5-2010

Genes involved in mushroom body development and behavior in Drosophila

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GENES INVOLVED IN MUSHROOM BODY DEVELOPMENT AND BEHAVIOR IN *DROSOPHILA*

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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

Doctor of Philosophy Degree in Biological Sciences
School of Life Sciences
College of Sciences

Graduate College
University of Nevada, Las Vegas
May 2010

UNLV
UNIVERSITY OF NEVADA LAS VEGAS

THE GRADUATE COLLEGE

We recommend that the dissertation prepared under our supervision by

Christine Nicole Serway

entitled

Genes Involved in Mushroom Body Development and Behavior in *Drosophila*

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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ABSTRACT

Genes Involved in Mushroom Body Development and Behavior in *Drosophila*

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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: *βFTZ-F1, Odorant receptor 42a, no extended memory, TAK1-associated binding protein, frizzled, Ecdysone-induced protein 75B, Casein Kinase 1γ* 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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ACKNOWLEDGEMENTS

I would like to start by thanking my advisor Steven de Belle. You have become my friend, my family, and one of my inspirations while remaining my boss. I am so thankful that I have had the opportunity to work for someone as unique and kind as you over these last 9 years. You have so generously taken me around the world, given me life-changing opportunities, and included me in your scientific family, which I now feel a part of. You have taught me countless invaluable lessons, that even though sometimes it seemed like I was crying too much to learn, I did. You have shown me how to become a scientist, how to express my ideas (verbally and on paper), and how to be myself while doing all of those things. By watching you, I have also learned how to treat people with patience, kindness and respect, and receive the same treatment while still remaining modest, an invaluable trait to have. You have always made time for me no matter what you were doing, or where in the world you were, and for that I am so thankful. You have also taught me that before you teach, your student must be ready to learn, and that you cannot learn unless you are willing to listen, a lesson whose value I truly didn't understand until my later years, but one, which I really will always remember. And finally I have seen the wisdom in your passion for science, and its every detail. Working with you has been an amazing gift, and I hope our relationship and friendship continues to grow over the years. I can't imagine the person I would be today had I not met you all those years ago in Colorado. Thank you for sharing your work, and your life with me, it has been a blessing!

Thank you to all the members of my committee, past and present: Thank you to Andy Andres, who has been like a stepfather to me over the years, giving me great advice, always being honest, and genuinely being concerned for my well being no matter what the situation. Your input on my work as well as my own personal development as a scientist has really been invaluable, and I am so thankful that you came to UNLV. Thank you to Ron Gary for joining my committee after the loss of Dr. Carper. Your scientific savvy paired with kindness is a perfect pair. Thank you to Jeff Shen for stepping in at the last minute, and for being a great member of the faculty at UNLV. I have learned so much from you over the years. Thank you to Allen Gibbs for you're input and interest in my work as well as your statistics guidance. Thank you to Steve Roberts. You were always one of my biggest supporters, giving me that extra boost of confidence. You also reminded me not to take my work or myself too seriously, and to choose to see the humor in life rather than the sadness, another invaluable lesson. And thank you to Steve Carper.

Thank you to my lab mates, past and present. Thank you to Brian Dunkelberger for always having the time to talk science with me as well as having the patience of a saint. My project just wouldn't have been the same if it weren't for all your interest and feedback. Thank you to Xia Wang for becoming a great friend who was always willing to help me out in a fix. I could have never finished these last 6 months without your help and amazing scientific skills. Your impeccable work ethic has also been an inspiration. I really believe you can do anything you want to Xia! Thank you to Chris Tabone for his willingness to work

so hard on the behavioral aspects of my thesis. You have also taught me that in understanding our differences we can learn a lot about ourselves. Thank you for always reminding me that excitement about your work is almost as valuable as the research itself! I hope we both remain excited to be doing interesting science. Thank you to Dave Green for all his help and great attitude. I hope to have colleagues like you around me, as it really made work a better place. Thank you to Mike Ginsburg for being an amazing friend that I am so glad to have shared this adventure with. Thank you Lisa Strobel. It was a pleasure to become your friend. Thank you to the undergraduates I have had the pleasure of mentoring: Denise Beck, Nicole Nolan, Stephanie Georges, and Stephanie Freer. Nicole, knowing it was your hard work that kept everything together when I was sick was a relief because you are great at everything you do. You will be an amazing doctor, and you have been a great friend. Denise, it has been a pleasure becoming your friend and teaching you about flies. I wish you all the luck on what ever you do in life.

Thank you to Frank van Breukelen for taking the time in my early days to teach me things that weren't being offered in a class. You are one of the most amazing scientists I know and a very special person. I am so glad that I got a chance to know you better here at UNLV. Thank you to Cheryl Vanier for making time again and again to explain statistics to someone who was not built to do complex math. Thank you to Lois Alexander for sequencing help. Thank you to all the members of the UNLV Genomics Core. Thank you to all the members of the Andres Lab & the Gibbs lab for their help and reagents. Thank you to

Bernard Mechler for Imp-α2 reagents that really made my *mbmB* chapter so interesting. Thank you to Roland Strauss for working with me on the walking paper. Thank you to Carl Thummel for *E75* reagents and helpful commentary. Thank you to Jerry Yin & Tom Tubin, who invited me into their lab with open arms and shared their scientific skills with me. Thank you to Andy Martin, who took a chance on me and changed my life. My love of science grew in your lab and flourished because of all of your patience, help and confidence in me.

Thank you to all the office staff in The SoLS, in particular Sharon Trotter, Pat Hunt, Ala Kiko and James McKoy. Thank you to the SEB staff, in particular Eric Knight and Cathy Willey. Thank you to Eric Lee in the graduate college. Thank you to Nicholle Booker who has kept me together on paper and for always making time to help me with whatever I needed. Thank you to the staff at the Utica College Library.

I also have a wonderful group of friends who have helped me make it through this PhD. Thank you to Jeremy Roche for EVERYTHING! Thank you to Nicole Bond for being an amazing friend, always telling me the truth when no one else would and for choosing to grow with me as a person. Sharing that part of my PhD with you was really great! Thank you to Stacy Mantooth for plenty of great talks about science over lots of cold beers and for becoming such a great friend. These last few years at UNLV have been much better because of you and for that I am thankful! Thank you to Derek Houston for always making me laugh and for being a good friend that I am lucky to have.

Thank you to my family for al of their love and support. Thank you Mom for always making sure I have had and seize every opportunity a child, adolescent, adult and scientist could ever hope for. You make it all possible with your love and support and have given me the tools to accomplish what ever I wanted to. Thank you Dad for sharing in the everyday adventures of this process with me. Our phone calls and all of your great advise paired with your interest in my work really helped me get through it all and remember why I am here. The confidence that you have both always had in me is where I find my strength. Your unconditional love and support has given me the room to acknowledge and use my creativity in the absence of fear or judgment. I know I am blessed to have had that experience, as it seems very rare. Thank you Emily for your amazing friendship, constant love, support, encouragement, and awesome cartoons. Our talks over these last nine years have really kept me going and helped me to grow into a better scientist and person. Thank you and I love you.

Thank you to Brian Hobbs, my spectacular husband, my statistician, my favorite lab assistant, my scientific advisor, my editor in chief, my financier, my cook, my best friend, the daddy of my weasels and my soul mate! Thank you for being so unbelievably patient with me these last 9 years and especially these last 5 months. Thank you for always seeing me through all of my work, even when I couldn't see myself, and loving me unconditionally. I could never have done this without your help and wouldn't have wanted to do it without you by my side. I love you Brian Hobbs!

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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	Elipsoid 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	Glutimate
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^{1}	*mushroom body miniature*
mbmB^{1}	*mushroom body miniature B*
mbmC^{1}	*mushroom body miniature C*
MCH	Methyl Cyclohexanol
MTM	Middle Term Memory
mud^{1}	*mushroom bodies deranged*
mud^{4}	*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^{1}	*small mushroom bodies*

STM	Short Term Memory
SNK	Student Newman Keuls
SNLS	Small Nuclear Localization Signal
SNLSB	Small Nuclear Localization Signal Binding Domain
SNP	Single Nucleotide Polymorphism
SOP	Sensory Organ Precursors
Tab2	TAk-associated binding protein
TAE	Tris-acetate ethylene-diamine-tetra-acetic acid
TIFR	Transient Interhemispheric Fibrous Ring
TLL	*Tailess*
TS	Temperature Sensitive
UAS	Upstream activating sequence
USP	*Ultraspiricle*
w^{1118}	*white 1118*
WT	Wild Type

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^{l},) and three medially projecting (β, β^{l} 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 5^{th} 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 (*shibirie*^{ts}) 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*^{ts}, 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 *mbmB* provided a unique opportunity to directly correlate the cellular requirements for MB development with its function. Our lab has shown that *mbmB* 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 *mbmB* 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 un-biased approach brought to light novel cellular and molecular pathways, and answered some very detailed questions regarding MB function.

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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 *shibirie^{ts}* 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; Livingstone 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 γ neurons are the first to develop, prior to the mid-third instar stage. The α' / β' neurons develop next between mid third instar and puparium formation, and finally the α / β 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 (α and α') and three medial (β, β' and γ) 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* (*mbm*^{1}), which showed significantly reduced MBs and female odor learning defects (Heisenberg et al., 1985; de Belle & Heisenberg, 1996). Cloning and characterization of *mbm*^{1} 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^{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-α2* and will be referred to here as *imp-$\alpha2^{0}$* (Gorjánácz et al., 2006). I performed rescue experiments with *imp-α2* 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;mbmB^{1}*(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, selaed 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-α2* cDNA) in a tissue specific manner (Brand & Perrimon, 1993). This method was used to investigate changes in MB cell number in the *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 μm 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 μm).

Mapping

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 *mbmB*. To accurately locate the physical position of *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 *mbmB* mutants.

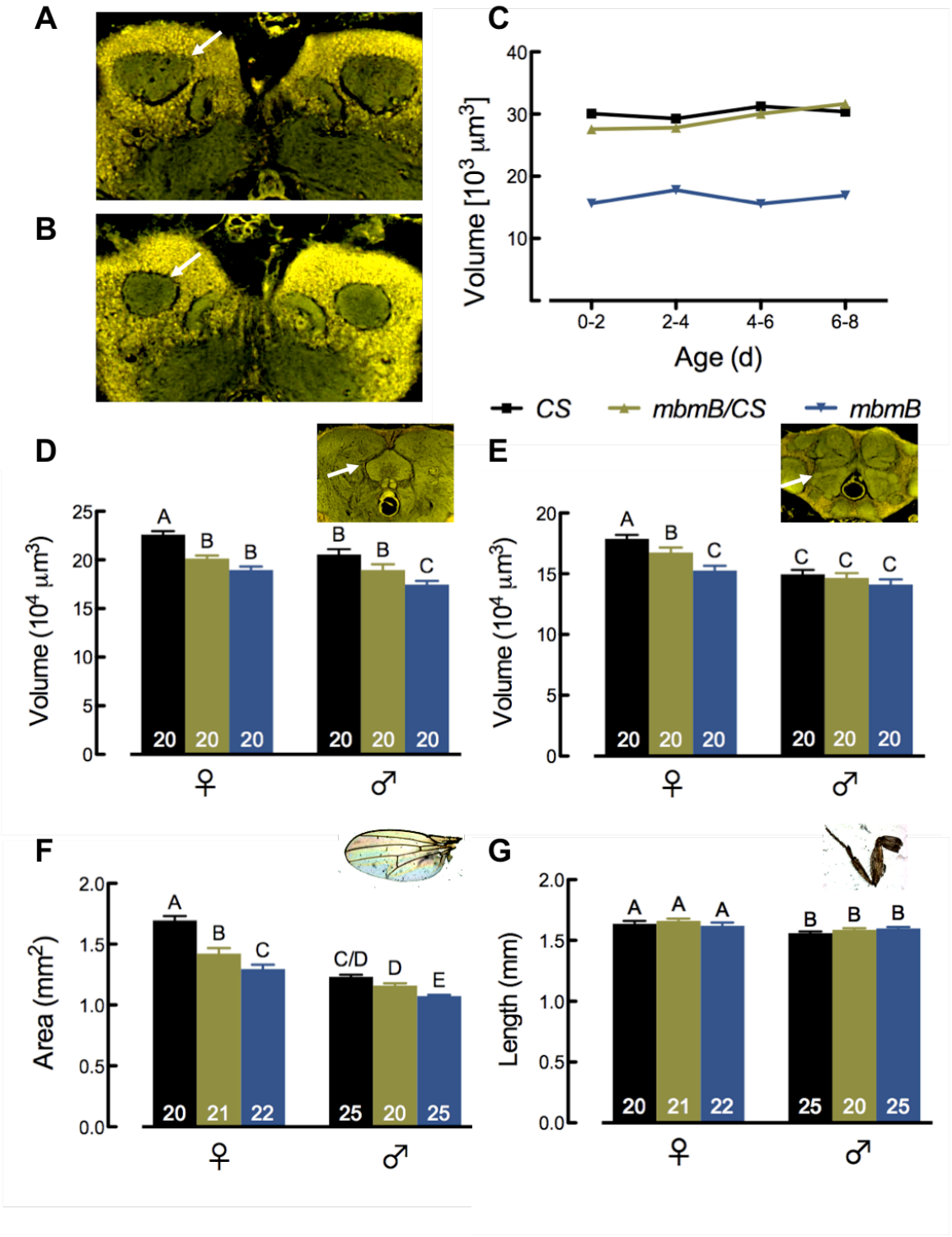

Figure 2.1 Anatomy of *mushroom body miniature B*. Serial sections of paraffin-embedded brains were used for planimetric measurements of several brain structures. (**A**) $CS^{wü}$ section and (B) $mbmB^{1}$*(CS)* section both showing MB

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, $P \leq 0.05$).

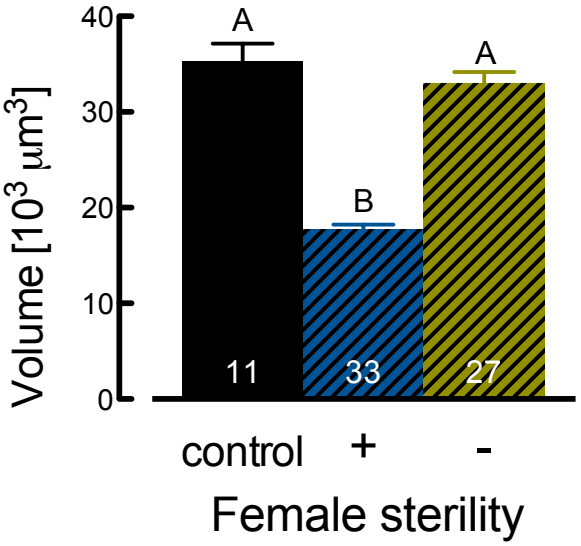

Figure 2.2 Sterility genetically linked to brain anatomy. All sterile flies also had significantly reduced MBs ($F_{[2,69]}$=70.59, *P*<0.0001). Bars represent mean ± SE of mean calyx volume for each genotype. *n* indicated on each bar. Different letters designate significant differences (SNK, *P*≤0.05).

Progeny expressing mutant phenotypes carry a deletion that uncovers the haplo-insufficient mutant *mbmB* allele. As deficiencies from Bloomington Stock Center became available I crossed them to *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 *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 *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 *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 *w;mbmB^{1}(CS)* and *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 20μl sequencing reactions using 2μl gel purified PCR product as template and 0.3μl of [10μM] primers (Table 2.2) under the following

Table 2.1 Fly strains.

A. Chromosome-2L re-arrangements

Name	Source	Description
$CS^{wü}$	Steve de Belle [1]	wildtype
$mbmB^{1}$(CS)/Sm5	Steve de Belle [1]	MB mutant
w;$mbmB^{1}$(CS)/Sm5	Steve de Belle [1]	MB mutant
yw;D14/y+Cy0;Tm6/+	Bernard Mechler [2]	Impα2 null
yw;Sp/y+Cy0;Impα2-cDNA/Tm6	Bernard Mechler [2]	Impα2-cDNA transgene
yw;D14/y+Cy0;$Impα2^{\Delta IBB}$ /Tm6	Bernard Mechler [2]	Impα2 IBB^{-} transgene
yw;Sp/y+Cy0;$Impα2^{S37A}$/Tm6	Bernard Mechler [2]	Impα2 S37A transgene
yw;Sp/y+Cy0;$Impα2^{S56A}$/Tm6	Bernard Mechler [2]	Impα2 S56 transgene
yw;Sp/y+Cy0;$Impα2^{S98A}$/Tm6	Bernard Mechler [2]	Impα2 S98 transgene
yw;Sp/y+Cy0;$Impα2^{3xSA}$/Tm6	Bernard Mechler [2]	Impα2 3xSA transgene
yw;Sp/y+Cy0;$Impα2^{SNLSB}$/Tm6	Bernard Mechler [2]	Impα2 $SLNSB^{-}$ transgene
yw;Sp/y+Cy0;$Impα2^{NLSB}$/Tm6	Bernard Mechler [2]	Impα2 $NLSB^{-}$ transgene
$Impα2^{DIM}$;Sp/y+Cy0;Tm6/+	Bernard Mechler [2]	Impα2 DIM^{-} transgene
yw;Sp/y+Cy0;$Impα2^{CASB}$;Sb/Tm6	Bernard Mechler [2]	Impα2 $CASB^{-}$ Transgene
w;+;+;P[Ok07::Gal4]	Connolly et al., 1996 [3]	MB driver (all lobes)
w;+;P[247::Gal4]	Robert Schulz [4]	MB driver (all lobes)
w;P[c772::Gal4]	Douglas Armstrong [5]	MB driver (all lobes)
P[elav::Gal4];+;+	Mani Ramaswami [3]	Pan-neuronal driver
w;P[nanos::$Gal4^{VP16}$]	B (Stock# 4937)	Ovary driver
w;+;P[GFP::LacZnls]	B (Stock# 6397)	Nuclear GFP
w;$mbmB^{1}$(CS)/Sm5;P[247::Gal4]/+	Brian Dunkelberger [6]	MB mutant with MB driver
w;$mbmB^{1}$(CS)-P[c772::Gal4]/Sm5	Brian Dunkelberger [6]	MB mutant with MB driver
w;$mbmB^{1}$(CS)/Sm5;+; P[Ok07::Gal4]/+	Brian Dunkelberger [6]	MB mutant with MB driver
w;+;Impα2-RNAi-5	VDRC	Impα2 RNAi
w;+;Impα2-RNAi-6	VDRC	Impα2 RNAi
w;+;Tm3/Tm6b	Brian Dunkelberger [6]	3d chromosomal balancer
wCyO;;Tm3/MKRS	Brian Dunkelberger [6]	Double balancer

B. Chromosome-2L re-arrangements

Stock #	Cytology	Source	Sequence Location
1641	21A;23E31-2	B	
6130	21A1;21B1-2	B	
7488	21A4;21B1	B§	2L:67166;129261
9353	21B1;21B3	B‡	2L:67365;161120
7778	21B1;22B5	B§	2L:7637689;7660390
7772	21B4;21B7	B§	2L:7140259--7140502;7202317
8672	21B7;21C2	B	2L:291728--291846;417947
6283	21B7-C1;21C2-3	B	
6608	21C3-4;21C6-8	B	
7774	21D1;21D2	B§	2L:7202317;7418003--7418128
7489	21D2;21D3	B§	2L:203089;264275--289931
7775	21D2;21D4	B§	2L:7364976;7495492
7490	21D3;21E3	B§	2L:559139;715085
7491	21E3;21F2	B§	2L:715084;826285
7776	21F2;21F4	B§	2L:7576630;7702880
5449	22A1;22B6-9, 42D	B	
7492	22A3;22B1	B§	2L:777148;868373

7779	22B1;22B8	B§	2L:8071311;8205166
8000	22B5;22D1	B§	2L:1911627;2175599
7780	22B8;22D1	B§	2L:8438123;8528528
7493	22D1;22E1	B§	2L:826173;1074079
1313	22D1-2;33F5-34A1	B	
6232	22D3-22D6;34A8-34A9	B	
7782	22D4;22E1	B§	2L:8529124;8801960
7783	22E1;22F3	B§	2L:8797995;8984993
7494	22F3;23A3	B§	2L:1074079;1158137
90	22F3-4;23C3-5	B	
7744	23A2;23B1	B§	2L:6922143;7022660--7022707
8038	23B8;23C5	B‡	2L:2873954;3055717
7784	23C4;23D1	B§	2L:8989308;9176164
7785	23D1;23E3	B§	2L:9388129;9448660--9448833
7786	23E3;23E5	B§	2L:9415663;9431473
7787	23E5;23F5	B§	2L:9447643;9560489
6506	23F3-4;24A1	B	
7789	24A1;24C2	B§	2L:9522946;9622987
7495	24C3;24C8	B§	2L:1158197;1311170--1311516
7790	24C8;24D4	B§	2L:9613611;9782218
3080	24D3-4;24F7-25A3	B	
9270	24F4;25A7	B‡	2L:4477085;4821294
7496	25A7;25B1	B§	2L:1555098;1737249
7792	25B1;25B1	B§	2L:9613665;9622528
7793	25B1;25B8	B§	2L:9782218;9897536
7794	25B10;25C 3	B§	2L:9860016;9940209
7795	25B3;25B9	B§	2L:10134181;10198945--10198992
7796	25B8;25B10	B§	2L:10276871;10333704
7497	25C8;25D5	B§	2L:1716977;1909976
7498	25D5;25E6	B§	2L:1737960;2010136
7797	25E5;25F1	B§	2L:10443323;10544859
7724	25E6;25F2	B§	2L:6664818;6786906
7798	25E6;25F2	B§	2L:10516675;10861982
7499	25F2;25F5	B§	2L:1911627;2175599
7500	25F5;26A3	B§	2L:1989057--1989058;2152458
7799	26A1;26A8	B§	2L:10853446--10853462;10975285
2340	26A4-6;26C1-2	B	
9297	26B2-26D7	B‡	2L:6000124;6465772
7501	26B9;26C1	B§	2L:2175607;2362917
7502	26C1;26D1	B§	2L:2221020;2362808
7800	26C2;26C3	B§	2L:11067029;11155825
7801	26F5;27B1	B§	2L:11155825;11358603
8940	27A1;27C4	B‡	2L:6709099;6921292
7802	27C4;27D4	B§	2L:11358603;11445762
6790	27D1-2;27F1-2	B	
7803	27E2;27E4	B§	2L:11807409;11971081
7503	27E4;27F5	B§	2L:2362917;2492447
7804	27F3;28A1	B§	2L:11971081;12066847
9189	27F4;28B1	B‡	2L:7423266;7576637
7147	28A4-B1;28D3-9	B	
7504	28B1;28C	B§	2L:2494660;2755377
7805	28B4;28C1	B§	2L:12066846--12066969;12270844
140	28DE (within)	B	

7807	28E1;28F1	B§	2L:12423459;12655793
179	28E4-7;29B2-C1	B	
179	28E4-7;29B2-C1	B	
7808	29C1;29D1	B§	2L:12655793;12854729
2892	29C1-2;30C8-9	B	
7809	29C4;29D4	B§	2L:12832803;12896409
384	29D1-2;30C4-D1	B	
7810	29D5;29F1	B§	2L:12872617;13165936
7811	29F1;29F6	B§	2L:13800829;13878188
7505	29F7;30A2	B§	2L:2677694;2808100
3702	29F7-30A1;30C3-5	B	
368	30A1-2;30D1-2	B	
6368	30A9-B1;30D2-F4	B	
7812	30B10;30C1	B§	2L:14300969;14470247
7813	30B3;30B5	B§	2L:14409711;14490657
7814	30B4;30B5	B§	2L:14455715--14455716;14997588
7506	30B5;30B11	B§	2L:2979654;3056809
7815	30C1;30C1	B§	2L:15264714;15439965
7507	30C1;30C9	B§	2L:3046635;3310250
556	30C1-2;30F	B	
12533	30C2	B◊	
12826	30C2	B◊	
12515	30C5	B◊	
12752	30C6	B◊	
7508	30C9;30E1	B§	2L:3302636--3302646;3354856--3354858
7816	30D1;30F1	B§	2L:15426051;15744445
1045	30D-30F;31F	B	
8469*	30F5;31B1	B	2L:9984170;10200998
7817	31A2;31B1	B§	2L:15912343;16042754
7818	31A3;31B1	B§	2L:16457328;16727482
6117	31B;31D	B	
3366	31B;32A	B	
7819	31C3;31D9	B§	2L:16685211;16886557
9495	31C-D;32D-E	B	
4367	31D1-11;31E1-7	B	
7999	31E3;31F5	B§	2L:10443323;10544859
7820	31F5;32B1	B§	2L:16728375;16824908
7821	32B1;32C1	B§	2L:16791487;17450255
7510	32D2;32D5	B§	2L:3354818;3473493
7511	32D5;32E4	B§	2L:3602642;3730180
7512	32E4;32F2	B§	2L:3771368;3888977
7513	33A2;33B3	B§	2L:3887981;4031325
7514	33B3;33C2	B§	2L:4820718;4887766
7515	33C2;33D4	B§	2L:4846961;4887766
7516	33E4;33F2	B§	2L:4846961;4977638
7517	33F2;34A1	B§	2L:4915628;4979299
7822	34A1;34A2	B§	2L:17382988;17495992
7823	34A2;34A7	B§	2L:17482011;17773525
7826	34D3;34E1	B§	2L:17502487--17502514;17604760
7518	35A3;35B2	B§	2L:4975605;5000943
7519	35B1;35B2	B§	2L:5000837--5000838;5058522
7828	35B1;35B8	B§	2L:17903087--17903187;18161791
6084	35B1-2;35B1-2 + 35D1-	B	

