1-1-2008

The effects of mushroom body lobe disruption on learning and memory

Brian S Dunkelberger
University of Nevada, Las Vegas

THE EFFECTS OF MUSHROOM BODY LOBE DISRUPTION ON LEARNING AND MEMORY

By

Brian S. Dunkelberger

Bachelor of Science
The Pennsylvania State University
1994

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

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

Graduate College
University of Nevada, Las Vegas
December 2008

UNLV
UNIVERSITY OF NEVADA LAS VEGAS

Dissertation Approval

The Graduate College
University of Nevada, Las Vegas

November 5, 20 08

The Dissertation prepared by

Brian S. Dunkelberger

Entitled

The Effects of Mushroom Body Lobe Disruption on Learning and Memory

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

Doctor of Philosophy in Biological Sciences

Examination Committee Chair

Dean of the Graduate College

Examination Committee Member

Examination Committee Member

Examination Committee Member

Graduate College Faculty Representative

ABSTRACT

The Effects of Mushroom Body Lobe Disruption on Learning and Memory

by

Brian S. Dunkelberger

Dr. J. Steven de Belle, Examination Committee Chair
Associate Professor of Life Sciences
University of Nevada, Las Vegas

Animal models have been used for centuries to study learning and memory in simple systems with many applications to humans (Chapter 1). The fruit fly *Drosophila melanogaster* has added greatly to our current understanding of learning and memory and its underlying biology (Chapter 2). The research described here focuses on the relationship between learning and memory and the brain using three mutant strains of flies: *mushroom body miniature B (mbmB), small mushroom bodies (smu), and mushroom bodies reduced (mbr).* Mushroom bodies are paired neuronal structures found in most invertebrate brains involved in learning and memory consolidation. All three mutations studied were initially isolated based on a reduced dendritic volume in the mushroom body calyx.

In chapter 3, GAL4 driven membrane bound and nuclear localized GFP expression revealed that adult *mbmB* and *smu* flies had intact γ lobes with the rest severely reduced in size; while *mbr* flies had severe disruption in all lobes. A β lobe midline fusion is seen in *mbmB* flies. Adults of all three mutants have a reduction in Kenyon cell number. They all show normal bifurcation and pathfinding of MB γ neurons in wandering third instar larvae; while cell counts of *mbmB* and *smu* Kenyon cell bodies during development show cell number is consistent with wild type until approximately mid-third instar.

I have shown that both *mbmB* and *smu* have impaired learning scores consistent with other fly mutations causing mushroom body calyx volume reductions. Both have reduced long term memory (LTM) and anesthesia resistant memory (ARM) as well. LTM and ARM are generated using two distinct training protocols, massed for ARM and spaced for LTM. Some reports state that these are additive processes while others say ARM is disrupted by spaced training. My studies support the hypothesis that ARM is disrupted by spaced training.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AC	Adenylyl Cyclase
AL	Antennal Lobe
ARM	Anesthesia Resistent Memory
BP	Base Pair
cAMP	cyclic Adenosine Monophosphate
CCX	Central Complex
cGMP	cyclic Guanine Monophosphate
CNS	Central Nervous System
CR	Conditioned Response
CS	Conditioned Stimulus
DAG	Diacylglycerol
DPM	Dorsal Paired Medial cells
EMS	Ethyl Methane Sulfonate
GAL4	Yeast Transcription Factor, Binds Upsteam Activation Sequence
GFP	Green Fluorescent Protein
IP_3	Inositol Triphosphate
KC	Kenyon Cells
LFP	Local Field Potential
LTM	Long Term Memory
MB	Mushroom Body
MTM	Middle Term Memory
NCAM	Neural Cell Adhesion Molecule
NMDAR	N-Methyl-D-Aspartate Receptor
NMJ	NeuroMuscular Junction
OR	Olfactory Receptor
OSN	Olfactory Sensory Neuron
PBS	Phosphate Buffer Saline
PBT	Phosphate Buffer Saline, Tween
PCAP	Pituitary-adenylyl-Cyclase-Activating Peptide
PER	Proboscis Extension Response
PKA	Protein Kinase A
PKC	Protein Kinase C
PNS	Peripheral Nervous System
R	Response
S	Stimulus
SNS	Stomatogastric Nervous System
SOC	Second Order Conditioning
STM	Short Term Memory
UR	Unconditioned Response

US	Uncondtioned Stimulus
WT	Wild Type

ACKNOWLEDGEMENTS

Support for this project was provided by a National Science Foundation grant, awarded to J. S. de Belle, # 0237395 to JSdB, the microscope facility was supported by NIH Grant Number 2 P20 RR016463 from the INBRE Program of the National Center for Research Resources, the School of Life Sciences, GSPSA, and UNLV.

I would like to thank my advisor J. Steven de Belle for his guidance and support. Also, I would like to thank the current members of my committee: Stephen Roberts, Andrew Andres, Ralph Greenspan as well as former members Jeffrey Shen and Mark Guadagnoli. I could not have completed this work without the people in the de Belle lab past and present particularly current members Christine Serway, Xia Wang, Chris Tabone, Denise and Stephanie. The office staff and members of the faculty of the School of life sciences have also provided a great deal of help including: Dennis Bazylinski, Sharon Trotter, Pat Hunt and James Mckoy.

Finally I would like to thank my Family for the unswerving support they have shown me. My parents Oscar and Grace and my brothers Michael and Kevin provided all the support I could ask for. Thanks to Falynn, Jaedon and Rian, Peg, Stormy and Forest for all your help over the years. And last but certainly not least, thank you Megan. I truly could not have done this without you.

CHAPTER 1

A BIOLOGICAL BASIS FOR ANIMAL MODEL STUDIES OF LEARNING AND MEMORY

Plato proposed that humans are born with innate knowledge of all things and only need the correct keys to unlock the secrets of the universe. Others, including Aristotle, believed we are blank canvases waiting to be painted by the events in our lives[1]. Humans are profoundly curious, particularly about how we are put together, how we are biologically related to other animals, and how our minds and personalities compare with those of other people. Much of the scientific endeavor in the biological and social sciences focuses on these issues. Perhaps one of the oldest and still most perplexing questions is, "How do we remember?" As humans, we have the ability not only to form and retain memories of events in which we have partaken or witnessed, but also to extrapolate, assimilate, and create novel or abstract ideas from fragments of previous experiences. Although these are indeed fantastic achievements of neural evolution, they are not uniquely human qualities and have been demonstrated in several animal taxa.

In this chapter, we will discuss a classification of behavioral phenomena and give examples of how behavior is measured in animal systems. This will be followed by a historical account of animal model organisms used to investigate

the neural mechanisms of learning and memory. Finally, we discuss homology as the biological basis for the comparative approach using animal model systems.

Learning and Memory Classification

Dozens of different forms of animal learning have been described[2]. It is likely that some reflect unique biological mechanisms while others are based on universal processes found across taxa. The assays selected for discussion here establish fundamental aspects of learning, which are likely based on these common learning processes. We will focus on nonassociative and associative learning, the two main classes of behavioral plasticity commonly studied in animals.

