an attempt to count all perikarya in
each image only once (diameter < 6 \( \mu \)m).

Mapping

\textit{mbmB} was mapped by recombination to the left arm of chromosome 2 (de
Belle & Heisenberg, 1996; Heisenberg et al., 1985). MB calyx volume was
initially used as the screening phenotype to further map \textit{mbmB}. To accurately
locate the physical position of \textit{mbmB} in the genome I embarked on a systematic
deficiency mapping study that exploits the recessive nature of the mutant
phenotypes (Figure 2.1 C and Figure 2.2) (Dunkelberger, 2008). Collections of
strains each bearing known chromosomal deletions were acquired (Ryder et al.,
2004; Parks et al., 2004; Bellen et al., 2004) and crossed with \textit{mbmB} mutants.
Figure 2.1 Anatomy of *mushroom body miniature B*. Serial sections of paraffin-embedded brains were used for planimetric measurements of several brain structures. (A) CS<sup>nu</sup> section and (B) mbmB<sup>1</sup>(CS) section both showing MB
calyx (arrowheads). (C) Homozygous \( mbmB^1(CS) \) flies had a 45% reduction in MB calyx volume compared to wildtype and there was no influence of sex or age. (D) Central body (arrowhead) volume was reduced by 16% in \( mbmB^1(CS) \) females, 11% in \( mbmB^1(CS)/CS \) females, 15% in \( mbmB^1(CS) \) males and 8% in \( mbmB^1(CS)/CS \) males. (E) Antennal lobe (arrowhead) volume was reduced by 15% in \( mbmB^1(CS) \) females and 6% in \( mbmB^1(CS)/CS \) females compared to \( CS \) females. Males, regardless of genotype, were not significantly different. (F) Wing area showed a 24% reduction in \( mbmB^1(CS) \) females, a 16% reduction in \( mbmB^1(CS)/CS \) females, a 13% reduction in \( mbmB^1(CS) \) males and a 6% reduction in \( mbmB^1(CS)/CS \) males compared to \( CS \) females and males, respectively. (G) Forelimb length was not significantly influenced by genotype although males had shorter forelimbs than females, an expected sexual dimorphism. For C-G, bars represent mean ± SE of mean calyx volume for each genotype. \( n \) indicated on each bar. Different letters designate significant differences (SNK, \( P \leq 0.05 \)).
Figure 2.2 Sterility genetically linked to brain anatomy. All sterile flies also had significantly reduced MBs ($F_{[2,69]} = 70.59, \ P < 0.0001$). Bars represent mean $\pm$ SE of mean calyx volume for each genotype. $n$ indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$).
Progeny expressing mutant phenotypes carry a deletion that uncovers the haplo-insufficient mutant \textit{mbmB} allele. As deficiencies from Bloomington Stock Center became available I crossed them to \textit{mbmB} for more detailed mapping (Table 2.1 A). Paraffin mass histology was performed to measure the MB calyx volume on the heterozygotes. After several unsuccessful mapping attempts (Appendix Figure A.1 A-C, F-G), I sought a less laborious method for scoring mutant phenotypes than paraffin mass histology. Homozygous \textit{mbmB} females were reported to be sterile (de Belle & Heisenberg, 1996; Ginsburg, 2002). I verified this using a backcrossing scheme (Appendix B.2) designed to separate the genetic basis of sterility and MB anatomy by recombination. After verifying tight linkage for these phenotypes in \textit{mbmB} flies, I then used female sterility to screen additional second chromosomal re-arrangements. Upon finding a deficiency that failed to complement the sterility phenotype of \textit{mbmB}, I continued the mapping efforts screening P-elements and gene disruptions in that region for sterility (Table 2.1 C) (Appendix Figures A.2-3, Table A.4).

Concurrently, I began mass sequencing coding regions of genes uncovered by the deficiency, starting with those identified in a microarray as up-regulated in brain tissue (Lyne et al., 2007). DNA was extracted from \textit{w;mbmB}^{1}\text{(CS)} and \text{CS} using Wizard genomic DNA Isolation Kit (Promega) and amplified using PCR. Samples were run out on 1.5% Tris-acetate ethylene-diamine-tetra-acetic acid (TAE) agarose gels and gel purified PCR product using QIAEX II (Qiagen) (Table 2.2). I performed 20\(\mu\)l sequencing reactions using 2\(\mu\)l gel purified PCR product as template and 0.3\(\mu\)l of [10\(\mu\)M] primers (Table 2.2) under the following
## Table 2.1 Fly strains.

### A. Chromosome-2L re-arrangements

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<th>Name</th>
<th>Source</th>
<th>Description</th>
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### B. Chromosome-2L re-arrangements

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| 7517 | 33F2;34A1 | B³ | 2L:16791487;17450255 |
| 7822 | 34A1;34A2 | B³ | 2L:16791487;17450255 |
| 7823 | 34A2;34A7 | B³ | 2L:16791487;17450255 |
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| 7518 | 35A3;35B2 | B³ | 2L:17502487;17502514;
| 7519 | 35B1;35B2 | B³ | 2L:17502487;17502514;
| 7828 | 35B1;35B8 | B³ | 2L:17903087;17903187;
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C. Chromosome-2L P-elements and gene disruptions
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**KEY:**

* = sterile & mutant MB volume  
B = Bloomington’s Stock Center  
H = Harvard Stock Center  
VDRC = Vienna Drosophila RNAi Center (Dietzl et al., 2007)  
‡ = DrosDel collection (Ryder et al., 2004)  
§ = Exelixis collection (Parks et al., 2004)  
◊ = BDGP collection (Bellen et al., 2004)  
1 = de Belle & Heisenberg, 1996  
2 = Gorjánácz et al., 2006  
3 = Connolley et al., 1996  
4 = Schulz et al., 1996  
5 = Armstrong et al., 1998  
6 = Dunkelberger, 2008
### Table 2.2 Primers for mapping and sequencing in chromosome-2L 30F5-31A1.

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Pen
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Primer KEY:
P = PCR primer
S = sequencing primer
T = top strand
B = bottom strand
conditions: 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. Steps 1-3 were repeated 25 times, then held at 4°C Dye terminator was removed using Centri-Sep™ Columns (Applied Biosystems), and samples were run on an ABI 3130 Genetic analyzer at The University of Nevada Las Vegas. I sequenced the coding regions of *big brain* (*bib*) (12,757Bp), *CG13131* (960 Bp) and *Pen* (3,429 Bp). All DNA sequences were assembled and analyzed using Lasergene (DNA*).

To verify that the single nucleotide polymorphism (SNP) identified in *mbmB* (*Pen; imp-α2*) was not due to human error or genetic background effects, I replicated the sequencing as follows: 7 *w;mbmB* (CS) samples, 3 CS samples and 2 Berlin samples.

**Analysis of Imp-α2 Brain Expression**

Whole brains were dissected from adult heads (as described above) for immunohistochemical analysis. I used the rabbit-anti Imp-α2 primary Ab at a 1:50 dilution (Gorjánácz et al., 2006). Goat anti-rabbit Alexa Flour 568 (Invitrogen) was used as a secondary Ab at 1:1,000 dilution. The staining procedure outlined by Dunkelberger (2008) was followed. Briefly, brains were dissected in Phosphate buffered saline (PBS), fixed in 4% Paraformaldehyde (PFA) for 3 hours at 4°C, washed in 1x PBS + 0.2% Triton X-100 (PBT) 3 x 30 minute at 4°C, blocked with 1x PBS + 0.2% Triton X-100 + 0.1% BSA (PBSBT) for 1 hour at 4°C, then incubated overnight at 4°C in 50 μl of Imp-α2 Ab diluted in PBSBT. Brains were washed 4 x 30 minute in PBSBT, incubated at room temperature for 4 hrs in secondary Ab covered in foil, then washed 3 x 30 minute in PBS. Slides were made as described above. CS and *mbmB* flies carrying the *P[Gal4]Ok107*
MB driver were crossed to CS and mbmB P[GFP]nls(LacZ) flies. GFP expression in MB Kenyon cells seen in green, and Imp-α2 expression seen in red.

For Western blot analysis, proteins were extracted from ten whole bodies (5 females and 5 males). Briefly, tissue was collected in DPBS, pelleted in a microcentrifuge and re-suspended in lysis buffer containing multiple protease inhibitors (Laemmli, 1979). Tissue was homogenized, boiled for 5 minutes then stored at 22°C for a maximum of one week. Samples were resolved on 10% SDS polyacrylamide gels, then transferred to Immobilon P membranes (Millipore) as described by Vaskova et al (2000). Blots were incubated with the following antibodies: rabbit anti-Imp-α2 (Török et al., 1995; Gorjánácz et al., 2006) in a 1:900 dilution, mouse anti-αTub (Sigma) in 1:15,000 dilution, goat-anti-rabbit and goat-anti-mouse secondary antibodies conjugated to HRP (Jackson Immuno Research) in a 1:7,500 dilution. Chemiluminescence ECL(+) Western-blotting detection system (GE Healthcare) and a Typhoon 8600 Variable Mode Phosphorimager (GE Healthcare) allowed us to visualize protein levels.

Impact of imp-α2 Functional Domains on MB calyx volume

imp-α2 has several domains known to give rise to its role in nuclear cytoplasmic trafficking. Bernard Mechler was kind enough to provide me with mutant transgenic flies disrupting a single imp-α2 domain, created with the PCR based quick change Site-Directed Mutagenesis Kit (Stratagene). Each imp-α2 mutant transgene consisted of either an alanine substitution at a critical amino acid residue, or a small deletion removing an entire domain. After driving each transgene in the null background, they were all verified to still produce the Imp-
α2 protein (Table 2.1 A, Figure 2.6 A; described in detail in Gorjánácz et al., 2006). To determine the role of these domains in MB development, I expressed nine individual UAS driven imp-α2 transgenes in mbmB mutant flies in the MBs using the UAS-Gal4 system and MB specific driver P[GAL4]c772 (Appendix B.7). MB calyx volume was measured to assess whether any of the constructs rescued MB anatomy.

Statistical Analysis

All measured parameters were analyzed for significant effects of genotype, gender, and MB disruption (volume or cell number), as well as any possible interactions, using analyses of variance (ANOVAs). The Student-Newman-Keuls (SNK) multiple range test was used to make comparisons between means for multiple groups (Zar, 1996) (SAS Institute software).

Results

mbmB Phenotype: Gross Brain Anatomy

Changes in morphology (size, position, cell number, tracts, or innervation) can be relatively minor, yet have a marked effect on behavioral outputs (e.g.: the effects of just 16 Pigment Dispersing Factor (PDF)-expressing neurons on circadian rhythms) (Review: Nitabach & Taghart, 2008). Although abnormalities in gross brain morphology of the mbmB mutant allele have been documented (Heisenberg, 1980; Heisenberg et al., 1985; de Belle & Heisenberg, 1996; Dunkelberger, 2008; Serway et al., 2009), in this study I have quantified it in a dosage-dependent manner performing paraffin mass histology on flies ranging
from one to seven days old. Adult neurogenesis (post eclosion) has recently been shown in *Drosophila* as a mechanism of experience dependant structural plasticity (Rokia-Mille et al., 2008). I wanted to investigate whether this was occurring in the brains, more specifically in the MBs of CS or *mbmB* mutant alleles, and if so were they different from one another. Representative images of CS and *mbmB*<sup>1</sup>(CS) used to collect MB calyx volumes are shown in Figures 2.1 A-B. Mean MB, CCX and AL volumes were calculated for CS, *mbmB*<sup>1</sup>(CS) and *mbmB*<sup>1</sup>(CS)/CS (Figure 2.1 C-E). There was a significant influence of genotype on MB calyx volume ($F_{[2,265]}=67.42$, $P<0.0001$). The most extreme differences were seen in the MBs of homozygous *mbmB*<sup>1</sup>(CS) flies, which showed a 45% reduction in MB calyx volume compared to wildtype. There was no influence of sex ($F_{[1,265]}=0.05$, $P=0.831$), age ($F_{[3,265]}=0.88$, $P=0.452$), or their interaction ($F_{[3,265]}=0.149$, $P=0.930$) on MB calyx volume (Figure 2.1 C). There was a significant influence of sex ($F_{[1,114]}=19.559$, $P<0.0001$) and genotype ($F_{[2,114]}=30.226$, $P<0.0001$) on CCX volumes, while there was no interaction effect ($F_{[2,114]}=0.508$, $P=0.603$). CCX volume was reduced by 16% in *mbmB*<sup>1</sup>(CS) females, 11% in *mbmB*<sup>1</sup>(CS)/CS females, 15% in *mbmB*<sup>1</sup>(CS) males and 8% in *mbmB*<sup>1</sup>(CS)/CS males compared to CS females and males respectively (Figure 2.1 D). There was a significant influence of sex ($F_{[1,114]}=42.896$, $P<0.0001$), genotype ($F_{[2,114]}=7.450$, $P=0.0001$) and their interaction ($F_{[2,114]}=5.371$, $P=0.0006$) on AL volumes. *mbmB*<sup>1</sup>(CS) females showed a 15% decrease in AL volume and *mbmB*<sup>1</sup>(CS)/CS females had a 6% reduction compared to CS. All males were smaller than wildtype and heterozygous females (Figure 2.1 E).
**mbmB Phenotype: External Anatomy**

Wing area and forelimb length were measured in homozygous and heterozygous *mbmB* flies to measure possible effects of the mutation on external anatomy. There was a significant influence of sex ($F_{[1,120]}=146.129$, $P<0.0001$), genotype ($F_{[2,120]}=29.292$, $P<0.0001$), and their interaction ($F_{[2,120]}=3.496$, $P=0.033$) on wing area. It was reduced by 24% in *mbmB*1(CS) females, 16% in *mbmB*1(CS)/CS females, 13% in *mbmB*1(CS) males and by 6% in *mbmB*1(CS)/CS males compared to CS females and males respectively (data not shown), I sequenced *Pen* because of its female sterility (Gorjánácz et *F*12,120=29.29, $P<0.0001$) (Figure 2.1 F). There was only a significant influence of sex ($F_{[1,127]}=14.705$, $P<0.0001$) on forelimb length. Genotype ($F_{[2,127]}=0.934$, $P=0.396$), and their interaction ($F_{[2,127]}=1.433$, $P=0.242$) had no effect on forelimb length. Males had shorter forelimbs than females, an expected sexual dimorphism (Figure 2.1 G).

**mbmB Phenotype: Female Sterility**

Homozygous *mbmB*1(CS) flies have previously been reported to be female sterile (de Belle & Heisenberg, 1996; Ginsburg, 2002). I was interested in using sterility as a simple screening phenotype for mapping *mbmB*. Verification that it shares a common genetic basis with mutant brain anatomy was necessary, so I devised a crossing scheme allowing an assessment of linkage (Appendix B.2). In the parental generation, *mbmB*1(CS)/SM5 females were crossed to CS males. The *F*1 *mbmB*1(CS)/CS female progeny were then crossed to *mbmB*1(CS)/*mbmB*1(CS) males. All *F*2 female progeny were sampled randomly.
for single female mating to CS males for 4 days and then subjected to paraffin mass histology to calculate MB calyx volumes. All fertile females had wildtype MBs, (presumably \textit{mbmB}^1(CS)/CS), and all sterile females had mutant MBs that were 50% smaller than CS and 46% smaller than non sterile flies (presumably \textit{mbmB}^1(CS)/\textit{mbmB}^1(CS)) ($F_{[2,68]}=70.59$, $P<0.0001$) (Figure 2.2). This was convincing evidence that sterility and brain anatomy are caused by the same genetic disruption in \textit{mbmB} flies and facilitated the use of sterility as the screening phenotype to map \textit{mbmB}.

\textit{mbmB}^1 is An Allele of The \textit{Drosophila importin-\alpha}2

I began mapping \textit{mbmB} with complementation analysis using a series of second chromosome rearrangements on the left arm (Table 2.1 B-C). Together the 164 lines I screened had 96% coverage of chromosome-2L. Female \textit{mbmB} flies were crossed to each line, and the female offspring were single female mated and screened for sterility. A subset of these flies were also tested for reduced MB calyx volume (Appendix Figure A.2, Table A.3). The deficiency \textit{Df(2L)8469} failed to complement \textit{mbmB}, as all heterozygous flies were both female sterile (data not shown), and displayed a reduced MB calyx volume ($F_{[8,153]}=237.59$, $P<0.0001$) (Figure 2.3 A, D). Analysis of both negative and positive complementation data revealed that \textit{mbmB} was located in region 30F4-30F6, which contained only 12 genes (Figure 2.3 A). I first began sequencing \textit{big brain} and \textit{CG13131} due to their known expression in the brain (Lyne et al., 2007). After finding no single nucleotide polymorphisms (SNPs) in these genes (data not shown), I sequenced \textit{Pen} because of its female sterility (Gorjánácz et
Figure 2.3 Mapping mbmB. (A) I used sterility to screen 168 second chromosome Dfs and found that Df 8469 (red) failed to complement mbmB. Sequencing of several genes in 30F4-30F6 revealed that mbmB had a point
mutation in Pendulin (Pen), the *Drosophila Importina2* gene. (B) *imp-a2* transcriptional unit with *imp-a2* null *P-c05212* and mbmB nonsense mutation (red asterisk) locations noted. (C) Chromatographs of sequence alignment made in DNA* for *Importina2*. Sequencing revealing that *mbmB* had an G to A transition in *Importina2* changing Tryptophan (TGG) to a pre-mature stop codon (highlighted in grey). This transition was unique to *mbmB* when compared to CS and Berlin wildtypes and the published wildtype sequence for *Importina2* (AAF52853). (D) MB calyx volume for *mbmB* flies heterozygous for *Df8469, P-c05212* and *imp-a2* as well as homozygous *imp-a2* flies were reduced (*F*[8,153]=237.59, *P*<0.0001). Bars represent mean ± SE of mean calyx volume for each genotype. *n* indicated on each bar. Different letters designate significant differences (SNK, *P*≤0.05).
The mutant allele of \textit{mbmB} had a G-to-A transition in \textit{Pen}, the \textit{Drosophila importin-\(\alpha\)2} at base pair 3,712. This nonsense mutation changed Tryptophan (TGG) to a premature stop codon (TGA) when compared with the two different wildtype strains I also sequenced (CS and \textit{Berlin}) and the published wildtype sequence for \textit{imp-\(\alpha\)2} (AAF52853) (Figure 2.3 C). Additional P-element inserts from collections at Bloomington and the Harvard stock centers in 30F4-30F6 were analyzed concurrently (Table 2.1 C). Our complementation tests further confirmed this finding in that \textit{P-c05212} (inserted in the second exon of \textit{imp-\(\alpha\)2}) failed to complement \textit{mbmB}, as the \textit{mbmB/P-c05212} heterozygotes were female sterile (data not shown) and had reduced MB calyx volumes \((F_{[8,153]}=237.59, P<0.0001)\) (Figure 2.3 B, D). \textit{imp-\(\alpha\)2\(^0\)} homozygotes and \textit{mbmB/imp-\(\alpha\)2\(^0\)} were female sterile (data not shown) and displayed reduced MB calyx volumes \((F_{[8,153]}=237.59, P<0.0001)\) (Figure 2.3 D).

\textit{mbmB}\(^1\) Mutants Lack Full Length Imp-\(\alpha\)2 Protein

To determine whether this nonsense mutation caused a functional change at the protein level, I performed western blots on whole body extracts from \textit{w\(^{1118}\)} (CS), \textit{w;mbmB}\(^1\)(CS) and \textit{imp-\(\alpha\)2\(^0\)}. I used a rabbit anti-Imp-\(\alpha\)2 primary antibody raised against amino acids #279-522 (Török et al., 1995; Gorjánácz et al., 2006). I have previously shown (above) that the nonsense mutation in \textit{mbmB} is located at amino acid #261, upstream of the epitope binding site for the antibody. On our western blot, the 56 and 58 kDa bands present in \textit{w\(^{1118}\)} (CS) control flies were absent in both \textit{w;mbmB}\(^1\)(CS) and \textit{imp-\(\alpha\)2\(^0\)} (Figure 2.4 C). I concluded that the
Figure 2.4 Immunohistochemistry shows Imp-α2 expression in central brain neuropil and western blot analysis reveals mbmB is lacking the second half of Imp-α2. For A and B: GFP (green) expressing in MB Kenyon cells. (A) CS; P[GFP]nls;; P[Gal4]Ok107 showed Imp-α2 expression throughout the neuropil of the adult brain (red), with some overlap in MB Kenyon cells (yellow). (B) mbmB-P[GFP]nls;;P[Gal4]Ok107 has no expression of Imp-α2 throughout the brain. (C) Two SDS-Page gels were run with samples of whole body protein extracts, blotted, trimmed and incubated with either the Imp-α2 Ab, or the α-Tub Ab (loading control). These blots reveal that mbmB, like the imp-α20 extract, is lacking the full length Imp-α2 protein.
stop codon sequenced in *mbmB* is responsible for *mbmB* mutant alleles lacking a full length Imp-α2 protein.

**Imp-α2 Expresses In Neuropil Throughout The Brain**

It is known that many genes central to learning and memory express their protein products in the MBs (Crittenden et al., 1996). I felt that looking at the expression patterns of Imp-α2 in the brain and more specifically in the MBs would provide us with valuable information regarding its cellular function during MB development and possibly during associative behaviors. I found that in CS Imp-α2 is expressed in neuropil throughout the adult brain, including many MB cells (Figure 2.4 A), and is essentially absent in *mbmB* (Figure 2.4 B). Preliminary data on the expression pattern of Imp-α2 in wandering third instar larvae support its possible role in MB development (Appendix Figure A.4).

**Rescue of mbmB Mutant Phenotypes With imp-α2 cDNA**

In addition to a reduced MB dendritic volume, *mbmB* mutants display axonal patterning defects as well as a reduction in MB cell number (Dunkelberger 2008). To verify that *imp-α2* is responsible for generating these MB phenotypes as well as female sterility, I performed a rescue using transgenic flies with a cDNA corresponding to *imp-α2* (Gorjánácz et al., 2006) under Gal4/UAS control. To look at the MB phenotypes, I used several MB specific drivers that express in all subsets of MB lobes: *P[Gal4]c772* (Armstrong et al., 1995), *P[Gal4]247* (Schultz et al., 1996) and *P[Gal4]Ok107* (Connolly et al., 1996) (Table 2.1 A). All drivers and the cDNA transgene were placed in the *mbmB* background and then crossed together to obtain the “rescue” flies (*mbmB* + Driver + cDNA) (Appendix B.4).
**P[Gal4]c772** driving *imp-\(\alpha\)2* cDNA in homozygous *mbmB* flies leads to a rescue of the MB calyx volume. *mbmB*-c772 has a MB calyx volume that is 54% of the wildtype volume, while the rescue flies (*P[Gal4]c772* driving *imp-\(\alpha\)2* cDNA in homozygous *mbmB* flies) have a MB calyx volume that is 75% of the wildtype volume (*F*\(_{4,42}\)=89.60, *P*<0.0001) (Figure 2.5 A).