	2;35D5-E1		
6085	35B1-2;35B2-4 + 35D1-2;35E2	B	
7830	35C5;35D2	B§	2L:18123514;18455586
7831	35D2;35D4	B§	2L:18294845;18299279
7521	35D6;35E2	B§	2L:5147258;5305646
3602	36A(?);77B1	B	
7833	36A1;36A12	B§	2L:18571864--18571867;18732675
7522	36A10;36B3	B§	2L:5305646;5555049
7834	36A12;36B2	B§	2L:18689053;18795820
7835	36B1;36C9	B§	2L:18753432--18753444;18943942
7836	36C10;36C11	B§	2L:18859186;19022139
7837	36C10;36D1	B§	2L:18973942;19161727
3592	36C2;35C5	B	
7838	36C7;36C10	B§	2L:18995784;19044446
7839	36D2;36E1	B§	2L:19110141;19161708
7840	36D3;36E3	B§	2L:19161727;19423559--19423709
7841	36E1;36E1	B§	2L:19320414--19320415;19452918
8834	36E1-3;37A	B	
7523	36F5;37A2	B§	2L:5524375--5524385;5594234
7843	37A1;37A7	B§	2L:19426459;19586375
7844	37A2;37B6	B§	2L:19438065;19452918
7845	37B1;37B9	B§	2L:19576108--19576133;19764726
7846	37B8;37B11	B§	2L:19764726;19935139
7524	37B8;37C5	B§	2L:5555049;5658629
7847	37C1;37C5	B§	2L:19918015;20072236
7525	37C5;37D7	B§	2L:5555049;5659285
7848	37D2;37E1	B§	2L:20205107;20449190--20458307
7849	37D7;37F4	B§	2L:20449190--20458307;20680624
7913	37E1;37E1	B§	2L:19438065;19452918
7526	37F2;38A4	B§	2L:5658629;5805324
7527	38A4;38A7	B§	2L:5805324;5944680
7850	38A7;38B2	B§	2L:20770538;20874804
9222	38B4;38C6;	B‡	2L:20085397;20382385
7528	38C2;38C7	B§	2L:5898291;5980153
7851	38C7;38D4	B§	2L:20861544;21102742
9175	38D1;38F5	B‡	2L:20638580;20917519
7852	38E6;38F3	B§	2L:21102742;21244119
7853	38F3;39A2	B§	2L:21237271;21309519
7529	39A2;39B4	B§	2L:6088361;6200227--6262082
7530	39B4;39D1	B§	2L:6253010;6411492
7855	39D1;39E6	B§	2L:21309519;21662938
7531	40A5;40D3	B§	2L:6292895;6338855

C. Chromosome-2L P-elements and gene disruptions

Stock#	Cytology	Source	Sequence Location
9459	2	B	
17883	21E2	B§	2L:603023..603210
8647	26B2	B	2L:5981836..5983009
4257	27F1-31E7	B	EMS induced allele of *paternal loss inducer*
5282	30C5	B	Antimorphic allele of *PKA–C1* 2L:9684656..9699293
4101	30C5	B	Loss of function : *PKA-C1*

			2L:9684656..9699293
14478	30F5	B◊	2L:9984663..9984663
15175	30F5	B◊	2L:9984543..9984543
15227	30F5	B◊	2L:9996617..9996617
17035	30F5	B§	2L:9984170..9984170
18759	30F5	B§	2L:10017648..10017648
d03624	30F5	H§	2L:9984563..9984563
d11066	30F5	H§	2L:9984624..9984624
f02453	30F5	H§	2L:10018003..10018003
f04310	30F5	H§	2L:10017648..10017648
1595	30F5	B	EMS induced Loss of function : *big brain* 2L:9984647;9995545
11078	30F5	B◊	2L:10010347..10010347
17183	30F5	B§	2L:10004802..10004802
c03479	30F5	H§	2L:10012086..10012089
e02569	30F5	H§	2L:9990278..9990278
c01735	30F5	H§	2L:9987945..9987945
f02066	30F5	H§	2L:9983928..9983928
13572	30F6	B◊	2L:10032530..10032530
19774	30F6	B◊	2L:10032623..10032623
f07077	30F6	H§	2L:10022115..10022115
d07603	30F6	H§	2L:10032677..10032677
16010	31A1	B	2L:10053842..10053842
16039	31A1	B	
d07004	31A1	H§	2L:10056941..10056941
f02264	31A1	H§	2L:10056588..10056588
c02130	31A1	H§	2L:10052323..10052323
11125	31A1-2	B◊	2L:10056948..10056948
15654	31A2	B◊	2L:10057508..10057508
20036	31A2	B◊	2L:10057031..10057031
c05212*	31A2	H§	2L:10057749..10057749
f00038	31A2	H§	2L:10102108..10102108
f04829	31A2	H§	2L:10071488..10071488
10210	31B	B	
3088	31B1	B	EMS induced mutation in *basket* 2L:10248232..10248232
10635	31B1	B◊	2L:10239309..10239309
10738	31B1	B	2L:10242510..10247064
12753	31B1	B◊	2L:10255891..10255891
13881	31B1	B◊	2L:10207313..10207313
13926	31B1	B◊	2L:10226357..10226357
14337	31B1	B◊	2L:10247020..10247020
14449	31B1	B◊	2L:10220952..10220952
14758	31B1	B◊	2L:10231683..10231683
14876	31B1	B◊	2L:10250497..10250497
15671	31B1	B◊	2L:10200998..10200998
16032	31B1	B	2L:10207735..10207735
16275	31B1	B	2L:10260712..10260827
17882	31B1	B§	2L:10198946..10198992
18425	31B1	B§	
19982	31B1	B◊	2L:10220945..10220945
20133	31B1	B◊	2L:10250060..10250060

20449	31B1	B	2L:10220316..10220316
21484	31B1	B	2L:10199172..10199172
6233	31B1	B	Allele of *Suppressor of veriegation 2-1*
7101	31B1	B	Loss of function : *trunk* 2L:10271443..10272223
10872	31B1	B§	2L:10,264,669..10,264,669
15456	31B1	B◊	2L:10206929..10206929
2369	31B1-31F2	B	Naturally occurring allele of *Malate dehydrogenase 1*
4006	31B1-31F2	B	Loss of function : *Malate dehydrogenase 1*
10617	32A2	B†	2L:10056945..10056945

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)
§ = Exelisis 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.

Gene	Primer	Sequence	T_m (ºC)	Size (Bp)	Location in gene
CG13131	P-130 T	5'- ATGGAAATCTGCAAGCAAAAACAA -3'	55	960	2196-2219
	P-130 B	5'- GAAAATGTGAACCGGTGAGAATGG -3'			3133-3156
	S-131 B	5'- GTGTTCCCCATCATCATCATCATC -3'			2651-2674
	S-131 T	5'- GCGCAGCACTGATCAATAACAC -3'			2562-2583
bib	P-bib T A	5'- ACTCATGTATGGCGGTAAA -3'	53	2009	1513-1531
	P-bib B A	5'- GTTCTCTGCTCCCCACTAA -3'			3504-3522
	S-bib B A1	5'- CCTTCTACTTTGACTTTGACTTCG -3'			2078-2101
	S-bib T A1	5'- AACCTGACTCTGACTCGAC -3'			2010-2028
	S-bib B A2	5'- GATAGGGTTCGATAGCTCTGGTA -3'			2685-2707
	S-bib T A2	5'- ACGCTGGAGTTTTGGAGG -3'			2484-2501
	S-bib B A3	5'- AGCGTTCAGACAAAGCCAG -3'			3223-3241
	S-bib T A3	5'- AGTCATTATCTGCACTTGC -3'			3162-3180
	P-bib B B	5'- AGGTTCTTTGGACAGCCT -3'	53	2015	5502-5519
	P-bib T B	5'- TTAGTGGGGAGCAGAGAAC -3'			3504-3522
	S-bib B B1	5'- ATGTGGAGGAAGCACTGC -3'			4287-4304
	S-bib T B1	5'- ATTCCAGATCCATCATCAGCGAG -3'			4136-4158
	S-bib B B2	5'- TGGTCTTTGGTCTGTTTTCAT -3'			4677-4698
	S-bib T B2	5'- TCATTTGCGTTGACATTCAAGG -3'			4616-4637
	S-bib B B3	5'- ACGCATAGAGCCAGGGTTA -3'			4976-4994
	S-bib T B3	5'- ATAGGCGCCCACATCAATC -3'			4951-4969
	P-bib T C	5'- AGGCTGTCCAAAGAACCT -3'	53	2031	5502-5519

P-bib B C	5'- AGTTCGCTTTGTTGCAGT -3'			7516-7533
S-bib B C1	5'- ACTCCTCTTTCCCGTTTCGT -3'			5975-5994
S-bib T C1	5'- ATCTGTCTGCCTGTCACG -3'			5915-5932
S-bib B C2	5'- TCCTGCATTCCAAACATTCTAC -3'			6634-6655
S-bib T C2	5'- TCGTCGTGATTTTATGAAGGGTG -3'			
S-bib B C3	5'- TCTTTGCATTGCTTGGCTG -3'			7192-7210
S-bib T C3	5'- ACGTTCCAAAAATGCGAGTA -3'			7113-7132
P-bib T D	5'- ACTGCAACAAAGCGAACT -3'		2150	7516-7533
P-bib B D	5'- AGCAGCGCTATGTGAGAT -3'			9649-9666
S-bib B D1	5'- ATGAGGATGGTGGTGAGG -3'			8005-8022
S-bib T D1	5'- ACTCACTACTACGGCAGG -3'			7916-7934
S-bib B D2	5'- TGCTCTTCCTGCGCAAATTG -3'			8650-8669
S-bib T D2	5'- ACGATTCCGGTTCACAGTTC -3'			8571-8590
S-bib B D3	5'- ACATACCGCAATCCTTTACC -3'			9105-9124
S-bib T D3	5'- TGAACCTTACGCCCACCAC -3'			8967-8985
S-bib B xtra	5'- GAGGGTTGATTGCCGAACT -3'			9417-9435
P-bib T E	5'- ATCTCACATAGCGCTGCT -3'	53	1680	9649-9666
P-bib B E	5'- TCTGATTCTGGACATTTTGGTTC -3'			11307-11329
S-bib B E1	5'- TGTCCGTCCGTTATGCCA -3'			10141-10158
S-bib T E1	5'- GCAAAATCGCAGCATGACAA -3'			9987-10006
S-bib B E2	5'- TCAGCCAGTCAATGTCGTTTG -3'			10522-10542
S-bib T E2	5'- ACTTCTTTCACAAGTATCCTTTG -3'			10469-10491
S-bib T E3	5'- AATCTGTACACTGCTCCGC -3'			11014-11032
P-bib T F	5'- GAACCAAAATGTCCAGAATCAG -3'	52	2015	11307-11328
P-bib B F	5'- ACTGTATCTTCCAAGCGC -3'			13305-13322
S-bib B F1	5'- ACTAACTTTTCGCTCCGAC -3'			11802-11820
S-bib T F1	5'- ATGCTGATGTATGCCCCG -3'			11740-11757

	S-bib B F2	5'- TGCTGCGTCTATCTAAACTAG -3'			12219-12239
	S-bib T F2	5'- AGCAGTTTGTTTTAGTCGTAGTCG -3'			12121-12144
	S-bib B F3	5'- TCCGATCCTAGGGTTGTAAG -3'			12731-12749
	S-bib T F3	5'- ATGTACTCTTCCCATTTTCCG -3'			12630-12650
	P-bib T G	5'- GCGCTTGGAAGATACAGT -3'	52	857	13305-13322
	P-bib B G	5'- ACCCAGGAATGGGTTATG -3'			14145-14162
	S-bib T G1	5'- AGGTGGCTGCCTGTTTTC -3'			13788-13805
	S-bib B G1	5'- AAAACAGGCAGCCACCTTG -3'			13786-13804
Pen	P-Pen T A	5'- AGATAACATGCGATATTAGGCACC -3'	55	1601	1952-1975
	P-Pen-B A	5'- AGAGCGGTGTCTCATTGTTG -3'			3534-3553
	S-Pen B A1	5'- AACTACGCCTTTGGTTGGC -3'			2447-2465
	S-Pen T A1	5'- ACTAGCGTTCATCAATTTGACC -3'			2354-2375
	S-Pen B A2	5'- AGCTCGATGGTCACCTCATG -3'			2934-2953
	S-Pen T A2	5'- ACTGCGTTCGGAACTAACC -3'			2881-2899
	P-Pen T B	5'- ATTGTCATCCACCACAACG -3'	53	1828	3490-3508
	P-Pen B B	5'- ATTCGATTGCCTGCATCG -3'			5301-5318
	S-Pen B B1	5'- ATCTGCTTCTGGTTACCTGC -3'			4050-4069
	S-Pen T B1	5'- ACTCCTTCTACAGCACAAC -3'			3983-4001
	S-Pen B B2	5'- AGAACGTGTAGCCACCTTC -3'			4542-4560
	S-Pen T B2	5'- AAGGTGGCTACACGTTC -3'			4542-4560

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*) *w*as not due to human error or genetic background effects, I replicated the sequencing as follows: 7 *w;mbmB*^{1}*(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 immunohistochamical 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 4ºC, washed in 1x PBS + 0.2% Triton X-100 (PBT) 3 x 30 minute at 4ºC, blocked with 1x PBS + 0.2% Triton X-100 + 0.1% BSA (PBSBT) for 1 hour at 4ºC, then incubated overnight at 4ºC 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 microcenterfuge and re-suspended in lysis buffer containing multiple protease inhibitors (Laemmli, 1979). Tissue was homogenized, boiled for 5 minutes then stored at 22ºC 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*^{1}*(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*^{1}*(CS) and mbmB*^{1}*(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*^{1}*(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*^{1}*(CS)* females, 11% in *mbmB*^{1}*(CS)/CS* females, 15% in *mbmB*^{1}*(CS)* males and 8% in *mbmB*^{1}*(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*^{1}*(CS)* females showed a 15% decrease in AL volume and *mbmB*^{1}*(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 ($F_{[2,120]}$=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 *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 *mbmB*^{1}*(CS)/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 *mbmB* flies and facilitated the use of sterility as the screening phenotype to map *mbmB*.

mbmB^{1} is An Allele of The *Drosophila importin-α2*

I began mapping *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 *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 *Df(2L)8469* failed to complement *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 *mbmB* was located in region 30F4-30F6, which contained only 12 genes (Figure 2.3 A). I first began sequencing *big brain* and *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 *Pen* because of its female sterility (Gorjánácz et

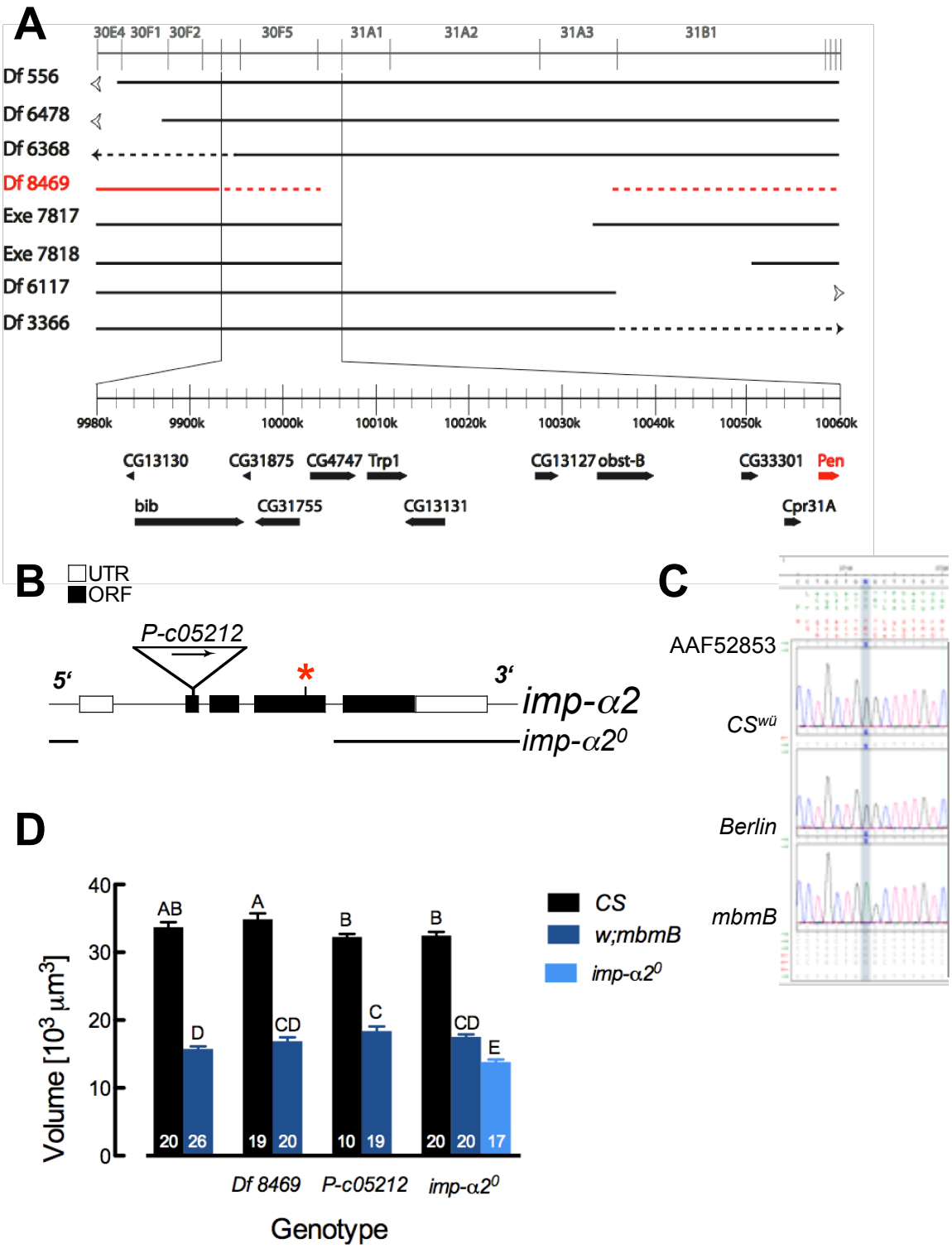

Figure 2.3 Mapping *mbmB*. (**A**) I used sterility to screen 168 second chromosome Dfs and found that *Df 8469* (red) failed to complement *mbmB*. Sequencing of several genes in 30F4-30F6 revealed that mbmB had a point

mutation in Pendulin (Pen), the *Drosophila Importinα2* gene. (**B**) *imp-α2* transcriptional unit with *imp-α2* null *P-c05212* and mbmB nonsense mutation (red asterisk) locations noted. (**C**) Chromatographs of sequence alignment made in DNA* for *Importinα2*. Sequencing revealing that *mbmB* had an G to A transition in *Importinα2* 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 *Importinα2 (AAF52853)*. (**D**) MB calyx volume for *mbmB* flies heterozygous for *Df8469, P-c05212 and imp-α2^{0}* as well as homozygous *imp-α2^{0}* 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).

al., 2002). The mutant allele of *mbmB* had a G-to-A transition in *Pen*, the *Drosophila importin-α2* 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 *Berlin*) and the published wildtype sequence for *imp-α2 (*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 *P-c05212* (inserted in the second exon of *imp-α2*) failed to complement *mbmB*, as the *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). *imp-α2^0* homozygotes and *mbmB/ imp-α2^0* were female sterile (data not shown) and displayed reduced MB calyx volumes ($F_{[8,153]}$=237.59, P<0.0001*)* (Figure 2.3 D).

mbmB^1 Mutants Lack Full Length Imp-α2 Protein

To determine whether this nonsense mutation caused a functional change at the protein level, I performed western blots on whole body extracts from *w^{1118} (CS)*, *w;mbmB^1(CS)* and *imp-α2^0.* I used a rabbit anti-Imp-α2 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 *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 *w^{1118} (CS)* control flies were absent in both *w;mbmB^1(CS)* and *imp-α2^0* (Figure 2.4 C). I concluded that the

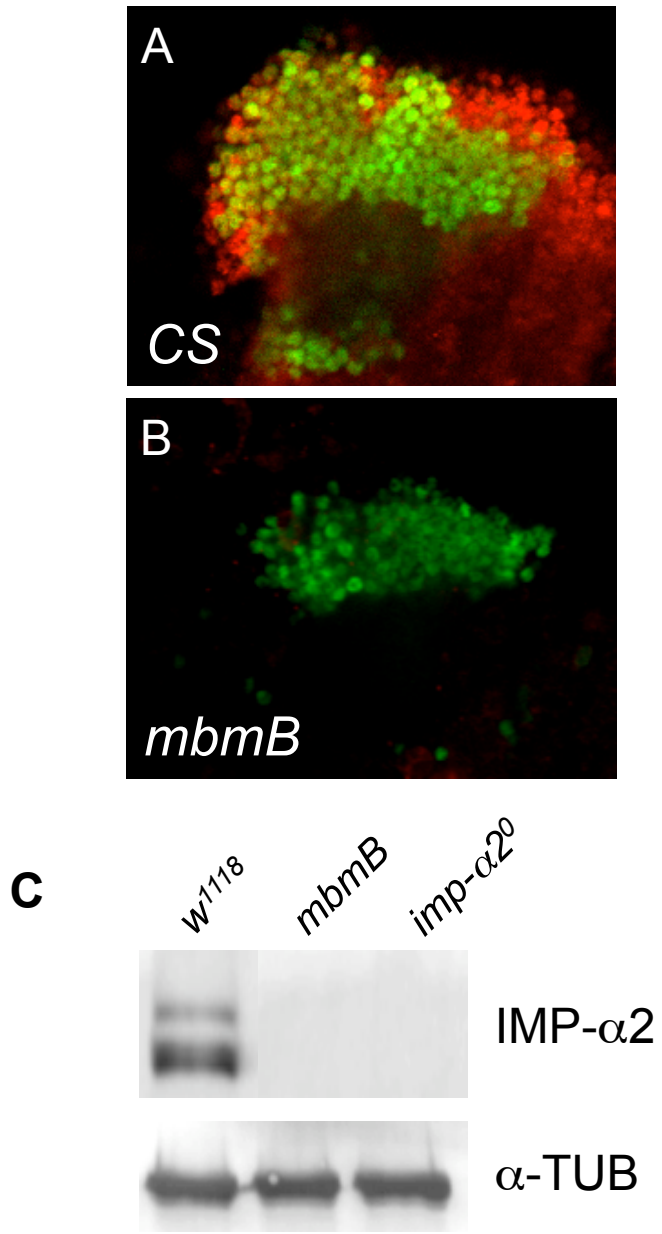

Figure 2.4 Immunohistochemistry shows Imp-α2 expression in central brain neuropil and western blot analysis reveals *mbmB* is lacking the second half of *Imp-α2*. For A and B: GFP (green) expressing in MB kenyon cells. (**A**) *CS; P[GPF]nls;; P[Gal4]Ok107* showed Imp-α2 expression throughout the neuropil of the adult brain (red), with some overlap in MB Kenyon cells (yellow). **(B)** *mbmB-P[GPF]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-α2*^{0} 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-α2* 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-α2* 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-α2* 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-α2* 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-α2* 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