Nonassociative Learning

Nonassociative learning is the response to a change in the salience of an object or event with interpretations ranging from benign to fearful[3]. The two most frequently discussed subsets of nonassociative learning are habituation and sensitization. Habituation is a decrease in the speed or severity of a response to a repeated stimulus, whereas sensitization is an increase. Typically, the difference in behavior is caused by the relative noxious nature of the stimulus with habituation elicited by a neutral stimulus and sensitization induced by negative stimuli. For example, habituation can be elicited in the gill withdrawal reflex of the marine mollusk *Aplysia californica* by the repeated application of a light tap to the body[4]. As this is not a harmful stimulus, a decrease in gill

withdrawal speed and duration is seen over time. However, if the animal receives a tail shock before receiving the repeated light taps, the animal exhibits sensitization as the gill withdrawal is faster and persists for longer periods than an animal that has not received a tail shock. As recently as the early 1900s, it was commonly believed that reflex behavior was invariant. One of the earliest signs of habituation of a reflex was observed in spiders[5,6]. When a tuning fork was used to vibrate their web, a spider would drop away, hanging at some distance on a single thread. After repeated exposure to this stimulus, they became habituated and discontinued this behavior. Today habituation and sensitization have been demonstrated as basic forms of learning throughout the animal kingdom, and include examples such as defensive withdrawal reflexes in Annelid worms [7,8], the gill withdrawal reflex in marine mollusks A. californica[9,10], as well as the umbilical abdominal reflex in humans Homo sapiens[11,12].

Associative Learning

Associative learning entails the pairing of two or more objects or events to provide new meaning to the previously novel stimuli[3]. For example, the color green has no inherent meaning. However, with repeated conditioning to traffic laws, green has acquired the meaning "go." The green light is associated with the task of moving forward, and therefore elicits that behavior. The two common forms of associative learning are Pavlovian or classical conditioning and instrumental or operant conditioning. We will briefly discuss some of the classic studies demonstrating these types of learning.

The pioneering work of Ivan Pavlov in the early twentieth century gave rise to

classical (Pavlovian) conditioning. Dogs very reliably salivate in response to the presentation of food. Based on this, Pavlov designed a simple experiment in which a bell [the conditioned stimulus (CS)] would ring just before a dog was presented with food [the unconditioned stimulus (US)] in an attempt to provide a meaningful prediction of the pairing of food with the bell. Normally the bell on its own does not elicit salivation [an unconditioned response (UR)]. But after a few training events, the dogs began to salivate at the sound of the bell in the absence of food [the conditioned response (CR)]. This response was seen only in conditioned dogs, as those that were not exposed to the pairing did not salivate to the sound of the bell[13]. Classical Pavlovian conditioning has been successfully adapted to induce learning events in a wide variety of animals including honeybees (*Apis mellifera)*[14,15], the common fruit fly (*Drosophila melanogaster)*[16,17], canaries (*Serinus canaries)*[18], and many other model systems.

Instrumental or operant conditioning creates a situation where an animal's voluntary behavior operates on the environment, which subsequently influences future behavioral outcomes. Animals form an association between their response (behavior) and the stimulus that follows (consequence). When Pavlov was developing his classical conditioning procedures, ground-breaking work on instrumental conditioning by Edward Thorndike and B. F. Skinner was also being conducted. Thorndike built puzzle boxes for domestic cats, with a built-in escape mechanism consisting of a looped string the cat could pull. When placed into the box, cats showed signs of discomfort and attempted escape until successfully

pulling the string either by accident or trial and error. Interestingly, as the same animals were repeatedly tested, they rapidly improved their escape time as they learned the task. The opposite is true for undesirable responses, which were weakened and occurred less frequently after repeated testing[19].

One drawback to Thorndike's puzzle box design was that upon solving the puzzle, the animal was no longer in the box, so he had to artificially control when a new experiment began. Skinner wanted to look at the rate at which an animal would perform a learned response on its own. His "Skinner box" was a small chamber with a lever inside attached to an electrical monitoring system. It provided a reinforcer when depressed by the animal, eliminating handling by the experimenter. Instrumental conditioning can involve positive or negative reinforcement that can be either given or withheld, creating many possible experimental situations. Skinner showed that animals would learn how to maximize a reward or minimize a punishment[20]. He also developed fixed-interval schedules using a timing device that allowed only small unit amounts of food reward delivery during a specified period of time. Interestingly, rats tended to "pace" themselves, with more attempts immediately before receiving reinforcement and performing fewer attempts immediately after it. Instrumental conditioning, like the others discussed so far, has been demonstrated in a wide variety of organisms including marine mollusks[21], the cockroach *Periplaneta americana*[22], various farm animals[23], and many others.

Memory Classification by Time

One defining feature of memory is the amount of time required for its loss.

This memory decay can often be divided into phases having distinct behavioral, physiological, or cellular properties revealed through experimentation. For example, mechanisms of short-term memory (STM) and long-term memory (LTM) can be separated through genetic and pharmacological methods in many model systems. The fly (*Drosophila)* has been an important source of information about learning and memory mechanisms for over 30 years, in both nonassociative and associative learning paradigms[17,24]. Dozens of characterized mutations have facilitated the genetic dissection of memory phases using an associative assay that pairs a mild electric foot shock with a novel odor. Two of the first memory mutants isolated in flies, *dunce* and *rutabaga*, were examined in an olfactory conditioning assay. Both exhibited some decrement in initial learning, but had much sharper decreases in memory retention within the first hour after training compared with normal wild-type flies[25,26]. After this time, their memory decay was relatively normal. *dunce* and *rutabaga* were thus categorized as STM mutants [27,28].

Genetic and pharmacological studies in *Drosophila* also established two distinct longer forms of memory. Early experiments demonstrated an anesthesia-resistant phase of memory (ARM) lasting up to 1 day after a single training session[27,29,30]. Massed training (10 training sessions administered one immediately after the other) produces even stronger memory retention, lasting about 3 days, and this memory is insensitive to the protein synthesis inhibitor cycloheximide. In contrast, spaced training (10 training sessions with a 15-min rest interval between each) yields a protein-synthesis-dependent memory lasting

at least 1 week[28]. As has been found in many model systems, repetition produces better memory, and spaced repetition results in the best memory of all.

Along with short and long forms of memory, intermediate memory processes bridging the gap between them have been described in flies as well as in several other species. Interestingly, *amnesiac* mutant flies show near-normal memory retention immediately after a single training session and again 7 hours later. In between these time points, memory retention in the mutants is appreciably lower than in normal flies [31].

Often human and model organism research is conducted independently with little exchange of information. However, there is much to be gained from merging ideas between the fields. Figure 1-1a shows a simplified Atkinson and Shiffrin model for human learning, which describes three distinguishable memory phases based on behavioral observations[32]. Figure 1-1b illustrates temporal features of memory phases in *Drosophila* based on genetic/transgenic dissection and pharmacological disruptions[28,33]. In the biological sciences, we hope to describe the neural mechanisms of behavioral phenomena well described in humans and other systems not amenable to invasive experimentation.

A Brief History of Animal Models

Animal models have been useful in demonstrating how neural mechanisms give rise to behavior and behavioral plasticity, as well as how the nervous system changes in response to experience and memory consolidation.