**P[Gal4]Ok107** is located on the 4th chromosome (where there are no chromosomal balancers), and exhibits expression not only in the MBs, but also in the ovaries (Serway, unpublished). Unfortunately flies with 2 copies are unstable as homozygotes. Therefore to obtain a “rescue” fly I was able to trace *imp-\(\alpha\)2* by selecting against the third chromosome balancer. To verify the presence of **P[Gal4]Ok107**, I screened for female sterility. Sterility was rescued in a subset of female flies, assumed to be *w; mbmB; imp-\(\alpha\)2* cDNA; **P[Gal4]Ok107**. I then calculated MB calyx volumes for these females as well as the respective controls. (Appendix B.4). *mbmB;Ok107* has a MB calyx volume that is 59% of the wildtype volume, while the “rescue flies” have a MB calyx volume that is 89% of the wildtype volume (*F*\(_{4,39}\)=67.46, *P*<0.0001) (Figure 2.5 A).

**P[Gal4]247** driving *imp-\(\alpha\)2* cDNA in homozygous mbmB flies leads to a partial rescue of the MB calyx volume as well. *mbmB;247* had a MB calyx volume that is 42% of the wildtype volume. The 247 “rescue flies” had a MB calyx volume that is 70% of the wildtype volume (*F*\(_{4,40}\)=83.06, *P*<0.0001) (Figure 2.5 A).

Similar results were observed with MB cell number when two different MB specific Gal4 drivers were used to drive UAS-GFP.nls in MB kenyon cells. *w;Ok107* showed GFP expression in 1470 cells while *w;mbmB;Ok107* expressed
Figure 2.5 *imp-α2* cDNA driven in the MBs rescues anatomical phenotypes. (A) MB calyx volume was rescued with three different MB specific Gal4 lines driving *imp-α2* cDNA in the MBs. *P*Gal4*]c772* driving *imp-α2* cDNA
in homozygous mbmB flies rescued 75% of the wildtype MB calyx volume. Using
P[Gal4]Ok107, 89% of the wildtype volume is rescued. P[Gal4]247 rescued 70% of the wildtype volume. Bars represent mean ± SE of mean calyx volume for each genotype. n indicated on each bar. Different letters designate significant differences (SNK, P≤0.05). (B) Representative images from confocal Z-stacks of CS and mbmB showing GFP driven in the nucleus of MB Kenyon cells. (C) MB Kenyon cells were counted from every 7th section of a z-stack using Image J. imp-α2 cDNA driven in mbmB with Ok107 rescued 91% of the wildtype cell count. Imp-α2-cDNA driven in w;mbmB;247 showed a 71% rescue of the wildtype cell count. Bars represent mean ± SE of mean calyx volume for each genotype. n indicated on each bar. Different letters designate significant differences (SNK, P≤0.05).
GFP in 700 MB cells, 48% of the wildtype ($F_{[2, 34]} = 135.42$, $P<0.0001$). When the
imp-$\alpha 2$ cDNA was driven in mbmB with Ok107, there was a partial rescue to
1298± cells, an increase to 91% of the wildtype ($F_{[2, 34]} = 135.42$, $P<0.0001$)
(Figure 2.5 B-C; Appendix B.5). w;247 showed GFP expression in 960 cells,
while w;mbmB;247 expressed GFP in 454 cells, 47% of the wildtype ($F_{[2, 35]} = 148.89$,
$P<0.0001$). imp-$\alpha 2$ cDNA driven in w;mbmB;247 showed a partial rescue as well expressing in 686 cells, 71% of the wildtype ($F_{[2, 35]} = 148.89$, $P<0.0001$) (Figure 1.5B-C; Appendix B.5).

I was unsuccessful in my attempted to rescue the behavioral phenotypes
associated with mbmB with a full length imp-$\alpha 2$ cDNA driven in the MBs due to
technical issues (see details in Appendix Figure A.5).

My rescue data for sterility, MB calyx volume and MB cell number in mbmB
mutants suggests that: 1.) imp-$\alpha 2$ expression in the ovaries is sufficient to rescue
the sterility defects seen in mbmB, and 2.) imp-$\alpha 2$ expression in the MBs is
necessary but not sufficient for complete rescue of the MB defects observed in
mbmB mutants.

All imp-$\alpha 2$ Domains Influence MB Development

imp-$\alpha 2$ consists of several well-characterized domains with highly conserved
functions across species, facilitating its role as an adaptor protein. It is capable of
binding other imp-$\alpha '$s, imp-$\beta '$s and NLS-bearing cargo for nucleo-cytoplasmic
taxficking (Figure 2.6 A) (review: Goldfarb et al., 2004). Each domain was
disrupted in a suite of UAS imp-$\alpha 2$ transgenes driven in the MBs by c772 in the
homozygous mbmB background. I found that there was no influence of sex
Figure 2.6 Analysis of imp-α2 Domain function on MB development. (A) Schematic diagram of imp-α2 transgenes used to disrupt individual domains of imp-α2 (diagram modified from Gorjánácz et al. 2006). (B) Histological data shows that all domains of imp-α2 are necessary for proper MB development. Bars represent mean ± SE of mean calyx volume for each genotype. 8 ≤ n ≤ 12 /bar. Different letters designate significant differences (SNK, P≤0.05).
(F[1,163]=2.854, P=0.093) or the interaction of sex and genotype (F[19,163]=0.775, P=0.733) on MB calyx volume. There was however an effect of genotype (F[19,163]=10.548, P<0.0001). Most of the domains in a homozygous mbmB background with the driver only showed reduced MBs, indicating that the transgenes themselves had no effect on MB structure when they were not driven (Figure 2.6 B). This reduced MB phenotype remained in the presence of the c772 MB driver. The S56A and SNLSB- strains displayed intermediate MB calyx volumes in the absence of the driver, yet showed significant reductions in the presence of the c772 MB driver. This indicates that all of imp-α2’s domains are necessary for MB development.

Discussion

The structure function relationship between the brain and behavior is deeply rooted in molecular neurobiology, allowing researchers to assign physiological processes occurring in neuronal networks to functional outcomes at the whole organism level. I was interested in determining how the molecular composition of the mutant allele mbmB gave rise to its interesting anatomical and behavioral phenotypes. First, I characterized the brain anatomy of mbmB. This included measurements of MB, CCX and AL. I quantified the MB defect showing that their reductions were the site of the most severe anatomical defect seen in mbmB mutants, as the MBs were reduced by roughly 50% in dendritic volume and cell number. I then went on to show that mbmB is Pen, the Drosophila importin-α2, a carrier protein central to nuclear cytoplasmic transport. I rescued the anatomical
phenotypes by driving an *imp-α2* cDNA in the MBs. The expression pattern of Imp-α2 in the adult brain was investigated, as well as the role each domain of the protein plays on MB development. My work represents the first molecular characterization of this 25-year old mutant with severe anatomical and behavioral defects, and also brings to light a new role for *imp-α2* in MB development and associative conditioning.

*imp-α2* belongs to a multigene family of evolutionarily conserved proteins called karyopherins. They have been known to function as soluble nuclear transporters taking cargos across the nuclear pore complex (Review: Mossammaparast & Pemberton, 2004; Tran & Wente, 2006; Stewart, 2007). Within the karyopherin family, Imp-αs are part of the Armadillo (ARM) domain protein family. Members of this family have 10 ARM repeats, each a 42 amino acid motif initially found in the *Drosophila* segment polarity gene *armadillo* (Andrade et al., 2001). Usually, Imp-αs bind classical NLS-bearing cargos directly at either one or both of their NLS binding sites. The larger NLS binding site is referred to as a mono-partite or large NLS motif (LNLSB) and is found at ARM 3-4. The smaller one is commonly called the bi-partite or small NLS binding motif (SNLSB) and is found between ARM 7-8. Once the NLS-cargo is bound to Imp-α2, it usually binds Imp-β at a carboxyl domain called the IBB domain, and the entire complex then passes through the NPC into the nucleus (Weis, 2003). Upon arrival in the nucleus, the Imp-α2/CARGO/Imp-β complex is disassociated through the binding of RanGTP to Imp-β. Imp-α releases its cargo and is recycled back into the cytoplasm via interactions with a complex of RanGTP and
cellular apoptosis susceptibility protein (CAS) (Hood & Silver, 1998; Kutay et al., 1997). When it returns back into the cytoplasm, the Imp-α2/CAS/RanGTP complex disassociates as RanGTP is converted into RanGDP allowing the cycle to start over again (Kuerston et al., 2001).

Importin’s do more than just transport proteins across the NPC, as they are involved in neuronal development and functional connectivity (Ting et al., 2007), retrograde injury signaling to the nucleus from distal axons (Hanz et al., 2003; Review: Yudin et al., 2008), eye development (Kumar et al., 2001), and even long term synaptic plasticity (Thompson et al., 2004). It is likely though that these additional roles of Importins are not independent of their central role in nuclear-cytoplasmic trafficking, but may be an extension of it.

The characterization of mbmB as imp-α2 provides a novel, mechanistic explanation for the neuronal defects and behavioral plasticity our lab has seen in mbmB mutants. It implicates biochemical processes including nuclear-cytoplasmic trafficking and actin-cytoskeletal movement in the axon as components of MB development, learning and long term memory formation. mbmB has previously been shown to have disrupted associative olfactory conditioning, in particular learning, ARM and LTM defects (Dunkelberger, 2008). The molecular characterization of mbmB as imp-α2 will allow us to associate known cellular functions of imp-α2 with anatomical and behavioral phenotypes seen in mbmB mutants.

The Relationship Between MB Cell Number and Learning

Our lab has used two approaches to show that a critical number of MB
cells are required for normal learning. Dunkelberger (2008) used behavioral and structural analysis of several MB structural mutants to show that the percent reduction in MB cell number was directly correlated to the reduction in learning. Wang et al., (2007) exposed wildtype flies to heat stress causing a reduction in MB cell number which was also shown to be directly correlated with poor olfactory associative conditioning scores. I believe that MB cells function together in an additive way providing the necessary salience for learning. Therefore when there are fewer MB cells, there is a reduction in performance because the overall signal is reduced. Each MB is derived from only four MB neuroblasts dividing continuously throughout pre-adult development (Lee et al., 1999; Ito & Hotta, 1992). In contrast, most other neuropil arise from increased neuroblast division during defined developmental time-points (Truman, 1990; Ito & Hotta, 1992). This small number of MB progenitor neuroblasts in combination with their continuous division leaves MB cells extremely susceptible to developmental abnormalities (Heisenberg et al., 1995) and environmental influences (Technau, 1984; Wang et al., 2007) that may change their cell fates and or lifespan. The reduction in cell number and olfactory associative learning defect observed in mbmB provide further support to the argument that there is a critical MB cell number necessary for learning.

I have rescued the reduced MB cell number through UAS driven imp-α2 transgene expression in the MBs. Previous work from our lab has shown that this reduction in MB cell number does not happen until late third instar (Dunkelberger 2008; Ginsburg, 2002). Characterization of mbmB as imp-α2 indicates several
possible molecular mechanisms responsible for this reduction discussed below.

Several groups have shown that Imp-αs are required for the transport of proteins involved in cell cycle regulation (Middeler et al., 1997; Thomas et al., 1996; Wang et al., 1997; Kim et al., 2000). Nuclear cytoplasmic recycling is mediated by CAS, which has a central role in the regulation of cell cycle checkpoints and apoptosis (Review: Behrens et al., 2003). If there is no Imp-α2 to shuttle CAS from the nucleus back into the cytoplasm, the cell cycle may be disrupted, initiating the apoptotic pathway, and potentially causing the reduction in MB cell number seen in mbmB mutants. My histological analysis of domain specific disruptions in imp-α2 driven in the MBs has shown that the CAS binding domain is necessary for proper MB development. Cell cycle regulation and prevention of apoptosis have also been implicated in MB neuroblast proliferation through mutant analysis of the orphan nuclear receptor, Tailless (TLL) (Kurusu et al., 2009). To test this hypothesis, TUNEL staining could be used to detect cells undergoing apoptosis during late third instar in mbmB mutants, the developmental time when the reduction in MB cell number occurs (Dunkelberger, 2008; Ginsburg, 2002).

Another possible explanation for the reduction in MB cell number and associated reduction in learning seen in mbmB may be the role that imp-α2 plays in mitotic spindle development. Correct orientation of the spindles is central to accurate cell fate determination of sensory organ precursors (SOP) (Tekotte et al., 2002) and neuroblast homeostasis (Calbernard & Doe, 2009). Interestingly, Imp-α2 regulates spindle formation in Xenopus egg extracts (Gruss et al., 2001),
and Imp-β, RanGTP and CAS are also known to have a functional role in mitotic spindle formation (Dasso, 2001). Imp-α2 is also required for assembly of the ring canal during oogenesis (Gorjánácz et al., 2002; Gorjánácz et al., 2006) causing the null to be female sterile. Female sterility has been observed in several genes with known roles in MB development as well: mbmB, small mushroom bodies (smu), mushroom body defect (mud) and calyx bulging (cbx) (de Belle & Heisenberg, 1996; Dunkelberger, 2008). Until now mud was the only one of these mutants that had been cloned and molecularly characterized (Raabe et al., 2004). mud, like imp-α2, is associated with meiosis II spindle formation in oocytes where it shuffles between the spindle and the nuclear envelope (Yu et al., 2003). I propose that imp-α2 utilizes the same mechanism to regulate MB number and oocyte development, either through the regulation of cell cycle/apoptosis during development (as mentioned above) or regulating the transition from asymmetric to symmetric cell division. Both possibilities likely involve regulation of spindle formation, as either could cause the observed reduction in MB cell number or non-functional oocytes and consequent sterility. Siller & Doe (2009) developed a model for the role of mitotic spindles in asymmetric cell division of neuroblasts. In this model Mud is part of complex anchored to the centrosome. A dynein complex is bound to the mitotic spindle, and it is the interaction between these two complexes that pull the mitotic spindles to the apical pole eventually giving rise to two neuroblasts. The complete mechanism for their contact is unknown. I propose that Imp-α2 is the unknown link between Mud and the dynein complex. Interestingly, mutations in
the dynein light chain (*roadblock*), the dynein heavy chain (*Dhc64*) and the linker between the two (*Lis1*) all display reduced cell number in neuroblast clones, reduced dendritic growth and branching and defective axonal transport in MB neurons (Reuter et al., 2003, Liu et al., 2000). In vertebrates, Imp-α’s are associated with the dynein motor proteins (review: Perry & Fainzilber, 2009). The interaction between Imp-α2, Mud and the dynein complex in MB neuroblast proliferation is an attractive model for the regulation of MB cell number that our lab is actively pursuing.

### The Relationship Between *imp-α2* Signaling and LTM

Imp-αs are known to be associated with neurological disorders including Schizophrenia (Wei et al., 2004; Wei et al, 2005; Wu et al., 2008; Liu et al., 2007) and Alzheimer’s disease (Zhang et al., 2006; Ogawa et al., 2003; Lee et al., 2006). A fly model for Alzheimer’s disease has recently been used to investigate the genetic and molecular contributions to the regulation of LTM (Song et al., 2009; Presente et al., 2004, Ge et al., 2004). The CREB family of transcription factors are also central to the formation of LTM through transcriptional regulation (Yin et al., 1995a; Yin et al 1995b; Yin & Tully 1996; Perazzona et al., 2004; Tully et al., 1994). CREB2, the transcriptional repressor known to modulate synaptic plasticity and LTM, has recently been identified at distal dendrites in rodent hippocampal neurons where it is also bound to Imp-α. This interaction is necessary for its movement from the synapse to the nucleus to regulate transcription (Lai et al., 2008). Dunkelberger (2008) has shown that *mbmB* mutants are deficient in LTM, and I have shown that the NLS binding sites in imp-
α2 are necessary for MB development, yet whether or even how CREB and imp-α2 interact to generate LTM is not known. One possibility is that Imp-α2 is necessary for binding CREB and then mobilizing it to the nucleus along microtubules, as CREB has an NLS signal (Waeber & Habener, 1991). Once it arrives at the nuclear envelope, it is transported across the NPC into the nucleus to initiate transcription of its downstream target genes. CREB may be stored in the axons as mRNA and transcribed only in the presence of a signal (i.e. LTM). Imp-αs are constitutively associated with retrograde motor dynein along axons, and in response to an injury signal, facilitate the movement of NLS cargos from the axon to the nucleus after local translation of Imp-β mRNA (Hanz & Fainzilber, 2006). CREB may be one of these NLS cargos whose binding to Imp-α2 is initiated by LTM.

A microarray experiment immediately following LTM training and testing as well as single gene mutant analysis revealed that staufen, oskar, CPEB, eIF-2G, eIF-5C and pumilio are involved in local control of RNA translation and LTM (Dubnau et al., 2003). eIF-2α has also been shown to play a central role in translational control of hippocampal synaptic plasticity and memory formation in mice (Costa-Mattioli et al., 2005). Pumilio expresses in the ovary and border follicle cell (Barker et al., 1992; MacDonald et al., 1992), has a female sterile allele similar to mbmB (Forbes & Lehmann, 1998) and is necessary for correct dendritic development and remodeling in DA neurons (Ye et al., 2004). These translational repressors may also be interacting, either aiding or competing, with CREB for its binding site on Imp-α2. They may even represent additional
signaling pathways that interact with Imp-α2 in response to LTM. Identification of the binding partners will provide us with a better understanding of what these factors are and how they may or may not interact with CREB to initiate LTM in the axons. My lab is currently working with the Yin lab at the University of Wisconsin Madison to address whether CREB and Imp-α2 are bound in cytoplasmic and nuclear fractionations (Appendix Figure A.6).

The Relationship Between Imp-α2 Signaling and ARM

mbmB mutants are also deficient in ARM (Dunkelberger 2008), a phase of memory known to be independent of protein synthesis, and the cAMP signaling cascade, unlike LTM, which is protein synthesis dependent and involves CREB (Tully et al., 1994). I propose that in addition to translational regulation of transcription necessary for LTM, mbmB also plays a role in transcriptional initiation, which is necessary for ARM. mbmB does so through Imp-α2 binding of NLS-bearing transcription factors (TFs) for localization into the nucleus. This can then initiate transcription of new mRNAs, to be transported out of the nucleus, where they can be stored for translation at a later time. This is a new way to think about the relationship between LTM and ARM. ARM may depend on transcriptional initiation between the cytoplasm and the nucleus, while LTM may reflect translational regulation in the axons that then influences transcription in the nucleus in a cyclic fashion based on the salience (strength, duration, and pattern) of the stimuli (training). Identification of Imp-α2 binding partners will shed light onto additional signaling pathways outside of the cAMP cascade, providing new candidate TFs that may be responsible for the regulation of ARM and its
possible influence on transcription.

Adult *mbmB* Mutants Exhibit No Neurogenesis or Neurodegeneration In the First Week of Life

To investigate whether adult neurogenesis, neurodegeneration, or even experience dependent structural plasticity is a component of either MB development or function, I measured MB calyx volume over time in flies aged from <1 to 7 days old. I have shown that there is a significant reduction in MB calyx volume in homozygous *mbmB* flies compared to wildtype and heterozygotes, and that this remains constant for up to one week after eclosion. Since the CS MB calyx volume did not increase with age, I suggest that under normal conditions there is probably no adult neurogenesis or neurodegeneration in the MBs during the first week of adult life. I also conclude that the defects in MB morphology observed in *mbmB* adult flies, specifically at the level of the dendrites, must happen prior to eclosion. This is consistent with the findings of Dunkelberger (2008) and Ginsburg (2002). The MB defect occurs during the third instar, and there are likely no further disruptions to neurogenesis in the *mbmB* mutants.

**Influence of imp-α2 on External Anatomy**

An analysis of leg length and wing area was conducted to determine whether the reductions observed in the MBs of *mbmB* mutants were structure specific or were present in other structures of the fly. I found no change in leg length compared to CS, and a slight, yet significant reduction in wing area. The discrepancies between these two structures may be explained by the fact that
the *Drosophila Importin 7* (*DIM-7*), the *Drosophila* homolog to mammalian Importin 7 (a member of the Imp-β family), has been shown to have effects on cross vein placement in the wing, inducing blistering when over-expressed (Baker et al., 2002). This work also indicates a functional connection between Imp-β and Integrins for nuclear cytoplasmic transport of growth factor signals critical for proper wing development. I occasionally observed wing blistering in *mbmB* mutant flies, although its frequency was not quantified. It would be interesting to investigate the frequency of blistering and possible cross vein placement defects in the wings of *mbmB* mutants, therefore making a more direct link between wing disc development and an *imp-α*. I would then be able to determine whether the reduction in wing area in *mbmB* mutants is a wing specific phenotype, or if it is a pleiotrophic effect of cell proliferation or guidance defects throughout the fly.

**All Domains of *imp-α2* are Necessary for MB Development**

I have shown that all of the functional domains of *imp-α2* are necessary for MB development, suggesting that the protein functions as a whole. I have previously discussed a possible role for the CASB, NLSB, and SNLSB domains. My data for the IBB domain, taken together with the literature, indicates that Imp-β binds to Imp-α2, and that this heterodimer is necessary for MB development. The involvement of the DIM domain indicates that correct Imp-α2 confirmation is also a necessary component of MB development. Phosphorylation of Imp-α2 is also required for MB development as indicated by data for all of the phosphorylation sites in *imp-α2* (*S^37_A, S^56_A, S^98_A* and *3xSA*). In our current
experimental set up, I only analyzed the role of all imp-\(\alpha2\) domains on MB development. In the future, we would like to distinguish the importance of each domain post developmentally and possibly assign each a role in different aspects of associative conditioning (as outlined above). Disruption of Imp-\(\alpha2\) binding partners, like CAS, Imp-\(\beta\) or it’s NLS-bearing cargos will be necessary to yield more detailed information about how Imp-\(\alpha2\) functions at a molecular and cellular level to regulate brain development and behavior.

*mbmB* is Missing the Full Length Imp-\(\alpha2\)

My results show that *mbmB* is lacking the full length Imp-\(\alpha2\) protein, which could be interpreted in one of two ways. *mbmB* is either an Imp-\(\alpha2\) protein null or is degraded perhaps through nonsense mediated decay machinery in the cell (Hanson et al., 2009). To distinguish between these two possibilities, I would need an antibody raised against any group of amino acids upstream of #261 (where *mbmB*’s premature stop is located). If *mbmB* was truncated, a western blot of *mbmB* with this Ab would show a smaller size band.