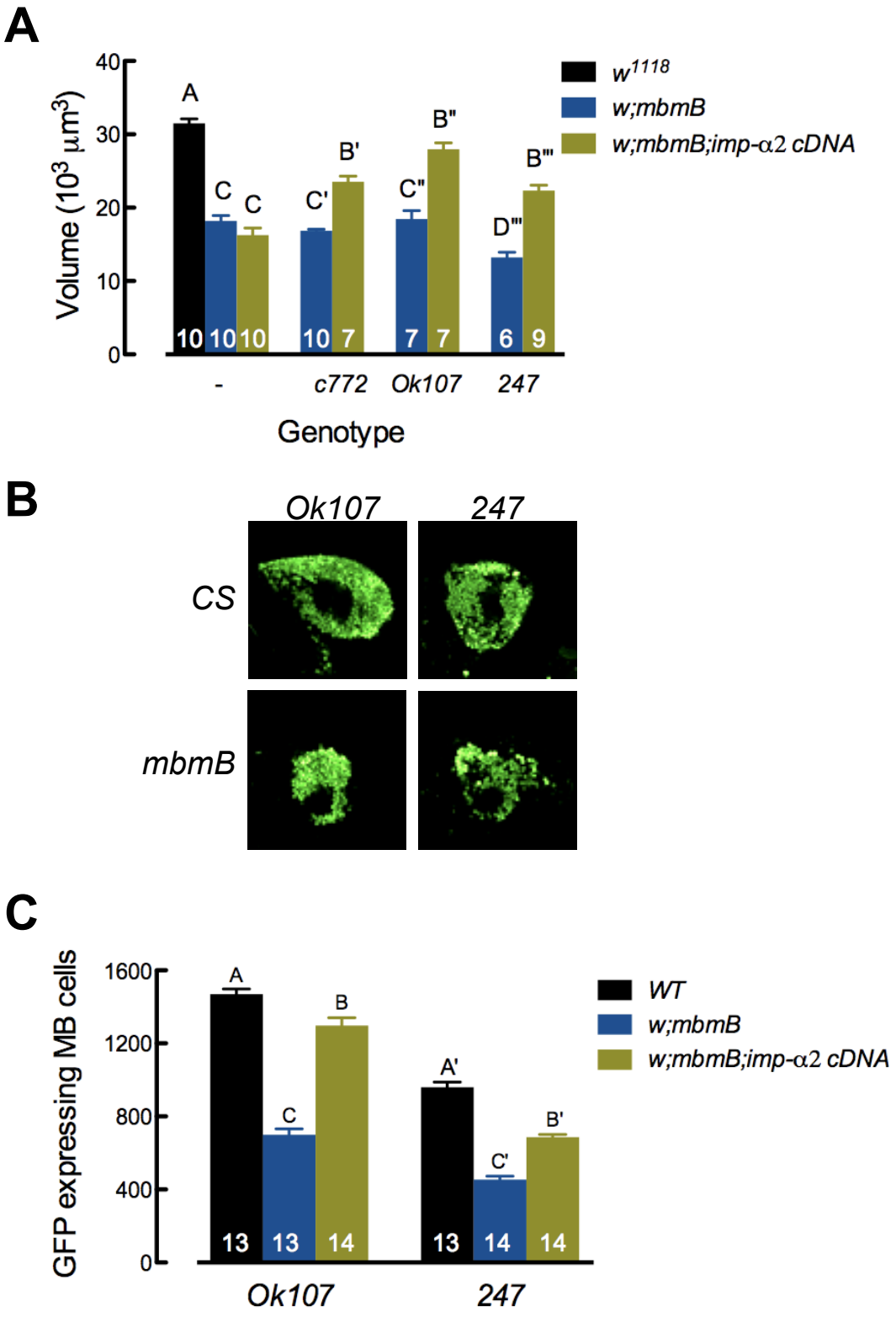

Figure 2.5 *imp-α2* cDNA driven in the MBs rescues anatomical phenotypes. (A) MB calyx volume was rescued with three different MB specific Gal4 lines driving *imp-α2* cDNA in the MBs. *P[Gal4]c772* driving *imp-α2* cDNA

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-α2* 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-α2* 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-α2* 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-α2* expression in the ovaries is sufficient to rescue *the* sterility defects seen in *mbmB*, and 2.) *imp-α2* expression in the MBs is necessary but not sufficient for complete rescue of the MB defects observed in *mbmB* mutants.

All *imp-α2* Domains Influence MB Development

imp-α2 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-α*'s, *imp-β*'s and NLS-bearing cargo for nucleo-cytoplasmic trafficking (Figure 2.6 A) (review: Goldfarb et al., 2004). Each domain was disrupted in a suite of UAS *imp-α2* transgenes driven in the MBs by *c772* in the homozygous *mbmB* background. I found that there was no influence of sex

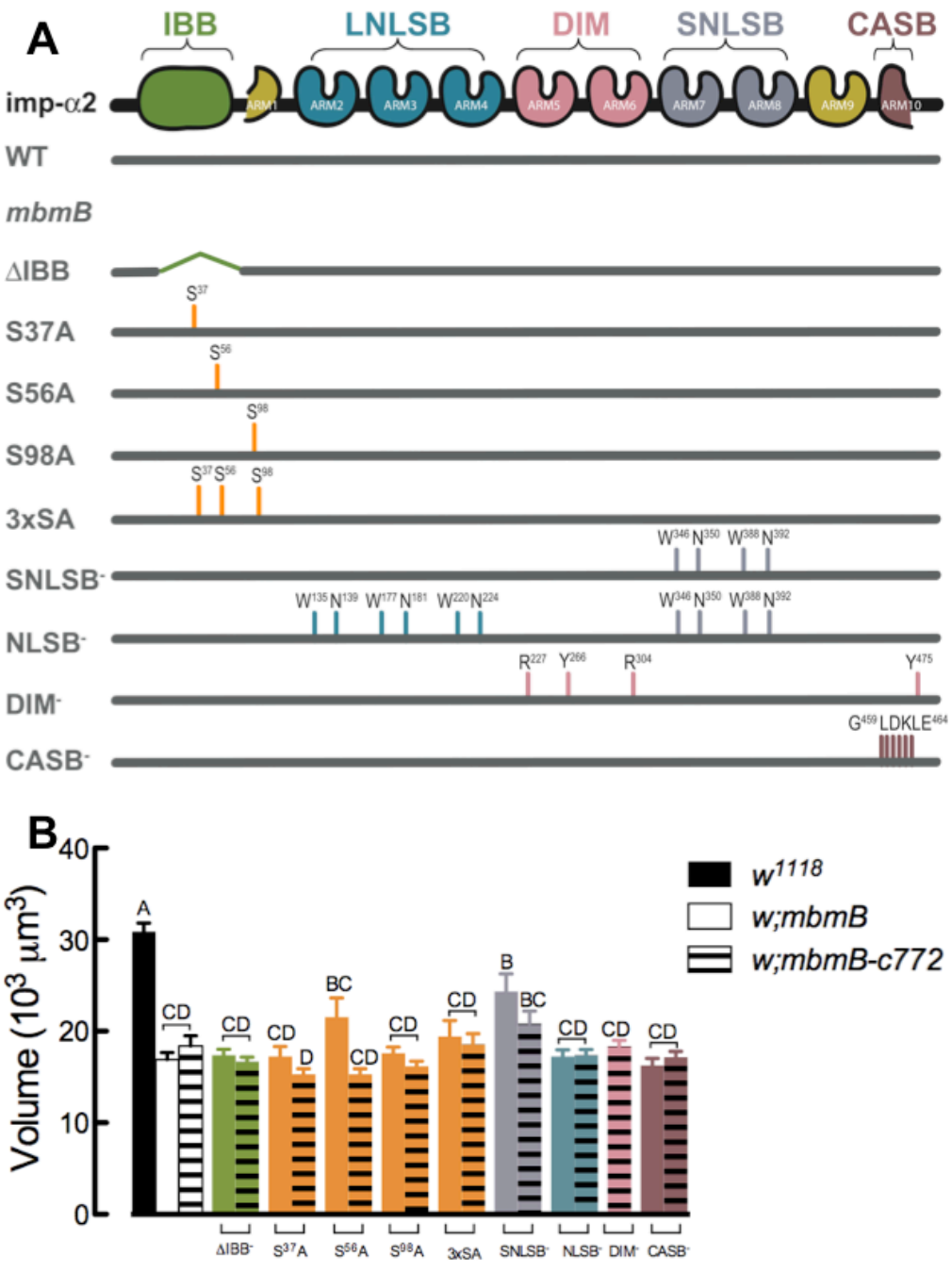

Figure 2.6 Analysis of *imp-α2* Domain function on MB development. (**A**) Schematic diagram of *imp-α2* transgenes used to disrupt individual domains of *imp-α2* (diagram modified from Gorjánácz et al. 2006). (**B**) Histological data shows that all domains of *imp-α2* are necessary for proper MB development. 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 $S^{56}A$ 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.

<u>The Relationship Between MB Cell Number and Learning</u>

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 he 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 down stream 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-α2* 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-α2 binding partners, like CAS, Imp-β or it's NLS-bearning cargos will be necessary to yield more detailed information about how Imp-α2 functions at a molecular and cellular level to regulate brain development and behavior.

mbmB is Missing the Full Length Imp-α2

My results show that *mbmB* is lacking the full length Imp-α2 protein, which could be interpreted in one of two ways. *mbmB* is either an Imp-α2 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-α2 Expression in The Adult Brain

Imp-α2 is expressed in neuropil throughout the adult brain, including MB cells. This analysis of the Imp-α2 expression pattern in the brain is very informative in concert with our preliminary L3 expression profile (Appendix Figure A.4). Imp-α2 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-α2). Similarly, it is interesting that Imp-α2 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 *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 it's 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 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 rosterally 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 with in 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.

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

- = 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*^{r} 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,

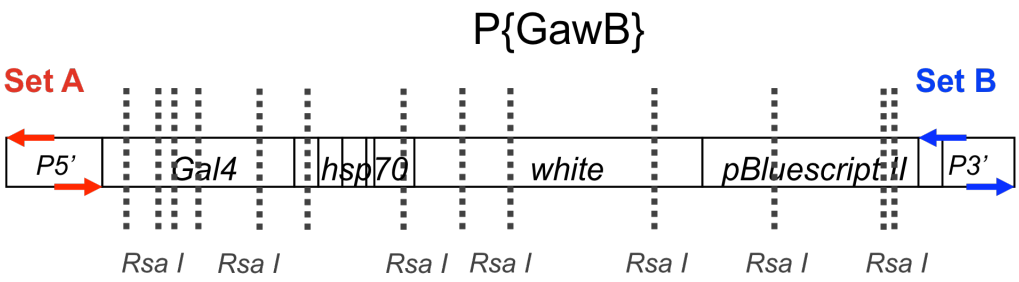


Figure 3.1 P[GawB] construct. Diagram of the P[GawB] construct (illustration modified from Phelps and Brand, 1998). Set A primers (red) located at the 5' end of the construct and Set B primers (blue) at the 3' end.

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.

Primer	Sequence	T_m (°C)	Relative Location / Strand
Set A for	5'-CTCAAGTGCTCCAAAGAAAAACCGA-3'	54	33 Bp from GawB *Rsa I* bottom strand
Set A rev	5'-ATCGACGGGACCACCTTATGTTATT-3'	54	8 Bp from end of GawB on top strand
Set B for	5'-CTCTTGCCGACGGGACCACCTTATG-3'	57	14 Bp from end of GawB on top strand
Set B rev	5'-GATTAACCCTTAGCATGTCCGTGGG-3'	57	70 Bp from GawB *Rsa I* bottom strand

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 22ºC 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 α = 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 [+] and [-] orientations (Table 3.3).

c739 is located in an intron roughly 5.8 Kb into the *βFTZ-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* (*Tab*2) ~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 γ* (*CK1γ*) in the

Table 3.3 GAL4 insertion positions and information regarding proximal genes.

Gal4 Line	Original map position	Insertion sequence location, and notes (genes in bold)	Proximal genes	Gene details	
				Sequence location & Orientation	Function & References
c739	2L: 40A	~6 Kb into 2nd exon of **βFTZ-1** & ~10 Kb upstream from its 3d exon.	βFTZ-1	2L:21,237,237..21,259,675 [+]	Orphan nuclear receptor involved in Ecdysone-mediated autophagy of the salivary gland (1).
c772	2RL: 42A	~2 Kb downstream from end of ***Or42a*** and 11.4 Kb upstream from ***CG11163***.	*Or42a*	2R:1,679,001..1,680,468 [-]	Olfactory associated G-protein coupled receptor necessary for olfactory sensory perception (2).
			CG11163	2R:1,662,279..1,670,360 [-]	Predicted zinc ion transmembrane transporter activity (3).
c492b	2R: 49C	4 Bp into ***CG8776*** & ~9.3 Kb into ***nemy***.	*CG8776*	2R:8,547,002..8,557,722 [-]	Predicted carbon-monoxide oxygenase activity (3).
			nemy	2R:8,557,988..8,567,094 [-]	male courtship conditioning & olfactory associative learning and 2 hr memory (4).
201Y	2R: 56C8-9	~1 Kb into 1st intron of ***Tab2***, & ~5.6 Kb upstream of 2nd exon.	*Tab2*	2R:15,180,073..15,191,529 [+]	antimicrobial (5) and bacterial (6) responses.
c35	2R: 44A	131 Bp upstream from ***fz***.	*fz*	3L:14,267,447..14,361,748 [+]	planner cell polarity, organ development, cell differentiation, cell division and axon guidance (7, 8)
30Y	3L: 70E	123 Bp upstream from ***fz***.			
238Y	2R: 48C	101 Bp upstream from ***fz***.			
247	3d chrom.	In an intron ~20 Kb downstream of the 1st exon of the B transcript of ***E75***.	*E75*	3L:17,945,063..18,052,698 [-]	Steroid hormone nuclear receptor involved in ecdysone signaling (9).
H24	3d chrom.	In an intron ~5 Kb downstream of the second exon of ***CK 1γ*** B, D, E & I transcripts.	*CK1γ*	3R:12,098,176..12,128,094 [+]	spermatogenesis and male sterility (10, 11).

Ok107	*4th chrom*	560 Bp into the first exon of ***ey***.	*ey*	4:718,315..741,787 [+]	Mushroom body development (12).

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

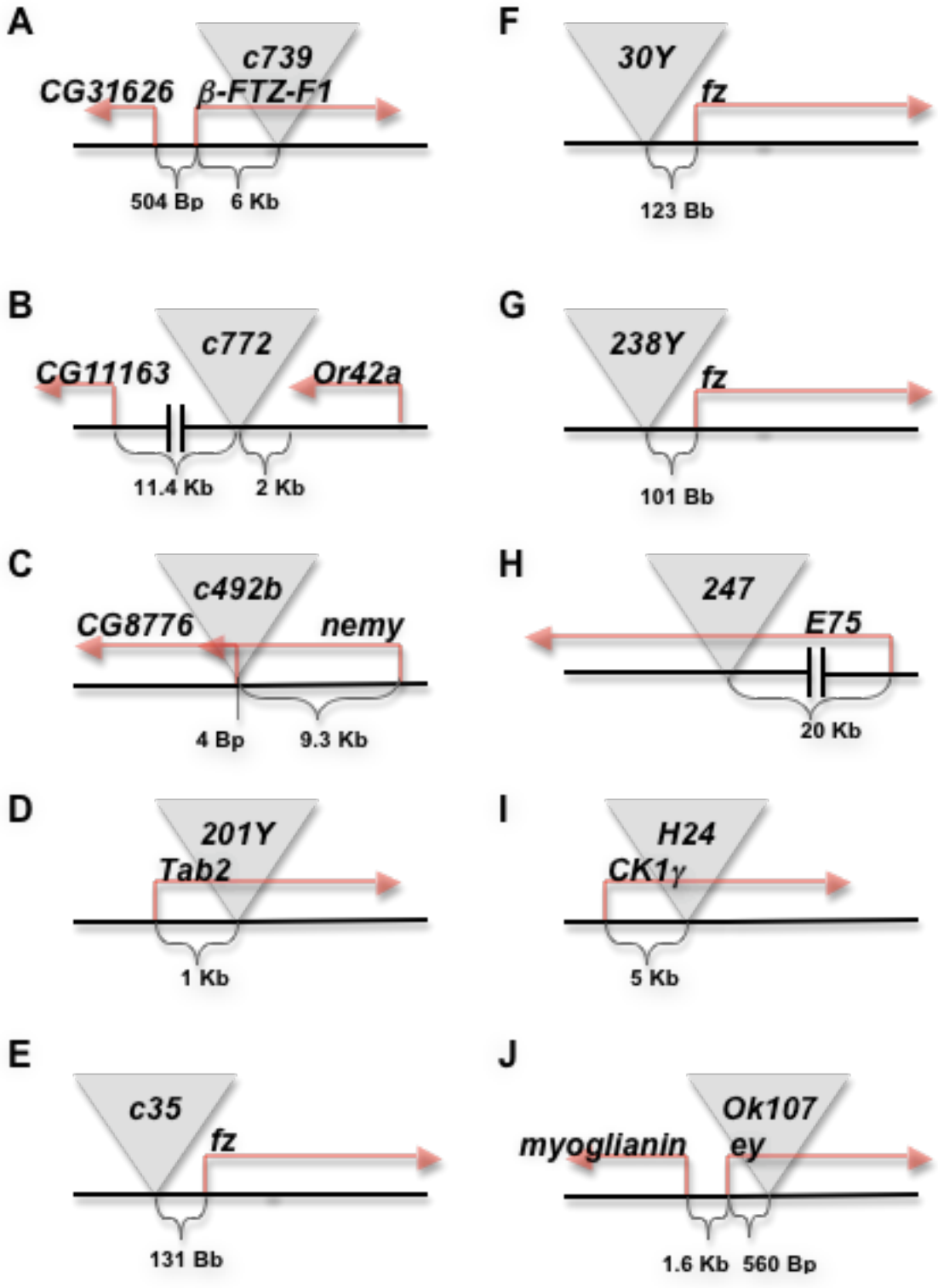


Figure 3.2 Sequence of insertion sites for 10 MB specific GAL4 lines. (A) c739 is located in the second intron of *β-FTZ-F1* ~6 Kb into the gene and ~10 Kb upstream of it's 3d exon (B) c772 is located ~2 Kb downstream from the end of

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) 201Y 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 (*CK1*γ) 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, 200.6.26). 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 α = 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

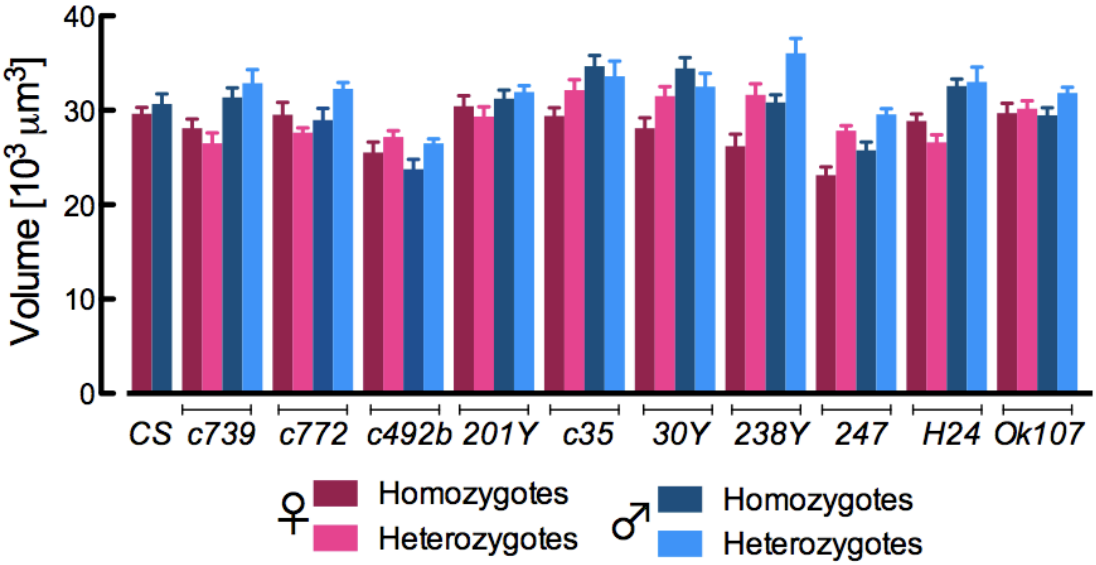


Figure 3.3 The influence of homozygous and heterozygous Gal4 lines on MB calyx volume. Serial sections of paraffin-embedded brains were used for planimetric MB measurements. *c492b* homozygous males had MBs that were 23% smaller than *CS* males, and 20% smaller than *CS* females. *c492b* homozygous females 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. $5 \leq n \geq 20$ 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: ★ < 0.05, ★★ < 0.01, ★★★ < 0.005, ★★★★ < 0.001. α = 0.05.

		CS F	*CS* M	*c492b* F	*c492b* M	*c492b/CS* F
	CS M	NS				
	c492b F	NS	★			
c492b	*c492b* M	★	★★★★	NS		
	c492b/CS F	NS	NS	NS	NS	
	c492b/CS M	NS	NS	NS	NS	NS

		CS F	*CS* M	*238Y* F	*238Y* M	*238Y/CS* F
	CS M	NS				
	238Y F	NS	NS			
238Y	*238Y* M	NS	NS	NS		
	238Y/CS F	NS	NS	NS	NS	
	238Y/CS M	★★★	NS	★★★★	NS	NS

		CS F	*CS* M	*247* F	*247* M	*247/CS* F
	CS M	NS				
	247 F	★★★★	★★★★			
247	*247* M	NS	★★★	NS		
	247/CS F	NS	NS	NS	NS	
	247/CS M	NS	NS	★	NS	NS

		CS F	*CS* M	*H24* F	*H24* M	*H24/CS* F
	CS M	NS				
	H24 F	NS	NS			
H24	*H24* M	NS	NS	NS		
	H24/CS F	NS	NS	NS	NS	
	H24/CS M	NS	NS	NS	NS	★

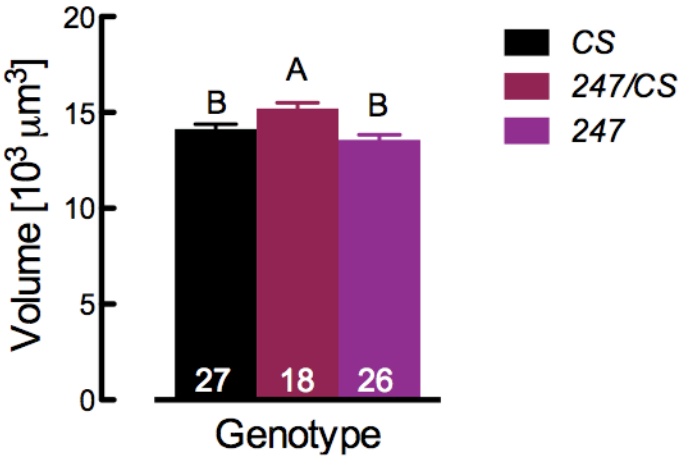
247 males had a 16% reduction in MB calyx volume compared to CS males. Finally the MBs in heterozygous *H24* females were 20% smaller than those of heterozygous *H24* males (Figure 3.3).

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

A



B

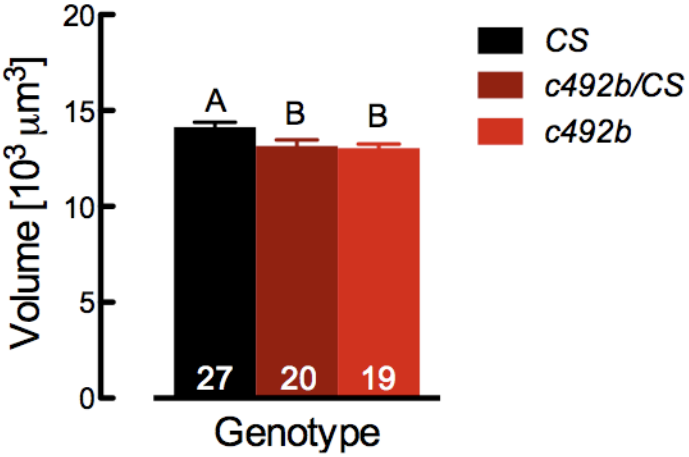


Figure 3.4 CCX volumes for *247* and *c492b*. Serial sections of paraffin-embedded brains were used for planimetric CCX measurements. (**A**) There was an influence of genotype and sex, yet no influence of the interaction of genotype and sex on CCX volumes for the *247* GAL4 line, so the sexes were pooled. 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*≤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*≤0.05).