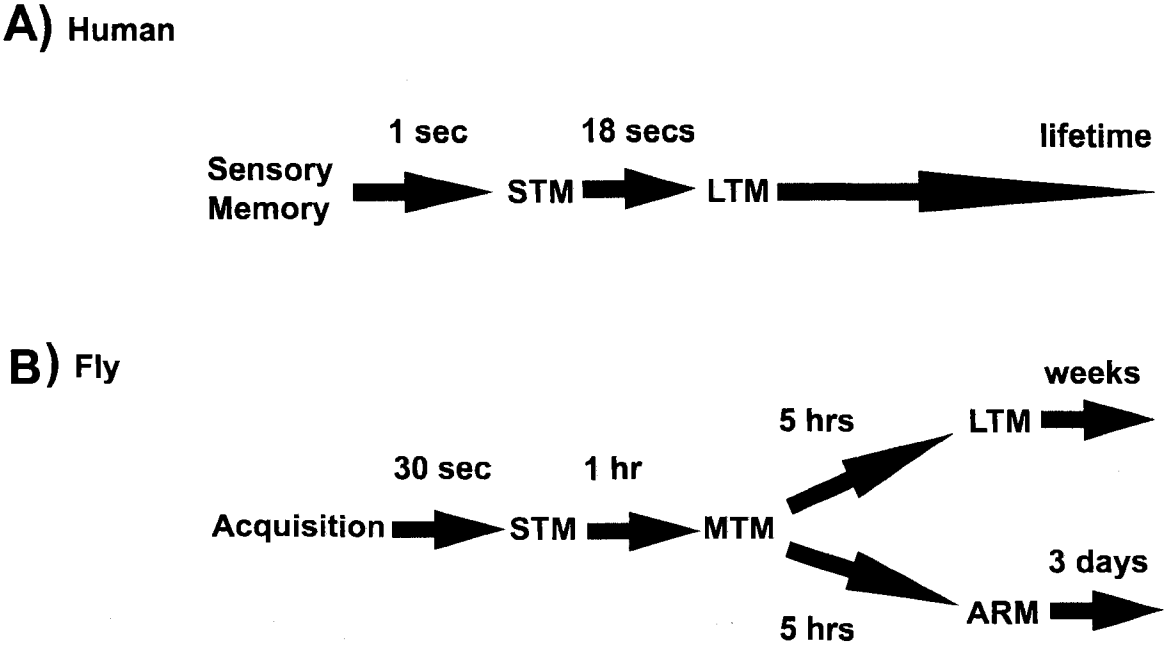

Figure 1. **Memory model comparison**. Comparison of human and fly memory models describing proposed duration of phases. (A) Simplified version of the three stage model of human memory phases as proposed by Atkinson and Shiffrin based on their behavioral observations[32]. They proposed that due to limited processing ability at higher levels most information is kept in a temporary buffer they referred to as sensory memory for approximately 1 second. Only relevant Information proceeds to short term memory (STM) and is retained for 18 seconds. Important information is stored in a more permanent fashion as long term memory (LTM) which can last a lifetime. (B) Proposed fly memory model showing genetically and pharmacologically defined memory phases and their estimated duration. Acquisition of a memory is achieved in the first 30 seconds, which is followed by short term memory (STM) processes which persist for the first hour. At this time intermediate mechanisms referred to as middle term memory (MTM) begin to further consolidate the memory. MTM is believed to continue until the 5th hour after acquisition. If a spaced learning protocol was used (10 rounds of training with 15 minute intervals between each round) this leads to long term memory (LTM) which is protein synthesis dependant and can still be detected weeks later. However, if a massed training protocol was used (10 rounds of successive training with no rest intervals) anesthesia resistant memory (ARM) is generated which has a duration of approximately 3 days[28].

The Importance of the Brain

In our early written history, it was debated which organ in the human body was the seat of memory and intelligence. The oldest written record containing the word "brain" is found in the Edwin Smith surgical papyrus written in 1700 BC. In this text, brain injuries are noticed to be associated with changes in the function of other parts of the body, especially the lower limbs[34]. Curiously, the Egyptians did not place such a great importance on the brain, as they discarded it during the mummification process while preserving other organs. Aristotle was also convinced that cognitive processes took place in the heart[35]. Alcmaeon used animal models to address this issue around 500 BC. He dissected the eye of an animal (of an unnamed species) and noted that the tract leading from the eye projected into the brain. From this observation he concluded that the brain was important for the collection of all sensory information[36]. The many philosophers and physicians who followed Alcmaeon began to attribute more behavioral and cognitive functions to brain activity[37].

Brain Functions

By the beginning of the nineteenth century, almost nothing was known about how the brain works. Marie-Jean-Pierre Flourens performed localized lesions in the brains of living rabbits and showed that the main divisions of the brain were responsible for largely different functions[38]. Since lesions and other accidental brain damage proved to be such useful tools to map out functionally relevant regions in human brains[39], people began to look for storage sites of learning and memory within the brain based on the same principles. Karl Lashley

systematically made various-sized lesions in diverse regions of the cerebral cortex of rats and examined their behavior in a series of mazes varying in difficulty[40]. Ultimately, he showed that the locus of the lesion was less important than the size of the lesion, particularly for the difficult mazes. Lashley's work helped to shape our current view of memory storage. It is currently believed that different aspects of memory including color and shape are stored in different locations, potentially accounting for the difficulty he encountered in finding traces of memory. Animal models continue to be important for studies of brain function in behavioral plasticity. They are especially useful in revealing the neural underpinnings of diseases that affect learning and memory.

Neurons

In the late nineteenth century, Golgi and Cajal developed staining methods that for the first time permitted the visualization of detailed fine structure of the brain (in birds). Cajal argued that the brain was made up of many small but interconnected cells[35]. These elements were given the name "neuron" in 1891 by Wilhelm Von Waldeyer[35] but it was not until many years later that people understood anything about how neurons actually functioned. In 1952, Hodgkin and Huxley published a computational model describing the flow of electrical current through neurons[41]. They recorded ionic currents in the giant axon fiber of the Atlantic squid, *Loligo pealei*. The sheer size of this neuron enabled them to conduct these experiments, which would have been impossible in most other organisms.

Homology and the Comparative Approach

Species homology has been the theoretical basis for why researchers have and continue to ask biologically interesting questions in model organisms. Structural and behavioral similarities among animals can result through common descent or through convergent evolution[42].

Genome Homology

Not surprisingly, the genomes of animals with common features and shared ancestry are homologous to some extent. Looking at this in another way, if sequence homology in related species contributes to the development of similar brains, these brains may also drive similar behavior and similar aspects of behavioral plasticity. Despite relatively long divergence times, both genome size and genes themselves can be highly conserved even among distantly related species[43,44]. Most mammals, for example, have similar genome sizes of ~3 billion base pairs (bp)[45]. Although humans have an estimated 25,000 genes and fruit flies have approximately 13,600, it is estimated that 60% of these are conserved between them[44]. Interestingly, many genes already known to be involved in human neurological diseases have fly homologues, and mutations in these genes appear to cause similar symptoms in both species[43].