Imp-\(\alpha2\) Expression in The Adult Brain

Imp-\(\alpha2\) is expressed in neuropil throughout the adult brain, including MB cells. This analysis of the Imp-\(\alpha2\) expression pattern in the brain is very informative in concert with our preliminary L3 expression profile (Appendix Figure A.4). Imp-\(\alpha2\) is required for accurate development of the MBs (and may express in all dividing neuroblasts as I have seen in my preliminary data), but may only be present in a subset of cells post-developmentally (which I see in the adult MB’s expression of Imp-\(\alpha2\)). Similarly, it is interesting that Imp-\(\alpha2\) expression was not observed in
the ring canals, although Kelch function (critical for ring canal development) was disrupted in the *imp-α2* null (Gorjánácz et al., 2002). These authors propose that *imp-α2* brings Kelch to the ring canals for its function, implying that the absence of *imp-α2* in the wildtype ring canal does not exclude it from being a necessary component of ring canal development (Gorjánácz et al., 2002). This same principle can be applied to MB development and function. A carefully timed developmental experiment of Imp-α2 expression in the brain would resolve these questions in addition to determining whether or not it was located in the cytoplasm or nucleus.

**Conclusion**

The findings of this study clearly establish that *mbmB* is *imp-α2*, encoding a novel factor associated with MB gross morphology and development as well as learning, LTM and ARM. I rescued the anatomical phenotypes by driving an *imp-α2* cDNA in the MBs, and investigated the expression pattern of Imp-α2 in the brain. All domains of the protein appear to play a critical role in MB development. This work provides the fuel for further investigations into how mechanisms like nucleo-cytoplasmic trafficking, mitotic spindle formation, retrograde axonal signaling, and the translational regulation of transcription influence brain development and its role in complex behaviors like learning and memory. Further examination of the spatial and temporal distribution and regulation of Imp-α2, and identification of its binding partners throughout development and during
associative behaviors will surely lead to the discovery of additional genes and mechanisms associated with MB development and function.
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CHAPTER 3
MOLECULAR LOCALIZATION OF MB-SPECIFIC GAL4 LINES IDENTIFIES CANDIDATE GENES ASSOCIATED WITH MB DEVELOPMENT

Abstract

The enhancer trap system is a versatile tool with unique applications in \textit{Drosophila} genetics facilitating the visualization of tissue-specific gene expression. It can serve as a cell type marker useful for looking at developmental morphologies, or it can be used to target the expression of known genes into specific tissue types. When transgenic constructs like P\[Gal4\]'s are created, reporter expression is determined by genomic enhancers located near the site of its insertion. This means that the spatial and temporal aspects of the expression pattern found in each line is unique to the site of its insertion, allowing the lines themselves to serve as molecular beacons for the identification of novel genes based on their expression patterns. In addition to serving as tools to locate novel genes, P\[Gal4\] insertions can disrupt gene function acting as more traditional transposon mutagens. Based on this logic, I evaluated the anatomical characteristics of 10 mushroom body specific Gal4 lines to determine their exact insertion sites in the genome as well as whether they affected MB structure. These lines have traditionally been used as cell type markers, allowing behavioral and structural analysis of the mushroom bodies (MB's) in mutant backgrounds. In this study, I characterized 10 Gal4 insertion sites using inverse PCR and then sequenced the flanking genomic DNA for each of MB Gal4 lines. An anatomical analysis of each line was also conducted to determine whether
they were affecting MB structure. This comprehensive analysis of Gal4 insertion sequence locations brought to light new candidate genes having potential roles in MB development and behavior. I have found a suite of interesting genes, several disrupted by the insertion, and several with MB structural defects caused by the insert itself. These genes range from learning and memory associated genes to those involved in axonal path finding or others with just a CG number and little to no associated research. They were all identified based on their marker expression in the mushroom bodies. I propose that these genes are exciting and potentially important candidates for future investigations into MB development and its associated role in associative conditioning.

Introduction

Systematic genetic screens have uncovered numerous genes over the years with functions at the single cell level all the way to that of the whole organism have given shape to the field of *Drosophila melanogaster* development and neurobiology. It began more that a century ago with Thomas Hunt Morgan’s studies on the white-eyed pigment mutation and the chromosomal theory of inheritance (review: Benson, 2001). Since then *Drosophila* research has evolved to offer numerous unique methods for the disruption of behaviors, development, anatomy, genetics, molecular pathways, and the cell itself, in defined space over the course of time. Forward genetic screens have allowed us to identify novel genes associated with a number of interesting phenotypes in an unbiased way (review: St. Johnston, 2002), while reverse screens give us the ability to
characterize new alleles of previously identified genes through detailed analysis of their sequence (review: Adams & Sekelsky, 2002). The battery of tools in the *Drosophila* geneticist’s toolbox facilitates analysis of relevant phenotype through enhancement, suppression, activation, repression, over-expression, mis-expression and even knock out of gene function. It is possible to look at dosage, or gene copy number and its effects on the organism (a physical phenotype) as well as the rest of the genome (regulation or influences on the expression of other genes). This is the intersection of genetic interaction and functional mapping, yet the full potential of this great suite of tools right at our fingertips has not been completely realized, as the field continues to move forward.

Perhaps the most influential component of *Drosophila* genetics is the Gal4/UAS system, which allows temporal and spatial control of gene expression (Brand & Perrimon, 1993). This bipartite system consists of the *Saccharomyces cerevisiae* transcription factor Gal4 (the driver) in one element, and the upstream activating sequence (UAS)-dependent transgene in a second element. These constructs are injected into the germlines of different strains and insert randomly into their genomes. The two strains are then crossed together and screened for expression patterns of interest. Transcription is induced (of a cDNA transgene consisting of either a reporter or any other cloned gene of interest) only in the presence of the Gal4 element (review: Elliott & Brand, 2008). As the UAS can be engineered in front of any gene, this system has driven a wide variety of genes for functional investigation in any tissue type. These have included toxic genes such as *ricin a* (Scuderi & Letsou, 2005), apoptotic regulators including *reaper* or
head involution defective (hid) (Hidalgo & Brand, 1997, Zhou et al., 1997) or tetanus toxin in the nervous system (review: Martin et al., 2002), numerous genes typically lethal during development and most commonly GFP (review: Duffy, 2002) providing targeted gene expression with exacting spatial resolution.

As most genes in Drosophila are pleiotrophic (Perrimon et al., 1989; Miklos & Rubin, 1996), the Gal4/UAS allows the analysis of gene functions at specific times during development through the use of a temperature sensitive (TS) promoter (Lis et al., 1983). Temporal resolution using defined promoters with known expression patterns such as the pan neuronal pattern of the embryonic lethal abnormal vision (ELAV) promoter (Yao & White, 1994) or ubiquitous promoters like tubulin (Bialojan et al., 1984) have facilitated a great deal of developmental analysis as well.

The P[Gal4] construct was inserted randomly in the fly genome under the control of local enhancers, whose expression patterns are determined by screening reporters like UAS-LacZ or UAS-GFP in a tissue of interest. I have focused on a subset of Gal4 lines that express preferentially (although not exclusively) in a fly brain structure called the corpora pedunculata or mushroom bodies (MBs), second order sensory integration centers of the insect brain known to be involved in olfactory associative conditioning in Drosophila (de Belle & Heisenberg, 1994; reviews: Heisenberg, 2003; Davis, 2005). MBs are paired neuropilar structures in the protocerebrum composed of approximately 2,500 densely packed intrinsic neurons called Kenyon cells. Each Kenyon cell sends out dendritic projections, collectively called the calyx. The calyx receives
olfactory information from the antennal lobe via the antennal cerebral tract (Heisenberg, 1998). The Kenyon cell axons project rostrally below the calyx as a structure called the pedunculus, which then bifurcates and gives rise to a series of lobes including the α, α’, β, β’ and γ, lobes. The commonly used MB-specific Gal4 lines show a wide range of expression patterns within the mushroom bodies, as some express in every class of MB neurons throughout the structure (e.g. OK107), while others express in a specific subset of the lobes (e.g. H24). Table 3.1 provides a detailed list of the MB lobe specific expression patterns for each Gal4 line used in this paper as well as their original cytological insertion site. Traditionally these lines have been used to drive genes in subsets of the MBs to investigate their roles in development of the MBs (Tomchik & Davis, 2009; Dunkelberger, 2008; Ito et al., 1997; Kurusu et al., 2000, Sentry et al., 1994, Yang et al., 1995). Additionally, behavioral analysis linked to MB functions including associative conditioning (Song et al., 2009; Krashes et al., 2007; de Belle & Heisenberg, 1996; Dubnau et al., 2001; McGuire et al., 2001;), sleep (Joiner et al., 2006, Pitman et al., 2006, Seugnet et al., 2008), aggression (Rollmann et al., 2008, Edwards et al., 2006), motor activity (Besson & Martin, 2005) and aspects of courtship memory (Joiner & Griffith, 2000; McBride et al., 1999) have been investigated using variations on the Gal4 system. Neurons within as well as those that innervate the MB are also manipulated with the Gal4 system for functional investigations of neurotransmitters (Krashes et al., 2009; Tsydzik & Wright, 2009; Andretic et al., 2008). Various modification of the Gal4 system have successfully been used to study the development of the MB through
single gene mutations (Raabe et al., 2004; Martini & Davis, 2004; Orihara-Ono et al., 2005), clonal analysis (Ito et al., 1997) and mosaic analysis (Reuter et al., 2003). More recently the Gal4-UAS system in combination with the FLP recombinase-FRT and fluorescent reporters, (known as G-TRACE) has been used to trace individual cell lineages and screen their expression patterns in real time (Evans et al., 2009).

The enhancer trap localization approach has facilitated the identification of many new genes based on their spatial and temporal expression patterns (O’Kane & Gehring, 1987; Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989; Molnar et al., 2006). Boquet et al., (2000) used this method to identify new genes involved in central brain and midline development, including the *alpha lobes absent* mutant (*ala*), which was later shown to be associated with long term memory (Pascual & Préat, 2001). Dura et al., (1993) also used this method to identify *linotte* (*derailed*) as a memory mutant, whose amorphic deletion was later characterized as having severe disruptions to the MB (Simon et al., 1998; Moreau-Fauvarque et al., 1998).

I proposed a similar method for identification of novel genes involved in MB structural development by localizing the site of insertion for ten MB specific Gal4 lines. I also propose that the inserts themselves may alter MB development by disrupting local gene expression. I have identified the sequence location of ten MB specific Gal4 lines and tested them for gross anatomical defects in the MBs. I found minimal reductions in the structure at the gross morphological level for most of the Gal4 lines. I also looked at the published expression patterns of the
genes that these Gal4 lines either insert in or fall in close proximity to, and found that several of them have expression either in the CNS or more specifically in the MBs. There was a reduction in protein levels for three Gal4 lines, indicating that they disrupt normal protein production and may influence its function, possibly related to that of the MBs. Over all, gene discovery based on expression analysis and localization of Gal4 lines has provided us with several new genes implicated in MB development and may also important for behavior.

Materials and Methods

**Fly Strains**

A wildtype *Canton Special* line derived from Würzburg stocks (CS), as well as *white*\(^{1118}\) (*w*\(^{1118}\)) (FBst0307124) backcrossed for seven generations to CS, were used as controls in all experiments listed. Ten Gal4 lines were selected based on their varying yet specific expression patterns in the MBs (Table 3.1). All Gal4 lines were from Würzburg stocks as well as from Chung Fang Wu’s lab and the Blomington stock center. *Df(3L)fz-D21*, *th*\(^{1}\) *st*\(^{1}/TM6B*, *Tb*\(^{1}\) completely disrupts *frizzled* (*fz*, Blomington Stock center; is hereafter referred to as *fz*\(^{0}\); Nambu & Nambu, 1996; Freeman et al., 1986; Adler et al., 1994; Park et al., 1994). Flies were grown on standard cornmeal and molasses food supplemented with live baker’s yeast (Bloomington, Indiana, United States). All lines were maintained in either plastic bottles with 40 ml of medium or vials with 8 ml of medium with cotton plugs, at 24°C with 50 % humidity in a constant 12:12 light dark cycle. For histological experiments, all flies were maintained at a concentration of 20 adults
Table 3.1 GAL4 line adult expression in MBs. This information was compiled from several different types of data including fluorescent marker and β-gal expression in the different subsets of the MB lobes in adult flies.

<table>
<thead>
<tr>
<th>Gal4 Line</th>
<th>α/β</th>
<th>α'/β'</th>
<th>γ</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>c739</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>Yang et al., 1995; Armstrong et al., 1998</td>
</tr>
<tr>
<td>c772</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yang et al., 1995; Armstrong et al., 1998</td>
</tr>
<tr>
<td>c492b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Tettamanti et al., 1997; Armstrong et al., 1998; Zars et al., 2000</td>
</tr>
<tr>
<td>201Y</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>Yang et al., 1995; Armstrong et al., 1998; Zars et al., 2000</td>
</tr>
<tr>
<td>c35</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yang et al., 1995; Dunkelberger 2008</td>
</tr>
<tr>
<td>30Y</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yang et al., 1995; Armstrong et al., 1998; Zars et al., 2000</td>
</tr>
<tr>
<td>238Y</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Yang et al., 1995; Armstrong et al., 1998; Zars et al., 2000</td>
</tr>
<tr>
<td>247</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>Schulz et al., 1996</td>
</tr>
<tr>
<td>H24</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Zars et al., 2000; Akalal et al., 2006</td>
</tr>
<tr>
<td>OK107</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Lee et al., 1999</td>
</tr>
</tbody>
</table>

- = no expression, + = expression, ++ = strong expression
(10 females and 10 males) in a single vial.

**Inverse Polymerase Chain Reaction**

Inverse Polymerase Chain Reaction (iPCR) was performed to determine the site of insertion for all MB Gal4 lines. Nine of the ten Gal4 lines were composed of the P[GawB] enhancer detection vector, an 11,279 Bp construct used to direct expression of Gal4 in a genomic integration site-specific manor (Brand & Perrimon, 1993). The main features of the P[GawB] vector are: P5 and P3 at each respective end, Gal4, hsp70 terminator, white, and pBluescript II containing amp’ and ori (Figure 3.1). The 247 Gal4 line was generated from the transformant line VII, harboring an enhancer fragment upstream of the *Drosophila myocyte enhancer factor-2* gene (*dMEF2*) fused to *lacZ*, with known β-galactosidase expression in the MBs (Schulz et al., 1996).

Genomic DNA (gDNA) was extracted from ten adult flies (5 females and 5 males) of CS and each Gal4 line using Wizard genomic DNA Isolation Kit (Promega). DNA was then digested with *Rsa I* (Promega) at a concentration of 10 u/µl for 2 hrs at 37°C. *Rsa I* is a 4 base cutter that recognizes a sequence of 4 bases and cuts at GT/AC which occurs at 13 sites in the P[GawB] construct (Figure 3.1). Phenol chloroform extraction of the digested product was performed and the aqueous phase was precipitated with ethanol. The sample was then ligated with T4 ligase at RT overnight (Promega). iPCR was performed with primers designed on either the 5’ (Set A) or 3’ (Set B) ends of the GawB construct (Table 3.2). Reactions were prepared at a final volume of 50 µl with 10 µl T4 ligated DNA as template under the following conditions: 94°C for 5 minutes,
Figure 3.1 P[GawB] construct. Diagram of the P[GawB] construct (illustration modified from Phelps and Brand, 1998). Set A primers (red) located at the 5’ end of the construct and Set B primers (blue) at the 3’ end.
Table 3.2 iPCR primers. Primer sets used for amplification and sequencing with locations in the P[GawB] construct listed. Dotted lines indicate the Rsa1 cut sites in the construct.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>T_m (°C)</th>
<th>Relative Location / Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set A for</td>
<td>5’-CTCAAGTGCTCCAAAGAAAAACCGA-3’</td>
<td>54</td>
<td>33 Bp from GawB RsaI bottom strand</td>
</tr>
<tr>
<td>Set A rev</td>
<td>5’-ATCGACGGGACCACCTTATGTATT-3’</td>
<td>54</td>
<td>8 Bp from end of GawB on top strand</td>
</tr>
<tr>
<td>Set B for</td>
<td>5’-CTCTTGCCGACGGGACCACCTTATG-3’</td>
<td>57</td>
<td>14 Bp from end of GawB on top strand</td>
</tr>
<tr>
<td>Set B rev</td>
<td>5’-GATTAACCCTTAGCATGTCCGTGGG-3’</td>
<td>57</td>
<td>70 Bp from GawB RsaI bottom strand</td>
</tr>
</tbody>
</table>
94°C for 1 minutes, either 54°C for 1 minutes, (primer set A) or 57°C for 30 seconds (primer set B), 72°C for 1 minutes, Steps 2-4 were repeated 29 more times, then held at 4°C. Samples were separated on 1.5% Tris-acetate ethylene-diamine-tetra-acetic acid (TAE) agarose gels and gel purified using QIAEX II gel extraction kit (Qiagen). Sequencing reactions were performed at a final volume of 20µl with 2µl gel purified iPCR product as template and 0.3µl of [10µM] primers under the following conditions: 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, Steps 1-3 25 were repeated 25 more times, then held at 4°C. The dye terminator was removed using Centri-Sep™ Columns (Applied Biosystems), and an ABI 3130 Genetic analyzer was used to run samples at University of Nevada Las Vegas. Sequences were aligned and analyzed using Sequencher 4.7 (Gene Codes Corporation).

**Anatomical Analysis**

I used paraffin mass histology to investigate brain morphology and the MBs in particular, for each of ten P[Gal4] insertions in both homozygous and heterozygous adult flies. Briefly, 2-6 day-old flies were cold-anesthetized, positioned in mass histology collars, fixed in Carnoy’s solution, dehydrated in ethanol and then embedded in paraffin (Heisenberg and Böhl, 1979). Heads were sliced in 7 µm serial sections and a fluorescent microscope was used for visualization and image capture (Zeiss, Thornwood, New York, USA). MB calyx volume and central complex (CCX) volume (fan shaped body (FB) + ellipsoid body (EB)) was calculated from planimetric measurements of the brains with
AXIOVISION software (Zeiss, Thornwood, New York, USA) (Serway et al., 2009; Wang et al., 2007).

**Genetic Backgound Control**

I wanted to verify that the significant changes I observed in MB calyx volume were a result of the inserts themselves rather than any possible modifiers present in an uncontrolled genetic background, as it is well established that genetic background influences MB morphology (de Belle & Heisenberg, 1996). Two Gal4 lines (c492b and 247) were crossed to $w^{1118}CS^{wu}$ for seven generations. I selected males with the red-eyed pigment to retain the mini-white marked Gal4 insert after each generation and crossed them to $w^{1118}$ virgins. After eight generations, >90% of their genetic background was replaced with wildtype. They were selected because they showed the most robust disruption in MB calyx volume prior to this backcrossing. Histological analysis was then repeated for homozygous and heterozygous flies.

**Protein analysis**

I investigated the levels of Fz protein using Western blot analysis. Seven wandering third instar larvae were sacrificed for protein extraction from CS$^{wu}$, 238Y, 30Y, c35, CS$^{wu}$/fz$^0$, 238Y/fz$^0$, 30Y/fz$^0$ and c35/fz$^0$. Fifteen head extracts were also used for Western blot analysis, but protein concentration was too low to see expression (data not shown). Briefly, tissues were collected in DPBS, pelleted in a microcenterfuge, and re-suspended in lysis buffer containing multiple protease inhibitors (Laemmli, 1979). Tissue was homogenized, boiled for 5 minutes then stored at 22°C for no longer than one week. Samples were
resolved on 9% SDS polyacrylamide gels, then transferred to Immobilon P membranes (Millipore) (Vaskova et al., 2000). Blots were incubated with 1C11 primary antibody, a mouse monoclonal Ab directed against the first 250 AA of fz (Developmental Studies Hybridoma Bank) in a 1:1,000 dilution, and goat-anti-mouse secondary antibody conjugated to HRP (Jackson Immuno Research) in a 1:7,500 dilution. Chemiluminescence ECL(+) Western-blotting detection system (GE Healthcare) and a Typhoon 8600 Variable Mode Phosphorimager (GE Healthcare) were used to look at protein levels. ImageQuantTM was used to determine protein concentrations (GE Healthcare).

**Statistical Analysis**

All MB calyx and CCX volumes were analyzed for significant effects of genotype, gender, zygosity and their respective interactions using analyses of variance (ANOVA). The Student-Newman-Keuls (SNK) multiple range test was employed for comparisons between means for multiple groups (Zar, 1996) (SAS Institute software). For the analysis of all MB calyx volumes and that of genetic background in 247 and c492b flies, I performed multiple pairwise t-tests between all biologically relevant genotypes and sexes. To maintain an error rate of $\alpha = 0.05$ for both experiments, a Bonferonni correction was used to adjust the critical $P$ values (Sokal & Rohlf, 1981).
Results

Molecular location of Gal4 lines

I performed iPCR on 10 MB specific Gal4 lines localizing them at the sequence level in the genome. I found that overall there was no general pattern of the insert sites relative to gene position. Inserts were localized to exons, introns, and intragenic regions in both [+J and [-] orientations (Table 3.3).

\[c739\] is located in an intron roughly 5.8 Kb into the $\beta$FTZ-F1, yet still ~10 Kb upstream from the 3d exon (Figure 3.2 A). \[c772\] is inserted in a non-coding region between Odorant receptor 42a (Or42a) and CG11163, roughly 2 Kb downstream from the end of Or42a and ~11.4 Kb upstream from CG11163 (Figure 3.2 B). \[c492b\] is inserted in the last exon of no extended memory (nemy), found in all eight transcripts and four Bp into the gene CG8776 (Figure 3.2 C).

\[201Y\] is inserted in the first intron of TAK1-associated binding protein 2 (Tab2) ~1 Kb into the gene, and ~5.6 Kb upstream from the second exon (Figure 3.2 D). My work confirms the previously reported cytological location of 201Y in Tab2 (Yang et al., 1995; Tettamanti et al., 1997) while also providing an exact sequence location for the insert. Surprisingly, three Gal4 lines were found to be upstream of frizzled (fz): \[c35\] is located 131 Bp upstream (Figure 3.2 E), \[30Y\] is inserted 123 Bp upstream (Figure 3.2 F) and 238Y is inserted 101 Bp upstream of the start of transcription (Figure 3.2 G). \[247\] is inserted in an intron, approximately 20 Kb downstream from the first exon of the B transcript of Ecdysone-induced protein 75B (E75), (Figure 3.2 H). \[H24\] is inserted in an intron approximately 5 Kb downstream from the end of the second exon of Casein Kinase 1 $\gamma$ (CK1$\gamma$) in the
Table 3.3 GAL4 insertion positions and information regarding proximal genes.