<u>*c35*, *30Y* and *238Y* disrupt FZ protein levels and MB Anatomy</u>

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

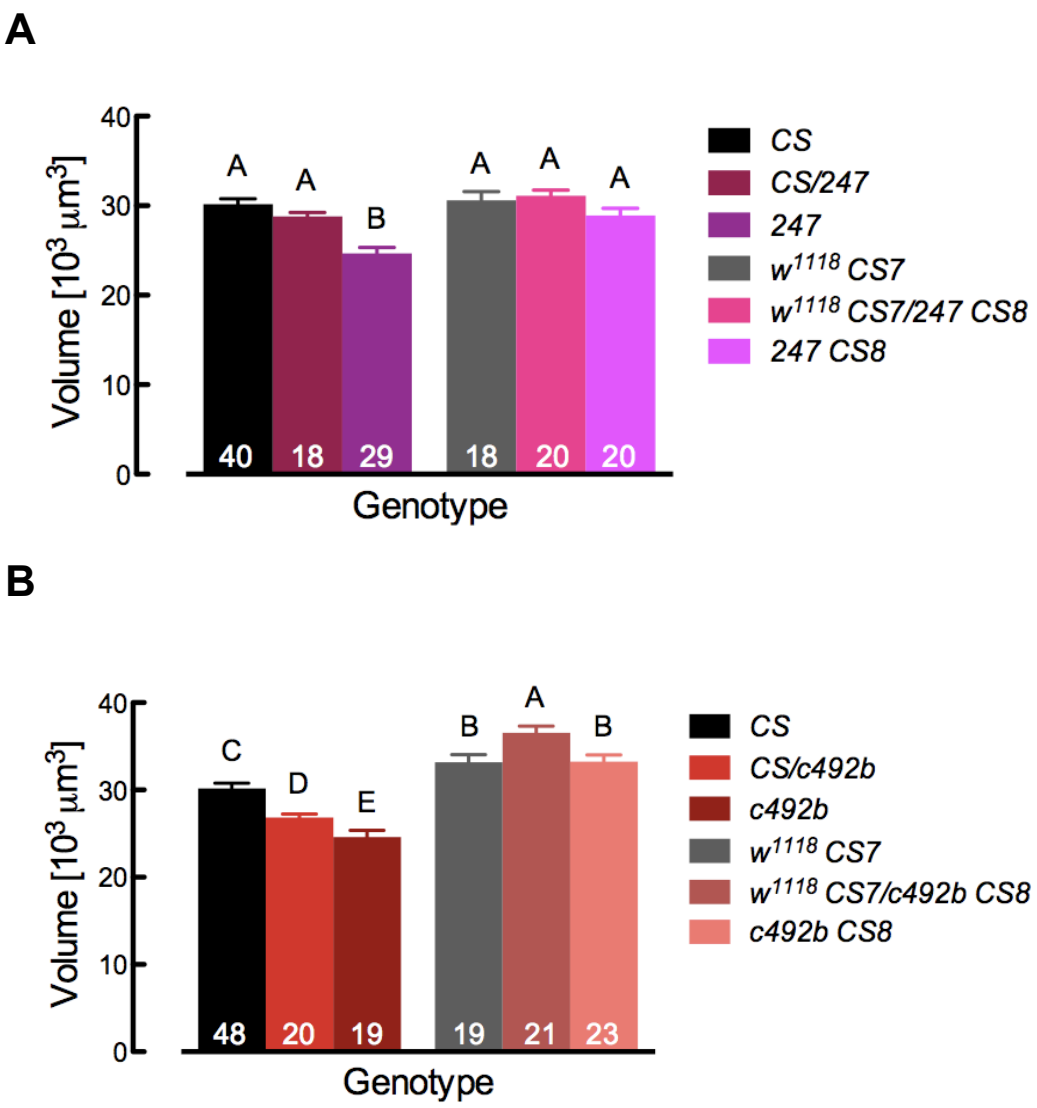


Figure 3.5 The effects of genetic background on MB calyx volume. (**A**) There was no influence of sex or the interaction of sex and genotype on MB calyx volume, so the sexes were pooled for *247.* There was a significant influence of genotype, as only non-cantonized homozygous *247* flies were ~18% 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

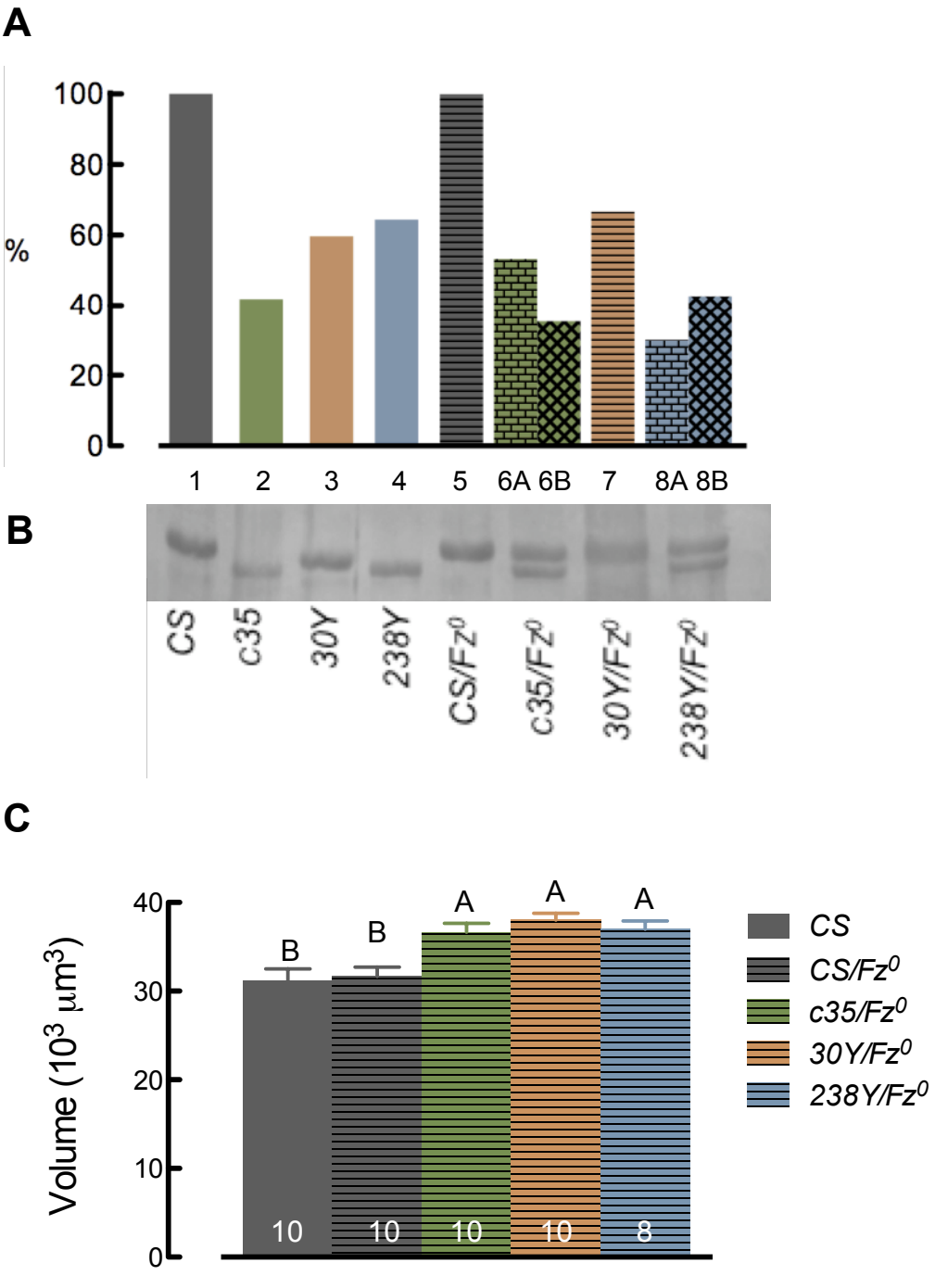


Figure 3.6 Fz western blot and anatomical analysis of MB calyx volume in Fz^0 crossed to *c35*, *30Y* and *238Y*. (**A**) This graph illustrates the quantification of bands from A using ImageQuant V5.1 (Molecular Dynamics). The *CS* band was set to 100% and used for comparisons to all other samples. There was a significant reduction in the amount of protein for all three homozygous GAL4 lines compared to wildtype, as well as all lines/Fz^0. (**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*/Fz^0 (lane 6) and *238Y*/Fz^0 (lane 8) displayed a doublet band. (**C**) There was a significant influence of genotype on MB calyx volume. *30Y*/Fz^0 was 17% larger that *CS*/Fz^0. *c35*/Fz^0 and *238Y*/Fz^0 were both 14% larger than *CS*/Fz^0. Bars represent mean ± SE of mean calyx volume for each genotype. *n* indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$).

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 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 fz^{0} to find that all three were

significantly larger than CS or the CS/ fz^{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*γ) 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 fz^0, there were small yet significant increase in MB calyx volume, with 30Y/ fz^0 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 $CS^{w\ddot{u}}$ 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*γ), 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:

Serway, C. N., Kaufman, R. R., Strauss, R. & de Belle, J. S. (2009). Mushroom bodies enhance initial motor activity in Drosophila. *Journal of Neurogenetics*. 23, 173-184.

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^{l}) and three medial (β, β^{l}, and γ) lobes, formed by the axonal projections of α/b, α^{l}/β^{l}, 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^{l}/β^{l} 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 miniature*^{1} (*mbm*^{1}). 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: *mbm*^{1}, *mushroom body miniature B* (*mbmB*^{1}), *mushroom body miniature C* (*mbmC*^{1}), *mushroom bodies reduced* (*mbr*^{1}), *small mushroom bodies* (*smu*^{1}), and two alleles of *mushroom bodies deranged* (*mud*^{1} and *mud*^{4}) (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). *mbm*^{1} 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

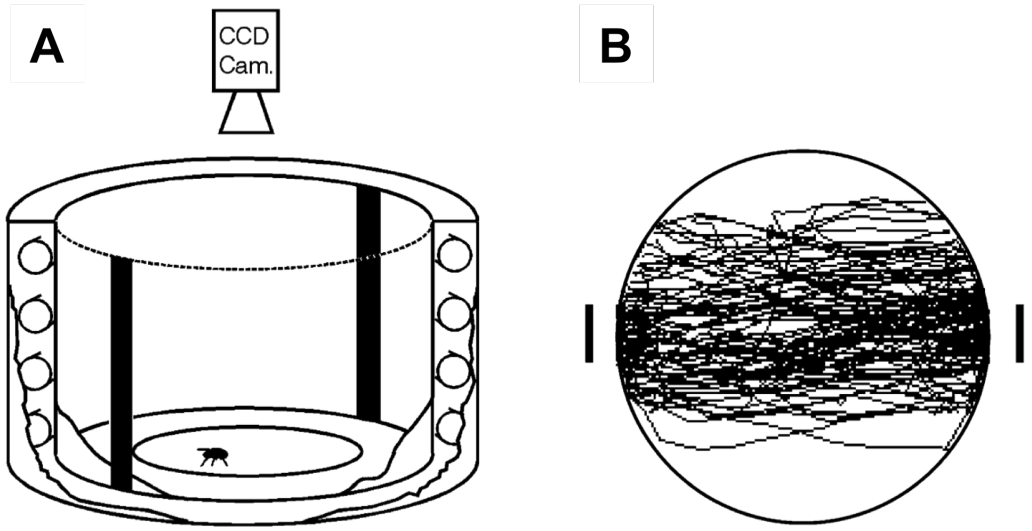

Figure 4.1 (A) Buridan's paradigm. **(B)** A sample tracing made by a *Berlin* male in 15 minutes.

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.

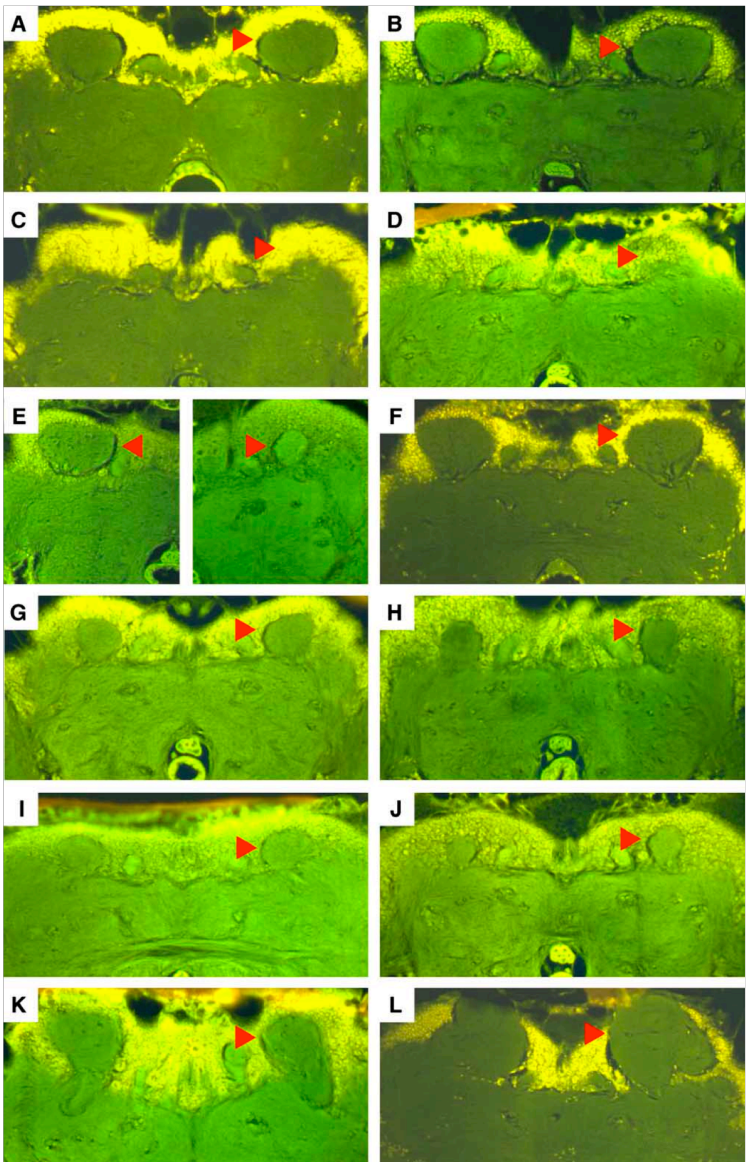

Figure 4.2 Brains of HU-treated flies and mushroom body (MB) structural mutants. Frontal 7-mm paraffin sections of *Drosophila* heads viewed under a fluorescence photomicroscope. Images are of sections approximately 40 mm from the caudal margin of the brain, showing MB calyces (arrows) at their broadest 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≤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≤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≤0.05), reflecting a strong influence of polymorphic gene interactions on the MB phenotype (de Belle & Heisenberg, 1996). MB calyces in $mbmB^1$*(CS)*, $mbmC^1$*(CS)*, mbr^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≤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

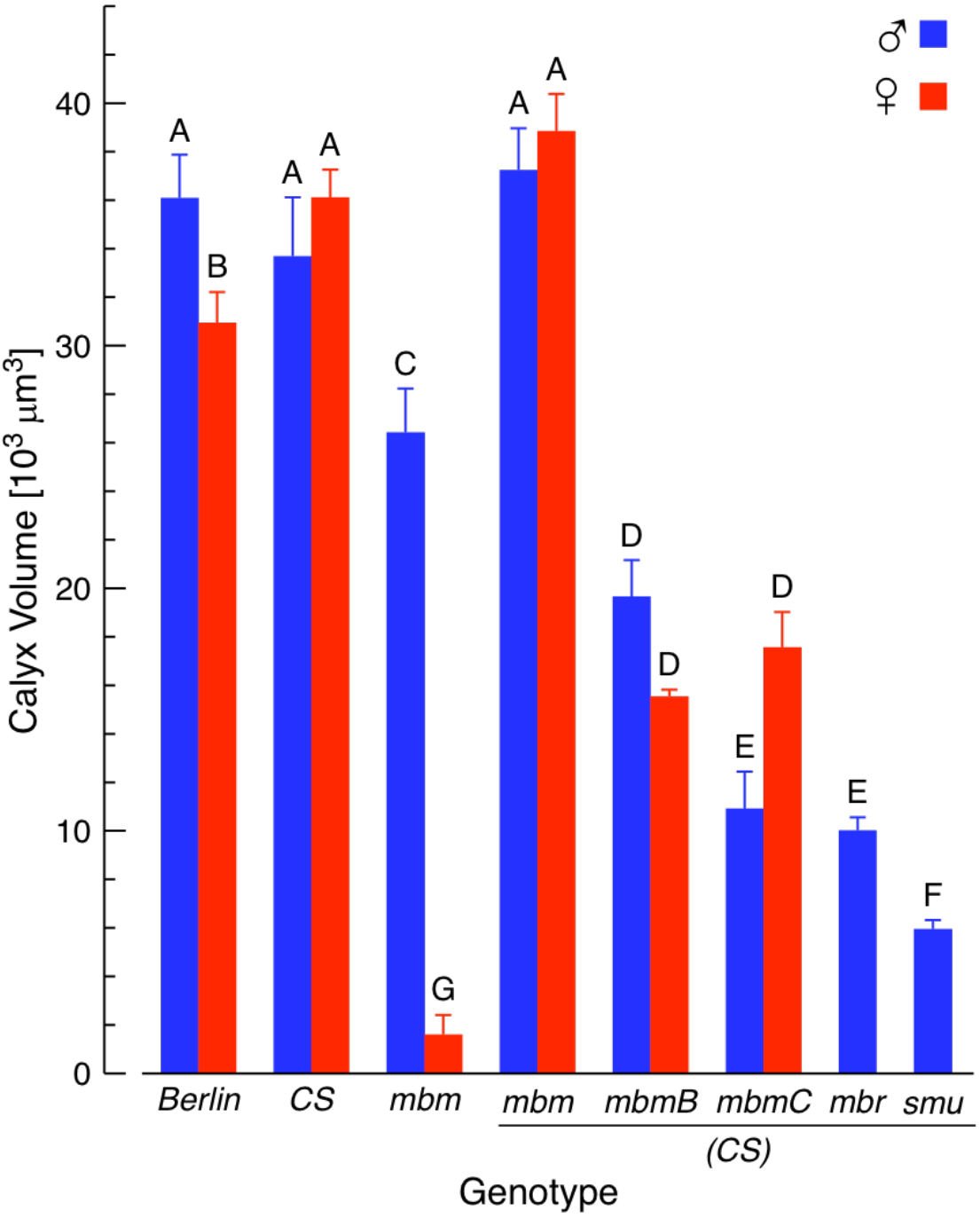

Figure 4.3 Mushroom body calyx volume was significantly different. Bars represent mean±SE of the mean calyx volume for each fly, 9 ≤ *n* ≤ 16/bar. Different letters designate significant differences (*P* ≤0.05). Values were derived from planimetric measurements of flies represented in Figures 5-7.

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). *mbr*^{1}*(CS)*, *smu*^{1}*(CS)*, *mud*^{1}*(CS)*, and *mud*^{4}*(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 *Berlin* and *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

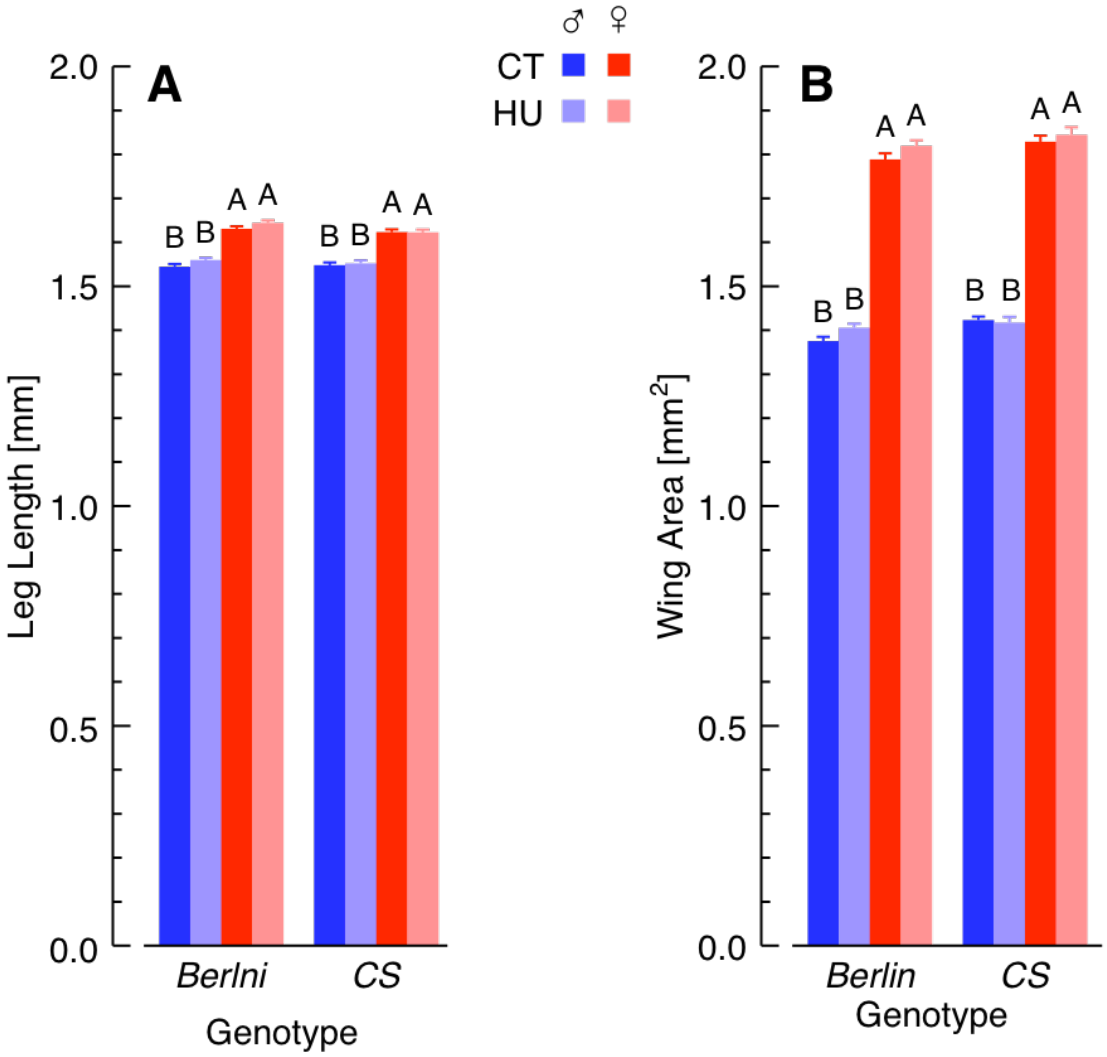

Figure 4.4 External anatomy was not influenced by genotype or HU treatment. Bars represent mean±SE, *n* = 30/bar. Different letters designate significant differences (*P*≤0.05). (A) Limb length was shorter in males than in females. (B) Similarly, wing area was smaller in males than in females.