Brain Homology

Upon initial observation, the brains of invertebrates (e.g., insects) and vertebrates appear vastly different. However, there is considerable evidence that these brains evolved from a common ancestor[46], from which both groups have retained many common features. All craniate brains develop from three primary

rostral–caudal segments (Fig. 1-2a) known as the forebrain, midbrain, and hindbrain[42]. Interestingly, higher invertebrate brains also develop in three primary rostal–caudal segments: protocerebrum, deutocerebrum, and tritocerebrum[42] (see Fig. 1-2b).

Further evidence of homology in the brain can be found by examining the genes known to direct aspects of nervous system development. The homeotic (Hox) genes produce proteins involved in establishing cellular identity in early *Drosophila* embryogenesis and are well conserved in all bilaterally symmetrical animals. The presence or absence of certain Hox proteins in very specific patterns drives the development of particular structures including the central nervous system (CNS) precursor cells. Mutational inactivation of two specific *Drosophila Hox* genes as well as their vertebrate homologs prevents cells from adopting their expected neuronal cell fate, indicating that these genes have similar neuronal functions in both fruit flies and mice[47].

The *Drosophila* gene *orthodenticle (otd)* is a "gap" gene that regulates the formation of two main regions of the brain: the protocerebrum and the deutocerebrum (see Fig. 1-2b). When mutated, the loss of *Otd* results in the loss of the rostral brain. Its mammalian ortholog, known as *Otx1*, produces a similar effect in mammals as mutations cause the loss of fore- and midbrain regions[48]. Remarkably, full restoration of the missing brain regions results when normal sequences of these genes are exchanged between mutants of both species[49,50]. This multispecies transgenic physiological rescue of brain defects is strong evidence for similarity in the development of the CNS in the animal kingdom.

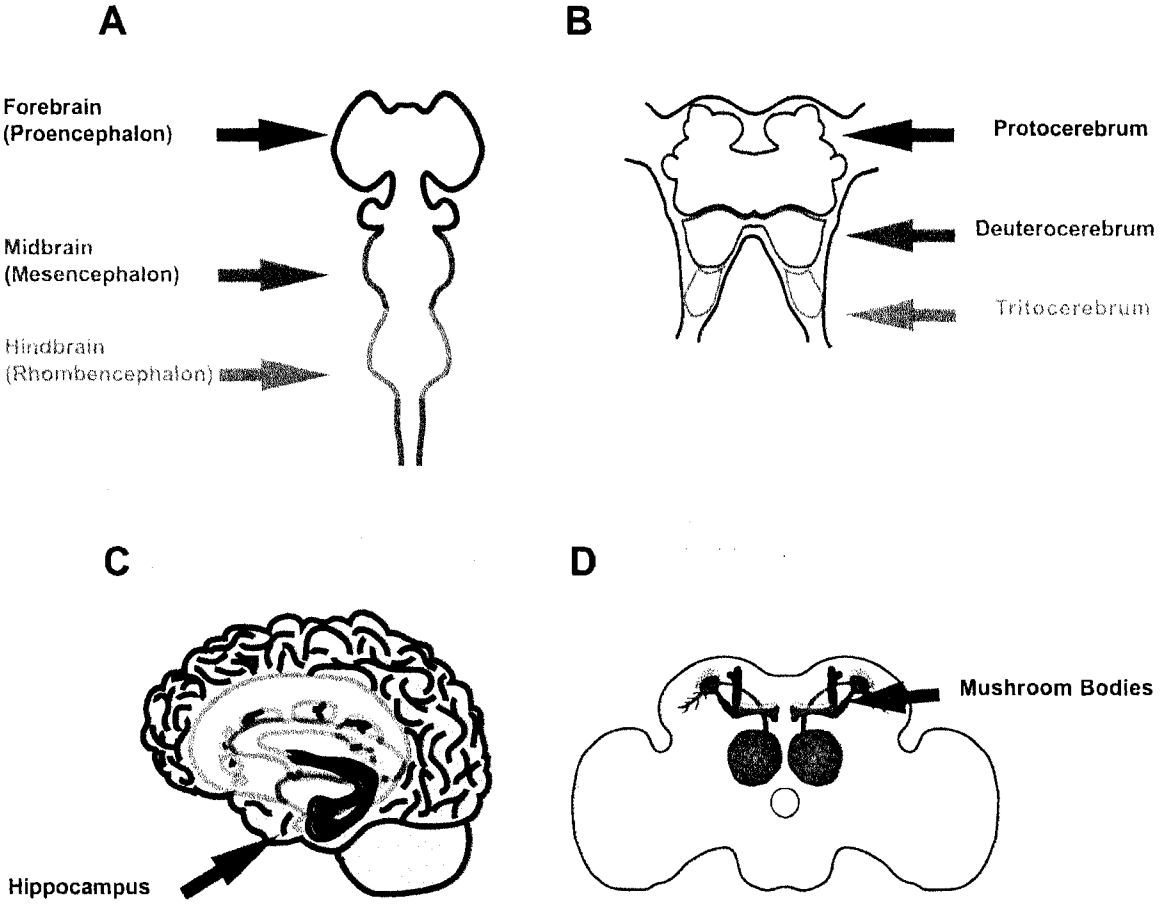

Figure 2. **Mammalian and insect brain comparison**. Comparison of mammalian and insect early brain development and functionally equivalent memory centers. (A) Early development in the mammalian brain establishes three major divisions called the forebrain, midbrain, and hindbrain. (B) Development of the insect brain also creates three divisions known as the protocerebrum, deuterocerebrum, and tritocerebrum. (C) This diagram of a human brain shows the relative location of the mammalian memory center known as the hippocampus located in the forebrain. (D) The mushroom bodies are invertebrate memory centers located in the protocerebrum shown here in a fly brain.

Functional Homology

The greatest differences between vertebrate brains of various species lie in their environmental adaptations. For example, Radinsky grouped multiple species of otters by how often they used their forepaws to manipulate food items and then compared this behavior with the somatosensory area in the cortex (forebrain) where these limbs were represented. The species with the greatest use of their forepaws use had the largest area devoted to forepaw control[51,52].

Many brain structures found in vertebrates and invertebrates have similar connectivity and organization. For example, the sensory systems of humans map spatial information from the external world onto the brain in an orderly way, generating topographic maps. In the visual system, cells in the retina that receive input from adjacent positions in the visual field have synaptic connections at neighboring positions in the brain[53]. Topographic organization of neural circuits is also commonly found in other vertebrates such as the audio and visual systems in the barn owl[54] and the mechanosensory and olfactory systems in mice[55,56]. This type of organization has also been demonstrated in higher-order invertebrates such as the honeybee and fruit fly mechanosensory and olfactory systems[57,58].

Functional homology between vertebrate and invertebrate brains is supported by a comparison of structures known to mediate aspects of behavioral plasticity. The vertebrate hippocampus (see Fig. 1-2c) constitutes part of the limbic system in the forebrain[59] . The functional equivalent of a hippocampus is the arthropod mushroom body located in the protocerebrum (see Fig. 1-2d)[60]. Although not

obviously similar physically, they are critical for both establishing memories[59,61,62] showing elevated

expression of similar learning-related molecules[63,64].