<table>
<thead>
<tr>
<th>Gal4 Line</th>
<th>Original map position</th>
<th>Insertion sequence location, and notes (genes in bold)</th>
<th>Proximal genes</th>
<th>Sequence location &amp; Orientation</th>
<th>Gene details</th>
</tr>
</thead>
<tbody>
<tr>
<td>c739</td>
<td>2L: 40A</td>
<td>~6 Kb into 2nd exon of βFTZ-1 &amp; ~10 Kb upstream from its 3rd exon.</td>
<td>βFTZ-1</td>
<td>2L:21,237,2 37..21,259,6 75 [+]</td>
<td>Orphan nuclear receptor involved in Ecdysone-mediated autophagy of the salivary gland (1).</td>
</tr>
<tr>
<td>c772</td>
<td>2RL: 42A</td>
<td>~2 Kb downstream from end of Or42a and 11.4 Kb upstream from CG11163.</td>
<td>Or42a</td>
<td>2R:1,679,00 1..1,680,468 [-]</td>
<td>Olfactory associated G-protein coupled receptor necessary for olfactory sensory perception (2).</td>
</tr>
<tr>
<td>c492b</td>
<td>2R: 49C</td>
<td>4 Bp into CG8776 &amp; ~9.3 Kb into nemy.</td>
<td>CG8776</td>
<td>2R:8,547,00 2..8,557,722 [-]</td>
<td>Predicted zinc ion transmembrane transporter activity (3).</td>
</tr>
<tr>
<td>201Y</td>
<td>2R: 56C8-9</td>
<td>~1 Kb into 1st intron of Tab2, &amp; ~5.6 Kb upstream of 2nd exon.</td>
<td>Tab2</td>
<td>2R:15,180,0 73..15,191,5 29 [+]</td>
<td>antimicrobial (5) and bacterial (6) responses.</td>
</tr>
<tr>
<td>c35</td>
<td>2R: 44A</td>
<td>131 Bp upstream from fz.</td>
<td>fz</td>
<td>3L:14,267,4 47..14,361,7 48 [+]</td>
<td>planner cell polarity, organ development, cell differentiation, cell division and axon guidance (7, 8)</td>
</tr>
<tr>
<td>30Y</td>
<td>3L: 70E</td>
<td>123 Bp upstream from fz.</td>
<td>fz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>238Y</td>
<td>2R: 48C</td>
<td>101 Bp upstream from fz.</td>
<td>fz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>247</td>
<td>3rd chrom.</td>
<td>In an intron ~20 Kb downstream of the 1st exon of the B transcript of E75.</td>
<td>E75</td>
<td>3L:17,945,0 63..18,052,6 98 [-]</td>
<td>Steroid hormone nuclear receptor involved in ecdysone signaling (9).</td>
</tr>
<tr>
<td>H24</td>
<td>3rd chrom.</td>
<td>In an intron ~5 Kb downstream of the second exon of CK1γ B, D, E &amp; I transcripts.</td>
<td>CK1γ</td>
<td>3R:12,098,1 76..12,128,0 94 [+]</td>
<td>spermatogenesis and male sterility (10, 11).</td>
</tr>
<tr>
<td>Ok107</td>
<td>4th chrom</td>
<td>560 Bp into the first exon of ey.</td>
<td>ey</td>
<td>4:718,315..7</td>
<td>Mushroom body development (12).</td>
</tr>
<tr>
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<td>----------------------------------</td>
</tr>
</tbody>
</table>

**KEY:**
1 = Takemoto et al., 2007
2 = Kreher et al., 2005
3 = Flybase, 1992
4 = Kamyshev et al., 2002
5 = Kleino et al., 2005
6 = Ferrandon et al., 2001
7 = review: Lawrence et al., 2007
8 = review: Bovolenta, et al., 2006
9 = review: King-Jones & Thummel, 2005
10 = Castrillon et al., 1993
11 = Nerusheva et al., 2009
12 = Callaerts et al., 2001
Figure 3.2 Sequence of insertion sites for 10 MB specific GAL4 lines. (A) c739 is located in the second intron of β-FTZ-F1 ~6 Kb into the gene and ~10 Kb upstream of it’s 3d exon (B) c772 is located ~2 Kb downstream from the end of
Odorant receptor 42a (Or42a) and ~11.4 Kb upstream from CG11163 (C) c492b is located in the last exon of no extended memory (nemy), and 4 Bp into CG8776. (D) 20Y is located in the first intron of TAK1-associated binding protein 2 (Tab2), ~1 Kb into the gene, yet ~5.6 Kb upstream from the second exon. (E) c35 is located 131 Bp upstream of the start of transcription of frizzled (fz). (F) 30Y is located 123 Bp upstream of the start of transcription of frizzled (fz). (G) 238Y is located 101 Bp upstream of the start of transcription of frizzled (fz). (H) 247 is located in Ecdysone-induced protein 75B (E75), in an intron ~20 Kb downstream from the first exon, yet still ~40 Kb upstream from the start of the next exon. (I) H24 is located in Casein Kinase 1 γ (CK1γ) in an intron ~5 kb downstream from the end of the second exon of CK1γ of the B,D, E and I transcripts and third exon of the F and H transcripts, yet still upstream from the first exon of the A, C and G transcripts. (J) OK107’s location in eyeless (ey) is confirmed 560 Bp into the first exon of transcript B while still upstream of the A, C and D transcripts.
B, D, E and I transcripts (Figure 3.2 I). And finally I confirmed the insertion site of OK107 as an internal iPCR control (Luo, 2006.26). It is 560 Bp into the first exon of transcript B of the eyeless gene (ey) (Figure 3.2 J).

Histological analysis showed 247 and c492b to have reduced MBs

Typically, Gal4 lines are used to drive transgenes in a temporal and or spatially specific manner in the MBs and look at their associated function. In this study I analyzed whether 10 MB independently generated Gal4 insertions have led to changes in the structure or size of the MBs. This would imply that an insertion has disrupted a genetic function critical for normal MB development. After a preliminary experiment demonstrating the influence of Gal4 lines on MB calyx volumes (Appendix Figure C.1), I performed paraffin histology in homozygous and heterozygous Gal4 lines with a larger sample size (Figure 3.3). I performed multiple pairwise t-tests between all biologically relevant genotypes and sexes with significant differences indicated in bold (Table 3.4). To maintain an error rate of $\alpha = 0.05$ for the experiment, the critical $P$ value was adjusted to 0.00012 (Sokal & Rohlf, 1981). For c492b, homozygous males had a 23% reduction in MB calyx volume compared to CS males, and a 20% reduction compared to CS females. c492b homozygous females had a 17% reduction compared to CS males. 238Y heterozygous males had a 22% increase in MB calyx volume compared to CS females and a 38% increase compared to homozygous 238Y females. Homozygous 247 females had a 22% reduction in MB calyx volume compared to CS females, a 25% reduction compared to CS males, and a 22% reduction compared to heterozygous 247 males. Homozygous
Figure 3.3 The influence of homozygous and heterozygous Gal4 lines on MB calyx volume. Serial sections of paraffin-embedded brains were used for planimetric MB measurements. c492b homozygous males had MBs that were 23% smaller than CS males, and 20% smaller than CS females. c492b homozygous females had MBs that were 17% smaller than CS males. 238Y heterozygous males had MBs that were 22% larger than CS females and 38% larger than homozygous 238Y females. Homozygous 247 females had MBs that were 22% smaller than CS females, 25% smaller than CS males, and a 22% smaller than heterozygous 247 males. Homozygous 247 males had MBs that were 16% smaller than CS males. And finally heterozygous H24 females had MBs that were 20% smaller than heterozygous H24 males. All significant differences are listed in bold in Table 3. Bars represent mean ± SE of mean calyx volume for each genotype. 5 ≤ n ≥ 20 for each bar.
Table 3.4 MB calyx volume multiple pairwise t-tests. Comparisons within each GAL4 line as well between each GAL4 and CS were made for all relevant genotypes and sexes. Significant differences are denoted as follows: \( \star \) < 0.05, \( \star \star \) < 0.01, \( \star \star \star \) < 0.005, \( \star \star \star \star \) < 0.001. \( \alpha \) = 0.05.

<table>
<thead>
<tr>
<th></th>
<th>CS F</th>
<th>CS M</th>
<th>c492b F</th>
<th>c492b M</th>
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<tbody>
<tr>
<td>c492b</td>
<td>NS</td>
<td>NS</td>
<td>( \star )</td>
<td>( \star \star \star \star \star )</td>
<td>NS</td>
</tr>
<tr>
<td>c492b F</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
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<table>
<thead>
<tr>
<th></th>
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<td>NS</td>
<td>NS</td>
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<td>238Y M</td>
<td>NS</td>
<td>NS</td>
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247 males had a 16% reduction in MB calyx volume compared to CS males. Finally the MBs in heterozygous H24 females were 20% smaller than those of heterozygous H24 males (Figure 3.3).

The 247 and c492b Gal4 lines were selected for further analysis as their reductions in MB calyx volume were the most robust, and consistently seen in both sexes and zygosities. I measured CCX volumes in individuals with previously measured MB calyx volumes. There was a significant influence of genotype ($F_{[2,65]}=8.189$, $P=0.001$) and of sex ($F_{[1,65]}=3.188$, $P=0.079$) on CCX volume in the 247 Gal4 line, while there was no influence of the interaction of sex and genotype ($F_{[2,65]}=0.224$, $P=0.800$) (Figure 3.4 A). I found that homozygous 247 flies were not significantly different from wildtype, while the heterozygotes showed a 7% increase in CCX volume (SNK, $P<0.05$). There was a significant influence of genotype ($F_{[2,60]}=7.120$, $P=0.002$) and of sex ($F_{[1,60]}=14.976$, $P=0.0001$) on CCX calyx volume in the c492b Gal4 line, while there was no influence of the interaction between sex and genotype ($F_{[2,60]}=2.250$, $P=0.114$) (Figure 3.4 B). Both homozygous and heterozygous c492b flies showed an 8% decrease in CCX volume compared to wildtype (SNK, $P<0.05$).

Placement of 247 and c492b Gal4 lines in genetic background derived from Canton Special wildtype strain eliminates their MB reduction

It was necessary to verify that any changes I observed in brain anatomy were due to the insert itself and not genetic background or the accumulation of genetic modifiers. Histological analysis of Gal4 lines c492b and 247 were repeated after they were backcrossed to CS for seven generations and then compare to the
Figure 3.4 CCX volumes for 247 and c492b. Serial sections of paraffin-embedded brains were used for planimetric CCX measurements. (A) There was an influence of genotype and sex, yet no influence of the interaction of genotype and sex on CCX volumes for the 247 GAL4 line, so the sexes were pooled. Heterozygous 247 flies showed a 7\% increase compared to CS. (B) There was a significant influence of genotype and sex, yet no influence of the interaction of genotype and sex on CCX volumes for the c492b GAL4 line, so the sexes were pooled. Both homozygous and heterozygous c492b flies showed an 8\% decrease in CCX volume compared to CS. Bars represent mean ± SE of mean calyx volume for each genotype. \( n \) indicated on each bar. Different letters designate significant differences (SNK, \( P \leq 0.05 \)).
original data. In the case of 247, there was no influence of sex ($F_{[1,138]}=0.153, P=0.696$), or the interaction of sex and genotype ($F_{[5,138]}=0.855, P=0.513$) on MB calyx volume, so sexes were pooled. There was an effect of genotype ($F_{[5,138]}=31.287, P<0.0001$) on MB calyx volume (Figure 3.5 A). I found that non-cantonized homozygous 247 flies were ~18% smaller than wildtype, while that decrease disappeared after cantonization. Heterozygous 247 flies were the same as wildtype both cantonized and non-cantonized (SNK, $P \leq 0.05$).

$c492b$ flies showed a similar trend, loosing their MB reduction once the insertion was placed in the CS genetic background. There was no influence of sex ($F_{[1,130]}=0.008, P=0.928$) or the interaction of sex and genotype ($F_{[5,130]}=0.783, P=0.563$) on MB calyx, allowing us to pool the sexes (Figure 3.5 B). There was a significant effect of genotype on MB calyx volume ($F_{[5,130]}=31.586, P<0.0001$). Homozygous $c492b$ flies showed an 18% reduction compared to wildtype, and heterozygotes showed an 11% reduction. These reductions were lost after out-crossing. Homozygous $c492b$ flies were no different from wildtype, and the heterozygotes surprisingly showed a slight hybrid vigor with a 9% increase in MB calyx volume compared to wildtype (SNK, $P \leq 0.05$).

$c35, 30Y$ and $238Y$ disrupt FZ protein levels and MB Anatomy

Although there were no significant changes to MB anatomy due to any of the three Gal4 insertions near the $fz$ gene, I was nonetheless interested to see whether the levels of the FZ protein were affected disrupted in the three Gal4 lines, $30Y$, $238Y$ and $c35$ (Figure 3.6 A-B), all inserted within 101 to 131 Bp of
There was no influence of sex or the interaction of sex and genotype on MB calyx volume, so the sexes were pooled for 247. There was a significant influence of genotype, as only non-cantonized homozygous 247 flies were ~18% smaller than wildtype. (B) In c492b flies, there was no influence of sex or the interaction of sex and genotype on MB calyx volume, so the sexes were pooled. There was a significant influence of genotype, as non-cantonized homozygous c492b flies showed an 18% reduction, and non-cantonized heterozygotes showed an 11% reduction compared to wildtype. Cantonized c492b heterozygotes, exhibited a slight hybrid vigor with a 9% increase in MB calyx volume compared to wildtype. Bars represent mean ± SE of mean calyx volume for each genotype. n indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$).
the start site of fz transcription (Figure 3.2 E-G). I analyzed each line as a homozygote as well as heterozygotes with fz\(^0\) (Freeman et al., 1986, Adler et al., 1994; Park et al., 1994) maintained over the third chromosomal larval balancer: TM6B,Tb, facilitating larval selection. I found a decrease in protein levels for all three homozygous Gal4 lines, as well as a slight reduction in the band size from the normal 62 kDa (Figure 3.6 A-B). There were 2 bands in the c35/ fz\(^0\) and 238Y/ fz\(^0\) samples. The smaller band seen in both groups is 60 kDa and is believed to be a phosphorylated version of the Fz protein (Park et al., 1994). MB calyx volume was measured in CS and all three lines crossed to fz\(^0\) (Figure 3.6 C). There was a significant influence of genotype ($F_{[4,38]}=11.769, P=0.001$) on MB calyx volume. All three Gal4 lines over fz\(^0\) were significantly larger (14-18%) than CS or CS/fz\(^0\).

Discussion

The Gal4-UAS system is likely the most widely used tool in Drosophila genetics due to it’s temporal and spatial resolution, and ability to be used in combination with many other tools for genetic and molecular manipulation. Gal4 inserts themselves have also been used (although much less frequently) for identification of novel genes associated with the structure they express in, as the screening process (usually the most laborious) is already done. The expression of ey in the MBs (Callaerts et al., 2001) and OK107’s identity in ey (Luo, 2000) was compelling evidence that localization of MB specific Gal4 lines would provide novel genes with possible roles in MB development. I have used this approach to
Figure 3.6 Fz western blot and anatomical analysis of MB calyx volume in Fz0 crossed to c35, 30Y and 238Y. (A) This graph illustrates the quantification of bands from A using ImageQuant V5.1 (Molecular Dynamics). The CS band was set to 100% and used for comparisons to all other samples. There was a significant reduction in the amount of protein for all three homozygous GAL4 lines compared to wildtype, as well as all lines/Fz0. (B) An SDS-Page gel was run with samples of wandering third instar larvae, blotted, trimmed and incubated.
with the 1C11 Ab. This blot revealed that c35 (lane 2) and 238Y (lane 4) homozygotes caused the FZ protein to be slightly reduced in size. c35/Fz0 (lane 6) and 238Y/Fz0 (lane 8) displayed a doublet band. (C) There was a significant influence of genotype on MB calyx volume. 30Y/Fz0 was 17% larger than CS/Fz0. c35/Fz0 and 238Y/Fz0 were both 14% larger than CS/Fz0. Bars represent mean ± SE of mean calyx volume for each genotype. n indicated on each bar. Different letters designate significant differences (SNK, P≤0.05).
identify the molecular location of 10 MB specific Gal4 lines (Table 3.3). These inserts were found in coding, non-coding and intragenic regions of the genome. As a control, I confirmed that OK107 was inserted in ey, which has been implicated in MB development and is expressed in adult MBs and the CCX (Callaerts et al., 2001). This data provides promising support that further investigation with more robust mutant alleles of genes proximal to each Gal4 insert may show their involvement in MB development and behavioral modulation.

Perhaps the most surprising result was the new molecular identification of the Gal4 lines c35, 238Y and 30Y. c35 has previously been cytologically mapped to the second chromosome at 44A, 238Y to the second chromosome at 48C, and 30Y to the third chromosome at 70E (Yang et al., 1995). All three lines have moderate expression in all lobes of the MBs, the calyx, cell bodies and the pedunculus (Yang et al., 1995; Armstrong et al., 1998; Zars et al., 2000; Dunkelberger et al., 2008) with additional expression in the antennal lobe, EB and FB of the CCX (Zars et al., 2000). To my surprise, I identified the sequence location of all three elements to be on the third chromosome within 30 Bp of each other, 101-131 Bp upstream from fz (Figure 3.2 E-G). As this result was so surprising, the experiment was repeated several times (with the same results) using flies from multiple sources.

fz has the cytological location of 70D4-5 and the molecular location of 3L:14,267,447..14,361,748 [+] . It is a 94 Kb gene with two transcripts encoding a G-protein coupled receptor with seven transmembrane domains. fz is involved in
many critical biological processes including planner cell polarity, organ
development, cell differentiation, cell division and axon guidance (reviews:
Lawrence et al., 2007; Bovolenta, et al., 2006; Schnorrer & Dickson, 2004;
Roegiers & Jan, 2005; Marques, 2005; Martinez Arias, 2005). Canonical
Wingless (Wnt) signaling is known to mediate many critical biological processes,
often through its binding to a member of the Frizzled family of receptors, and has
ev even been linked to a number of cognitive disorders, including schizophrenia and
Alzheimer’s disease (De Ferrari & Moon, 2006). More specifically, axon
extension in the lobula and medulla of the Drosophila eye, is regulated by WNT
binding to the FZ receptor (Srahna et al., 2006), while this binding of WNT to FZ-2
regulates synaptic architecture at the larval NMJ (Mathew et al., 2005). FZ is
expressed in the embryonic brain, ventral nerve cord, third instar larval brain,
pupal CNS, as well as the ovary, wing and eye-antennal disc (Park et al., 1994;
Adler et al., 1990; Zheng et al., 1995). Recently WNT5 has been implicated as a
necessary component for MB axon guidance (Grillenzoni et al., 2007). Currently
there is no known function for fz in MB development, although based on its early
expression in the embryonic brain it is possible that it is associated with CNS or
even more specifically MB development.

MB calyx volumes for all three Gal4 inserts were measured in both
homozygotes and heterozygotes. I observed a 22% increase in 238Y
heterozygous males compared to CS females and a 38% increase when
compared to homozygous 238Y females (Figure 3.3, Table 3.4). I also tested the
MB calyx volume of all three Gal4 lines crossed to fz0 to find that all three were
significantly larger than CS or the CS/ fz\textsuperscript{0} (Figure 3.6 C). To get an even more accurate analysis of the role of fz on MB development, more efficient alleles would need to be used. I drove four Fz-RNAi lines (as well as one for nemy and one for CK1\textgreek{y}) in the MB and looked at their calyx volumes, although the effectiveness of the lines is still in question as a new approach to our RT-PCR data is currently being discussed (Appendix Figures C.6-C.7, Tables C.2-C.3). Overall it appears that fz may play a very interesting role in MB development, but to see this at the level of the calyx, a more robust group of alleles should be used to initiate a disruption to this entire signaling pathway. Knowledge regarding the expression of Fz in the brain over development would also be very helpful in determining which avenue of investigation is most likely biologically relevant.

I was interested in whether this suite of MB specific Gal4 lines had an impact on MB development when represented in the genome as either heterozygotes or homozygotes. In general, these Gal4 lines did not appear to be ideal mutant alleles of each gene, as I did not see a trend of disruption to the MBs (Figure 3.3, Table 3.4). There are a several possible reasons for this. The first, and most likely is that the inserts themselves do not serve as adequate disruptions to the endogenous genes. This may be due to the fact that many of the insertions were located in non-coding regions with little or no influence on the expression of each gene. There is evidence that insertions outside of a gene’s coding region can induce severe phenotypes (Bejsovec, 2006), as regulatory elements are being identified in many unexpected places in the genome often far from the gene they are regulating (review: Bulger & Groudine, 2009) or in enhancer regions
upstream of genes (Rebeiz et al., 2009; Wittkopp et al., 2009). These regulatory
regions also appear to be driving genome evolution in a gene specific fashion
(review: Stern & Orgogozo, 2009). The c35, 30Y, and 238Y inserts were located
in the promoter region of fz, and further supported this idea that for an insert to
cause a significant change to MB anatomy, it had to be located in either a
regulatory or coding region, as they did in fact disrupt Fz protein levels as well as
size (Figure 3.5 A-B). Interestingly, when the lines were tested alone, they
caused minor and mostly insignificant changes to MB calyx volume (Figure
Table 3), yet when they were crossed to the fz0, there were small yet significant
increase in MB calyx volume, with 30Y/ fz0 showing the greatest increase (Figure
3.5 C). Overall, the fz data indicates that iPCR as a method to identify genes
based on their Gal4 expression pattern is only a good way to ID genes, and that
better alleles exist to further test the function of the newly identified genes.

A second reason why I did not see a significant disruption to the MBs across
all the Gal4 lines could be that these genes are not involved with MB dendritic
development. Our method for analyzing MB anatomy (MB calyx volume
calculations from planimetric measurements of the MB area of sequential
sections) may not have picked up a subtle change to the MB, or even a change
at a functional level. I looked at the morphology of the lobes in the histological
sections and found no gross defects in all cases. This does not however rule out
defects at a functional or molecular level. And finally, as it is known that
enhancers can act on genes located tens of thousands of base pairs away
(review: Sipos & Gyurkovics, 2005), it is possible (although unlikely) that the
enhancer I identified through iPCR are not related to the closest proximal genes. OK107’s insertion in ey is evidence to the contrary, and indicates that the closest gene, or the one the Gal4 is inserted in may in fact play a functional role in MB development. Each individual line must be further analyzed for its expression patterns as well as strongest alleles influence on MB anatomy.

The 247 and c492b Gal4 lines did display significant disruptions to MB calyx volume (Figure 3.5, Table 3.4). I wanted to verify that the inserts were responsible for this reduction rather than the accumulation of any genetic modifiers, so I out-crossed them to CS\textsuperscript{wü} and repeated this experiment. Unfortunately, once this was done, these insert lines lost the reduced MB phenotype, indicating that they were both extremely sensitive to genetic background (Figure 3.5 A-B). 247 and c492b were also selected for analysis of CCX to determine if their reductions in MB calyx volume were MB specific or found throughout the brain. Although there were minor differences observed, the pattern in reduction was not consistent with that seen in the MBs, nor was the phenotype as severe (Figure 3.4 A-B). Unfortunately this became a moot point when the MB phenotype was lost due to backcrossing. Again, to investigate either of these lines and the candidate genes they may disrupt/mimic, it will be necessary to use more efficient tools. I used a nemy-RNAi line to look at MB calyx volumes but found no differences when driven in the MBs (Appendix Figure C.6-C.7, Table C.2-C.3).