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 ± 0.005; HU, r = 0.7479 ± 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

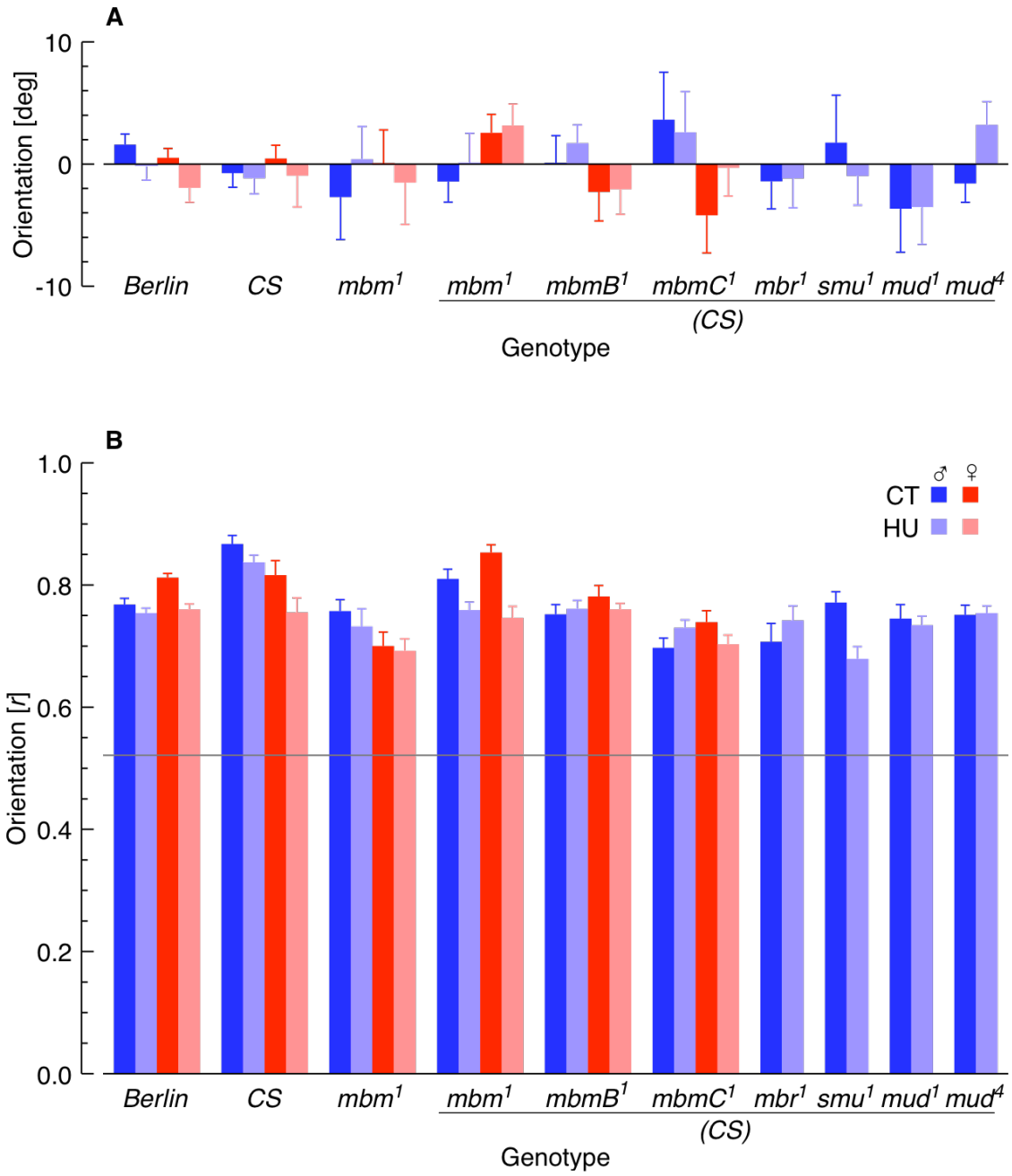

Figure 4.5 All groups of flies demonstrated comparable patterns of landmark orientation, indicating similar responses to visual stimulation in Buridan's paradigm. Bars represent mean±SE, 13 ≤ *n* ≤ 23/bar for all groups except *CS*, which is 39 ≤ *n* ≤ 56/bar. (A) There was no difference in the mean 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

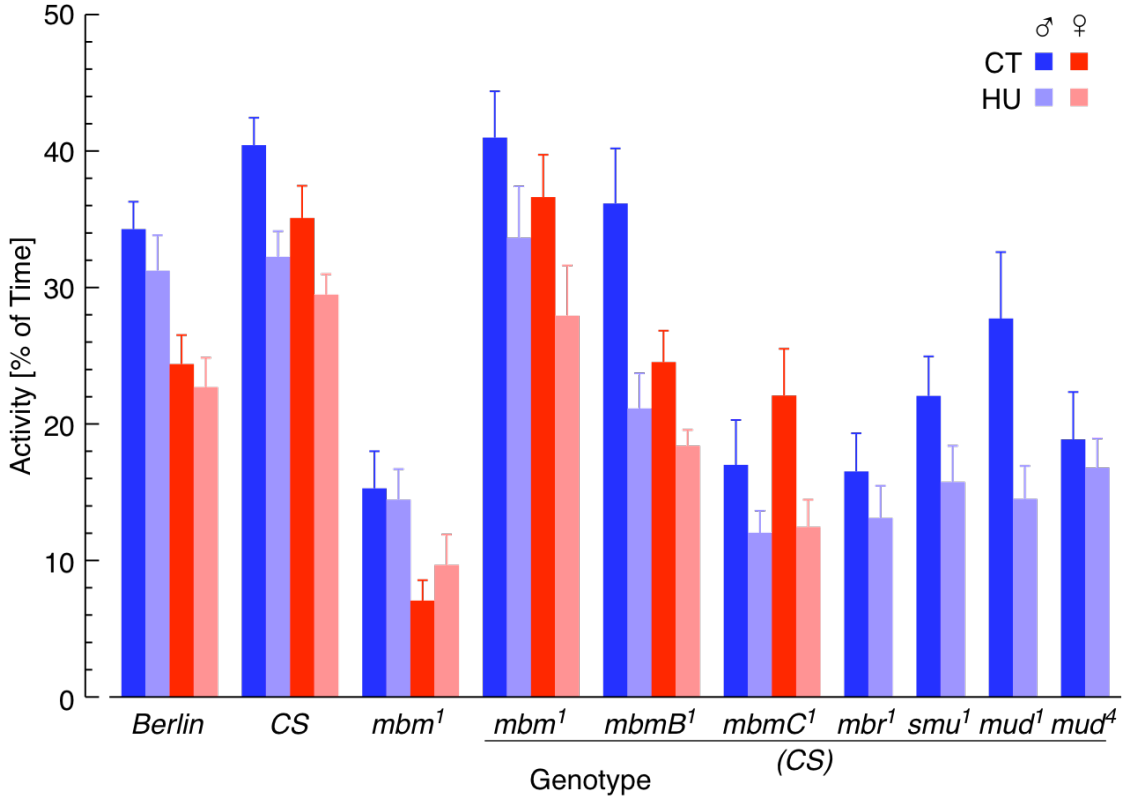

Figure 4.6 The mean percent of time flies were actively walking during 15 minutes in Buridan's paradigm was influenced by genotype, gender, and HU ablation. Bars represent mean±SE, *n* same as Figure 5.

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*^{1}*(CS)* and *mbmB*^{1}*(CS)* walked slower than control CS flies. In the original Berlin background, walking velocity in *mbm*^{1} males was comparable with that of other mutants having similar MB phenotypes. However, *mbm*^{1} 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

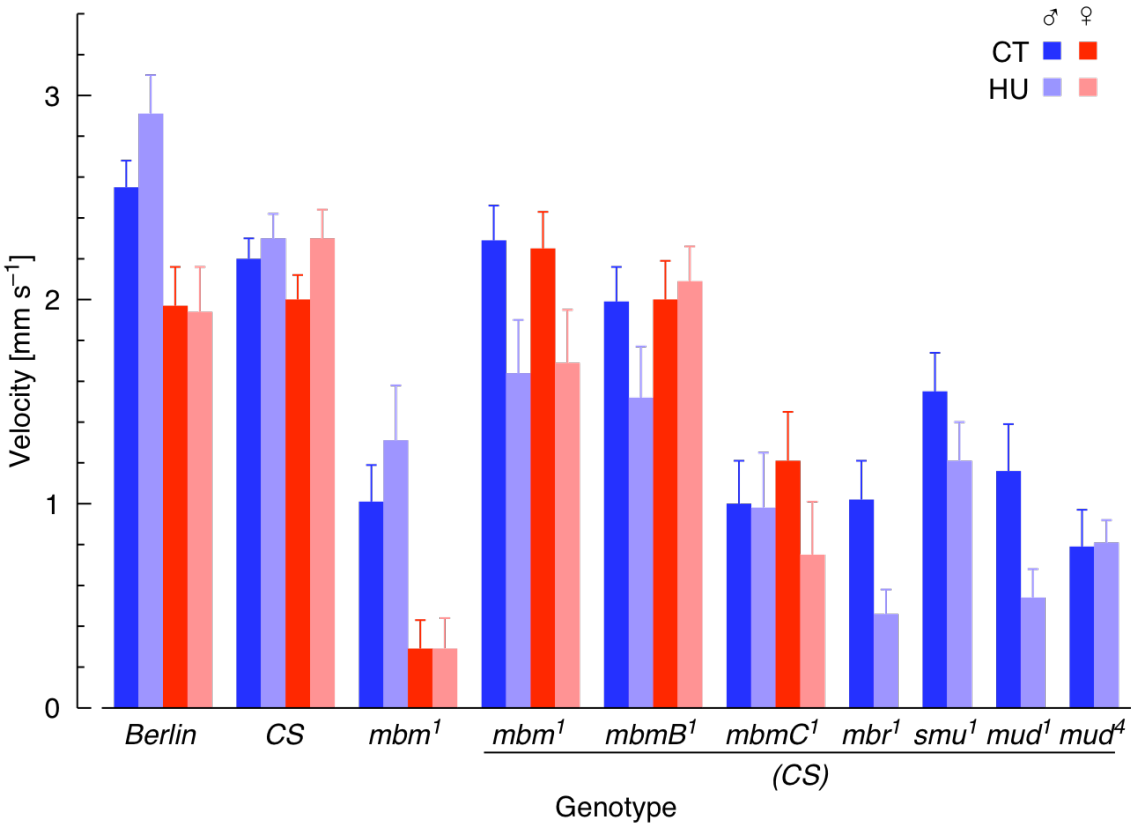

Figure 4.7 The mean velocity of flies walking during 15 minutes in Buridan's paradigm was influenced by genotype and the interaction of genotype and gender. Bars represent mean±SE, *n* same as Figure 5.

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 *mbm*^{1} 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^{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^{1} in the original Berlin genetic background showed very low activity and velocity in Buridan's paradigm. In mbm^{1}, this was not strictly a function of MB defects, since ablation of these mutant structures had no impact on behavior. However, outcrossing mbm^{1} with CS flies [generating $mbm^{1}(CS)$] 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.

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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 spermatotogenesis 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

(Goldman et al., 2005). The molecular function of *CG11163* is inferred from sequence similarity as having zinc ion transmembrane transporter activity, (Flybase, 1992) 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 glutimate (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* homozyotes 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* (*CK1*γ) (also known as *gilgamesh)* in an intron ~5 kb downstream from the end of *CK1*γ'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. *CK1*γ 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). *CK1*γ 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 *CK1*γ 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). Re-visitation 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.

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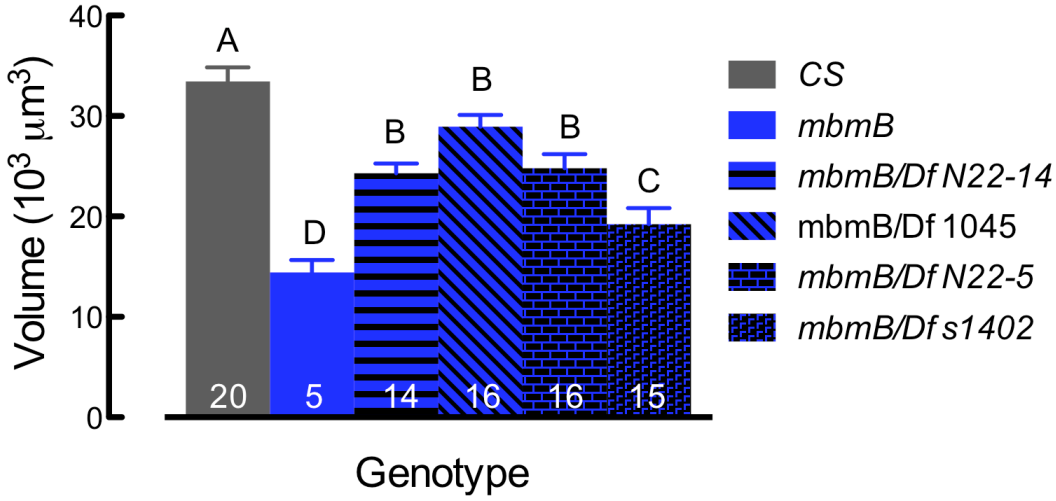
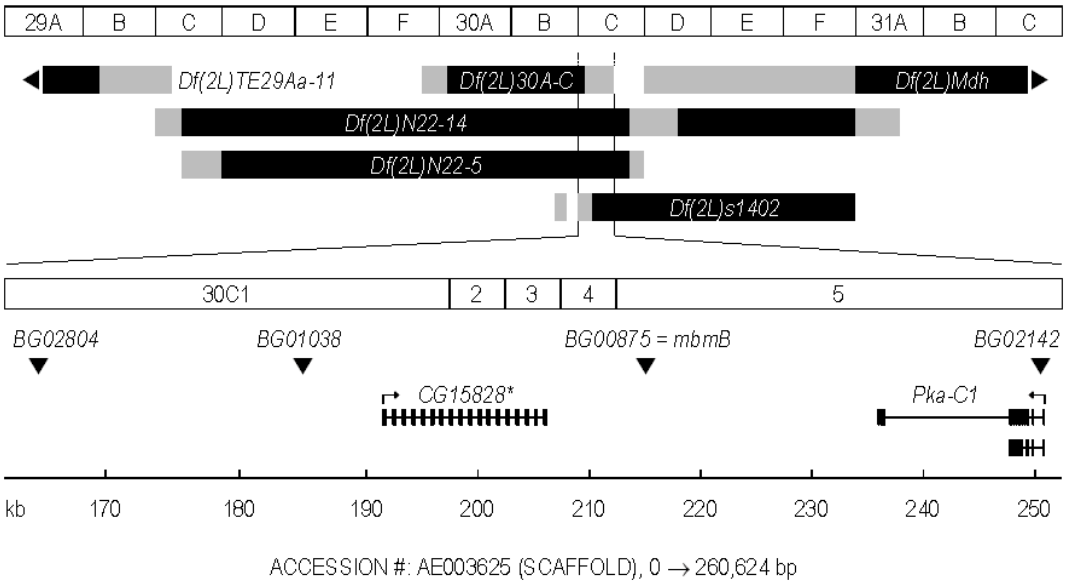
APPENDIX A

MBMB

A



B



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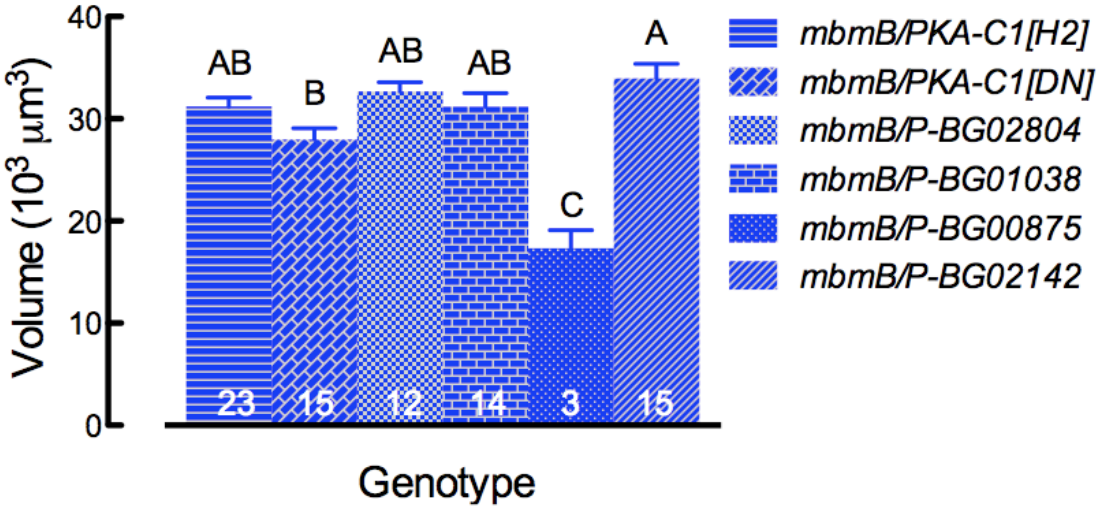
C


Volume (10^3 µm^3)
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0
AB
B
AB
AB
C
A
23
15
12
14
3
15
Genotype
mbmB/PKA-C1[H2]
mbmB/PKA-C1[DN]
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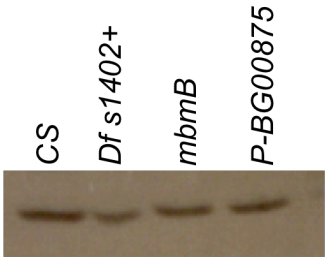
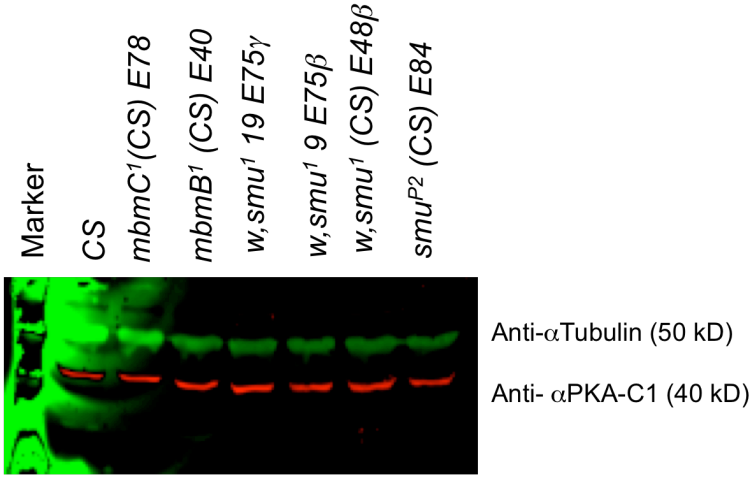
mbmB/P-BG01038
mbmB/P-BG00875
mbmB/P-BG02142

D


CS
Df s1402+
mbmB
P-BG00875

E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mbmB1 (CS) E40
w,smu1 19 E75γ
w,smu1 9 E75β
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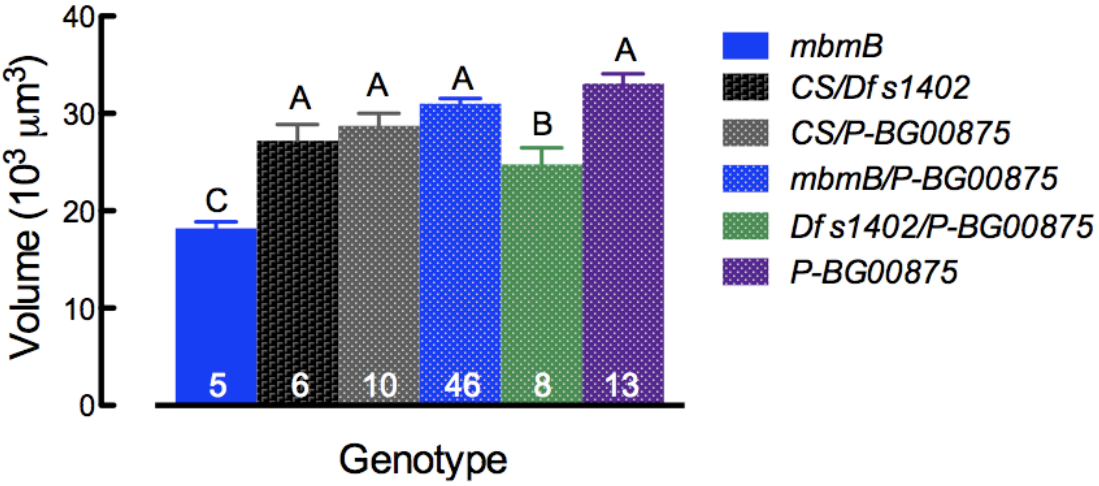
Anti- αPKA-C1 (40 kD)

F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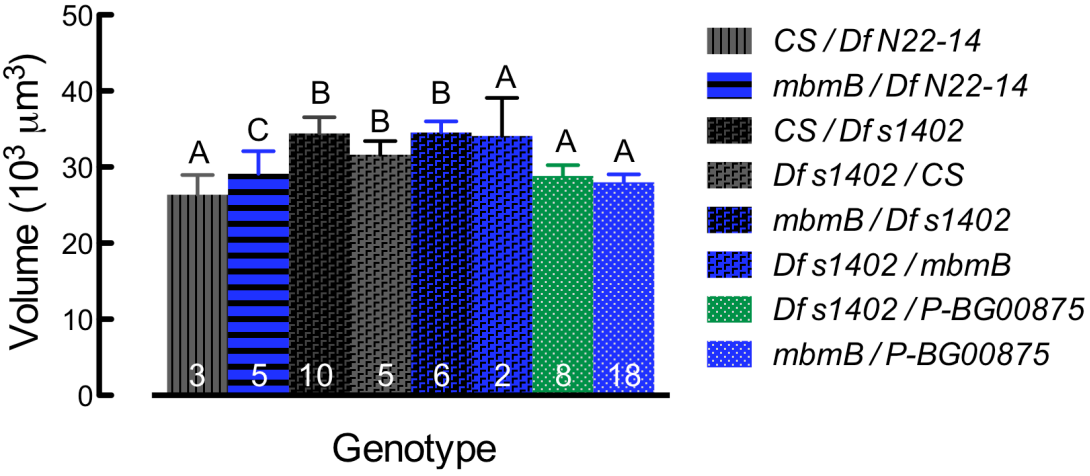
G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B
B
A
A
A
3
5
10
5
6
2
8
18
Genotype
CS / Df N22-14
mbmB / Df N22-14
CS / Df s1402
Df s1402 / CS
mbmB / Df s1402
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mbmB / P-BG00875

H

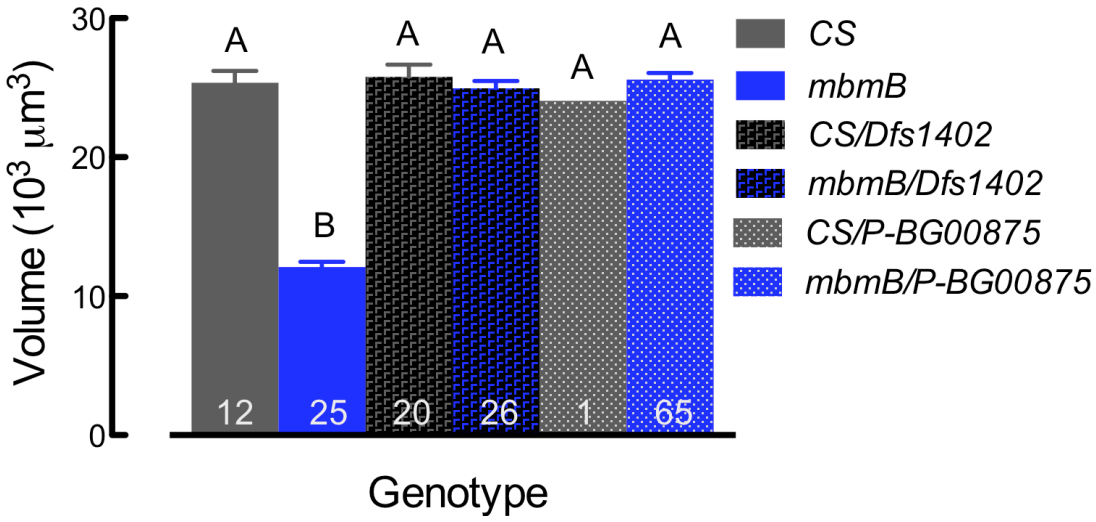


Figure A.1 Mapping of *mbmB*: Early data and experimental approaches from 2001-2003. (A) I began my mapping efforts of *mbmB* using complementation analysis and histology with all available Dfs near its previously reported recombination map site of 2-31. Serial mass histology was performed on a series 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 μm^3), yet not quite as small as *mbmB* (14 x 10^3 μm^3) ($F_{[5,78]}$=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 μm^3) ($F_{[5,58]}$=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 *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 *Pen*, a likely distance away for a jump, thereby disrupting *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 *Pen*. Finally, I can imagine two different scenarios where the P-BG00875 line would falsely compliment *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. *n* indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$).