Neuron Homology

Neurons perform essentially the same tasks and utilize similar mechanisms across species. Sensory neurons relay information to interneurons or perhaps to motor neurons directly through either electrical gap junctions or chemical synapses using neurotransmitters. Human embryonic stem cells implanted into the brains of embryonic mice and chicks[65,66] differentiate into neurons and integrate into the host forebrain. This argues that cells are functionally similar and interchangeable among species, lending further support to the comparative approach using animal model systems.

Behavioral Homology

Homology across species is also seen on a behavioral level. Certainly most animals perform the same basic behavioral repertoire as humans: they all feed, sleep, move, and reproduce. Therefore, it should not be surprising that we share at least some neural mechanisms that drive these common behaviors. However, we are often astonished when encountering examples of complex behavior thought to be exclusive to humans, such as Chimpanzees learning sign language[67] and honeybees that dance to communicate[68]. Even more complex behavioral interactions have been described in nature. For example, the white-fronted bee-eaters (*Merops bullockoides)* are a type of monogamous bird species that upon losing its brood, frequently abandon further breeding attempts

and begins to help a closely related pair rear their brood[69,70]. Knowing that animals share some higher-order cognitive ability with humans makes them ideal candidates for research into the nervous systems giving rise to these behaviors.

Conclusion

There is extensive support for the use of model systems to further our understanding of learning and memory in all animals, including humans. This is based on the preponderance of homology at all levels of biological organization among species, allowing for meaningful comparisons of behavioral plasticity and brain mechanisms from which it is derived. For as long as there have been paintings on cave walls, tales passed down from generation to generation, and words written on clay tablets, papyrus, or paper, we have looked to animals to tell us a little more about ourselves. All evidence suggests that we are not mistaken in doing so.

Bibliography

1. Bennett, M. R. Development of the concept of mind. *Australian and New Zealand*

   Journal of Psychiatry **41**, 943 - 956 (2007).

2. Moore, B. R. The evolution of learning. *Biological Reviews* **79**, 301-335 (2004).

3. Walker, S. *Animal Learning* (ed. Coltheart, M.) (Routledge & Kegan Paul, New York, 1987).

4. Carew, T. J., Pinsker, H. M. & Kandel, E. R. Long-term habituation of a defensive withdrawal reflex in Aplysia. *Science* **175**, 451-454 (1972).

5. Christoffersen, G. R. J. Habituation: Events in the history of its characterization and linkage to synaptic depression. A new proposed kinetic criterion for its identification. *Progress in Neurobiology* **53**, 45-66 (1997).

6. Peckham, G. W. & Peckham, E. G. Some observations on the Mental Powers of Spiders. *j. Morphol.* **1**, 383-419 (1887).

7. Boulis, N. M. & Sahley, C. L. A behavioral analysis of habituation and sensitization of shortening in the semi-intact leech. *J Neurosci* **8**, 4621-7 (1988).

8. Ratner, S. C. & Gilpin, A. R. Habituation and retention of habituation of responses to air puff of normal and decerebrate earthworms. *J Comp Physiol Psychol* **86**, 911-8 (1974).

9. Carew, T. J., Castellucci, V. F. & Kandel, E. R. An analysis of dishabituation and sensitization of the gill-withdrawal reflex in Aplysia. *Int J Neurosci* **2**, 79-98 (1971).

10. Pinsker, H., Kupfermann, I., Castellucci, V. & Kandel, E. Habituation and dishabituation of the gill-withdrawal reflex in Aplysia. *Science* **167**, 1740-2 (1970).

11. Hagbarth, K. E. & Kugelberg, E. Plasticity of the Human Skin Reflex. *Brain* **81**, 305-318 (1958).

12. Lehner, G. F. J. A Study of the Extinction of Unconditioned Reflexes. *J. Expl. Psychol.* **29**, 435-455 (1941).

13. Pavlov, I. *Conditioned Reflexes* (Oxford University Press, Oxford, 1927).

14. Hammer, M. & Menzel, R. Learning and memory in the honeybee. *J Neurosci* **15**, 1617-30 (1995).

15. Menzel, R. & Muller, U. Learning and memory in honeybees: from behavior to neural substrates. *Annu Rev Neurosci* **19**, 379-404 (1996).

16. Quinn, W. G., Harris, W. A. & Benzer, S. Conditioned behavior in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **71**, 708-12 (1974).

17. Tully, T. & Quinn, W. G. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J Comp Physiol [A]* **157**, 263-77 (1985).

18. Jarvis, E. D., Mello, C. V. & Nottebohm, F. Associative learning and stimulus novelty influence the song-induced expression of an immediate early gene in the canary forebrain. *Learn. Mem.* **2**, 62-80 (1995).

19. Thorndike, E. L. Animal Intelligence: An Experimental Study of the Associative Processes of Animals. *Psychological Review* **2**, 1-109 (1898).

20. Ferster, C. S., BF. *Schedules of Reinforcement* (Appleton-Century-Crofts, Inc, New York, 1957).

21. Baxter, D. A. & Byrne, J. H. Feeding behavior of Aplysia: A model system for comparing cellular mechanisms of classical and operant conditioning. *Learn. Mem.* **13**, 669-680 (2006).

22. Sakura, M., Okada, R. & Mizunami, M. Olfactory discrimination of structurally similar alcohols by cockroaches. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **188**, 787-797 (2002).

23. Arave, C. W. Assessing sensory capacity of animals using operant technology. *J. Anim Sci.* **74**, 1996-2009 (1996).

24. Corfas, G. & Dudai, Y. Habituation and dishabituation of a cleaning reflex in normal and mutant Drosophila. *J Neurosci* **9**, 56-62 (1989).

25. Byers, D. A review of the behavior and biochemistry of dunce, a mutation of learning in Drosophila. *Basic Life Sci* **16**, 467-74 (1980).

26. Livingstone, M. S. Genetic dissection of Drosophila adenylate cyclase. *Proc Natl Acad Sci U S A* **82**, 5992-6 (1985).

27. Margulies, C., Tully, T. & Dubnau, J. Deconstructing Memory in Drosophila. *Current Biology* **15**, R700-R713 (2005).

28. Tully, T., Preat, T., Boynton, S. C. & Del Vecchio, M. Genetic dissection of consolidated memory in Drosophila. *Cell* **79**, 35-47 (1994).

29. Quinn, W. G. & Dudai, Y. Memory phases in Drosophila. *Nature* **262**, 576-7 (1976).

30. Tempel, B. L., Bonini, N., Dawson, D. R. & Quinn, W. G. Reward learning in normal and mutant Drosophila. *Proc Natl Acad Sci U S A* **80**, 1482-6 (1983).

31. Quinn, W. G., Sziber, P. P. & Booker, R. The Drosophila memory mutant amnesiac. *Nature* **277**, 212-4 (1979).

32. Atkinson, R. C. S., R. M. The control of short-term memory. *Scientific American*, 82-90 (1971).

33. Tully, T. et al. Genetic dissection of memory in Drosophila. *J Physiol Paris* **90**, 383 (1996).

34. Gross, C. G. *Brain, Vision, Memory: Tales in the history of neuroscience* (MIT press, cambridge, Mass, 1998).

35. Finger, S. *Origins of neuroscience: a history of explorations into brain function* (Oxford University Press, New York, 1994).