Overall, I found that several of the genes associated with Gal4 insertion sites were expressed in the CNS (ex: fz and CK1\textgamma), some were known to express in
the MBs (ey), and the rest were poorly characterized. Lack of expression for each gene in the particular subset of MB lobes represented by each Gal4 lines may reflect several things. It could be something as simple as research not yet done on expression in the MBs, or something as complicated as a re-evaluation of how enhancers function in the genome. In spite of the reason why, there is some compelling evidence that these lines may have associative conditioning defects either in combination or independent of any MB structural defects they may possess. Amnesiac for example is not expressed in the MBs but rather in the DPM cells and still has a memory phenotype (Waddell et al., 2000). This same principal can be applied to the candidate genes I uncovered in this paper. Expression in the MBs may have very little to do with possible associative conditioning defects or disruptions to the structure beyond the gross morphological level in which it was evaluated.

Conclusion

Molecular localization of this group of MB specific Gal4 lines has provided us with a suite of candidate genes whose roles in MB development and associative conditioning have yet to be thoroughly realized, yet appear quite promising. Surprisingly, out of the >13,000 genes in the fly genome this work has centered around three categories of genes: those involved in hormone signaling, glial cell function and zinc ion binding. I have also provided further examples that illustrate the strong, yet gene specific roles genetic background can have on brain
development, in the MBs in particular. And finally, this work sheds light on the complicated routes enhancers use to function in the genome.
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CHAPTER 4

MUSHROOM BODIES ENHANCE INITIAL MOTOR ACTIVITY IN DROSOPHILA

This chapter has been published in the Journal of Neurogenetics and is presented in the style of that journal. The complete citation is:


I made the following contributions to this paper: I collected and analyzed the MB calyx volume dataset, screened histological slides of all flies used in the behavioral experiment to identify and capture MB images and wrote the manuscript.

Abstract

The central body (or central complex, CCX) and the mushroom bodies (MBs) are brain structures in most insect phyla that have been shown to influence aspects of locomotion. The CCX regulates motor coordination and enhances activity while MBs have, thus far, been shown to suppress motor activity levels measured over time intervals ranging from hours to weeks. In this report, we investigate MB involvement in motor behavior during the initial stages (15 minutes) of walking in Buridan’s paradigm. We measured aspects of walking in flies that had MB lesions induced by mutations in six different genes and by chemical ablation. All tested flies were later examined histologically to assess MB neuroanatomy. Mutant strains with MB structural defects were generally less active in walking than wild-type flies. Most mutants in which MBs were also ablated with hydroxyurea (HU) showed additional activity decrements. Variation in measures of velocity and orientation to landmarks among wild-type and mutant flies was attributed to pleiotropy, rather than to MB lesions. We conclude that
MBs upregulate activity during the initial stages of walking, but suppress activity thereafter. An MB influence on decision-making has been shown in a wide range of complex behaviors. We suggest that MBs provide appropriate contextual information to motor output systems in the brain, indirectly fine tuning walking by modifying the quantity (i.e., activity) of behavior.

Introduction

Insect locomotion has been studied for over a century in a wide variety of species, revealing generally conserved mechanisms of motor control. Coordinating a suite of complex behaviors, including foraging, courtship, and predator avoidance, is necessary for survival. Behavioral investigations focused on the mechanics of walking have shown varying leg coordination and gait at different walking speeds (Wilson, 1966; Graham, 1972). Insect thoracic ganglia, like the vertebrate spinal chord, are responsible for this type of basic motor control (Bässler, 1983; Graham, 1985), which is then further regulated by the brain. Integration of neural activity in the thorax with signals from the brain gives rise to appropriate motor activities. In stick insects for example, severed neck connectives largely inhibited walking, while severed circumoesophageal connectives had minimal affects (Graham, 1979). Electrical stimulation of severed neck connectives induced various types of walking in tethered locusts as well (Kien, 1983). In decapitated Drosophila, leg movement can be stimulated with biogenic amines applied at the cervical connective, substituting for signals from the brain (Yellman et al., 1997). Exactly how the brain is able to override the
central pattern generators (CPGs) of the thoracic ganglia to fine tune walking for course control has yet to be determined. However, several structures in the insect brain have been implicated as influences or centers of motor control, providing sensory integrated descending signals to CPGs in the thorax. Here, we use genetic dissection and chemical ablation in *Drosophila* to examine the role of the MBs in regulating walking behavior.

Studies on higher control of motor activity in the insect brain have historically focused on the central body or CCX in some phyla (reviews: Homberg, 1987; Heisenberg, 1994; Strauss, 2002). In Dipterans, the CCX is located centrally between the two hemispheres of the adult brain and is composed of four structures: the fanshaped body, the ellipsoid body, the paired noduli, and the protocerebral bridge, each enclosed by a thin glial lamella (Hanesch et al., 1989). While distinct input and output tracts are not obvious, the CCX receives sensory input from a large portion of the brain and sends motor outputs diffusely. The emergence of legs and the timing of CCX differentiation are correlated during development. Larval hemimetabolous insects have legs and develop a CCX similar to the adult form during prelarval stages (Wegerhoff & Breidbach, 1992). Holometabolous species, on the other hand, including the Diptera, remain legless during larval development, with CCX differentiation delayed until pupation. CCX precursor fibers of *Drosophila* larvae have been isolated in the interhemispheric commissure (Hanesch, 1987; Hanesch et al., 1989). In crickets, surgical disruption (Huber, 1960) and electrical stimulation (Otto, 1971) of CCX provided
evidence for its role in motor control. Strauss and Heisenberg (1993) later confirmed this in *Drosophila* through behavioral analysis by using CCX structural mutants, all of which showed irregular aspects of walking that include turning, start/stop maneuvers, shorter step size, and overall reduced walking speeds. Martin et al. (1999) further demonstrated a CCX role in locomotion by driving tetanus toxin expression in the CCX, which led to decreased activity. This study showed that the protocerebral bridge and fan-shaped body are required for maintenance, but not initiation, of locomotion in flies (Martin et al., 1999). Another recent study localizes CCX influences on walking activity and velocity to the protocerebral bridge (Poeck et al., 2008). In addition to upregulating walking speed, the CCX is also involved with several other aspects of motor control, including landmark orientation, balancing, and across-body symmetry via right-left bargaining (review: Strauss, 2002). Although CCX structures are formed during pupal development in *Drosophila*, mutants with adult CCX phenotypes nonetheless have reduced larval motor and feeding activity (Varnam et al., 1996). Several studies have also implicated the *Drosophila* CCX in olfactory and place memory (Heisenberg et al., 1985; Liu et al., 2006; Neuser et al., 2008; Wang et al., 2008).

The MBs have long been associated with insect locomotion as well. In adult flies, these paired neuropils are each composed of approximately 2,500 intrinsic Kenyon cell (KC) neurons per hemisphere. Three morphologically and spatially distinct classes of KC differentiate in sequence from four neuroblasts dividing throughout preimaginal development (Lee et al., 1999). KCs in the posterodorsal
protocerebrum send dendritic arborizations into a region called the calyx and project axons into a large bundle known as the pedunculus. The pedunculus bifurcates anteroventrally into two dorsal (α and α′) and three medial (β, β′, and γ) lobes, formed by the axonal projections of α/b, α′/β′, and γ KC classes (Heisenberg, 2003).

The functional role of the MB has been a focus of discussion for over 150 years (Dujardin, 1850). In the 1950s, MBs were implicated in the control of motor activity (Huber, 1955). Electrical stimulation in, or proximal to, the MBs induced singing (i.e., stridulation) in crickets (Huber, 1960; Otto, 1971). In honeybees, MBs receive prominent visual (Gronenberg & López-Riquelme, 2004), gustatory, and mechanosensory (Schroter & Menzel, 2003) inputs. These connections likely provide mixed-modality signals that lead to experience dependent structural changes documented in freely behaving bees (e.g., Farris et al., 2001). Using extracellular recordings of freely moving cockroaches, Mizunami et al. (1998a) identified MB neurons with a potential role in motor activity as well as those involved in place memory (Mizunami et al., 1998b). Numerous studies have established that the MB is necessary for associative olfactory learning and memory in honeybees (review: Giurfa, 2007), locusts (Perez-Orive et al., 2002), and Drosophila (review: Margulies et al., 2005). MBs have also been implicated in other complex aspects of locomotion, including centrophobism and thigmotaxis (Besson & Martin, 2005). While MBs play no significant role in circadian activity rhythms (Helfrich-Förster et al., 2002) or visual-based association tasks (Wolf et al., 1998), they are necessary for context generalization in memory recall (Liu et
al., 1999). MBs also mediate aspects of aggressive behavior (Baier et al., 2002) and sleep (Joiner et al., 2006; Pitman et al., 2006; Seugnet et al., 2008). MB-ablated flies show normal courtship and courtship conditioning, but have impaired memory of courtship events after 30 minutes (Joiner & Griffith, 2000; McBride et al., 1999). In late third instar larvae, which already have well established α′/β′ and γ lobes (Lee et al., 1999), MBs were found to have no significant influence on feeding behavior (Osborne et al., 2001).

Several studies have focused on the role of Drosophila MBs in motor behavior. Heisenberg et al. (1985) initially described elevated motor activity of the MB structural mutant, mushroom body miniature1 (mbm1). Martin et al. (1998) later used chemical ablation, as well as genetic and transgenic techniques, to show that MBs suppress walking activity measured over several hours by regulating the termination of walking bouts. During an even longer period of time (days to weeks), male flies with chemically ablated MBs also showed an increase in activity (Helfrich-Förster et al., 2002). These reports all indicate that MBs down-regulate aspects of motor behavior. Interestingly, this effect is only well described for activity measured over longer time scales. Within the first 15 minutes of activity, assessment of MB influences on behavior has not been well described. This information is significant, since we know very little about how the brain regulates motor output, particularly during the initial stages of activity. Curiously, MBs appear to have no significant impact on general locomotion in Drosophila larvae (Osborne et al., 2001), suggesting that connections to larval motor systems are not established.
Here, we report a detailed analysis of *Drosophila* walking behavior in Buridan’s paradigm during 15 minutes of activity. To assess the role of MBs in this behavior, we examined seven genetically independent mutants with MB structural defects: *mbm¹*, *mushroom body miniature B (mbmB¹)*, *mushroom body miniature C (mbmC¹)*, *mushroom bodies reduced (mbr¹)*, *small mushroom bodies (smu¹)*, and two alleles of *mushroom bodies deranged (mud¹ and mud⁴)* (Heisenberg, 1980; Heisenberg et al., 1985; de Belle & Heisenberg, 1996). All mutations were outcrossed to the Canton Special (CS) wild-type strain to control for effects of genetic background (de Belle & Heisenberg, 1996). *mbm¹* was also examined in the Berlin genetic background for comparison. In addition, MBs were ablated by using the DNA synthesis inhibitor, hydroxyurea (HU), in all allele and genetic background combinations to control for nonspecific effects of genetic lesions.

We found that both Berlin and CS wild-type strains showed a decrease in walking activity after MB ablation. Most MB structural mutants were even less active and/or walked significantly slower than both MB-ablated and intact wild-type flies. We also observed a consistent additional decrease in motor activity after MB ablation in most mutant lines. From these experiments, we conclude that 1) MBs enhance the initial stages (at least 15 minutes) of walking activity and 2) pleiotropic functions outside the MBs account for the reduced activity and velocity observed for most MB mutants included in this study. Our results indicate that MBs provide fine tuning of walking behavior by modifying the quantity of walking (i.e., activity) rather than the quality (i.e., velocity and orientation).
Materials and Methods

Fly Strains

We used wild-type Berlin and CS, both derived from Würzburg stocks, as standard control strains in all anatomical and behavioral analyses. MB structural mutants and their anatomical phenotypes are described elsewhere (Heisenberg, 1980; Heisenberg et al., 1985; de Belle & Heisenberg, 1996). Briefly, the MBs of $mbm^1$, $mbmB^1$, $mbmC^1$, $mbr^1$, and $smu^1$ are reduced in size. In $mbm^1$, the reduction is more pronounced in females than in males. MBs in both $mud^1$ and $mud^4$ have enlarged calyces but reduced pedunculi and lobes. All mutant strains were outcrossed with CS to control for background effects (de Belle & Heisenberg, 1996). $mbm^1$ was also tested in its original Berlin genetic background. All flies were grown in 180-mL plastic bottles at equal concentrations, with 40 mL of Drosophila medium (cornmeal, molasses, agar, yeast, and nipagin) at 25°C with a 16-hour light/8-hour dark light regime (standard conditions).

Mushroom Body Ablation

HU fed to newly hatched larvae selectively deletes MB neuroblasts, resulting in complete, precise ablation of all postembryonically derived MB structures in adult flies (de Belle & Heisenberg, 1994; Sweeney et al., 2000). Briefly, larvae were collected 0-1 hour after hatching, and incubated for 4 hours in a yeast-HU mixture (50 mg mL $^{-1}$). They were then rinsed in distilled water, transferred to normal medium, and reared in standard conditions. Control larvae (CT) were treated similarly, except that HU was omitted.
Histology and Anatomy

All flies tested in behavioral experiments were examined histologically to assess brain anatomy. Flies were cold anesthetized, placed in mass histology collars, fixed in Carnoy’s solution, dehydrated in ethanol, and embedded in paraffin (Heisenberg & Böhl, 1979). Heads were cut in 7 mm frontal serial sections and viewed with a fluorescent microscope (Zeiss, Thornwood, New York, USA). MB calyx volumes were derived from planimetric measurements of these brains by using AXIOVISION software (Zeiss, Thornwood, New York, USA) (Wang et al., 2007). We measured right wing area and right forelimb length to compare external anatomy of CT and HU-treated wildtype flies (Wang et al., 2007). Flies were cold anesthetized and their appendages were removed with microscissors. These were mounted on glass microscope slides with cover slips sealed with nail polish. Images were photographed under a light microscope with an AXIOCAM digital camera and measured by using AXIOVISION software (Zeiss, Thornwood, New York, USA).

Walking Behavior

We measured aspects of walking in Buridan’s paradigm (Götz, 1980; see Strauss et al., 1992; for details, see Figure 4.1). Single flies with clipped wings were confined to an elevated circular disk (8.5 cm in diameter) surrounded by a water-filled moat between two opposing and inaccessible landmarks (vertical black stripes) on an otherwise uniformly illuminated white background. We recorded the walking track of each fly for 15 minutes with a video-scanning device sampling at 5 Hz (similar to that described by Bülthoff et al., 1982). This
Figure 4.1 (A) Buridan’s paradigm. (B) A sample tracing made by a Berlin male in 15 minutes.
visual stimulation elicits spontaneous alternation of walking between the competing targets that can persist for hours (Bülthoff et al., 1982; Götz, 1998). Angles of orientation toward landmarks (deg; Strauss & Pichler, 1998), activity (the percent of time walking), and walking velocity (mm s\(^{-1}\)) for transitions between landmarks were calculated in two 6-day-old flies.

**Statistical Analysis**

All measured parameters were tested for significant influences of genotype, gender, and MB ablation by using analyses of variance (ANOVAs). Comparisons between means for multiple groups were made by using the Student-Newman Keuls (SNK) multiple range test (Zar, 1996). Appropriate procedures for circular data were used to analyze mean angles of orientation and concentration (\(r\)) of orientation vectors about the mean (Zar, 1996).

**Results**

**Brain Anatomy**

In this study, we characterized the influence of MBs on motor behavior during 15 minutes of activity in Buridan’s paradigm (Figure 4.1). All flies tested were subsequently sacrificed for assessment of gross brain morphology, allowing a correlation of anatomy with behavior for 685 subjects. We verified complete MB ablation in all 355 HU-treated flies included in the behavioral analysis (Figure 4.2 A-D). A small number of flies developed with partial MB structures, as noted in other studies (de Belle & Heisenberg, 1994; Armstrong et al., 1998), and were not included in the behavioral analysis.
**Figure 4.2 Brains of HU-treated flies and mushroom body (MB) structural mutants.** Frontal 7-mm paraffin sections of *Drosophila* heads viewed under a fluorescence photomicroscope. Images are of sections approximately 40 mm from the caudal margin of the brain, showing MB calyces (arrows) at their broadest point. (A) Berlin male. (B) CS female. (C) MB-ablated Berlin male. (D) MB-ablated CS male. (E) mbm\(^{1}\) male (left) and female (right). (F) mbm\(^{1}\) (CS) male. (G) mbm\(^{1}\) (CS) male. (H) mbmC\(^{1}\) (CS) male. (I) mbr\(^{1}\) (CS) male. (J) smu\(^{1}\) (CS) male. (K) mud\(^{1}\) (CS) male. (L) mud\(^{4}\) (CS) male. For most genotypes, calyx volume accurately reflects the condition of the pedunculus and lobes (not shown). In both mud mutants, MB axonal components that normally contribute to the pedunculus and lobes are misrouted to form the enlarged structures observed where the calyces are found in wild-type brains.
Gross brain morphology has been described previously for the MB structural mutants used in this study (Heisenberg, 1980; Heisenberg et al., 1985; de Belle & Heisenberg, 1996). Representative images of mutant flies that we examined for walking behavior are shown in Figure 4.2 E-L. Mean MB calyx volume was measured in samples of flies tested for behavior (Figure 4.3). Differences among all groups were significant ($F_{[13,154]} = 111.29; P < 0.0001$). HU-treated flies (having no visible MBs under a fluorescent microscope) and mud(CS) mutant flies were not included in this analysis. For all other groups, calyx volume provided an accurate proxy for whole MB anatomy. Berlin females had significantly smaller MBs, compared with males and CS flies of either gender ($P \leq 0.05$). The pronounced sexual dimorphism described for mbm$^1$ (Heisenberg et al., 1985) was clearly evident, as mbm$^1$ females had the smallest MBs of any mutant, while calyx volume in mbm$^1$ males was roughly 80% of that measured in Berlin males (Figure 4.2E; $P \leq 0.05$). The same mbm$^1$ allele in a CS genetic background supported wild-type MB anatomy in both genders that was not significantly different from CS (Figure 4.2 F; $P \leq 0.05$), reflecting a strong influence of polymorphic gene interactions on the MB phenotype (de Belle & Heisenberg, 1996). MB calyces in mbm$^1$(CS), mbm$C^1$(CS), mb$^1$(CS), and smu$^1$(CS) were reduced to between 20 and 50% of those in CS flies (Figure 4.2 G 4.2J; $P \leq 0.05$). As documented elsewhere (de Belle & Heisenberg, 1996), we noted an ellipsoid body phenotype with low expressivity and penetrance in many of these reduced MB mutants (data not shown), but otherwise, their gross brain morphology appeared relatively normal. In mud(CS) flies, enlarged and misshapen MB
Figure 4.3 Mushroom body calyx volume was significantly different. Bars represent mean±SE of the mean calyx volume for each fly, $9 \leq n \leq 16$/bar. Different letters designate significant differences ($P \leq 0.05$). Values were derived from planimetric measurements of flies represented in Figures 5-7.
structures are formed by excessive KC proliferation (Figure 4.2 K and 4.2 L). We also observed enlarged antennal lobes in most flies, as documented elsewhere (data not shown; Prokop & Technau, 1994). \textit{mbr}^{CS}, \textit{smu}^{CS}, \textit{mud}^{CS}, and \textit{mud}^{CS} females are semilethal (de Belle & Heisenberg, 1996) and could not be reliably collected for testing.

External Anatomy

HU treatment used to ablate MBs in flies has a minor effect on antennal lobe development and no obvious effects on external anatomy (de Belle & Heisenberg, 1994; Stocker et al., 1997). But, since performance in motor tasks might be influenced by subtle allometric differences caused by HU, we measured and compared limb length (Figure 4.4 A) and wing area (Figure 4.4 B) in CT and HU-treated \textit{Berlin} and \textit{CS} flies. Neither HU treatment nor genotype had significant effects on the dimensions of either structure (\(P \leq 0.05\)). The only significant differences were found between genders, with males having shorter legs (\(F_{[1,232]} =349.5; P<0.0001\)) and smaller wings (\(F_{[1,232]} =2122.97; P<0.0001\)) than females. This was expected, since male flies are normally smaller than females.

Walking Behavior

A variety of behavioral paradigms measure slightly different aspects of locomotor behavior (review: Martin, 2003). In this study, we used Buridan’s paradigm (Götz, 1980), which induces spontaneous, robust alternation of walking between competing visible targets (Bülthoff et al., 1982; Götz, 1998; Strauss & Pichler, 1998; Figure 4.1). Although this behavior can persist for hours, we were
Figure 4.4 External anatomy was not influenced by genotype or HU treatment. Bars represent mean±SE, n = 30/bar. Different letters designate significant differences (P≤0.05). (A) Limb length was shorter in males than in females. (B) Similarly, wing area was smaller in males than in females.
interested in the initial 15 minutes of activity. We examined walking in 7
_Drosophila_ mutants that have predominant MB structural defects. To control for
pleiotropic effects in these mutants, we used HU to ablate their MBs and
compared behavior with CT and wild-type flies (Figures 4.5-4.7).

**Orientation**

Measures of a fly’s orientation toward landmarks in Buridan’s paradigm reflect
visual acuity, visual signal processing, and motivation for walking. We sampled
the direction of motion for each fly at a frequency of 5 Hz (4,500 measurements
in 15 minutes). The means of mean angles of orientation toward landmarks
among genotypes, genders, and HU treatment groups were not significantly
different ($F_{[27,598]} =0.69; P=0.8795$; Figure 5A). We also calculated and compared
the means of mean concentrations ($r$) of orientation angles toward landmarks for
all groups of flies (Strauss & Pichler, 1998; Zar, 1996; Figure 5B). Analysis
showed that $r$ was influenced by genotype ($F_{[9,606]} =13.25; P<0.0001$), HU
ablation ($F_{[1,606]} =24.48; P<0.0001$), genotype ablation interaction ($F_{[9,606]} =2.93; P
=0.0021$), genotype gender interaction ($F_{[5,484]} =6.31; P<0.0001$), and gender
ablation interaction ($F_{[1,484]} =8.89; P =0.003$). Gender and interactions among the
three main effects had no significant impact on $r$ ($P 0.01$). In spite of this complex
result, we suggest that the lack of obvious pattern and relatively minor variation
in orientation (e.g., CT, $r =0.7759 \pm 0.005$; HU, $r = 0.7479 \pm 0.004$) does not limit
our assessment of other components of walking behavior (see below). All groups
of flies demonstrated comparable landmark orientation in Buridan’s paradigm,
indicating that MBs are not critical for visual stimulus recognition and general
Figure 4.5 All groups of flies demonstrated comparable patterns of landmark orientation, indicating similar responses to visual stimulation in Buridan’s paradigm. Bars represent mean±SE, $13 \leq n \leq 23$/bar for all groups except CS, which is $39 \leq n \leq 56$/bar. (A) There was no difference in the mean angle of orientation toward landmarks. (B) Mean concentration ($r$) of orientation angles to landmarks was influenced by genotype, HU ablation, and interactions among the main effects. The grey line represents a reference value of $r$ generated by a random walk.
motivation for walking.