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^{tm} 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

Stock#	Genotype	Sequence location
4101	Pka-C1[H2]	2L:9,696,959..9,696,959
5282	Pka-C1[DN] : antimorph/dominant negative	
12826	BG02804: w^{1118}; P{GT1}Trx-2^{BG02804}	2L:9,613,333..9,613,333
12533	BG01038: w^{1118}; P{GT1}BG01038	2L:9,634,152..9,634,152
12515	BG00875: w^{1118}; P{GT1}BG00875	2L:9,665,081..9,665,081
12752	BG02142: w^{1118}; P{GT1}Pka-C1^{BG02142}	2L:9,699,218..9,699,218

B

Primer	Sequence	T_m (°C)	Size (Bp)	Location in *PKA-C1*
1F pka-c1	5'-AGGGGGAGGAGGACCTA -3'	55	379	6,453
1R pka-c1	5'-CGTTCAGCGTGTGCTCCA-3'	55		6,832
2F pka-c1	5'-AAGGTGGTCAAGCTGAAGCA-3'	53	368	6,790
2R pka-c1	5'-CCGCACAGTGTCCAGGT-3'	53		7,158
3F pka-c1	5'-AGGTGACGGACTTCGGTTTT-3'	53	308	7,101
3R pka-c1	5'-GATTGCCGTAGCGCTTGGT-3'	53		7,409
4F pka-c1	5'-ACTGCGCAACCTGCTGCA-3'	53	447	7,363
4R pka-c1	5'-AGCAGGAGCAGTTGCAGATA-3'	53		7,810

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

"Original Recipe" Ingredients	**Amount**
Water	770 ml
Yeast (S.c. II)	80 g
Yeast Extract	20 g
Agar	10 g
Peptone (soy based)	20 g
Sucrose	40 g
Glucose	50 g
$MgSO_4$ x $7H_2O$	0.5 g
$CaCl_2$ x $2H_2O$	0.5 g
Propionic Acid	6 ml
Tegosept (10% p-hydroxy-benzoic acid, CH3 ester in 95% ethanol)	10 ml
TOTAL	**1 L**

B

Bloomington's Food Ingredients	**Amount**
Water	917 ml
Yeast	15.9 g
Soy Flour	9.18g
Yellow Corn meal	67.1 g
Agar	5.29 g
Light Corn Syrup	71 mL
Propionic Acid	4.42 mL
TOTAL	**1 L**

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.

Stock#	Cytology	Sequence location
7507	30C1;30C9	2L:3046635;3310250
7508	30C9;30E1	2L:3302636--3302646;3354856--3354858
7512	30B10;30C1	2L:14300969;14470247
7515	30C1;30C1	2L:15264714;15439965

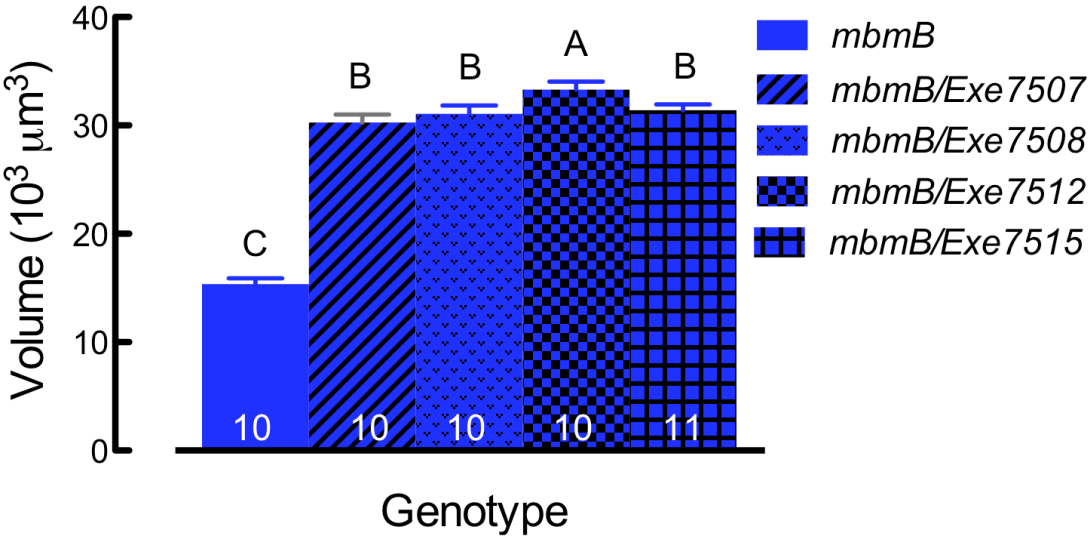


Figure A.2 Histology data for Exelixis lines in 30B10-30E1. I performed paraffin mass histology on *w;mbmB/Exe* flies and found a significant difference between these flies and homozygous *mbmB* flies (($F_{[4,41]}$=136.16, *P*<0.0001)). Bars represent mean ± SE of mean calyx volume for each genotype. *n* indicated on each bar. Different letters designate significant differences (SNK, *P*≤0.05).

Table A.4 Sequence location of additional *imp-α2* alleles. After mapping *mbmB* to *importin-α2*, I obtained additional P-elements in the gene. Their positions relative to *importin-α2* (located between 2L:10,056,906 - 10,060,097 in the [+] orientation) are listed. Each line was crossed to virgin *w; mbmB/Sm5* and *mbmB/Imp-α2 P*'s were checked for female sterility and MB calyx volume.

Stock#	Cytology	Sequence location	Position in *imp-α2*
D07004	31A1	2L:10056941..10056941	35 Bp in 5'UTR (1st exon)
20036	31A2	2L:10057031..10057031	125 Bp in 5' UTR (1st exon)
15654	31A2	2L:10057508..10057508	602 Bp into 1st intron, 114 Bp upstream of second exon

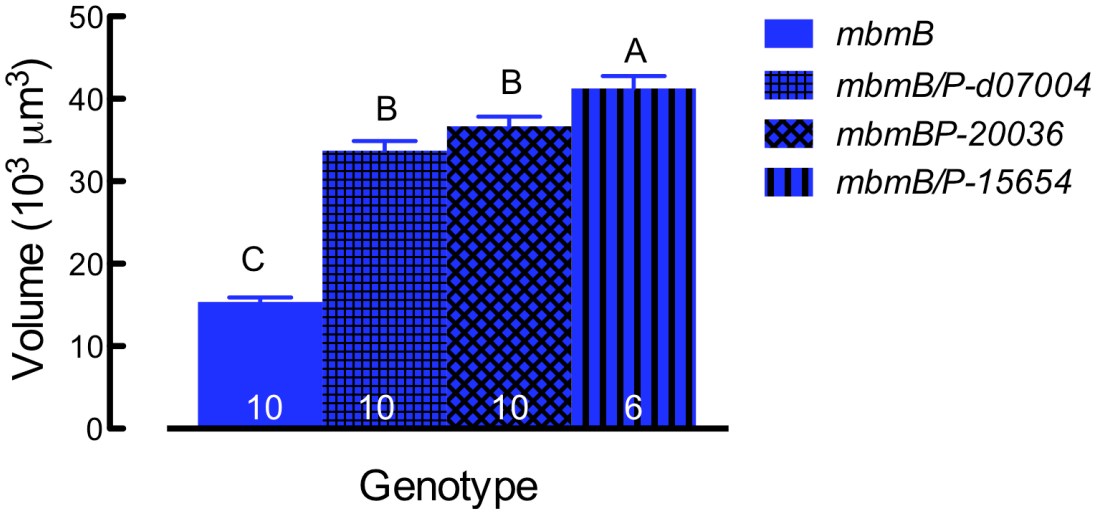


Figure A.3 Histology on additional *imp-α2* alleles. All females heterozygous for each of the three P-element lines and *mbmB* were fertile (data not shown), and had significantly larger MB calyx volumes (119-168%) than homozygous *mbmB* females ($F_{[3,28]}$=96.90, *P*<0.0001). Bars represent mean ± SE of mean calyx volume for each genotype. *n* indicated on each bar. Different letters designate significant differences (SNK, *P*≤0.05).

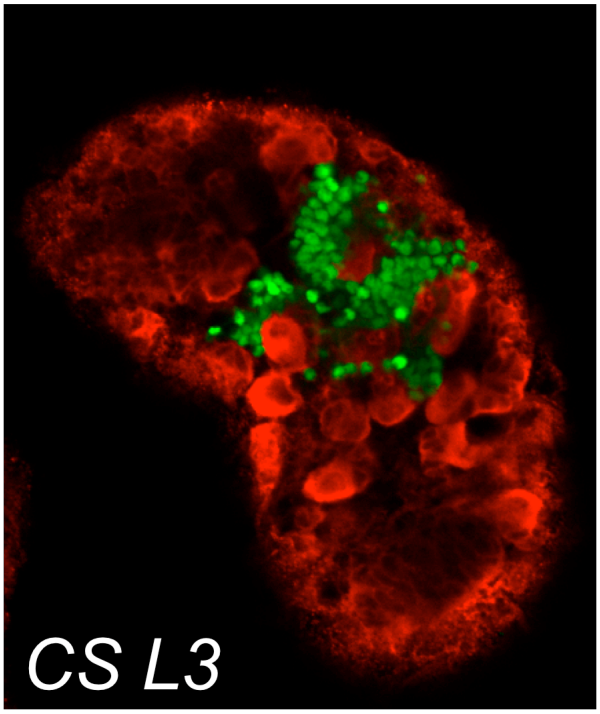
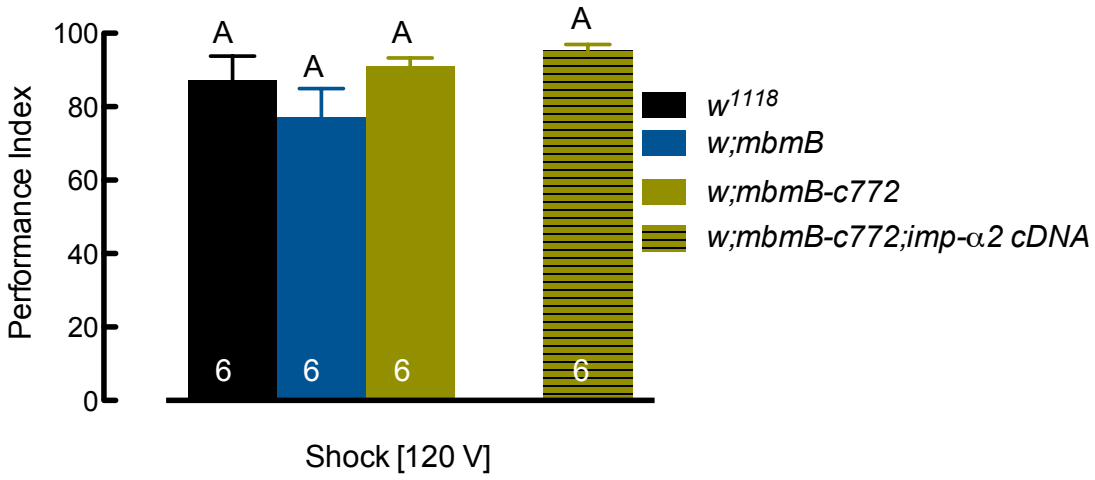


Figure A.4 Imp-α2 expression pattern in the larval brain. I analyzed the expression pattern of Imp-α2 in *CS* L3 expresing cytoplasmic GFP in the MBs with the Ok107 driver. There was no overlap in MB cells that had already undergone mitotic division (green), while Imp-α2 expressed in dividing neuroblasts 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.

A


Performance Index
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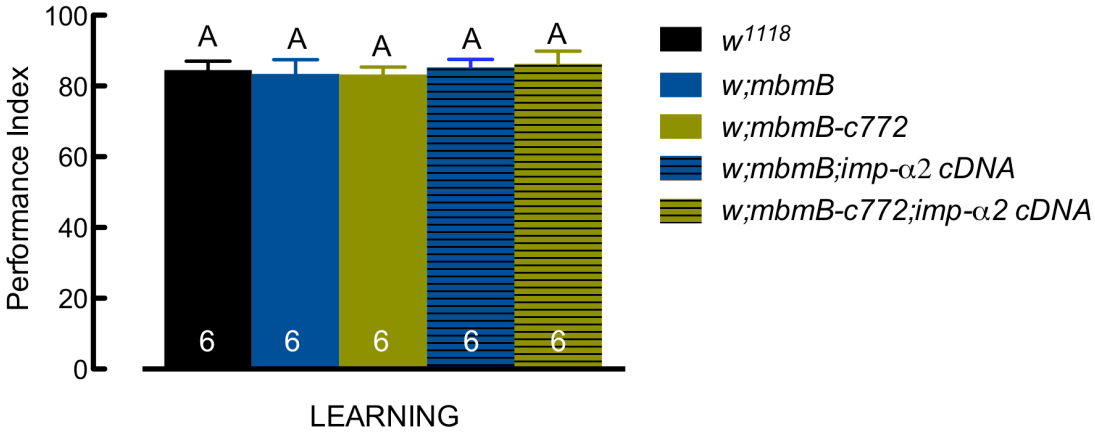
80
60
40
20
0
A
A
A
A
6
6
6
6
Shock [120 V]
w1118
w;mbmB
w;mbmB-c772
w;mbmB-c772;imp-α2 cDNA

B


Performance Index
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60
40
20
0
A
A
A
A
A
6
6
6
6
6
LEARNING
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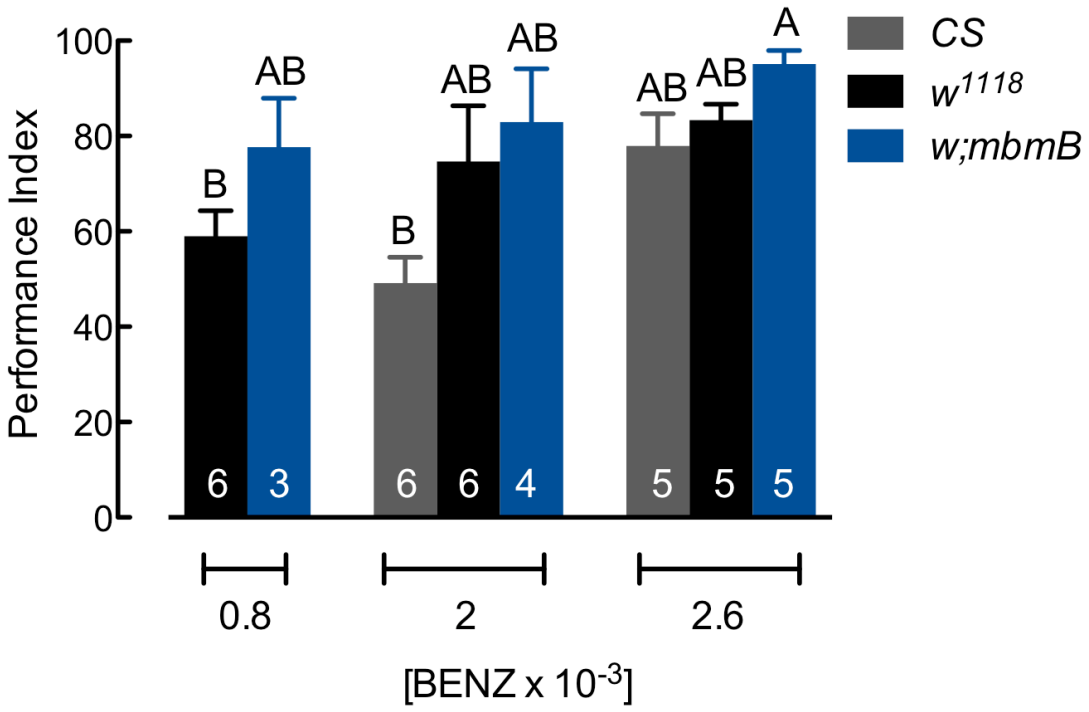
w;mbmB
w;mbmB-c772
w;mbmB;imp-α2 cDNA
w;mbmB-c772;imp-α2 cDNA

C


Performance Index
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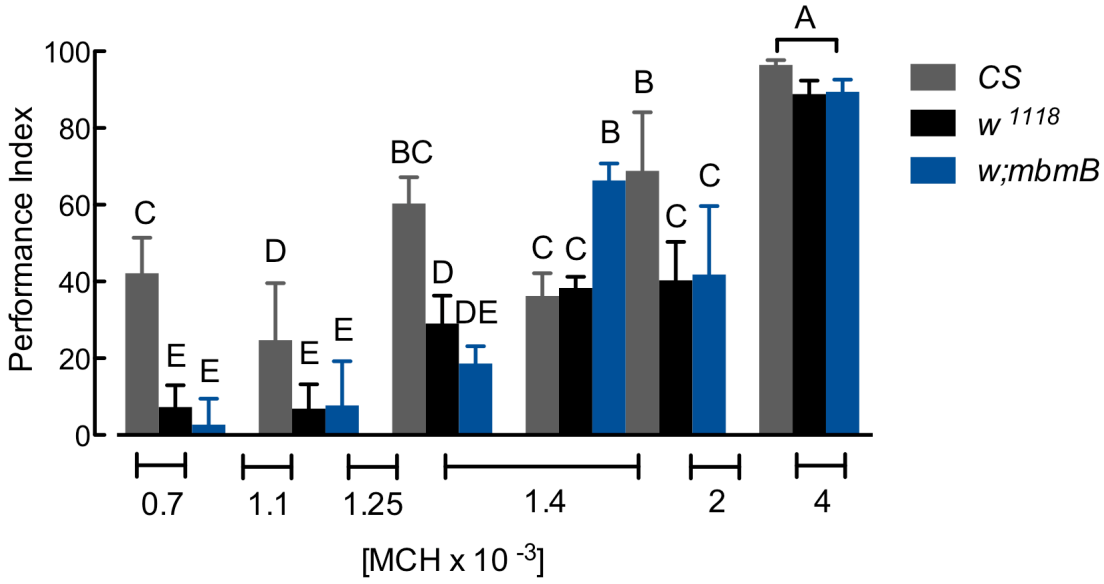
40
20
0
B
AB
B
AB
AB
AB
AB
A
6
3
6
6
4
5
5
5
0.8
2
2.6
[BENZ x 10-3]
CS
w1118
w;mbmB

D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20
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C
E
E
D
E
E
BC
D
DE
C
C
B
B
C
C
A
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1.1
1.25
1.4
2
4
[MCH x 10 -3]
CS
w 1118
w;mbmB

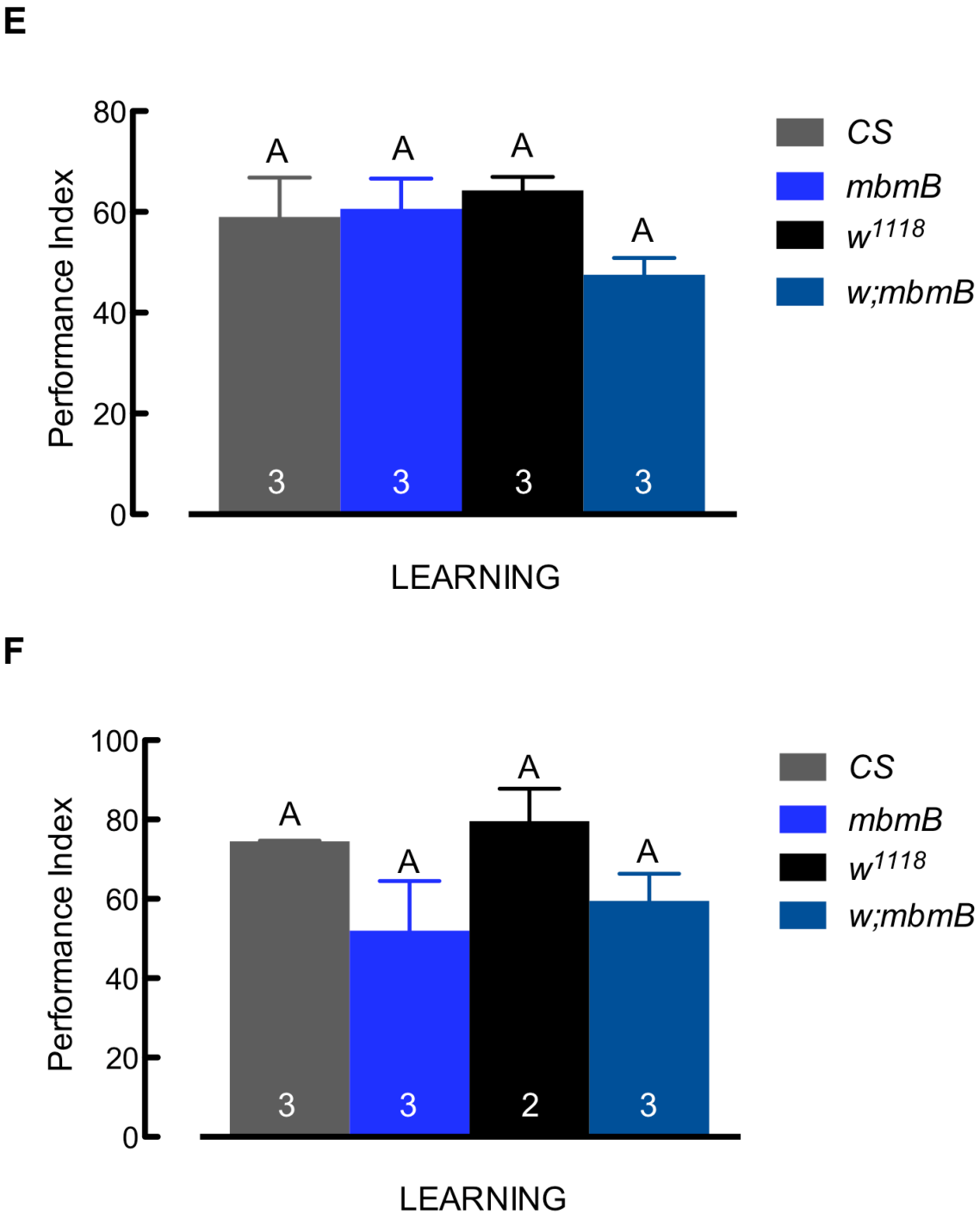
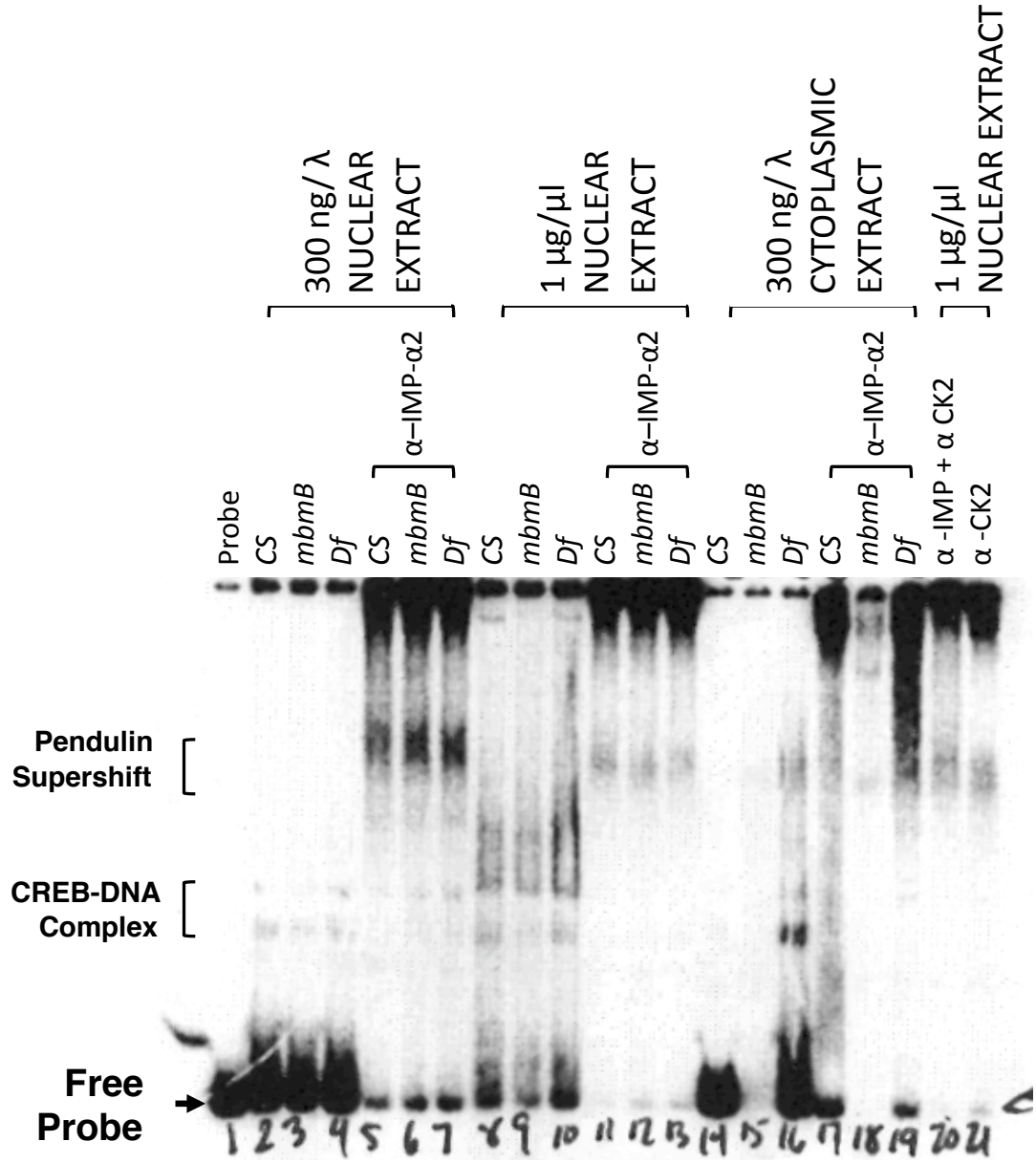


Figure A.5 Preliminary rescue experiments for *mbmB* and odor balancing. A modified Pavlovian conditioning T-maze paradigm was used to assay olfactory associative learning and sensory controls (Tully & Quinn, 1985; de Belle & Heisenberg, 1994; de Belle & Heisenberg, 1996; Tully et al., 1994). Briefly, 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 ul 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; mbmB* 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).