36. Lloyd, G. *Alcmeon and the Early History of Dissection* (Sudhoffs Archive, New York, 1975).

37. *Encyclopedia Britannica* (Encyclopedia Britannica online, 2007).

38. Yildirim, F. B. & Sarikcioglu, L. Marie Jean Pierre Flourens (1794 1867): an extraordinary scientist of his time. *J Neurol Neurosurg Psychiatry* **78**, 852- (2007).

39. Marshall, J. C. & Fink, G. R. Cerebral localization, then and now. *NeuroImage: Convergence and Divergence of Lesion Studies and Functional Imaging of Cognition* **20**, S2-S7 (2003).

40. Lashley, K. *Brain mechanisms and intelligence: A quantitative study of injuries to the brain* (University of Chicago Press, Chicago, 1929).

41. Hodgkin, A. H., A. Propagation of electric signals along ginat nerve fibers. *Proc R Soc Lond B Biol Sci*, 177-83 (1952).

42. Butler, A. B. Chordate evolution and the origin of craniates: An old brain in a new head. *The Anatomical Record* **261**, 111-125 (2000).

43. Hedges, S. B. The origin and evolution of model organisms. *Nature Reviews Genetics* **3**, 838-849 (2002).

44. Rubin, G. M. et al. Comparative Genomics of the Eukaryotes. *Science* **287**, 2204-2215 (2000).

45. Pennisi, E. The Human Genome. *Science* **291**, 1177-1180 (2001).

46. Kammermeier, L. R., H. Common developmental genetic mechanisms for patterning invertebrate and vertebrate brains. *Brain Research Bulletin*, 657-682 (2001).

47. Hirth, F. H., B; Reichert, H. Homeotic gene action in embryonic brain development of Drosophila. *Development* **125**, 1579-89 (1998).

48. Acampora, D. et al. Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121**, 3279-3290 (1995).

49. Acampora, D. et al. Murine Otx1 and Drosophila otd genes share conserved genetic functions required in invertebrate and vertebrate brain development. *Development* **125**, 1691-1702 (1998).

50. Leuzinger, S. et al. Equivalence of the fly orthodenticle gene and the human OTX genes in embryonic brain development of Drosophila. *Development* **125**, 1703-1710 (1998).

51. Butler, A., Hodos W. *Comparative vertebrate neuroanatomy : evolution and adaptation* (Wiley-Liss, New York, 1996).

52. Radinsky, L. Evolution of somatic sensory specialization in otter brains. *J Comp Neurol* **134**, 495-506 (1968).

53. Kandel, E. J., Schwartz, J. H. & Jessell, T. M. *Principles of neural science* (Appleton & Lange ; Prentice Hall, East Norwalk, Conn.; Hemel Hempstead, 1999).

54. Maczko, K. A., Knudsen, P. F. & Knudsen, E. I. Auditory and Visual Space Maps in the Cholinergic Nucleus Isthmi Pars Parvocellularis of the Barn Owl. *J. Neurosci.* **26**, 12799-12806 (2006).

55. Andermann, M. L. & Moore, C. I. A somatotopic map of vibrissa motion direction within a barrel column. *Nature Neuroscience* **9**, 543-551 (2006).

56. Strotmann, J. B., H. Formation of glomerular maps in the olfactory system. *Seminars in Cell & Developmental Biology Olfaction; Animal Stem Cell Types* **17**, 402-410 (2006).

57. Ai, H., Nishino, H. & Itoh, T. Topographic organization of sensory afferents of Johnston's organ in the honeybee brain. *The Journal of Comparative Neurology* **502**, 1030-1046 (2007).

58. Vosshall, L. B. W., A. M.; Axel, R. An Olfactory Sensory Map in the Fly Brain. *Cell* **102**, 147-159 (2000).

59. Ekstrom, A. D. et al. Cellular networks underlying human spatial navigation. *Nature* **425**, 184-188 (2003).

60. Zars, T. Behavioral functions of the insect mushroom bodies. *Curr Opin Neurobiol* **10**, 790-795 (2000).

61. Muller, R. A Quarter of a Century of Place Cells. *Neuron* **17**, 813-822 (1996).

62. Scoville, W. M., B. Loss of recent memory after bilateral hippocampal lesions. *J Neurol Neurosurg Psychiatry* **20**, 11-21 (1957).

63. Crittenden, J. R., Skoulakis, E. M., Han, K. A., Kalderon, D. & Davis, R. L. Tripartite mushroom body architecture revealed by antigenic markers. *Learn Mem* **5**, 38-51 (1998).

64. Kandel E, A. T. Neuropeptides, adenylyl cyclase, and memory storage. *Science* **268**, 825-826 (1995).

65. Goldstein, R. D., M; Reubinoff, B; Benvenisty, N. Integration and differentiation of human embryonic stem cells transplanted to the chick embryo. *Developmental Dynamics* **225**, 80-86 (2002).

66. Muotri, A. R., Nakashima, K., Toni, N., Sandler, V. M. & Gage, F. H. Development of functional human embryonic stem cell-derived neurons in

mouse brain. *Proceedings of the National Academy of Sciences* **102**, 18644-18648 (2005).

67. Fouts, R. S. & Waters, G. S. Chimpanzee sign language and Darwinian continuity: Evidence for a neurological continuity for language. *Neurological Research* **23**, 787-794 (2001).

68. Menzel, R. & Giurfa, M. Dimensions of Cognition in an Insect, the Honeybee. *Behav Cogn Neurosci Rev* **5**, 24-40 (2006).

69. Cheney, D. ANIMAL BEHAVIOR:Only Unthinking Intelligence? *Science* **283**, 333- (1999).

70. Emlen, S. T. W., P. H. ; Demong, N. J. Making decisions in the family – an evolutionary perspective. *American Scientist*, 148-157 (1995).

CHAPTER 2

GENERAL INTRODUCTION TO DROSOPHILA LEARNING AND MEMORY

In chapter 1 I showed how animal models can be used as a proxy to help us understand how learning and memory processes might function in humans. In this chapter I will introduce information we have gathered from one of those model organisms, the fruit fly *Drosophila melanogaster*.

Neurons and neuronal assemblies can be probed in a variety of ways to determine relevant structures to learning and memory behavior on a more macro scale. From a geneticist's point of view, two broad classes of genes can be expected to perturb learning and memory when mutated: first, genes essential for the development and maintenance of neuronal ensembles which support these behaviors, second genes involved in the biochemistry of memory formation, storage and retrieval. For a number of years people have been studying the biological underpinnings of learning and memory in *Drosophila* at all of these levels. In this chapter we will begin by discussing what learning behaviors have been demonstrated in fruit flies. Next we will introduce relevant neuronal structures which have been implicated in learning behavior and conclude by detailing the major classes of genes whose products support learning and memory storage as well as other relevant features such as memory extinction and attention.