Activity

Previous work has implicated the MBs as negative regulators of motor activity (Heisenberg et al., 1985; Martin et al., 1998; Helfrich-Förster et al., 2002). However, none of these studies collected high-density data at the beginning of walking, when decisions about initiation of behavior are critical. We measured activity as a percentage of time that a fly is in motion (Figure 4.6). Our results showed that mean activity levels were significantly influenced by genotype ($F_{9,606}=32.51; P<0.0001$), with males being more active than females ($F_{1,483}=21.65; P<0.0001$), and HU-treated flies more active than CT ($F_{1,606}=33.87; P<0.0001$). All interactions among main effects were not significant ($P>0.01$). Most mutants showed decreased activity, compared to wild-type controls. An exception was $mbm^1(CS)$, which was not surprising, since MBs in these flies appeared anatomically normal (Figure 4.2). Despite developing with a 50% reduction in MB volume (Figure 4.3), $mbmB^1(CS)$ males showed normal activity levels. MB ablation in all groups of wild-type and mutant flies led either to additional decreases or it had no significant influence on activity. Together, these results suggest that MBs are positive regulators of activity during the initial stages of walking, and that additional reductions in activity are pleiotropic effects of most mutations that cannot be attributed to MBs.

Velocity

We measured the speed of walking in every fly for each transition made between landmarks in Buridan’s paradigm (Figure 4.7). Mean velocity was
Figure 4.6 The mean percent of time flies were actively walking during 15 minutes in Buridan’s paradigm was influenced by genotype, gender, and HU ablation. Bars represent mean±SE, n same as Figure 5.
significantly influenced by genotype ($F_{[9,606]} =42.13; P<0.0001$) and genotype
gender interaction ($F_{[5,484]} =6.31; P<0.0001$), while effects of gender, HU ablation,
and all other interactions were not significant ($P \leq 0.01$). Berlin males walked
slightly faster than all other groups measured. All mutants except $mbm^{T}(CS)$ and
$mbmB^{T}(CS)$ walked slower than control CS flies. In the original Berlin
background, walking velocity in $mbm^{T}$ males was comparable with that of other
mutants having similar MB phenotypes. However, $mbm^{T}$ females were extremely
slow, in addition to being the least active of all groups (Figure 4.6). Given that HU
ablation had no significant influence on velocity, we conclude that MBs are not
important for this aspect of walking. Velocity differences among genotypes and
genders are attributed to pleiotropic influences outside of the MBs.

Discussion

The aim of this study was to investigate whether Drosophila MBs function is
modulating walking when motivated by a visual stimulus. Our results
demonstrate that MBs enhance motor activity under these conditions during
relatively short (15-minute) tests of behavior, but are not important for regulating
visual orientation to landmarks or walking velocity. This outcome appears to
contrast the findings of Martin et al. (1998), who recorded walking in MB-
defective flies over several hours and concluded that MBs suppress motor
activity. Similarly, Helfrich-Förster et al. (2002) measured motor behavior in MB-
defective flies over a period of weeks and showed that MBs suppress activity of
males (but not females). It is worth noting that flies have not been observed
Figure 4.7 The mean velocity of flies walking during 15 minutes in Buridan’s paradigm was influenced by genotype and the interaction of genotype and gender. Bars represent mean±SE, n same as Figure 5.
walking for extended periods of time in their natural habitat. Our comparatively brief assay already exceeds the duration of walking that would normally be abbreviated by bouts of inactivity, preening, flight, foraging, fighting, or courtship in the field. Although flies with clipped wings are not likely to feel at home in an illuminated Buridan's arena, they have freedom of movement (in two dimensions) and are not entirely deprived of visual cues. By comparison, in both Martin et al. (1998) and Helfrich-Förster et al. (2002), flies are confined to narrow tubes for extended periods of time (hours to weeks), and their behavior in some experiments is measured in complete darkness. We were thus not surprised by the apparent contradictory results from these three studies of behavior measured 1) in different environmental contexts, 2) over different time scales, and 3) using different recording methods.

A closer look at the time course of walking data in Martin et al. (1998) reveals that most groups of flies with MB lesions were actually less active than control flies during the first 10-20 minutes, followed by a reversal of this relationship that persisted for the remainder of each experiment. This effect was especially pronounced in flies that expressed the tetanus toxin light chain driven by the GAL4 enhancer trap line, 201Y (γ-lobe and, to a lesser extent, in α/β-lobes), and was also observed in mbm¹ and HU-treated flies. Elevated activity levels were recorded throughout experiments in which tetanus was driven by GAL4 line H24 (γ-lobe) and by 17D (α/β-lobes). As suggested by the authors, these data do not support the notion that an MB influence on walking is associated with specific subsystems of KCs projecting to different lobes (Martin et al., 1998). Helfrich-
Förster et al. (2002) recorded activity over a period of weeks at low temporal resolution, which did not permit analysis of behavior in short time intervals. However, it is possible that flies with MB lesions may also have reversed their patterns of behavior early in this experiment, as observed in Martin et al. (1998) and in the current study.

Flies in our study were presented with opposing unattainable visible landmarks to provide a stimulus for walking. In consideration of MB involvement in mediating this behavior, direct anatomical evidence of visual input to the MBs in *Drosophila* has yet to be firmly established (Heisenberg, 2003). This is reflected by several reports of normal visual learning and memory in flies lacking intact MBs (e.g., Wolf et al., 1998). One recent study by Neuser et al. (2008) showed that MB-less flies had normal spatial visual memory in a modified version of Buridan’s paradigm. Functional evidence of a visual input to the MBs was provided by Liu et al. (1999), who demonstrated that flies lacking MBs are unable to retrieve spatial memories under conditions of variable lighting. In terms of neural projections from MBs to motor centers in the brain, these are reported in some insects (e.g., Strausfeld & Li, 1999) but have yet to be described in *Drosophila*. We suggest that activity differences among groups of flies in our study may be a consequence of differing levels of arousal in response to the arena environment context.

Many of the mutants in our experiments were observed to be less active and slower in walking than both Berlin and CS wild-type controls. Chemical ablation of MBs in these flies tended to further reduce activity, but had no significant
influence on their walking velocity. This is consistent with the notion that many of
the brain structure mutants also have additional pleiotropic phenotypes (de Belle
& Heisenberg, 1996). For example, all mud mutant alleles affect neuroblast
proliferation throughout the nervous system, including those that generate
excessive numbers of Kenyon cells and lead to enlarged calyx-like structures at
the expense of pedunculi and lobes (Prokop & Technau, 1994; Guan et al.,
2000). The severity and variability of brain defects in mud flies are extreme and
confound our ability to attribute variation in behavior to a specific structure. Like
mud, many other MB mutants have multiple malformed brain structures as well,
most notably in the CCX (de Belle & Heisenberg, 1996), which is known to
mediate aspects of motor behavior (review: Strauss, 2002). We suggest that this
may account for walking decrements in many of the MB mutants.

In mbm\textsuperscript{1} flies, reduced calyces are due to an interaction between the gene
and the genetic background, since outcrossed flies have MBs of normal size
(Figures 2F and 3; de Belle & Heisenberg, 1996). Consistent with the findings of
Helfrich-Förster et al. (2002), mbm\textsuperscript{1} in the original Berlin genetic background
showed very low activity and velocity in Buridan’s paradigm. In mbm\textsuperscript{1}, this was
not strictly a function of MB defects, since ablation of these mutant structures had
no impact on behavior. However, outcrossing mbm\textsuperscript{1} with CS flies [generating
mbm\textsuperscript{1}(CS)] completely rescued mutant brain and behavioral phenotypes,
indicating that both are under polygenic control.

It is well established that Drosophila MBs mediate odor learning and memory
(Heisenberg, 2003; Fiala, 2007). They have also been shown to function in
various other simple and complex behaviors, including courtship conditioning (McBride et al., 1999; Joiner & Griffith, 2000), sleep (Joiner et al., 2006; Pitman et al., 2006; Seugnet et al., 2008), aggression (Baier et al., 2002), and temperature preference (Hong et al., 2008). A common observation in many behavioral studies of MB function is an influence on decision making and timing (e.g., Tang & Guo, 2001). We suggest that MBs integrate sensory information from the environment and provide contextual signals that modulate motor centers in the brain. In this regard, MBs are important for normal initiation and termination of behavior (Martin et al., 1998). The temporal context and appropriate “reactive” components of behavioral output for these events are MB dependent. By comparison, the CCX affects coordination and other aspects of walking that modulate velocity, contributing an “active” component to motor output (Strauss, 2002).

Conclusion

We have shown, through ablation of wild-type and mutant Drosophila strains, that MBs enhance the initial stages of walking activity. A goal of future studies will be to examine the timing of the MB-dependent switch from enhancement to suppression of motor functions. The value of this work is in shaping a more comprehensive view of sensory integration with the neural actuation of motor output.
References


CHAPTER 5

GENERAL CONCLUSION

The studies presented in this dissertation represent a multi-pronged approach to investigating the relationship between brain structure and function, with an emphasis on the molecular nature of this relationship in the *D. melanogaster* MBs. My work has also laid the foundation for a wide variety of additional questions whose answers will provide even more detail to our understanding of the molecular mechanisms behind the brain-behavior relationship.

In the first study, I characterized the *mbmB* gene, showing that it encodes Imp-α2, a central component of the NPC expressed in neurons throughout the brain. The results associate MB development, learning, LTM and ARM with novel cellular processes known to be dependent on Imp-α2. These include mitotic spindle organization, nuclear cytoplasmic trafficking and axonal transport along microtubules, and offer exciting new avenues for investigations of neural and behavioral plasticity mechanisms. Powerful reagents are now available to address long standing problems, such as studying distributed phases of memory consolidation in the brain, particularly the roles of different MB lobes in these processes (Pascual & Préat, 2001; Dunkelberger, 2008) as well as distinguishing a role during development and or during a behavior.

I rescued MB cell number as well as calyx volume in adult *mbmB* flies using a UAS driven *imp-α2* cDNA expressing in the MBs with the Gal4 line c772 that expresses in all MB lobes. Behavioral experiments have attempted to assign functions to each lobe for different phases of memory. For example, analysis of
the different alleles of the mutant *alpha lobes absent (ala)*, revealed a role for vertical lobes in LTM, while the medial lobes appeared unnecessary (Pascual & Préat, 2001). Repeating the *mbmB* rescue experiments with more spatial resolution (*i.e.* additional drivers with more restricted lobe specific expression patterns) will allow us to determine which portions of the MB support Imp-α2-dependent learning, LTM or ARM. Additionally repeating my rescue experiments using Gal4 drivers that come on earlier during development may increase the percent of rescue for cell counts and MB calyx volume.

Exciting questions about the temporal requirements for Imp-α2 expression in MB development and behavior can now be addressed with a number of modified UAS-Gal4 elements as well (review: McGuire et al., 2004). With the use of the Gal4/UAS system under temporal control via the *hs* promoter (Lis et al., 1983), MB’s could be allowed to develop correctly and then test Imp-α2’s role in learning/LTM/ARM. Imp-α2 function could be disrupted only during development to verify that a reduction to the MBs gives rise to the learning defect independent of Imp-α2’s role in signal transduction post developmentally.

My work on *mbmB* brings to light the importance of possible transport across the NPC and up and down axonal tracts, but I have only looked at the function of a single Imp-α in MB development and classical conditioning. There are three conserved clades of Importin-α’s in most animals as well as in *Drosophila*: α1, α2 and α3 (Kohler et al., 1997; Malik et al., 1997; Tsuji et al., 1997; Mason et al., 2002; Hogarth et al., 2006). Importin-α’s have been shown to have both overlapping and distinct roles in spermatogenesis and oogenesis in *Drosophila*. 
Imp-α2 induced defects in spermatogenesis are due to the loss of activity in a region of α2 conserved between all three paralogs, which can be rescued using transgenes derived from α1 and α3. Conversely, the role of Imp-α2 in oogenesis is not conserved between paralogs, as it is only rescued by its own transgene (Mason et al., 2002). Imp-α1 also performs paralog-specific functions necessary for gametogenesis (Ratan et al., 2008). Imp-α3’s role in the transition to second instar larvae can be rescued with α1 or α3, while its role in the development of the adult and photoreceptor cells is unique to α3 (Mason et al., 2003). All of this work suggests that each Imp-α has tissue specific and developmentally regulated roles worthy of investigating in both time and space. Research into the interplay between all three Imp-α’s and their influence on brain development and associative behavior may provide additional exciting research avenues to explore.

I have also demonstrated that all of Imp-α2’s functional domains are necessary for MB development. This implicates another suite of genes whose binding or interactions with Imp-α2 at each of these sites is critical for MB development and possibly associative conditioning. These include (but are not limited to) Imp-β, CAS, and NLS bearing cargos including CREB. My domain experiment testing the function of Imp-α2’s IBB domain demonstrated a role for Imp-β in MB development and possibly classical conditioning. Although this interaction has yet to be shown in vivo, Imp-α2 and Imp-β likely form a heterodimer in the Drosophila MBs. Kumar et al. (2001) implicated the Drosophila ortholog of imp-β, Ketel, in axon guidance in the developing eye, a
phenotype similar to the β lobe fusion seen in mbmB mutants.

My analysis of Imp-α2 expression in the adult fly brain as well as my preliminary expression data in third instar larvae provides compelling evidence for a more detailed investigation of Imp-α2 expression throughout development. Staining with apoptotic or cell cycle markers would aid in determining exactly why mbmB has a reduction in MB cell number.

Overall the identification of imp-α2 as mbmB provides many more questions than answers, as the cell’s transport system is now central to olfactory associative conditioning and MB development. Perhaps mbmB is the nuclear cytoplasmic gatekeeper, regulating transcription factors (like CREB for example) movement into the nucleus, where it is known to be associated with LTM (Yin et al., 1994). Identification of imp-α2’s binding partners may verify this, and could be taken a step further to investigate possible transient bindings only present after associative testing.

In the second study, I identify eight candidate genes having probable associations with MB development. This work relied on the principles of enhancer trapping, used in a non-traditional way. Typically enhancer traps function as cell-specific markers used for targeted gene expression based on local enhancer expression patterns. I used them as molecular beacons to identify genes associated with MB development. In doing so, I identified the following candidate genes: βFTZ-F1, Or42a, nemy, Tab2, Fz, E75, CK1γ, and ey. These genes are vastly different but a quick glimpse at their cellular function and genetic makeup demonstrates their potential as regulators of MB development.
and associative behaviors. My work on *Fz* (described in Chapter 3), and previous work on *ey* (Callaerts et al., 2001) have shown that these inserts can in fact disrupt genes necessary for MB development.

I mapped the *c739* Gal4 insert to ~6 Kb into the second intron of *βFTZ-F1* and ~10 Kb upstream of its third exon. *βFTZ-F1* is an orphan nuclear receptor and is involved in Ecdysone-mediated autophagy of the salivary gland (Takemoto et al., 2007) as well developmental regulation of the female reproductive system (Allen & Spradling, 2008). A role for *βFTZ-F1* in zinc ion binding has also been inferred from electronic annotations (Flybase curators et al., 2004). *βFTZ-F1* has been shown to express in the embryonic ventral nerve chord and brain (Ayer et al., 1993; Ohno & Petkovich, 1993), yet roles in MB development and behavior have not been investigated. The use of additional alleles of *βFTZ-F1* may distinguish these relationships more accurately as *c739* had no influence on MB calyx volume or gross morphology, yet did lead us to a very intriguing gene.

The Gal4 line *c772* was molecularly mapped to an intragenic region approximately 2 Kb downstream from the end of *Or42a* transcription and about 11.4 Kb upstream of *CG11163*. This may be an enhancer region for either gene, as the traditional view that enhancers must sit upstream of the start of transcription is no longer the only possibility (review: Bulger & Groudine, 2009). *Or42a* is a G-protein coupled receptor necessary for recognition of specific chemical stimuli required for aspects of olfactory sensory perception (Kreher et al., 2005; review: Hallem & Carlson, 2004) and odor specific chemotaxis (Fishilevich et al., 2005). It has been shown to express in the maxillary palp
The molecular function of CG11163 is inferred from sequence similarity as having zinc ion transmembrane transporter activity, and an implied role in cation transport based on electronic associations (Flybase Curators et al., 2004), yet there are no associated phenotypes or in vivo research on this very poorly understood gene. With this in mind, CG11163 may influence the properties of ion channel gating. Divalent cations including Zn$^{2+}$ have been shown to modulate A-type K+ channels in Drosophila CNS (Xu et al., 2005). Shaker, a well studied voltage gated K+ selected channel, is central to associative olfactory learning in Drosophila (Cowan & Seigel, 1986), and expresses in the MBs (Schwarz et al., 1990; Rogero et al., 1997). At this time it is impossible to directly implicate either Or42a or CG11163 in MB development or behavior, but both genes pose interesting possibilities that would be worth investigating directly.

I molecularly mapped c492b to 4 Bp into CG8776 and ~9.3 Kb into nemy. CG8776 is a poorly characterized gene predicted to have carbon-monoxide oxygenase activity, yet it has no associated biological process, or phenotypic data. nemy is a gene necessary for aspects of male courtship conditioning and olfactory based associative learning and middle term (2 hr) memory (Kamyshev et al, 2002). It has been implicated, yet not functionally tested for its role in aggressive behavior (Edwards et al., 2006) as well as moderate suppression of neuromuscular junction (NMJ) overgrowth (Laviolette et al., 2005). nemy is predicted to encode a product highly homologous with mammalian glutaminase, typically associated with glutamine metabolism (Sardiello et al., 2003).
Glutaminase is typically found in glial cells, where it is believed to convert excess pools of glutamate (Glu) (the primary excitatory neurotransmitter in vertebrates) into glutamine (Gln), which is then recycled back into the neurons (review: Márquez et al., 2009). There is compelling evidence that accurate quality and quantity of synaptic transmission of Glu is central for structural maintenance of the NMJ (Featherstone et al., 2005), as well as a necessary component of male courtship in *Drosophila* (Grosjean et al., 2007). It will be interesting to see whether more robust mutant alleles of *nemy* can induce similar structural disruptions to the MB or cause disrupted olfactory associative conditioning. The slight reduction seen in the CCX of *c492b* homozygotes and heterozygotes may be due to the fact that the CCX is ensheathed in a layer of glia known as the transient interhemispheric fibrous ring (TIFR) (Simon et al., 1998). When the TIFR is disrupted, it can cause abnormal CCX morphology (Hitier et al., 2000). Perhaps *nemy* is involved in TIFR formation, which may be disrupted in *c492b* flies, initiating the changes to CCX morphology that were observed.

The MB Gal4 line 201Y has been previously cytologically located as an insert in *TAK1-associated binding protein 2* (*Tab2*), yet there was no sequence location for the insert (Yang et al., 1995; Tettamanti et al., 1997). I verified this work and mapped 201Y to the first intron of *Tab2*, ~1 Kb into the gene, yet still ~5.6 Kb upstream from the second exon. Although there is minimal research on the function of *Tab2 in vivo* and no known expression data, it has been shown to be involved in antimicrobial (Kleino et al., 2005) and bacterial responses (Ferrandon et al., 2001). Like *CG11163* and *βFTZ-F1*, its capacity to bind zinc ions has also
been inferred from electronic annotation (Flybase Curators et al., 2004). Most notably, a Tab2-RNAi driven in class 1 da neurons caused defects in dendritic morphogenesis (Parrish et al., 2006). Although I did not see any significant reductions in MB calyx volume (the dendritic bundles of the MBS) this may be due to the fact that 201Y is not a very robust mutant allele of Tab2. Further investigation using other Tab2 alleles may be more interesting.

Unlike the other Gal4 constructs, Schulz et al. (1996) generated the 247 Gal4 line with an enhancer fragment normally found upstream of dMEF2 fused to lacZ. I mapped the 247 insert inside E75, a secondary gene involved in Ecdysone signaling (review: King-Jones & Thummel, 2005). Ecdysteroids play a critical role in the life cycle of Drosophila melanogaster as well as many other insects via signal transduction through the binding of 20 hydroxyecdysone (20E) typically to a heterodimer of ecdysteroid receptor (EcR), and Ultraspiracle (USP) (review: Thummel, 2001). Ligand binding to this receptor initiates a cascade of downstream gene expression including that of E75 (review: Riddiford et al., 2000). Pruning and degradation of larval MB neurons is necessary for the development of the adult MBs (Technau & Heisenberg, 1982; Lee et al., 1999) and requires the EcR/USP receptor, yet the role of E75 as well as several other Ecdysone primary response genes is dispensable for larval MB pruning (Lee et al., 2000). Our finding that 247 is inserted in E75 brings yet another signaling pathway to light in our attempt to unravel the secrets of MB development. E75 may not have a role in larval MB remodeling of the lobe, but it’s role in cell cycle regulation and cell proliferation has yet to be investigated, as pruning only occurs
at the dendritic level. *E75* is known to inhibit apoptosis in the egg chamber (Terashima & Bownes, 2006), therefore it could influence apoptosis during MB development as well. *E75* may also play a physiological role in signal transduction with impacts on adult behaviors that are independent of its developmental functions. Our preliminary olfactory Pavlovian conditioning data supports this argument, as homozygous 247 flies have a learning defect compounded by reduced olfactory acuity. I do not believe that an *E75* enhancer is solely responsible for the MB specific expression pattern of 247 (due to the MB specific *Mef2* enhancer engineered into the 247 construct). I cannot however rule out the possibility that *E75* regulation acts in concert with the 247 *dMef2* enhancer. Thorough investigation of the function of *E75* in MB development and associative behaviors would be more accurately performed with a more robust mutant allele insensitive to genetic background.