A


Probe
CS
mbmB
Df
CS
mbmB
Df
α–IMP-α2
300 ng/ λ NUCLEAR EXTRACT
CS
mbmB
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CS
mbmB
Df
α–IMP-α2
1 µg/µl NUCLEAR EXTRACT
CS
mbmB
Df
CS
mbmB
Df
α–IMP-α2
300 ng/ λ CYTOPLASMIC EXTRACT
α -IMP + α CK2
α -CK2
1 µg/µl NUCLEAR EXTRACT
Pendulin Supershift
CREB-DNA Complex
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

B

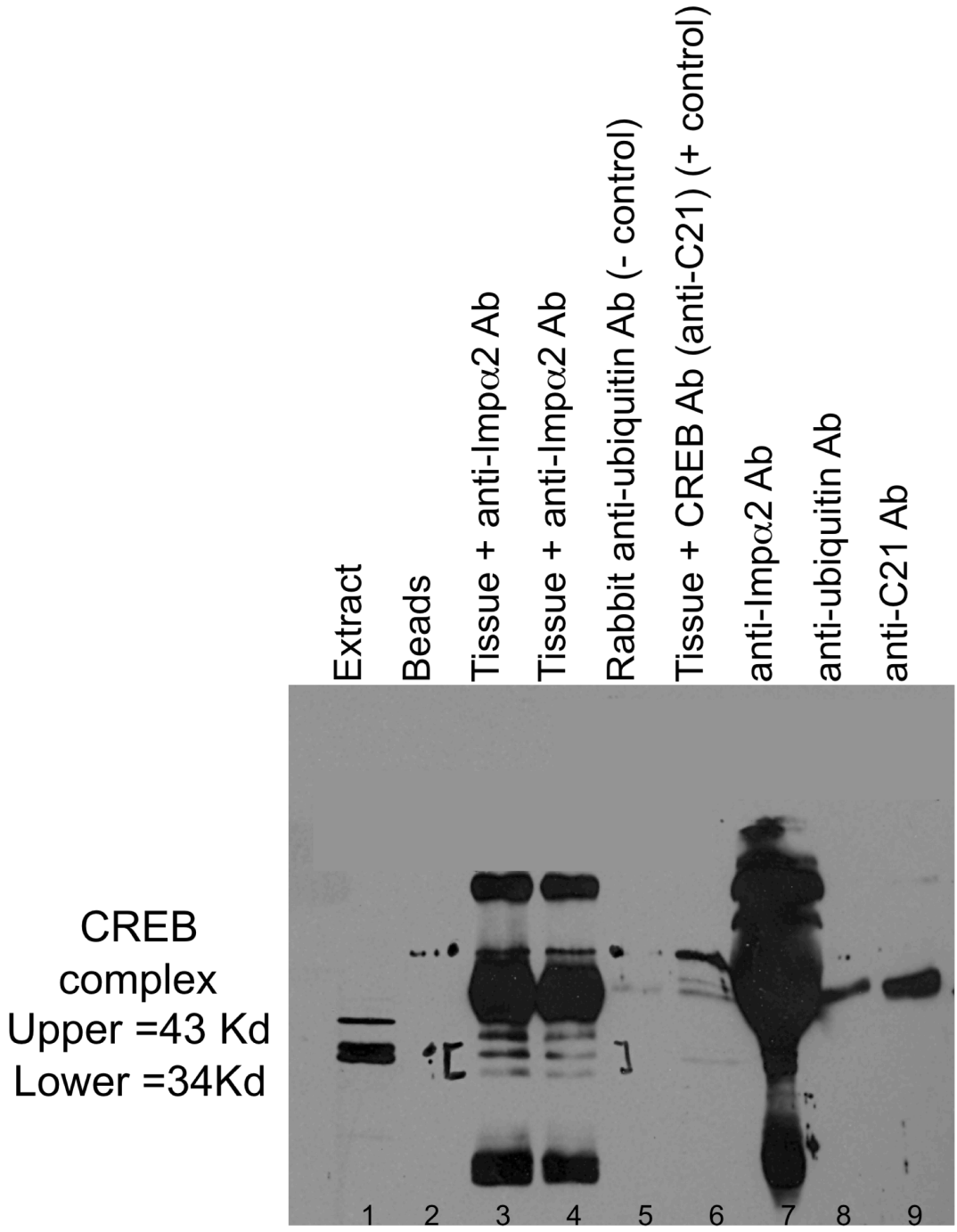

Figure A.6 Analysis of possible CREB-IMP-α2 interaction. A. It has been well established that IMP-α2 binds cargos containing an NLS tag, many of which are transcription factors whose presence in the nucleus is critical for their

function (Hogarth et al., 2005). It has also been shown that CREB has an NLS in it's 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 $MgCl_2$, 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-^{32}P]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(dI-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 (data not shown). 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 mono-partite 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 (Waeber & 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.

	UAS *imp-α2* RNAi 1	**UAS *imp-α2* RNAi 2**
P[Gal4]nanos	Fertile	Fertile

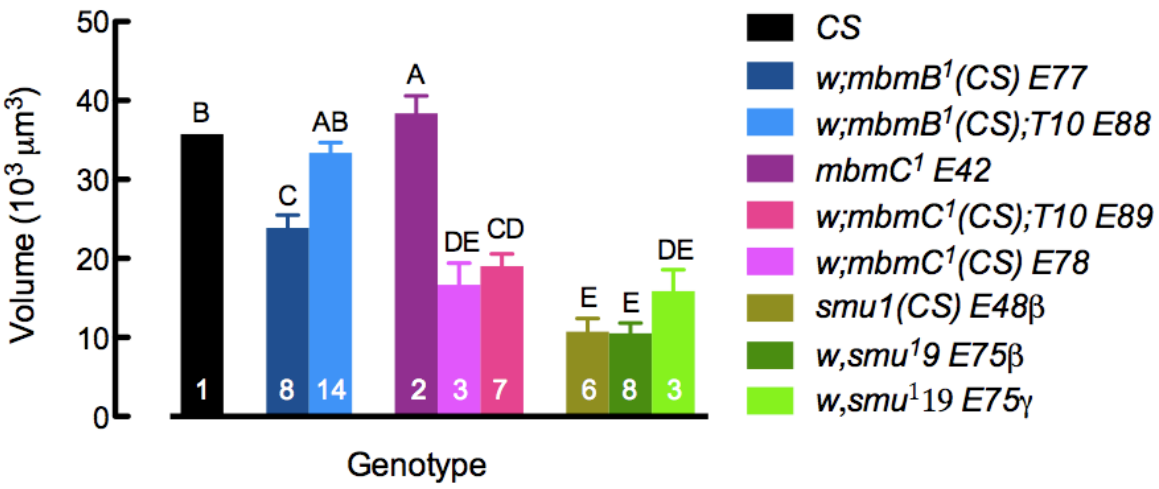

Figure A.7 Histological analysis for multiple alleles of several MB structural mutants. I analyzed the MB calyx volume of a few alleles of multiple MB structural mutants (*mbmB*, *mbmC*, and *smu*). Significant differences were observed in all of the MB structural alleles ($F_{[8,42]}$=26.21, P<0.0001).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

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APPENDIX B

CROSSING SCHEMES

Table B.1 Crossing schemes.

1. <u>Anatomy</u>

G_1: ♀ *CS* × ♂ *CS*

↓

G_2: ♀ & ♂ *CS*

G_1: ♀ *mbmB/SM5* × ♂ *CS*

↓

G_2: ♀ & ♂ *mbmB/CS*

G_1: ♀ *mbmB/SM5* × ♂ *mbmB/SM5*

↓

G_2: ♀ & ♂ *mbmB/mbmB*

<u>2. Sterility back crossing</u>

G_1: ♀ *mbmB/SM5* × ♂ *CS*

↙

G_2: ♀ *mbmB/CS* × ♂ *mbmB/mbmB*

↙

G_3: Single ♀'s *mbmB/CS* × ♂ *CS*
Single ♀'s *mbmB/mbmB* × ♂ *CS*

<u>3. Mapping</u>

G_1: ♀ *CS* × ♂ *w; Df* or *P/Balancer*

↓

G_2: ♀ & ♂ *CS/Df* or *P*

G_1: ♀ *w; mbmB/SM5* × ♂ *w; Df* or *P/Balancer*

↓

G_2: ♀ & ♂ *w; mbmB/Df* or *P*

G_1: ♀ w^{1118}*; CS; CS* × ♂ *w; imp-*$\alpha 2^{o}$*/CyO; Tm6/CS*

↓

G_2: ♀ & ♂ *w^{1118}; imp-α2^o/CS; CS*

G_1: ♀ *w; mbmB/SM5* × ♂ *w; mbmB/imp-α2^o*

↓

G_2: ♀ & ♂ *w^{1118}; imp-α2^o/CS*

4. Rescue Histology

G_1: ♀ *w^{1118}* × ♂ *w^{1118}*

↓

G_2: ♀ & ♂ *w^{1118}*

G_1: ♀ *w; mbmB/SM5* × ♂ *w; mbmB/SM5*

↓

G_2: ♀ & ♂ *w; mbmB/mbmB*

G_1: ♀ *w; mbmB/SM5; CS* × ♂ *w; mbmB/SM5; imp-α2 cDNA/MKRS*

↓

G_2: ♀ & ♂ *w; mbmB; imp-α2 cDNA/CS*

G_1: ♀ *w; mbmB/SM5* × ♂ *w; mbmB-c772/SM5*

↓

G_2: ♀ & ♂ *w; mbmB/mbmB-c772*

G_1: ♀ *w; mbmB/SM5; imp-α2 cDNA/MKRS* × ♂ *w; mbmB-c772/SM5*

↓

G_2: ♀ & ♂ *w; mbmB/mbmB-c772; imp-α2 cDNA/CS*

G_1: ♀ *w; mbmB/SM5; 247* × ♂ *w; mbmB/SM5; imp-α2 cDNA/MKRS*

↓

G_2: ♀ & ♂ *w; mbmB/mbmB; 247/ imp-α2 cDNA*

G_1: ♀ *w; mbmB/SM5; CS* × ♂ *w; mbmB/SM5; 247*

↓

G_2: ♀ & ♂ *w; mbmB; 247/CS*

G_1: ♀ *w; mbmB/SM5; CS; CS* × ♂ *w; mbmB/SM5; CS; Ok107/CS*

↓

G_2: ♀ & ♂ *w; mbmB; CS; Ok107/CS*

G_1: ♀ *w; mbmB/SM5; CS; Ok107/CS* × ♂ *w; mbmB/SM5; imp-α2 cDNA/MKRS; CS*

↓

G_2: ♀ & ♂ *w; mbmB; imp-α2 cDNA/+; Ok107/CS*

5. Rescue Cell count

G_1: ♀ *w; GFPnls; CS; CS* × ♂ *w; CS; CS; Ok107/CS*

↓

G_2: ♀ & ♂ *w; GFPnls; CS; Ok107/CS*

G_1: ♀ *w; mbmB::GFPnls/SM5; CS; CS* × ♂ *w; mbmB/SM5; CS; Ok107/CS*

↓

G_2: ♀ & ♂ *w; mbmB::GFPnls/mbmB; CS; Ok107/CS*

G_1: ♀ *w; GFPnls; CS* × ♂ *w; CS; 247/CS*

↓

G_2: ♀ & ♂ *w; GFPnls; 247/CS*

G_1: ♀ *w; mbmB::GFPnls/SM5; CS* × ♂ *w; mbmB/SM5; 247/CS*

↓

G_2: ♀ & ♂ *w; mbmB::GFPnls/mbmB; 247/CS*

6. *imp-α2* RNAi Lines

G_1: ♀ w^{1118}; *CS; CS* × ♂ *w; CS; imp-α2 RNAi-5* or *6*

↘

$G_{2\text{-}8}$: ♀ w^{1118}; *CS; CS* × ♂ w^{1118}; *CS; imp-α2 RNAi-5* or *6/CS*

↙

G_9: ♀ w^{1118}; *CS; imp-α2 RNAi-5* or *6/CS* × ♂ *w; CS/CS; Tm3/Tm6b*

↓

G_{10}: ♀ & ♂ w^{1118}; *CS; imp-α2 RNAi-5* or *6/Tm3*

G_1: ♀ w^{1118}; *CS; Impα2-RNAi-5* or *6/Tm3* × ♂ w^{1118}; *P*[*nanos::Gal4*^{VP16}];

CS

↓

G_2: ♀ & ♂ w^{1118}; P*[nanos::Gal4^{VP16}]/+; Impα2-RNAi-5* or *6/CS*

<u>7. *imp-α2* Domains</u>

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

G_1: ♀ *w; mbmB/SM5; imp-α2-cDNA/MKRS* × ♂ *yw; Sp/CyO; imp-α2$^{DOM\ 1\text{-}8}$/ Tm6*

↘

G_2: ♀ *w; mbmB-c772/SM5; CS* × *w; mbmB/y+Cy0; imp-α2$^{DOM\ 1\text{-}8}$/MKRS*

↓

G_3: ♀ & ♂ *w; mbmB-c772/mbmB; imp-α2$^{DOM\ 1\text{-}8}$/CS*

G_1: ♀ *w; mbmB/SM5; imp-α2 cDNA/MKRS* × ♂ *yw; Sp/y+CyO; imp-α2$^{DOM\ 1\text{-}8}$/Tm6*

↘

G_2: ♀ *w; mbmB/SM5; CS* × ♂ *w; mbmB/y+Cy0; imp-α2$^{DOM\ 1\text{-}8}$/MKRS*

↓

G_3: ♀ & ♂ *w; mbmB; imp-α2$^{DOM\ 1\text{-}8}$/CS*

X chromosome (*imp-α2^{DIM}* ; n=1)

G_1: ♀ *w; mbmBc-772/SM5* × ♂ *imp-α2^{DIM}; Sp/y+Cy0; Sb/TM6*

↘

G_2: ♀ *w; mbmB/SM5; CS* × ♂ *imp-α2^{DIM}/CS; mbmB-c772(CS)/Sp; Sb/CS*

↓

G_3: ♀ & ♂ *imp-α2^{DIM}/CS; mbmB-c772/mbmB; CS*

G_1: ♀ *w; mbmB/SM5* × ♂ *imp-α2^{DIM}; Sp/y+Cy0; Sb/TM6*

↙

G_2: ♀ *imp-α2^{DIM}/w; mbmB/Sp; Sb/CS* × ♂ *w; mbmB; CS*

↓

G_3: ♀ & ♂ *imp-α2^{DIM}/w; mbmB; CS*

G_1: ♀ w^{1118} × ♂ w^{1118}

↓

G_2: ♀ & ♂ w^{1118}

G_1: ♀ *w; mbmB/SM5* × ♂ *w; mbmB/SM5*

↓

G_2: ♀ & ♂ *w; mbmB*

G_1: ♀ *w; mbmB/SM5* × ♂ *w; mbmB-c772/SM5*

↓

G_2: ♀ & ♂ *w; mbmB-c772/mbmB*

APPENDIX C

GAL4

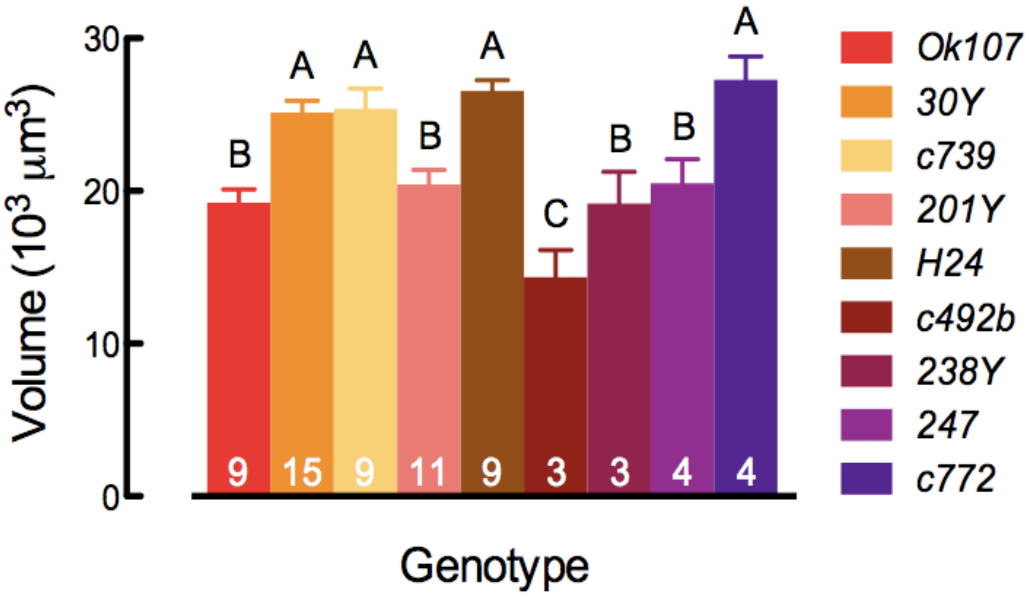

Figure C.1 Preliminary MB GAL4 calyx volumes. Serial sections of paraffin-embedded brains were used for planimetric MB measurements. This was a pilot study to determine if a thorough analysis of MB specific Gal4 lines would yield any interesting phenotypes. All genotypes are homozygous for the Gal4 insertions. 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≤0.05).

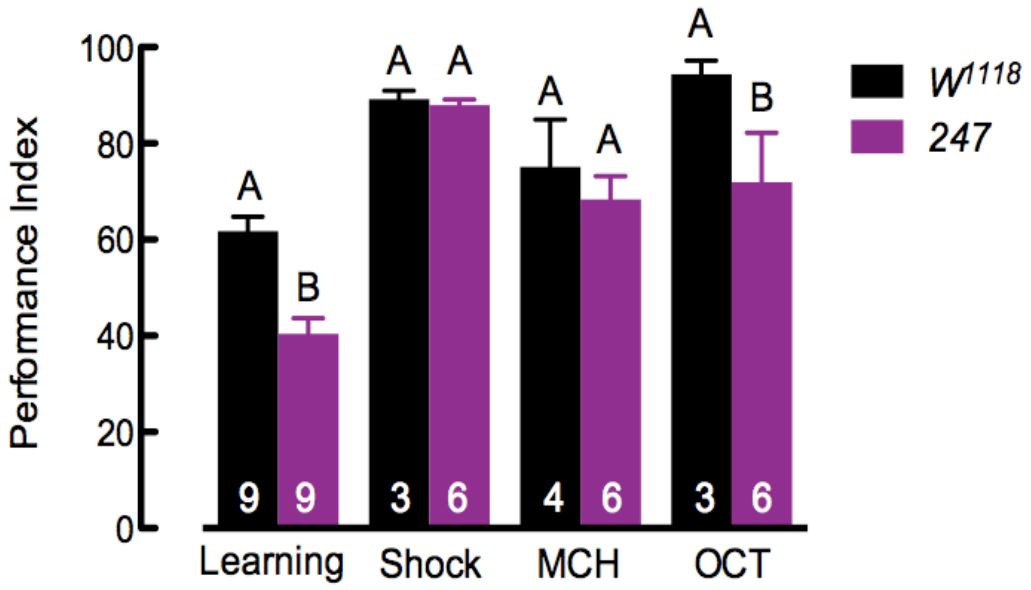

Figure C.2 *247* learning data. A modified Pavlovian conditioning T-maze paradigm was used to assay olfactory associative learning and sensory controls (Tully & Quinn, 1985; de Belle & Heisenberg, 1994; de Belle and Heisenberg, 1996; Tully et al., 1994). Described in detain in Appendix B Figure 4. There was a significant difference between *247* and *CS* for Octanol (OCT) avoidance at a concentration of 2 x 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 x 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 benzaldehyde. 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).

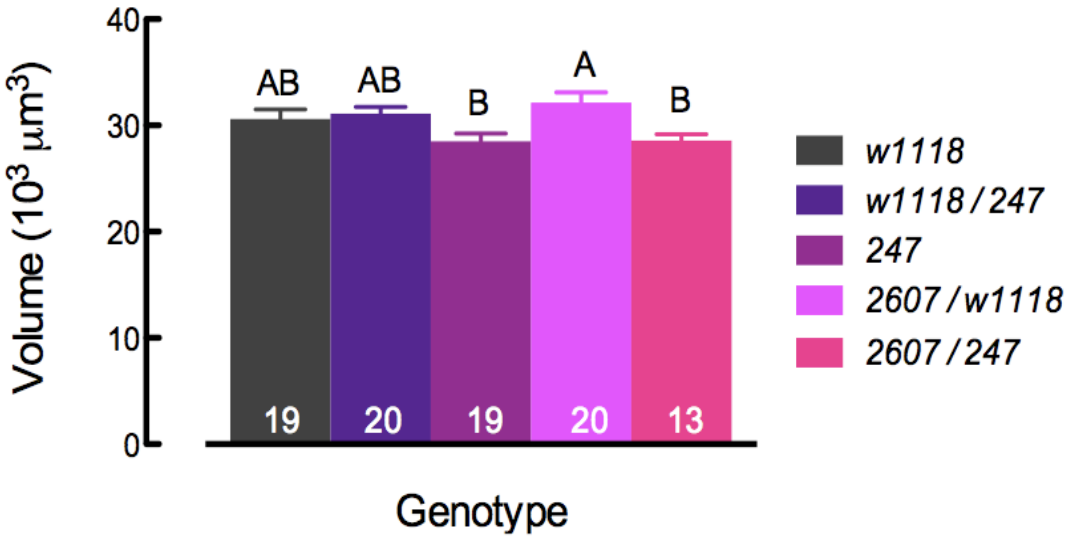

Figure C.3 *E75* null histological analysis. Serial sections of paraffin-embedded brains were used for planimetric MB measurements. I was interested in whether a null allele of *E75* would increase the reduced MB phenotypes I had previously seen with the *247* Gal4 insert (Chapter 2 Figure 2, Table 3). 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 that 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).