Fruit Fly Learning and Memory Behavior

The common fruit fly has been proven capable of learning and retaining new memories through a variety of behavioral paradigms. Learning itself is not the easiest thing to define. Some people have defined it based on its adaptive significance: "We can define learning as that process which manifests itself by adaptive changes in individual behavior as a result of experience"[1]. Some were very restricted in their definitions making it apply more specifically to their own work such as: "Learning is a relatively permanent increase in response strength that is based on previous reinforcement and that can be made specific to one out of two or more arbitrarily selected stimulus situations"[2]. Others believed not all learning necessarily was adaptively beneficial or context restricted. I tend to agree most with their definition which is more open to include a variety of behaviors and conditions: "We consider any systematic change in behavior to be learning whether or not the change is adaptive, desirable for certain purposes, or in accordance with any other such criterion"[3].

The level of complexity of an organism often dictates what kinds of behavioral plasticity can be found. For example, we would not expect fruit flies to be capable of declarative memory because this type of memory is operationally defined to require some form of language which has not been found in flies. Because of the innate differences between humans and flies it is often difficult to pinpoint the exact moment a learning event occurs but we infer that it did occur by assaying for modified behavior immediately after a training session has completed. In the following sections I will describe what forms of learning have been demonstrated

in fruit flies.

Nonassociative Learning

As was discussed in the first chapter, there are two primary classes of training paradigms, nonassociative and associative, which have been demonstrated to elicit learning in a wide variety of animals. Nonassociative learning represents the modification of basic species-typical behaviors through repeated exposure to a stimulus. This can further be broken down into two subclasses of nonassociative learning known as habituation and sensitization. Habituation is a response decrement of a behavior occurring as the result of repeated stimulation, and not attributable to fatigue or sensory adaptation[4]. Sensitization is an increased response of a behavior making this the theoretical opposite of habituation. But as Hilgard pointed out this is not exclusively a nonassociative response[5]. This increase can be seen in associative conditioning when a response to a conditioned stimulus is increased at later presentations. Some component of this increase is likely attributable to sensitization affects. More stringent definitions of sensitization were established describing both nonassociative and associative sensitization[6]. In the case of nonassociative sensitization, a stimulus (S^u) is repeatedly presented. Incremental sensitization tests for changes in the response (R^u) probability or amplitude elicited by that same stimulus (S^u) while pseudoconditioning looks at the change in R^u when presenting a novel stimulus (S^o). While some early work with *aplysia* used pseudoconditioning protocols the assays used in fruit flies were nonassociative sensitization.

Flies can exhibit nonassociative behavior. In fruit flies eight different reflexive

behaviors have been shown to exhibit habituation across a range of sensory modalities including (olfactory) proboscis extension reflex (PER)[7,8], olfactory startle[9-13], (mechanosensory) electroshock avoidance[14], cleaning reflex[15], leg position reflex[16] ,(visual) landing response[17,18], visual startle[19-21], while sensitization has been less explored with two reflexes exhibiting this behavior[7,13]. Nonassociative learning has been demonstrated in a large variety of organisms including human and rodent eyeblinking[22-24], spider and scorpion defensive withdrawal reflex[25,26], marine mollusks defensive gill withdrawal reflex[27-30]. This type of behavior does not require a complete nervous system or nerves at all because habituation can be elicited in spinal severed cats[31] and even single celled protozoa[32,33].

Associative Learning

Human memory frequently relies on association of objects repeatedly seen together to become linked in our mind; when we try to retrieve information, one thing reminds us of another, which reminds us of yet another, and so on. Not surprisingly, neurobiologists have been trying to uncover the underlying mechanisms for decades. Associative learning can be broken down into two general subtypes classical and instrumental learning.

Classical conditioning. Classical conditioning involves the temporal pairing of a stimulus with no previous innate meaning with a stimulus that does. An associative learning event is inferred to have occurred when presentation of the novel stimulus elicits the same species typical behaviors as the meaningful one. The classic demonstration of Pavlov's conditioning of dogs was discussed in

chapter 1. There are many different types of learning conditions which have been described as classical processes[34]. They range from the simple to more complex associations. For example, a basic process called garcia conditioning is based on the principle that an animal will more easily form associations between behaviorally relevant stimuli pairings such as flavor eliciting illness[35] , while associations between two neutral stimuli with no innate meaning signify a more complex associative process[36].

Classical conditioning was first demonstrated in fruit flies over thirty years ago[37]. The task presented was a population level olfactory discrimination learning assay. Two odors were presented to the flies in successive order. One of the stimuli (CS+) was paired with a mild electric shock while the other (CS-) was unpaired. To assay for learning, the CS+ and CS- were presented in tandem. All flies were placed at a choice point where they could walk toward either the CS+ or CS-. Learning was interpreted to occur if a statistically significantly higher percentage of animals favored the CS- choice than naive animals given the same choice. The reciprocal odor combinations were used to generate another learning index and the two scores are combined to create a learning score free of biases such as left right preference or color/odor preference. This type of discrimination assay (Apparatus seen in figure 2-1) has been the most frequently employed training procedure over the years and the one used in my work but others are used as well. Some groups have used the same T-maze apparatus but converted it for use as an olfactory appetitive assay rather than olfactory avoidance[38,39]. While these assays employ a population of flies to create a

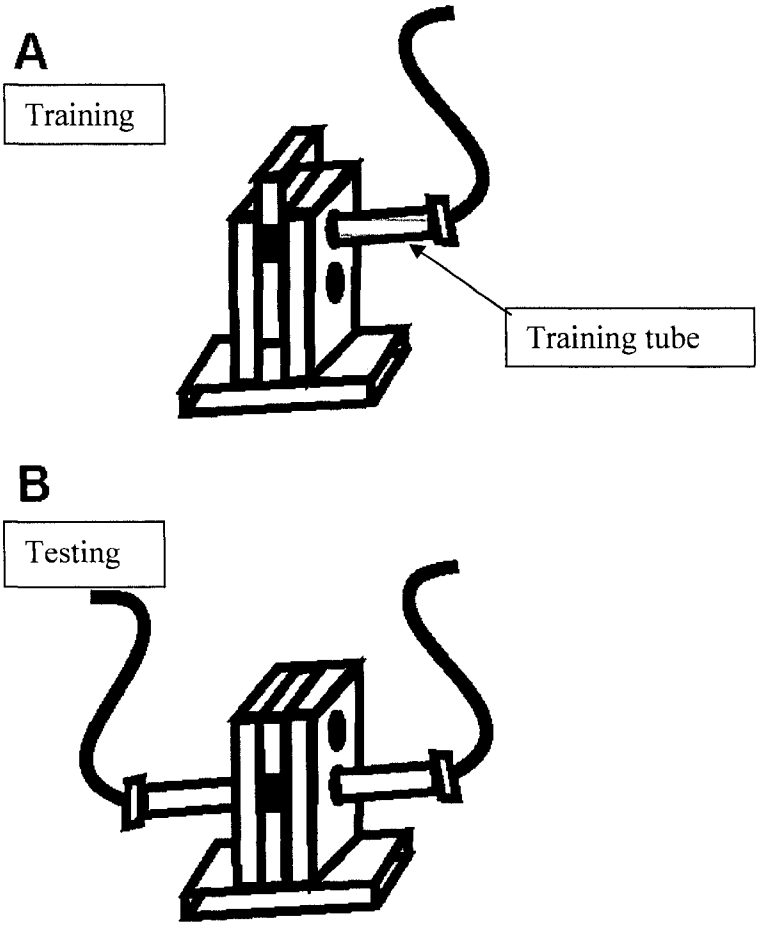

Figure 2-1. T-Maze apparatus used for classical training. A). Training phase of the T-maze. Aproximately 100 flies are placed in the training tube which is lined with electrifiable copper wire. Two odors are presented to the flies sequentially. The first is paired with shock while the second is unpaired. Flies are then nocked into an elevator in the middle of the apparatus (black rectangle) and lowered the the choice point below. B) Testing phase of the T-maze. Both odors are presented to the fly in converging currents for 2 minutes. Performance is measured as a function of shock-paired odor avoidance.

learning index, others have demonstrated adult learning in individuals including: the proboscis extension reflex (PER) assay[40], courtship conditioning[41], flight simulator assay[42] as well as larval olfactory discrimination assay[43] and a visual discrimination assay[44].