I mapped the H24 Gal4 line to *Casein Kinase 1 (CK1γ)* (also known as *gilgamesh*) in an intron ~5 kb downstream from the end of *CK1γ*’s second exon in the B, D, E and I transcripts and third exon of the F and H transcripts, yet still upstream from the first exon of the A, C and G transcripts. *CK1γ* plays a central role in spermatogenesis and male sterility (Castrillon et al., 1993; Nerusheva et al., 2009), as well as up-regulating *Wnt* signaling (Zhang et al., 2006). *CK1γ* was also identified as an uncharacterized neuronal precursor in a differential embryonic head cDNA screen (Brody et al., 2002), although no further work was done to verify its exact role in brain development or tissue localization. The expression of *CK1γ* in glial cells of the developing eye is necessary for the control
of glial cell migration, and happens in concert with ey (Hummel et al., 2002). Another glial gene, draper, is necessary for MB pruning (Awasaki & Ito, 2004), a process dependent on ubiquitin mediated degradation (Watts et al., 2003) that only occurs in the γ lobes. Interestingly CK1α has been shown to promote ubiquitin dependent degradation of the Drosophila homologue of the human c-myc proto-oncogene (dMyc), known to regulate growth, cell death and inhibition of ommatidial differentiation (Galetti et al., 2009). Spatial convergence of H24’s γ lobe specific expression pattern and larval MB pruning occurring only in the γ lobe, as well as the evidence linking glial cells to pruning and the CK1 family to ubiquitin dependent degradation provides strong evidence that CK1γ may be involved in MB pruning. Knowledge of CK1γ’s expression pattern in the central brain will greatly help in determining what role it may have in MB development and function. Although I believe the H24 insert to be a weak allele of CK1γ, it did induce a 20% decrease in MB calyx volume in heterozygous females compared to heterozygous males. Again a stronger allele may show even more interesting anatomical phenotypes in the MBs.

Further investigations using more temporal and spatial control of each gene’s expression should yield some interesting results regarding how each gene influences brain development and associative behaviors. My work has provided the building blocks for future studies on how these candidate genes function in the brain.

In the third study, I provide an analysis of the role of the MBs in regulating motor behavior in Drosophila. Experiments show that they have an impact on
motor activity in a time-dependent fashion. During the initial stages of walking, MBs up-regulate activity levels, followed by a switch to down regulation after approximately 4.5 hrs. MBs are associated with different behaviors including sleep (Joiner et al., 2006; Pitman et al., 2006; Seugnet et al., 2008), aggression (Baier et al., 2002), and some aspects of courtship memory (Joiner & Griffith, 2000; McBride et al., 1999). Our data provides evidence that MBs regulate behaviors differentially over time, and I suggest that this may be an important property of their impacts on other behaviors. Indeed, their role in responding to convergent sensory signals during associative learning is based on their capacity to recognize temporally-coded information (review: Berry et al., 2008). Revisitation of these sleep, aggression and courtship experiments with this in mind will likely provide a more accurate representation of how MBs serve to regulate behavior in real time.

It would also be very informative to repeat our work and assess walking over longer periods of time (like Martin et al., 1998) using a suite of Gal4 lines driving tetanus toxin, or shibirie ts to disrupt different subsets of the MB lobes. This type of structural and functional dissection may indicate that one subset of MB lobes initiates the behavior, while another terminates it in a context/time dependent fashion. There is compelling evidence from the olfactory associative conditioning work of Dunkelberger (2008) and Pascual and Préat (2001) indicating that different MB lobe subsets modulate different phases of learning and memory. I propose that MB lobe subsets modulate function in a more general way by fine-tuning the quantity, rather than the quality, of a behavior.
In *Drosophila*, technical advances in molecular and behavioral neurobiology are moving at a rapid pace. Guided by knowledge of which neural substrates support a particular behavior of interest, it is now possible to characterize the important cellular processes involved in (1) the development of relevant neural networks and neurons, and (2) their functions in generating behavior. Experiments can also be devised that identify processes that either share, or distinguish, developmental from functional properties.
References


FlyBase Curators. (2004-). Swiss-Prot Project Members, InterPro Project Members, Gene Ontology annotation in *FlyBase* through association of InterPro records with GO term.


Terashima, J. & Bownes, M. (2006). E75A and E75B have opposite effects on
the apoptosis/development choice of the *Drosophila* egg chamber. *Cell Death Differ*, 13, 454-64.


C

![Bar chart showing volume (10^3 μm^3) for different genotypes.]

Genotype

Volume (10^3 μm^3)

mbmB/PKA-C1[H2]
mbmB/PKA-C1[DN]
mbmB/P-BG02804
mbmB/P-BG01038
mbmB/P-BG00875
mbmB/P-BG02142

D

![Immunoblot with bands labeled CS, Df s1402+, mbmB, and P-BG00875.]

E

![Western blot showing anti-αTubulin (50 kD) and anti-αPKA-C1 (40 kD) signals.]
Figure A.1 Mapping of *mbmB*: Early data and experimental approaches from 2001-2003. (A) I began my mapping efforts of *mbmB* using complementation analysis and histology with all available Dfs near its previously reported recombination map site of 2-31. Serial mass histology was performed on a series of Dfs on chromosome 2L (methods described in Chapter 1 experimental procedures). It appeared that *Dfs1402* (BL#556) failed to compliment *mbmB*, as heterozygotes were significantly smaller than wildtype (19 x 10^3 µm³), yet not quite as small as *mbmB* (14 x 10^3 µm³) (F[5,78]=18.69, P<0.0001). There was no significant influence of sex (F[1,78]=2.97, P=0.089) or the interaction between sex and genotype on MB volume (F[5,78]=1.39, P=0.239). (B) This mapping led us to the region 30C1-2;30F. Volume data for *mbmB/Df(2L0Te29Aa-11* and *mbmB/Df(2L)30A-C* was collected prior to my arrival in the lab. Both Dfs complemented *mbmB*, displaying wildtype MB volumes as heterozygotes with *mbmB*, so they were included as negative complementation data in the map. I knew that *mbmB* was female sterile in addition to having MB and behavioral defects. *PKA-C1* is a very well characterized learning and memory mutant (Skoulakis et al., 1993) that expresses in the MBs, is sterile and falls in the cytological region believed *mbmB* to be in, specifically at 30C5. (C) I performed serial mass histology and found that all but one disruption (*P-BG00875*) failed to complement *mbmB* as heterozygotes had significantly smaller MBs (17 x 10^3 µm³) (F[5,58]=8.72, P<0.0001). There was no influence of sex (F[1,58]=4.02, P=0.050) or the
interaction between genotype and sex ($F_{[5,58]}=0.65$, $P=0.660$). Although this P-element is not located directly in PKA-C1, I sought additional routes to investigate two questions: 1) was mbmB in the same pathway of PKA-C1 (i.e. did $P$-$BG00875$ affect PKA-C1 expression levels) and 2) was mbmB PKA-C1? (D-E) To investigate the first question, I performed an SDS-page western blot using the following primary antibodies: rabbit anti-PKA-C1 Ab (O’Kane) at 1:5,000 dilution, as well as an anti-αTubulin Ab (Sigma) at 1:8,000 dilution, and the following secondary Abs: goat anti-rabbit HRP diluted 1:7,500 (Jackson Immuno Research), Alexa Fluor® 488 and Alexa Fluor® 594 (Invitrogen) both used at 1:1,000 dilution. I followed the same tissue collection, protein extraction and blotting protocols listed in Chapter 1 experimental methods except that I used a 12% gel. (D) Dfs1402 is known to uncover PKA-C1. I saw an estimated 50% reduction in PKA-C1 levels in the Dfs1402/+ sample, while the rest showed wildtype expression levels of PKA-C1, including mbmB. (E) Samples from multiple alleles of other uncharacterized MB structural mutants (mbmC and smu) were analyzed as well (de Belle & Heisenberg, 1996). Although there was bleeding from the molecular marker I used into the first few lanes of the gel (Precision Plus Protein Standard, Bio-Rad), I was able to determine that there was no difference in α-TUBULIN, or PKA-C1 levels in any of the MB mutant alleles, verifying our previous blot for mbmB. I interpreted this to mean that mbmB (as well as the other MB structural mutants) were likely not in the same molecular pathway as PKA-C1 as they did not have any influence on its adult expression level. (F) Unfortunately after I conducted the original mapping, there was an incubator meltdown and both the Dfs1402 and $P$-$BG00875$ strains of flies were lost. They were re-ordered (on several occasions) and the experiments were repeated (as well as the appropriate controls which were missing from the preliminary mapping data). I was unable to repeat any of the original complementation data, as Dfs1402, and $P$-$BG00875$ now complemented mbmB ($F_{[5,77]}=6.19$, $P<0.0001$). (G) I repeated the last experiment with several additional strains of flies from the original mapping, all of which were re-ordered a third time. I was still unsuccessful as no strain failed to complement mbmB ($F_{[7,41]}=2.06$, $P=0.071$). (H) I re-created the histology crosses on the “original recipe” (Table A.2) food and found no significant difference in MB calyx volume ($F_{[4,138]}=90.42$, $P<0.0001$). I concluded that changes in the food quality were not responsible for our inconsistencies in histology mapping data. Another parameter that I investigated was maternal contribution. All crosses listed in this food experiment were done in both directions. I observed significant differences in calyx volume for reciprocal crosses ($F_{[6,134]}=76.79$, $P<0.0001$) (Data not shown), however these changes did not restore any cross to the original mutant calyx volume. Therefore I pooled calyx volume data for all reciprocal crosses. In final conclusion of our original mapping data, I now feel that there were several contributing factors to these false positive results, unfortunately stalling the mapping of mbmB for several years. Firstly, the Dfs1402 originally had a copy of a P-element in it, as the eye color of the flies was orange. After re-ordering the strain, it arrived as a white-eyed fly. I believe that the original complementation data that uncovered mbmB was due to residual P-element activity, not the Df.
This fits with the verified location of \textit{mbmB} (30F6), only 443 Kb away from the start of Dfs1402 (30C1). The P-element (used to make Dfs1402) was likely still present in the stock and inserted in \textit{Pen}, a likely distance away for a jump, thereby disrupting \textit{Pen} transcription. It is also likely that the end point of all Dfs I used were not accurate, perhaps explaining why I would see some intermediate phenotypes with other Dfs like DfN22-14. Interestingly the end points of DfN22-14 to the right and DfMdh to the left both had uncertain regions, which perhaps did not completely overlap at 30F6, thereby missing \textit{Pen}. Finally, I can imagine two different scenarios where the P-BG00875 line would falsely compliment \textit{mbmB}. First if there was a second insert present (as described above). Second, if it disrupted another gene (perhaps PKA-C1) that was involved in the structural regulation of MBs, although this scenario is less likely as when the line was re-ordered, I was unable to replicate the complementation data. (A, C, F-H) Bars represent mean ± SE of mean PI for each genotype. \textit{n} indicated on each bar. Different letters designate significant differences (SNK, \textit{P}$\leq$0.05).
Table A.1 Complementation analysis of PKA-C1. (A) I decided to test the following lines of flies for complementation to mbmB, several of which are disruptions in the PKA-C1 gene (located between 2L:9,684,656 - 9,699,293 in the [-] orientation) (Flybase) (details in Chapter 1 experimental methods). (B) To investigate whether or not mbmB was in fact PKA-C1, I sequenced 1,240 Bp of the coding sequence (CDs) of PKA-C1 in mbmB mutants and CS. Primer sets and their respective T_m's are listed in D. I used the same methods for DNA extraction, PCR and sequencing as those listed in Chapter 1 experimental procedures. I replicated each sequencing reaction in the forward and reverse direction 7-8 times per primer set. Chromatographs were analyzed and sequences aligned to the wildtype sequence for PKA-C1 using Sequencher™ version 3.0 (Gene Codes, Ann Arbor Michigan). I found no SNPs in the sequence data for mbmB compared to CS (data not shown). Although there was preliminary evidence that mbmB could have been synonymous with the catalytic subunit of PKA, using a variety of techniques I found contradictory evidence verifying that they are not the same gene.

A

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Table A.2 Fly food recipes. I was interested in testing the food quality on MB plasticity as our lab switched fly food recipes during that time as well. MB calyx volume has been shown to be relatively plastic phenotype. (A) The “original” semi-defined medium recipe. (B) The new recipe used by Bloomington’s Stock center (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). In an attempt to re-create our original positive mapping data for mbmB, I decided to try the original recipe with the newly ordered flies.

A

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<td>Yeast Extract</td>
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<tr>
<td>Agar</td>
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<td>Peptone (soy based)</td>
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<tr>
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<tr>
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<tr>
<td>MgSO₄ x 7H₂O</td>
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<td>CaCl₂ x 2H₂O</td>
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<tr>
<td>Propionic Acid</td>
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<tr>
<td>Tegosept (10% p-hydroxy-benzoic acid, CH₃ ester in 95% ethanol)</td>
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B

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Table A.3 Exelixis lines. (A) Before I had switched to using sterility as our screening phenotype for mapping $mbmB$, and believed the region missing in Df556 (30C1-30F4) uncovered $mbmB$, I tested 4 Exelisis collection lines in that region for complementation to $mbmB$ (Parks et al., 2004). Their cytological and sequence positions are listed.

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<td>7508</td>
<td>30C9;30E1</td>
<td>2L:3302636–3302646;3354856–3354858</td>
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<tr>
<td>7515</td>
<td>30C1;30C1</td>
<td>2L:15264714;15439965</td>
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Figure A.2 Histology data for Exelixis lines in 30B10-30E1. I performed paraffin mass histology on \(w;mbmB/Exe\) flies and found a significant difference between these flies and homozygous \(mbmB\) flies \((F_{[4,41]}=136.16, P<0.0001)\). Bars represent mean ± SE of mean calyx volume for each genotype. \(n\) indicated on each bar. Different letters designate significant differences (SNK, \(P\leq0.05\)).
Table A.4 Sequence location of additional $imp-\alpha2$ alleles. After mapping $mbmB$ to $importin-\alpha2$, I obtained additional P-elements in the gene. Their positions relative to $importin-\alpha2$ (located between 2L:10,056,906 - 10,060,097 in the [+1] orientation) are listed. Each line was crossed to virgin $w; mbmB/Sm5$ and $mbmB/Imp-\alpha2$ P's were checked for female sterility and MB calyx volume.

<table>
<thead>
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<th>Stock#</th>
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<td>D07004</td>
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<td>15654</td>
<td>31A2</td>
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<td>602 Bp into 1st intron, 114 Bp upstream of second exon</td>
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Figure A.3 Histology on additional *imp-\textalpha{}2* alleles. All females heterozygous for each of the three P-element lines and *mbmB* were fertile (data not shown), and had significantly larger MB calyx volumes (119-168\%) than homozygous *mbmB* females ($F_{[3,28]}=96.90$, $P<0.0001$). Bars represent mean ± SE of mean calyx volume for each genotype. *n* indicated on each bar. Different letters designate significant differences (SNK, $P\leq0.05$).
Figure A.4 Imp-α2 expression pattern in the larval brain. I analyzed the expression pattern of Imp-α2 in CS L3 expressing cytoplasmic GFP in the MBs with the Ok107 driver. There was no overlap in MB cells that had already undergone mitotic division (green), while Imp-α2 expressed in dividing neuroblasts throughout the larval brain (red). This work is preliminary because the larvae needed to be staged much more specifically as these were just selected based on their position in the vial (wandering on the wall). I would also like to include mbmB in this profile as a negative control for the Ab.
Figure A.5 Preliminary rescue experiments for \textit{mbmB} and odor balancing. A modified Pavlovian conditioning T-maze paradigm was used to assay olfactory associative learning and sensory controls (Tully & Quinn, 1985; de Belle & Heisenberg, 1994; de Belle & Heisenberg, 1996; Tully et al., 1994). Briefly, two to
six day old flies were aspirated into training tubes lined with an electrifiable copper grid and tested in groups of ~100. To measure olfactory learning, flies were exposed to a 750 ml/min. air current bubbled through a single odor mixed with heavy mineral oil. Odors Benzaldehyde (BENZ) (Sigma) and 4 Methyl cyclohexanol (MCH) (Sigma) were temporally paired with 1.25 second pulse of 120V dc shock delivered every 5 sec. for one minute. Flies were then exposed to a second odor for an additional minute with no electric shock. To measure learning, trained flies were given the choice of both odors in converging air current for 2 min. Learning was measured as a function of shock paired odor avoidance one minute after training. Flies were trained in a reciprocal fashion as well, and scores from both tests were averaged to get a performance index (PI) accounting for any odor biases that may exist between populations of flies. Shock avoidance controls used a single arm of the T-maze with a 120V exposure for 2 min. Odor avoidance controls employed both arms of the T-maze with an exposure of a single odor in one arm and air in the other. Performance indices are the average normalized percent avoidance of either learning (shock paired with an odor) or a single stimuli (shock or odors alone). I performed rescue experiments using flies with the c772 MB driver (Appendix B) (A) Shock avoidance (120V) was normal for all genotypes tested (F_{[3,20]}=2.16, P=0.124). (B) Dunkelberger (2008) initially characterized the learning defect seen in mbmB in our learning room using the following odor concentrations: 4 μl of BENZ and 7 μl of MCH each in 5 ml of oil. I attempted a behavioral rescue of the learning defect using these concentrations, but mbmB did not show a mutant learning score, as all genotypes performed similarly (F_{[4,25]}=4.25, P=0.948). This also meant I was unable to determine if the rescue was successful. My rescue experiment differed from Dunkelberger’s data in that I used a w; mbmB and Dunkelberger had used mbmB. Interestingly, there is some evidence that w^{1118} may improve olfactory defects (Deiglemenn et al., 2006). (C-D) It then became necessary for us to re-balance the odors to find concentrations where w; mbmB would display its learning defect. I tested a variety of odor concentrations for both BENZ and MCH in CS, w^{1118} and w; mbmB flies. This would also allow us to see if there was an olfactory difference between CS and w^{1118} that may account for the normal learning PI initially observed in w; mbmB. All odor concentrations listed were diluted in 5 ml of oil. (C) Flies tested at higher concentrations had appropriate PIs greater than 80% and there were no significant differences across genotypes [13 μl BENZ (F_{[7,32]}=4.01, P=0.003) and 20 μl MCH (F_{[17,89]}=9.86, P<0.0001)]. Our data, although preliminary, indicates that at low MCH concentrations, w^{1118} may show a decrease in odor avoidance. (E) I repeated the learning experiment with 13 μl of BENZ and 20 μl of MCH to find that although the trend was that w; mbmB had a reduced PI, it was not statistically different from the other genotypes (F_{[7,32]}=4.01, P=0.003). Overall the PI’s were also on the low side. It should also be noted that at these very high odor concentrations, it is possible for the person conducting the tests to smell the odors. (F) I felt that the learning experiment should be repeated at slightly lower odor concentrations to see if the overall PI could be increased and therefore allow the expected reduction in PI in mbmB to become more obvious. I used 10 μl BENZ and 15 μl of MCH, and found that
although there was no significant difference in PI's, the trend was that both mbmB and w; mbm had a reduced PI [(F_{7,32}=4.01, P=0.003) and (F_{22,94}=9.86, P<0.0001), respectively). As this was only a pilot study, my sample size was quite low for this experiment (n=2-3/column). I would predict that at these concentrations with a higher sample size, the learning defect originally exhibited by mbmB would return and the rescue experiment could be successfully conducted. A-F: Bars represent mean ± SE of mean PI for each genotype. n indicated on each bar. Different letters designate significant differences (SNK, P≤0.05).
Figure A.6 Analysis of possible CREB-IMP-α2 interaction. A. It has been well established that IMP-α2 binds cargos containing an NLS tag, many of which are transcription factors whose presence in the nucleus is critical for their
function (Hogarth et al., 2005). It has also been shown that CREB has an NLS in its bZip domain (Waeber & Habener, 1991). Interestingly, mbmB and CREB are two of only several genes known to cause LTM defects (Dunkelberger, 2009, Yin et al., 1995). An electromobility supershift assay (EMSA) (as described by Horiuchi et al., 2004) was performed to investigate the possibility that CREB and IMP-α2 are binding partners. Briefly, to collect extracts, heads were crushed by adding 3µl/head cold homogenization buffer (15 mM Hepes, pH 7.5, 10 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). To remove debris, each extract was centrifuged two times at 14,000 x g. I then added equal volumes of 2 x HEMG (200 mM KCl, 40 mM Hepes, pH 7.5, 20 mM MgCl2, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). a spectrophotometer was used to determine protein concentrations. For probe generation, 200 ng of 3xCRE double-stranded oligonucleotide was used (Yin et al., 1995b), and radiolabeled using polynucleotide kinase + 100µCi of [γ-32P]ATP as recommended (New England Biolabs). I incubated the probe at room temperature for 1 h with 5 µg of Drosophila extract, (fractionated by centrifugation into nuclear, cytoplasmic and crude extracts) at a volume of 10 µl in: 12 mM Hepes, pH 7.9, 4 mM Tris-Cl, pH 7.9, 1 mM EDTA, 12% glycerol, 1 mM dithiothreitol, 5 mM MgCl2, 60 mM KCl, 1 mg/ml bovine serum albumin, and 0.4 mg/ml poly(dl-dC). Samples were run out on a 4% acrylamide gel (acryl:bis at 80:1 dilution), Tris-glycine (380 mM glycine, 50 mM Tris base, 2 mM EDTA, 3.6mM MgCl2, and 1% glycerol). Sample transfer to Whatman No. 3MM paper was done, and then it was exposed to film. Unlike my previous experiments, I used an IMP-α2 Ab generated by the Frasch lab for our preliminary EMSA (Kussel & Frasch, 1995). Of note is the fact in my hands this Ab was unsuccessful for use with western blot analysis. I observed a supershift of the CREB complex in the presence of IMP-α2 Ab in CS, mbmB and Df (null) lanes. This is seen the strongest in the nuclear extracts at the higher concentration (lanes 4-6), although it is also minimally observed in the cytoplasmic extracts (lanes 17-19). Unfortunately, I also observed high levels of background binding of the Ab to the probe, making the supershift results ambiguous. This experiment was repeated by a member of the Yin lab using a purified version of the Frasch IMP-α2 Ab and with the Mechler Ab (Török et al., 1995; Gorjánácz et al., 2006). Unfortunately, the super-shift I originally observed was not seen in either case (data not shown). B. I performed a immunoprecipitation (IP) western blot as another test to determine if IMP-α2 and CREB were forming a complex. Briefly, extracts were collected from 50 heads, flash frozen in liquid nitrogen, homogenized in homogenization buffer (HB buffer) + inhibitors + NP40 into a final concentration of 2 µl HB buffer/head. Samples were incubated on ice 30 min., spun and supernatant containing crude soluble homogenate was removed and used in IP. The Frasch IMP-α2 Ab was used for the IP (as well as a second unsuccessful set with CREB, data not shown) (Kussel & Frasch, 1995). Briefly, 25 µl of Protein A beads were added to 200 µl of each extract and rotated for 3 min. Samples were then spun at 3,000 x for 3
min. 2 µl of Ab and 100 µl extract were incubated on a rotator at RT for 3 hrs. 50 µl of Protein A beads in a 50% slurry were also incubated on a rotator for 3 hrs. Samples were spun at 3,000 x for 3 min. then supernatant saved and beads washed 5 x with 1 ml 0.15M HEMGN + protease inhibitors, with spins at 3,000x for 3 min. between washes to pellet beads. Beads were then re-suspended in 2x Laemmli buffer, boiled for 10 min. then 25 µl was loaded onto SDS page for western blotting. I used mouse anti-CREB 657 (Horiuchi, et al., 2004) (representing the blocker isoform) at 1:50 dilution as the primary Ab and goat anti-mouse antibodies conjugated to HRP (Jackson Immuno Research) at 1:3,000 dilution as the secondary Ab. See Chapter 1 experimental procedures for SDS-page, blotting and detection details. I observed the CREB DNA complex in the tissues with the IMP-α2 Ab present as well (lane 3-4 43 and 34 Kd bands), yet this result is confounded by the IMP-α2 Ab alone (lane 7) showing a giant smear. At this point I only have arguable evidence, not conclusive data about CREB IMP-α2 binding. I feel that both experiments were inconclusive mainly because of the Ab, yet they do not rule out the idea that CREB and IMP-α2 may be binding partners for several reasons. Firstly, it is possible that our extractions were not done at a biologically meaningful time, as our samples for both experiments came from adults ranging from 2-7 days old. The potential binding of CREB and mbmB may also be very short lived as well, as passage through the NPC is rapid and likely dependent on a stimulus. Loss of the supershift with the Mechler Ab can be explained by the fact that the Ab is missing part of the small NLSB, as it is from AA#279-522, while the original Frasch Ab (used here) is from AA#13-522. It is known that imp-α2 has two NLS binding residues distributed in separate domains; the large NLS binding site (LNLSB), and the small NLS binding domain (SNLSB). LNLSB spans ARM repeat 2-4 and can bind monopartite NLS motifs or the larger section of bipartite NLS motif. SNLSB spans ARM repeats 7-8 and binds the smaller portion of the bipartite motif (Conti & Kuriyan, 2000; Conti et al., 1998; Fontes et al., 2000; Kobe, 1999; Matsuura and Stewart, 2004). CREB has an NLS signal (Waebner & Habener, 1991), although it has not been established whether CREB binds at the LNLSB, the SNLSB or both in the MBs. This does not explain why there is so much background binding of the Ab in both the EMSA and the IP western. To resolve these issues, the Yin lab is currently working on generating a full length Ab to IMP-α2, which will be purified and used to repeat these experiments. It will also be interesting to investigate the role of IMP-α1 and 3 as well as IMP-β, as IMP-β has been shown to bind CREB in vivo (Forwood et al., 2001). This is not compelling enough to stop investigating the possible interactions between IMP-α2 and CREB due to our preliminary data and their shared LTM defects. In addition, the roles of Importins are often different between species and tissue types (Mason et al., 2002). A striking example of this can be found in the human IMP-α4 which is responsible for 1% of the protein in skeletal muscle, yet is virtually missing heart, kidney and spleen (Nachury et al., 1998). I conducted this work at the University of Wisconsin, Madison in collaboration with Tom Tubin in the Yin Lab.
Table A.5 *imp-α2* RNAi lines tested for sterility (data not shown). I was interested in using two potential *imp-α2* RNAi lines from Vienna Drosophila RNAi Center (VDRC) (Dietzl et al., 2007) for further analysis of *imp-α2*. First, I backcrossed each RNAi line to *w^{1118}(CS7)* for seven generations, then re-balanced them (Appendix B 6). The two lines containing the UAS *imp-α2* RNAi constructs (UAS *imp-α2* RNAi 1 and UAS *imp-α2* RNAi 2) were driven in the ovaries using the *P[Gal4]nanos* driver (Van Doren et al., 1998), which has previously been shown to rescue *imp-α2* sterility in the null (Gorjánácz et al., 2002). Female offspring were screened for sterility. Between 16-19 single female matings were set up with female offspring from the above mentioned crosses. Results indicated that neither RNAi line was sterile, as all female UAS *imp-α2* RNAi 1 and 2; P[Gal4]nanos flies were fertile.