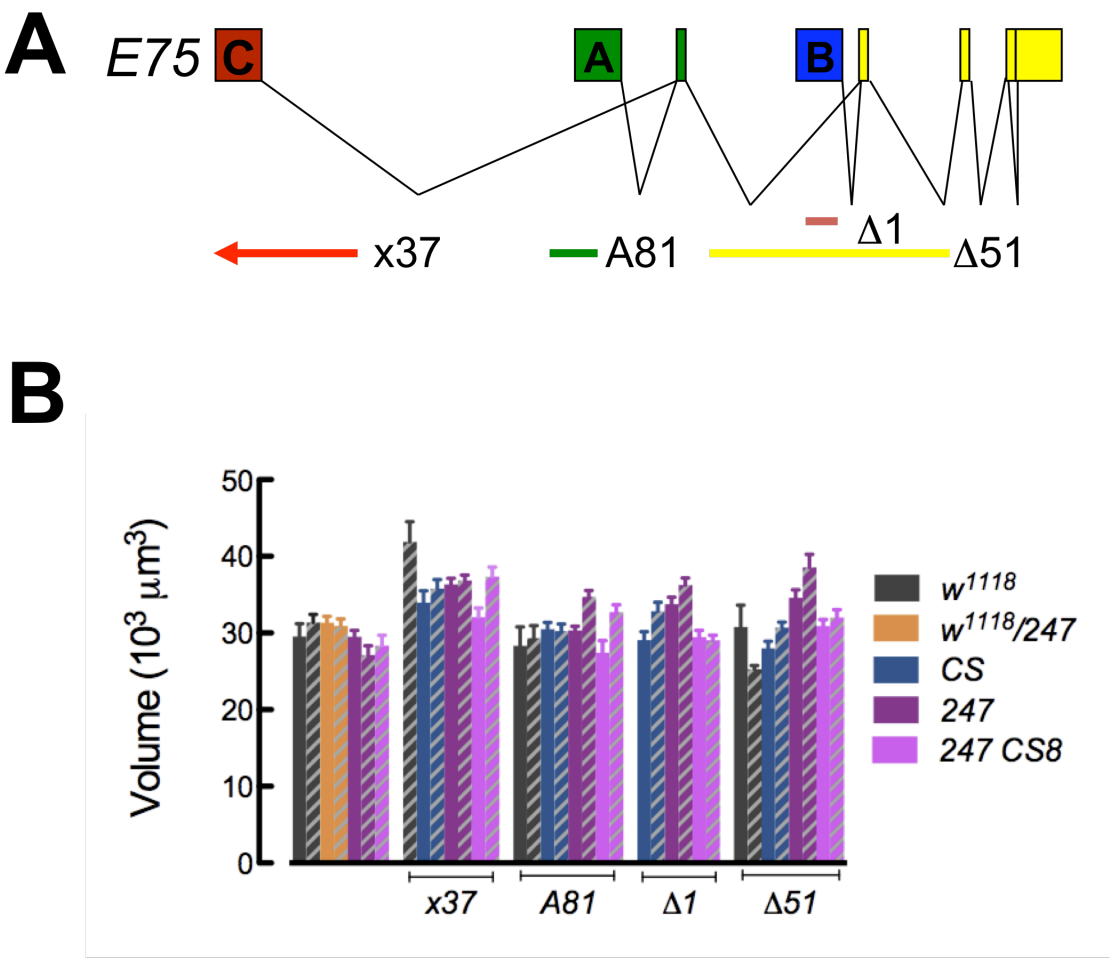

Figure C.4 *E75* splice variant histological analysis. There are three isoforms of *E75* (Segraves and Hogness, 1990) represented by the following isoform specific null mutations: *E75A* (*A81*), *E75B* (*Δ51*) and *E75C* (*x37*) as well as a protein null (*Δ1*) (Bialecki et al., 2002). I was interested 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 $4 \leq n \leq 20$.

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 α = 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: ★ < 0.05, ★★ < 0.01, ★★★ < 0.005, ★★★★ < 0.001). The most obvious trends from this very complex dataset are within *x37* and *Δ1*. *X37/w*^{1118} males appear to be larger than all other genotypes tested. *Δ1/247* males also appear larger that 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*

	w^{1118} F	w^{1118} M	w^{1118}/ 247 F	w^{1118}/ 247 M	247 F	247 M	247 CS8 F	247 CS8 M	x37/ w^{1118} F	x37/ w^{1118} M	x37/CS F	x37/CS M	x37/ 247 F	x37/ 247 M	x37/24 7 CS8 F
w^{1118} M	NS														
w^{1118}/24 7 F	NS	NS													
w^{1118}/24 7 M	NS	NS	NS												
247 F	NS	NS	NS	NS											
247 M	NS	NS	NS	NS	NS										
247 CS8 F	NA	NA	NA	NA	NA	NA									
247 CS8 M	NS	NS	NS	NS	NS	NS	NA								
x37/ w^{1118} F	NA	NA	NA	NA	NA	NA	NA	NA							
x37/ w^{1118} M	*** *	*** *	*** *	*** *	*** *	*** *	NA	*** *	NA						
x37/CS F	NS	NS	NS	NS	NS	NS	NA	NS	NA	***					
x37/CS M	NS	NS	NS	NS	NS	NS	NA	NS	NA	NS	NS				
x37/24 7 F	*	NS	NS	NS	**	*** *	NA	*** *	NA	NS	NS	NS			
x37/24 7 M	**	NS	NS	NS	*** *	*** *	NA	*** *	NA	NS	NS	NS	NS		
x37/24 7 CS8 F	NS	NS	NS	NS	NS	NS	NA	NS	NA	*** *	NS	NS	NS	NS	
x37/24 7 CS8 M	NS	NS	NS	NS	*	***	NA	**	NA	NS	NS	NS	NS	NS	NS

Table C.1.b *A81*

	w^{1118} F	w^{1118} M	w^{1118}/ 247 F	w^{1118}/ 247 M	247 F	247 M	247 CS8 F	247 CS8 M	A81/ w^{1118} F	A81/ w^{1118} M	A81/CS F	A81/CS M	A81/ 247 F	A81/ 247 M	A81/247 CS8 F
w^{1118} M	NS														
w^{1118}/247 F	NS	NS													
w^{1118}/247 M	NS	NS	NS												
247 F	NS	NS	NS	NS											
247 M	NS	NS	NS	NS	NS										
247 CS8 F	NA	NA	NA	NA	NA	NA									
247 CS8 M	NS	NS	NS	NS	NS	NS	NA								
A81/ w^{1118} F	NS	NS	NS	NS	NS	NS	NA	NS							
A81/ w^{1118} M	NS	NS	NS	NS	NS	NS	NA	NS	NS						
A81/CS F	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS					
A81/CS M	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS				
A81/247 F	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS	NS			
A81/247 M	NS	NS	NS	NS	NS	*	NA	NS	NS	NS	NS	NS	NS		
A81/247 CS8 F	NS	NS	*	NS	NS	NS	NA	NS	NS	NS	NS	NS	NS	*	
A81/247 CS8 M	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS	NS	NS	NS	NS

Table C.1.c *Δ1*

	w^{1118} F	w^{1118} M	w^{1118}/ 247 F	w^{1118}/ 247 M	247 F	247 M	247 CS8 F	247 CS8 M	Δ1/ w^{1118} F	Δ1/ w^{1118} M	Δ1/CS F	Δ1/CS M	Δ1/ 247 F	Δ1/ 247 M	Δ1/247 CS8 F
w^{1118} M	NS														
w^{1118}/247 F	NS	NS													
w^{1118}/247 M	NS	NS	NS												
247 F	NS	NS	NS	NS											
247 M	NS	NS	NS	NS	NS										
247 CS8 F	NA	NA	NA	NA	NA	NA									
247 CS8 M	NS	NS	NS	NS	NS	NS	NA								
Δ1/ w^{1118} F	NA	NA	NA	NA	NA	NA	NA	NA							
Δ1/ w^{1118} M	NA	NA	NA	NA	NA	NA	NA	NA	NA						
Δ1/CS F	NS	NS	NS	NS	NS	NS	NA	NS	NA	NA					
Δ1/CS M	NS	NS	NS	NS	NS	NS	NA	NS	NA	NA	NS				
Δ1/247 F	NS	NS	NS	NS	NS	NS	NA	NS	NA	NA	NS	NS			
Δ1/247 M	*	NS	NS	NS	**	*** *	NA	*** *	NA	NA	*	NS	NS		
Δ1/247 CS8 F	NS	NS	NS	NS	NS	NS	NA	NS	NA	NA	NS	NS	NS	**	
Δ1/247 CS8 M	NS	NS	NS	NS	NS	NS	NA	NS	NA	NA	NS	NS	NS	***	NS

Table C.1.d *Δ51*

	w^{1118} F	w^{1118} M	w^{1118}/ 247 F	w^{1118}/ 247 M	247 F	247 M	247 CS8 F	247 CS8 M	Δ51/ w^{1118} F	Δ51/ w^{1118} M	Δ51/CS F	Δ51/CS M	Δ51/ 247 F	Δ51/ 247 M	Δ51/247 CS8 F
w^{1118} M	NS														
w^{1118}/247 F	NS	NS													
w^{1118}/247 M	NS	NS	NS												
247 F	NS	NS	NS	NS											
247 M	NS	NS	NS	NS	NS										
247 CS8 F	NA	NA	NA	NA	NA	NA									
247 CS8 M	NS	NS	NS	NS	NS	NS	NA								
Δ51/ w^{1118} F	NS	NS	NS	NS	NS	NS	NA	NS							
Δ51/ w^{1118} M	NS	NS	NS	NS	NS	NS	NA	NS	NS						
Δ51/CS F	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS					
Δ51/CS M	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS				
Δ51/247 F	NS	NS	NS	NS	NS	*	NA	NS	NS	*	NS	NS			
Δ51/247 M	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS	NS	NS		
Δ51/247 CS8 F	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS	NS	NS	NS	
Δ51/247 CS8 M	NS	NS	NS	NS	NS	NS	NA	NS	NS	NS	NS	NS	NS	NS	NS

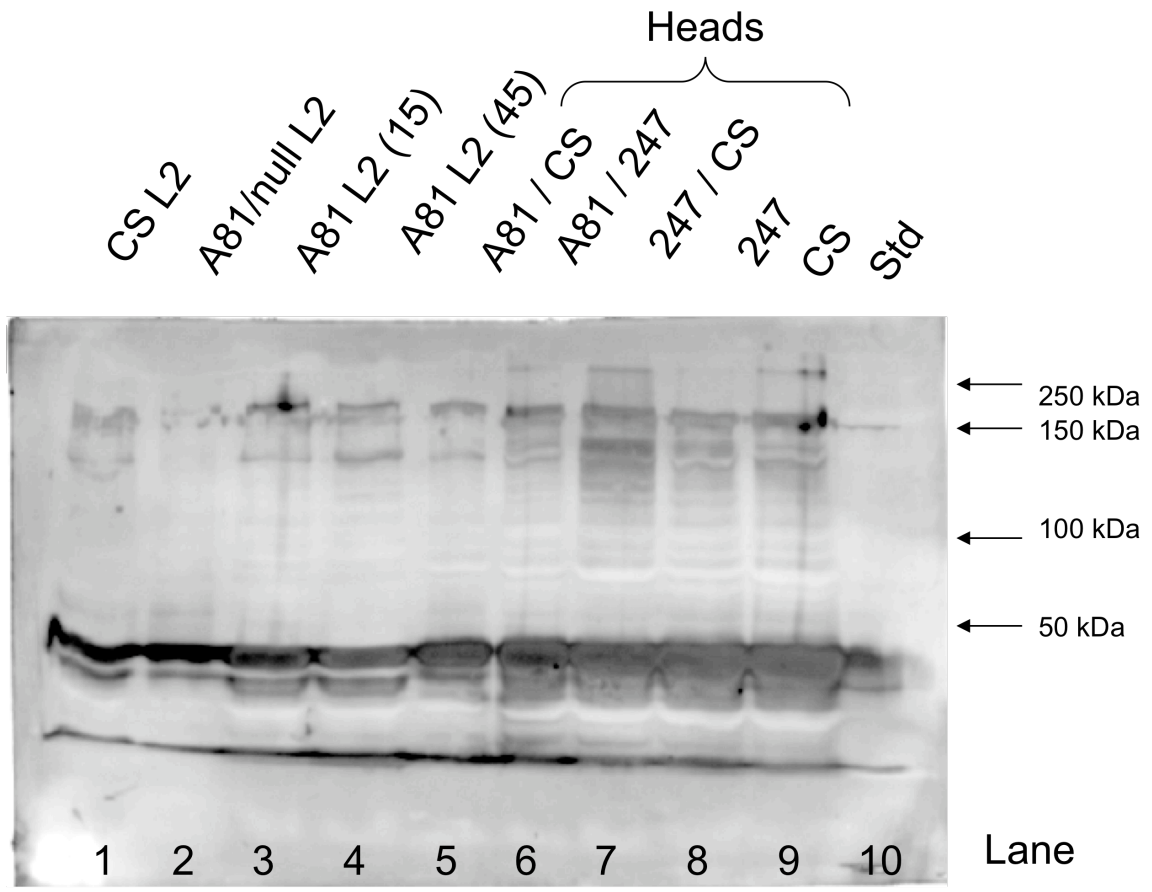

Figure C.5 E75 western blot. An SDS-Page 7.5% gel was run with samples of either second instar larvae with their concentrations listed (lanes 1-4) or heads of 1-day-old flies (lanes 5-9) (15 heads/sample). The gel was blotted, trimmed and incubated with the following primary Abs: mouse anti-E75A 1B12 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 *E75* data, I felt that *247* was an ineffective disruption of *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*=*CK1*γ

RT Primers	Sequence	T_m (°C)	Size (Bp)	Location
Fz-T E1	5' - ATGCGAGTCCGTATTATCGC - 3'	54	490	Exon 1
Fz-B E1	5' - ACGGGTAGTGACCTTAGC - 3'	54		
nemy-T E7	5' - ATGCGGAGGATGTGCTATTC - 3'	54	210	Exon 7
nemy-B E7	5' - AAGGTCTCTCGATTGAGATGC - 3'	54		
gish-T E10	5' - ATATTGACTGAGCCAACCG - 3'	53	264	Exon 10
gish-B E10	5' - ATTCGTTGAAGGCGGTAAAG - 3'	53		
gish-T E12/13	5' - ATGCCAAAGGAGGTGTTG - 3'	53	194	Exon 12, 13
gish-B E12/13	5' - ATCGACCACTTCGACTTCC - 3'	53		
a-Tub T E2	5' - ATGTTGGTCAGGCTGGTG - 3'	55	477	Exon 2
a-Tub B E2	5' - AGCTTGGACTTCTTGCCG - 3'	55		Exon 2

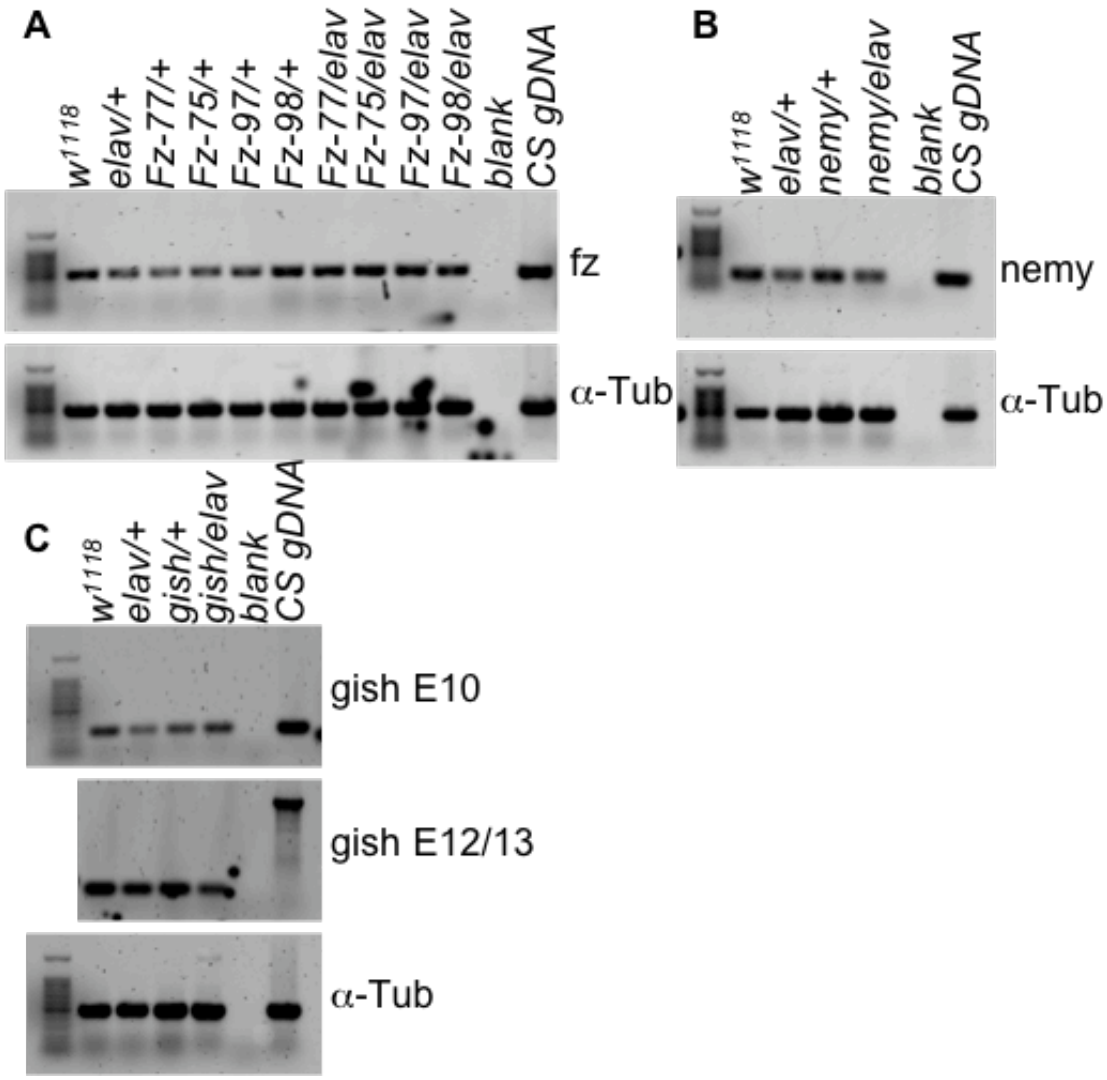

Figure C.6 *Fz*, *nemy* and *gish* RNAi RT-PCR. Tissue was collected from the whole bodies of 10-20 females, flash frozen in liquid nitrogen, and then RNA was extracted using RNAspin mini (GE Healthcare). Reverse transcription was performed with normalized amounts of RNA as template using qscript^{TM} 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 my 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: *gish*=*CK1*γ

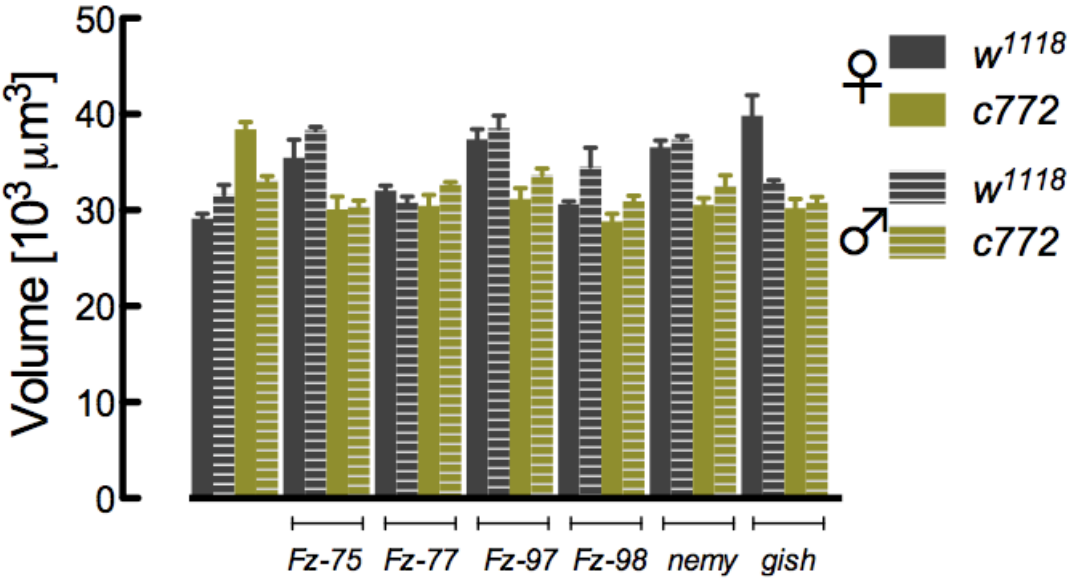

Figure C.7 *Fz*, *nemy* and *gish* RNAi histological analysis. Although I have data indicating that these RNAi lines are not ideal disruptions of *fz*, *nemy* or *gish*, I concurrently tested their role in MB anatomy by driving them in the MBs with the *c772* Gal4 driver. Serial sections of paraffin-embedded 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<0.0001) 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 α = 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: ★ < 0.05, ★★ < 0.01, ★★★ < 0.005, ★★★★ < 0.001). *c772* females were larger than both male and female w^{1118}. Please note: *gish=CK1γ*

		w^{1118} F	w^{1118} M	*c772* F	*c772* M	*fz-75* F	*fz-75* M	*c772;fz-75* F
	w^{1118} M	NS						
	c772 F	★★★★	★★★★					
fz-75	*c772* M	NS	NS	NS				
	fz-75 F	★	NS	NS	NS			
	fz-75 M	★★★★	★★★★	NS	NS	NS		
	c772;fz-75 F	NS	NS	★★★★	NS	NS	★★★★	
	c772;fz-75 M	NS	NS	★★★★	NS	NS	★★★★	NS

		w^{1118} F	w^{1118} M	*c772* F	*c772* M	*fz-77* F	*fz-77* M	*c772;fz-77* F
	w^{1118} M	NS						
	c772 F	★★★★	★★★★					
fz-77	*c772* M	NS	NS	NS				
	fz-77 F	NS	NS	★★	NS			
	fz-77 M	NS	NS	★★★★	NS	NS		
	c772;fz-77 F	NS	NS	★★★★	NS	NS	NS	
	c772;fz-77 M	NS	NS	NS	NS	NS	NS	NS

		w^{1118} F	w^{1118} M	*c772* F	*c772* M	*fz-97* F	*fz-97* M	*c772;fz-97* F
	w^{1118} M	NS						
	c772 F	★★★★	★★★★					
fz-97	*c772* M	NS	NS	NS				
	fz-97 F	★★★★	★	NS	NS			
	fz-97 M	★★★★	★★★★	NS	NS	NS		
	c772;fz-97 F	NS	NS	★★★ or ★★★★?	NS	★	★★	
	c772;fz-97 M	NS	NS	NS	NS	NS	NS	NS

		w^{1118} F	w^{1118} M	*c772* F	*c772* M	*fz-98* F	*fz-98* M	*c772;fz-98* F
	w^{1118} M	NS						
	c772 F	★★★★	★★★★					
fz-98	*c772* M	NS	NS	NS				
	fz-98 F	NS	NS	★★★★	NS			
	fz-98 M	NS	NS	NS	NS	NS		
	c772;fz-98 F	NS	NS	★★★★	NS	NS	★	
	c772;fz-98 M	NS	NS	★★★★	NS	NS	NS	NS

		w^{1118} F	w^{1118} M	*c772* F	*c772* M	*nemy* F	*nemy* M	*c772;nemy* F
	w^{1118} M	NS						
	c772 F	★★★★	★★★★					
nemy	*c772* M	NS	NS	NS				
	nemy F	★	NS	NS	NS			
	nemy M	★★★★	NS	NS	NS	NS		
	c772;nemy F	NS	NS	★★★★	NS	NS	NS	
	c772;nemy M	NS	NS	★	NS	NS	NS	NS

		w^{1118} F	w^{1118} M	*c772* F	*c772* M	*gish* F	*gish* M	*c772;gish* F
	w^{1118} M	NS						
	c772 F	****	****					
gish	*c772* M	NS	NS	NS				
	gish F	****	****	NS	***			
	gish M	NS	NS	NS	NS	***		
	c772;gish F	NS	NS	****	NS	****	NS	
	c772;Fz-75 M	NS	NS	****	NS	****	NS	NS

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APPENDIX D

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