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Figure A.7 Histological analysis for multiple alleles of several MB structural mutants. I analyzed the MB calyx volume of a few alleles of multiple MB structural mutants (mbmB, mbmC, and smu). Significant differences were observed in all of the MB structural alleles ($F_{8,42}=26.21, P<0.0001$). Bars represent mean ± SE of mean calyx volume for each genotype. $n$ indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$).
References


APPENDIX B
CROSSING SCHEMES

Table B.1 Crossing schemes.

1. **Anatomy**

   G₁: \( \Phi \text{ CS } \times \Phi \text{ CS} \downarrow \)  
   G₂: \( \Phi \text{ CS } \)

   G₁: \( \Phi \text{ mbmB/SM5 } \times \Phi \text{ CS} \downarrow \)  
   G₂: \( \Phi \text{ CS/mbmB} \)

   G₁: \( \Phi \text{ mbmB/SM5 } \times \Phi \text{ mbmB/SM5} \downarrow \)  
   G₂: \( \Phi \text{ CS/mbmB} \)

2. **Sterility back crossing**

   G₁: \( \Phi \text{ mbmB/SM5 } \times \Phi \text{ CS} \)  
   G₂: \( \Phi \text{ mbmB/CS } \times \Phi \text{ mbmB/mbmB} \)  
   G₃: Single \( \Phi \text{ s mbmB/CS } \times \Phi \text{ CS} \)

3. **Mapping**

   G₁: \( \Phi \text{ CS } \times \Phi \text{ w; Df or P/Balancer} \downarrow \)  
   G₂: \( \Phi \text{ CS/Df or P} \)

   G₁: \( \Phi \text{ w; mbmB/SM5 } \times \Phi \text{ w; Df or P/Balancer} \downarrow \)  
   G₂: \( \Phi \text{ w; mbmB/Df or P} \)

   G₁: \( \Phi \text{ w}^{1118}; \text{ CS; CS } \times \Phi \text{ w; imp-α2⁰/CyO; Tm6/CS} \)
4. Rescue Histology

G1:  
♀ w; mbmB/SM5 × ♂ w; mbmB/imp-α2⁰

G2:  
♀ & ♂ w¹¹¹⁸, imp-α2⁰/CS

G1:  
♀ w¹¹¹⁸ × ♂ w¹¹¹⁸

G2:  
♀ & ♂ w¹¹¹⁸

G1:  
♀ w; mbmB/SM5 × ♂ w; mbmB/SM5

G2:  
♀ & ♂ w; mbmB/mbmB

G1:  
♀ w; mbmB/SM5; CS × ♂ w; mbmB/SM5; imp-α2 cDNA/MKRS

G2:  
♀ & ♂ w; mbmB; imp-α2 cDNA/CS

G1:  
♀ w; mbmB/SM5 × ♂ w; mbmB-c772/SM5

G2:  
♀ & ♂ w; mbmB/mbmB-c772

G1:  
♀ w; mbmB/SM5; imp-α2 cDNA/MKRS × ♂ w; mbmB-c772/SM5

G2:  
♀ & ♂ w; mbmB/mbmB-c772; imp-α2 cDNA/CS

G1:  
♀ w; mbmB/SM5; 247 × ♂ w; mbmB/SM5; imp-α2 cDNA/MKRS

G2:  
♀ & ♂ w; mbmB/mbmB; 247/ imp-α2 cDNA

G1:  
♀ w; mbmB/SM5; CS × ♂ w; mbmB/SM5; 247

G2:  
♀ & ♂ w; mbmB; 247/CS
G₁: ♀ w; mbmB/SM5; CS; CS × ♂ w; mbmB/SM5; CS; Ok107/CS
G₂: ♀ & ♂ w; mbmB; CS; Ok107/CS

G₁: ♀ w; mbmB/SM5; CS; Ok107/CS × ♂ w; mbmB/SM5; imp-a2 cDNA/MKRS; CS
G₂: ♀ & ♂ w; mbmB; imp-a2 cDNA/+; Ok107/CS

5. Rescue Cell count
G₁: ♀ w; GFPnls; CS; CS × ♂ w; CS; CS; Ok107/CS
G₂: ♀ & ♂ w; GFPnls; CS; Ok107/CS
G₁: ♀ w; mbmB::GFPnls/SM5; CS; CS × ♂ w; mbmB/SM5; CS; Ok107/CS
G₂: ♀ & ♂ w; mbmB::GFPnls/mbmB; CS; Ok107/CS
G₁: ♀ w; GFPnls; CS × ♂ w; CS; 247/CS
G₂: ♀ & ♂ w; GFPnls; 247/CS
G₁: ♀ w; mbmB::GFPnls/SM5; CS × ♂ w; mbmB/SM5; 247/CS
G₂: ♀ & ♂ w; mbmB::GFPnls/mbmB; 247/CS

6. imp-α2 RNAi Lines
G₁: ♀ w¹¹¹⁸; CS; CS × ♂ w; CS; imp-α2 RNAi-5 or 6
G₂: ♀ w¹¹¹⁸; CS; CS × ♂ w¹¹¹⁸; CS; imp-α2 RNAi-5 or 6/CS
G₉: ♀ w¹¹¹⁸; CS; imp-α2 RNAi-5 or 6/CS × ♂ w; CS/CS; Tm3/Tm6b
G₁₀: ♀ & ♂ w¹¹¹⁸; CS; imp-α2 RNAi-5 or 6/Tm3
G₁: ♀ w¹¹¹⁸; CS; Impα2-RNAi-5 or 6/Tm3 × ♂ w¹¹¹⁸; P[nanos::Gal4VP16];
G_2: \( \varphi \text{ & } \varnothing \text{ w}^{1118}, \text{ P[nanos::Gal4}^{VP16} \text{]/+; Impα2-RNAi-5 or 6/CS} \)

7. \textit{imp-α2} Domains

3d chromosome (\( \text{imp-α2}^{\text{DOM 1-8}} ; \text{n=8} \))

G_1: \( \varphi \text{ w; mbmB/SM5; imp-α2-cDNA/MKRS} \times \varnothing \text{ yw; Sp/CyO; imp-α2}^{\text{DOM 1-8}/Tm6} \)

G_2: \( \varphi \text{ w; mbmB-c772/SM5; CS} \times \varnothing \text{ w; mbmB/ y/+; imp-α2}^{\text{DOM 1-8}/\text{MKRS}} \)

G_3: \( \varphi \text{ & } \varnothing \text{ w; mbmB-c772/mbmB; imp-α2}^{\text{DOM 1-8}/\text{CS}} \)

X chromosome (\( \text{imp-α2}^{\text{DIM}} ; \text{n=1} \))

G_1: \( \varphi \text{ w; mbmBc-772/SM5} \times \varnothing \text{ imp-α2}^{\text{DIM}/\text{Sp}/\text{CyO}; \text{Sb/TM6}} \)

G_2: \( \varphi \text{ w; mbmB/SM5; CS} \times \varnothing \text{ imp-α2}^{\text{DIM}/\text{CS}; \text{mbmB-c772(CS)/Sp; Sb/CS}} \)

G_3: \( \varphi \text{ & } \varnothing \text{ imp-α2}^{\text{DIM}/\text{CS}; \text{mbmB-c772/mbmB; CS}} \)

G_1: \( \varphi \text{ w; mbmB/SM5} \times \varnothing \text{ imp-α2}^{\text{DIM}/\text{Sp}/\text{CyO}; \text{Sb/TM6}} \)

G_2: \( \varphi \text{ imp-α2}^{\text{DIM}/\text{w}; \text{mbmB/Sp}; \text{Sb/CS} \times \varnothing \text{ w; mbmB; CS}} \)

G_3: \( \varphi \text{ & } \varnothing \text{ imp-α2}^{\text{DIM}/\text{w}; \text{mbmB; CS}} \)

G_1: \( \varphi \text{ w}^{1118} \times \varnothing \text{ w}^{1118} \)

G_2: \( \varphi \text{ & } \varnothing \text{ w}^{1118} \)

G_1: \( \varphi \text{ w; mbmB/SM5} \times \varnothing \text{ w; mbmB/SM5} \)
\( G_2: \)  
\[ \varphi \& \varrho \; w; \ mbmB \]

\[ G_1: \]  
\[ \varphi \; w; \ mbmB/SM5 \times \varrho \; w; \ mbmB-c772/SM5 \]

\[ G_2: \]  
\[ \varphi \& \varrho \; w; \ mbmB-c772/mbmB \]
Figure C.1 Preliminary MB GAL4 calyx volumes. Serial sections of paraffin-embedded brains were used for planimetric MB measurements. This was a pilot study to determine if a thorough analysis of MB specific Gal4 lines would yield any interesting phenotypes. All genotypes are homozygous for the Gal4 insertions. There was no effect of sex ($F_{[1,59]}=0.063$, $P=0.802$) or the interaction of sex and genotype ($F_{[2,59]}=2.213$, $P=0.054$) on MB calyx volumes, so sexes were pooled. There was a significant effect of genotype ($F_{[8,59]}=10.027$, $P<0.0001$) on MB calyx volume. I observed lines with reduced MB calyx volumes, and chose to continue on with the experiment, testing zygosity as well in a more thorough way (see Chapter 2 Figure 2, Table 3). Bars represent mean ± SE of mean calyx volume for each genotype. $n$ indicated on each bar. Different letters designate significant differences (SNK, $P\leq0.05$).
Figure C.2 247 learning data. A modified Pavlovian conditioning T-maze paradigm was used to assay olfactory associative learning and sensory controls (Tully & Quinn, 1985; de Belle & Heisenberg, 1994; de Belle and Heisenberg, 1996; Tully et al., 1994). Described in detain in Appendix B Figure 4. There was a significant difference between 247 and CS for Octanol (OCT) avoidance at a concentration of $2 \times 10^{-2}$ ($F_{[1,6]}=5.786$, $P=0.047$). There were no significant difference between 247 and CS for 4-Methylcyclohexanol (MCH) avoidance at a concentration of $4 \times 10^{-3}$ ($F_{[1,7]}=5.144$, $P=0.058$), or 80V dc shock avoidance ($F_{[1,6]}=0.320$, $P=0.592$). There was a significant difference between 247 and CS for learning ($F_{[1,15]}=24.852$, $P<0.0001$). I believe this defect in learning is compounded by the olfactory defect observed with OCT. To see if the learning defect is real, I can either change odor concentrations to get a normal avoidance for OCT, or I can switch to a different odor like benzenaldehyde. Bars represent mean ± SE of mean calyx volume for each genotype. $n$ indicated on each bar. Different letters designate significant differences (SNK, $P\leq0.05$).
Figure C.3 *E75* null histological analysis. Serial sections of paraffin-embedded brains were used for planimetric MB measurements. I was interested in whether a null allele of *E75* would increase the reduced MB phenotypes I had previously seen with the 247 Gal4 insert (Chapter 2 Figure 2, Table 3). The Bloomington line Df(3L)W4, ru[1] h[1] e[1] ca[1]/TM6B, Tb[1] 75B8-11;75C5-7 (BL#2607) (Pauli et al., 1995; Addison et al., 1995; Salzberg et al., 1997) has been shown to be an *E75* null (BDGP Project members 1994-1999). This line will be referred to as 2607 from here on. There was a significant influence of genotype (*F*[4,81]=3.942, *P*=0.006) on MB calyx volume. 247 was 7% smaller than *w*¹¹¹⁸ and 2607/247 was 9% smaller than 2607/*w*¹¹¹⁸. The homozygous null is lethal, so alternate approaches will need to be taken to get a stronger allele, as it appears that 247 is a weak disruption of *E75*. Bars represent mean ± SE of mean calyx volume for each genotype. *n* indicated on each bar. Different letters designate significant differences (SNK, *P*≤0.05).
Figure C.4 E75 splice variant histological analysis. There are three isoforms of E75 (Segraves and Hogness, 1990) represented by the following isoform specific null mutations: E75A (A81), E75B (∆51) and E75C (x37) as well as a protein null (∆1) (Bialecki et al., 2002). I was interested in whether any particular splice variants of E75 would influence MB development. I crossed the previously mentioned lines to 247 and cantonized 247 CS8 to analyze MB calyx volumes (A) Diagram representing the locations of the three splice variants and the null in the E75 gene (Modified from Bialecki et al., 2002). (B) Serial sections of paraffin-embedded brains were used for planimetric MB calyx volume measurements. Males indicated with hatched bars. There was a significant influence on sex ($F_{[1,315]}=13.503, P<0.0001$), genotype ($F_{[18,315]}=13.467, P<0.0001$) and the interaction between sex and genotype ($F_{[16,315]}=1.943, P=0.017$) on MB calyx volume. Bars represent mean ± SE of mean calyx volume for each genotype. For each bar $4 \leq n \leq 20$. 

\[\text{Volume (10}^3 \mu\text{m}^3)\]
Table C.1 *E75* splice variant histology multiple pair wise t-tests. I opted for a very conservative approach when analyzing this complicated *E75* splice variant data, and performed multiple pair wise t-tests within each splice variant line as well between each splice variant line and controls. To maintain an error rate of $\alpha = 0.05$, a Bonferonni correction was used to adjust the critical $P$ value to 0.00015 (Sokal & Rohlf, 1981). Significant differences are denoted as follows: $\star < 0.05$, $\star \star < 0.01$, $\star \star \star < 0.005$, $\star \star \star \star < 0.001$. The most obvious trends from this very complex dataset are within x37 and Δ1. X37/w1118 males appear to be larger than all other genotypes tested. Δ1/247 males also appear larger than most other genotypes. Repeating this work with a stronger allele that is not as sensitive to genetic background (in place of 247) may increase the strength of the phenotype and allow a more accurate portrait of the role each splice variant may have on MB development. It would also be interesting to look at the rest of the MB anatomy, more specifically axonal projection patterns and Kenyon cell number, as it is possible that *E75* is involved in another aspect of MB development outside of dendrite development.
Table C.1.a x37

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Figure C.5 E75 western blot. An SDS-Page 7.5% gel was run with samples of either second instar larvae with their concentrations listed (lanes 1-4) or heads of 1-day-old flies (lanes 5-9) (15 heads/sample). The gel was blotted, trimmed and incubated with the following primary Abs: mouse anti-E75A 1B12 Clone Ab used at 1:10,000 dilution (144 kDa) (specific to the A splice variant, and generously given to me by Carl Thummel) and mouse anti α-Tubulin Ab used at 1:4,000 dilution (~50 kDa) as a loading control. The secondary Ab was: Goat anti mouse conjugated to HRP used at 1:7,500 dilution. This blot revealed a band missing close to the expected 144 kDa for E75 in lane 2 of the A81 splice variant mutant/null L2 larval sample. I was unable to clearly detect the α-Tub from non-specific binding of the E75 Ab. Note: this was repeated several times, cut and incubated as 2 blots to distinguish the possible E75 background staining from α-Tub, and the results were the same (data not shown). The goal of this experiment was to determine whether 247 disrupted E75. Although the blot is not very clean, it does indicate that 247 (in any genetic combination) looks the same as wildtype. My western blot data was consistent with other labs, as per personal communication with Dr. Thummel, who indicated that this Ab has not worked in western blots or tissue stains with wildtype flies in several other labs (it is only detectable when over expressed). I also conducted a single preliminary northern
blot (data not shown), with similar inconclusive results. Taken together with the histological \textit{E75} data, I felt that 247 was an ineffective disruption of \textit{E75} for further investigation of its role in MB development.
Table C.2 Fz, nemy and gish RNAi RT-PCR primers. Tissue was collected from the whole bodies of 10-20 females, flash frozen in liquid nitrogen, and then RNA was extracted using RNAspin mini (GE Healthcare). Reverse transcription was performed with normalized amounts of RNA as template using qscript™ cDNA supermix (Quanta Biosciences). The resulting cDNA was then used as template in a PCR reaction. Primers used are listed with their associated Tm's and expected band size. PCR conditions were as follows: 94˚C for 5 minutes, 94˚C for 1 minute, 53-55˚C for one minute, 72˚C for one minute, repeat steps 2-4 30 times, 72˚C for 7 minutes, then a 4˚C hold. Please note: gish=CK1γ

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Figure C.6 *Fz, nemy* and *gish* RNAi RT-PCR. Tissue was collected from the whole bodies of 10-20 females, flash frozen in liquid nitrogen, and then RNA was extracted using RNAspin mini (GE Healthcare). Reverse transcription was performed with normalized amounts of RNA as template using qscript™ cDNA supermix (Quanta Biosciences). The resulting cDNA was then used as template in a PCR reaction. (A-C) RT-PCR products were resolved on 1% TAE agarose gels and imaged with a Typhoon 8600 Variable Mode Phosphorimager (GE Healthcare). The RNA levels were relatively equal across all samples (bottom gels for α-Tub), yet I saw no down-regulation of any gene’s RNA when the lines were driven in the CNS (top gels). Unfortunately our results from this experiment indicate that the RNAi lines were not completely functional. There are several reasons why the RNAi lines appeared non-functional. First, the driver elav only expresses in the CNS and I may not have had enough CNS tissue since our extracts were from the whole fly. Had I used heads or even dissected brains, I
may have seen a slight difference in the RT-PCR experiment. The age of our flies may have also presented a problem, as I used flies aged between 2 and 6 days old, and the expression profile of each gene may change drastically in that time period. Finally, endogenous RNA present in any tissue other than the CNS could have served as the template for RT-PCR. Further, if the elav driver did not turn on early enough, you would expect endogenous RNA's to be present. Please note: \textit{gish}=CK1\gamma
Although I have data indicating that these RNAi lines are not ideal disruptions of fz, nemy or gish, I concurrently tested their role in MB anatomy by driving them in the MBs with the c772 Gal4 driver. Serial sections of paraffin-embedded brains were used for planimetric MB measurements. There was a significant influence on genotype ($F_{(13,111)}=14.701, P<0.0001$) and the interaction between sex and genotype ($F_{(13,111)}=4.644, P<0.001$) on MB calyx volume. There was no significant influence of sex on MB calyx volume ($F_{(1,111)}=1.486, P=0.225$). Although several lines were decreased when driven in the MBs, the controls for each line were no different from wildtype. Taken together with the previous RT data, I believe these differences are negligible. Bars represent mean ± SE of mean calyx volume for each genotype. $n$ indicated on each bar. Please note: gish=CK1γ
**Table C.3 Fz, nemy and gish RNAi histology multiple pair-wise t-tests.**

Multiple pair wise t-tests within each RNAi line as well between each RNAi line and the controls were performed. To maintain an error rate of $\alpha = 0.05$, a Bonferonni correction was used to adjust the critical $P$ value to 0.0044 (Sokal and Rohlf, 1981). Significant differences are denoted as follows: $\star < 0.05$, $\star\star < 0.01$, $\star\star\star < 0.005$, $\star\star\star\star < 0.001$). c772 females were larger than both male and female $w^{1118}$. Please note: $gish=CK1\gamma$
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References


APPENDIX D
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March 2nd 2010

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Dissertation Title: Genes Involved in Mushroom Body Development and Behavior in *Drosophila*

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