Second order conditioning (SOC) is used to assess an organism's ability to form associations between two neutral stimuli. This form of conditioning is particularly relevant to real world scenarios the flies may encounter. In the wild animals will not merely encounter single stimuli pairings but many layered stimuli the animal must sort through in terms of significance. In order to examine SOC, paradigms were needed where behavioral change caused by the association of two neutral stimuli could be seen. To get around this difficulty, SOC pairs a conditioned stimulus (CS^1) with an unconditioned stimulus (US). Once this association is firmly trained a second training attempts to associate a second stimulus (CS^2) with CS^1. This should transfer CS^1's association with the US to CS^2. There have been few reported cases of research attempting to demonstrate SOC in flies. Using the visual flight simulator SOC has been demonstrated using an operant paradigm[45]. Recently the first reported case of a significant SOC affect in olfactory classical conditioning was demonstrated in flies using an olfactory associative paradigm[46].

Classical conditioning has been demonstrated in a wide variety of species and has great potential for cross species comparisons of learning. The PER assay has proven particularly useful as it has been employed on a variety of different insects including honeybees[47] and moths[48]. It may be possible to use results

from the PER assay to get a better understanding of learning capabilities across insect species.

Instrumental conditioning. Classical conditioning involves a temporal pairing of a conditioned stimulus and unconditioned stimulus and no behavior of the animal during its training affects the training regime. Its goal is to determine if animals can learn associations. Instrumental learning is different in that it tries to simulate more real world trial and error learning procedures. A particular predetermined behavior of an animal will be paired with a particular unconditioned stimulus often referred to as the reinforcer. Seven distinct subtypes of instrumental conditioning have been described[34]. Of these, the simplest variety has been demonstrated in flies.

Thorndikian conditioning involves species-typical, reinforcement-appropriate responses, elicited by environmental stimuli. Such responses, when reinforced, become more likely to recur in the particular situation[36]. This type of learning behavior has been demonstrated in flies using the heatbox[49] and flight simulator[50] assays. The heatbox is typically used in a place memory paradigm in which flies are punished by heat when entering a particular side of the box creating an association between walking in the punished side with heat. In the flight simulator animals are tethered in a flight arena and placed in front of a screen which mimics proper movement based on the torque rotation and strength placed on the tether by the fly. Animals are heated on the abdomen by a concentrated beam of light when they orient toward certain objects or colors placed on the screen in particular quadrants. Animals learn to associate certain

flight behaviors with punishment.

These relatively simple tasks have been used to ask a wide variety of questions about fly learning capabilities. In most applications of these assays, stimuli are kept simple in order to demonstrate a clear strong learning behavior. In some cases these assays have been used as quick throughput behavior assays for mutagenesis screens. However, many simple yet clever modifications allow researchers to ask a variety of questions. The flight simulator operant assay has been used to examine if flies can separate predictive stimuli from surrounding context by changing the background light[51]. Flies could still exhibit changed behavior when shifting from white light to a monochrome color but not between monochromes. Many other tests trying to determine a flies ability to properly associate stimuli among different kinds of distractions have been completed including visual feature extraction[52], visual context dependant olfactory learning[53], and choice behavior[54].

Thordikian instrumental learning has been demonstrated in a wide variety of species including pond snails (*Lymnaea stagnalis*)[55], honeybees[56,57], rats[58] and cats[36]. However, some species have been shown to perform more complex forms of instrumental learning. While thordikian learning elicits species typical behavior more complex forms such as operant instrumental learning require the subject to create non-species typical (novel) behavior. For example, chimpanzees can learn tool use skills to extract insects from logs[59] while dogs can learn rudimentary counting skills[60]. Even more complex instrumental learning is distinguished by the lack of an external reinforcer such as food and relies on

some internal reward such as we might feel once we have mastered a skill like riding a bike. So far only a handful of mammals and birds have demonstrated these advanced abilities[34]. However, this does not mean that flies are incapable of these types of behavior. We may have simply not phrased the proper question to allow them to demonstrate their abilities.

Aspects of Memory Maintenance

So far I have discussed different types of learning events and assays in which they can be conditioned. An interesting study revealed that it is possible for a fruit fly to demonstrate memory without demonstrating initial learning[61]. Temporal expression of protein kinase C (PKC) inhibitor caused flies to exhibit depressed initial learning scores but normal memory scores after 1 hour. Through many genetic and pharmacological studies researchers have been able to demonstrate that learning can be mechanistically separated from memory and that memory itself can be broken down into phases.

Memory retention. Memory can be mechanistically divided into four distinct phases (Figure 1-1) having distinct behavioral, physiological, and cellular properties revealed through experimentation. Mechanisms of short-term and long-term memories have been separated through genetic and pharmacological manipulation. The *dunce (dnc)* and *rutabaga (rut)* genes were examined using an olfactory associative test and both exhibited an extreme decrement in initial learning scores and a much sharper decrease in memory retention with in the first hour compared to wild type[62,63].However, after an hour the sharp drop in retention is curtailed and any persisting memory is retained. *dnc* and *rut* were

therefore categorized as short-term memory (STM) mutants[64,65].

Intermediate memory processes (also referred to as middle term memory MTM) bridging the gap between STM and more permanent forms of consolidated memory have been genetically defined in flies. Interestingly, flies mutant for the *amnesiac (amn)* gene show near-normal memory retention immediately after a single training session and again seven hours later. In between these time points, memory retention in the mutants is appreciably lower than normal[66].

Finally two distinct forms of long term memory have been established pharmacologically and genetically. Early experiments demonstrated an anesthesia-resistant component of memory (ARM) lasting up to one day after a single training session[65,67,68]. Flies which were anesthetized immediately after training after revival showed little memory retention. However, with increasing time between training and anesthetization when the flies recovered they demonstrated increasingly improved memory. It was hypothesized that over time memory was consolidated from a labile anesthesia sensitive form to a more stable anesthesia resistant form. Massed training (10 sessions administered one immediately after the other) produces even stronger ARM retention, lasting about three days, and this memory is insensitive to the protein synthesis inhibitor cycloheximide. In contrast, spaced training (10 sessions with a 15 minute rest interval between each) yields a protein-synthesis-dependent long term memory (LTM) lasting several weeks[64]. How these forms of long term memory interact is currently up for debate. Previous models proposed that ARM and LTM coexisted in independent mechanisms with an additive affect on memory but recent studies

have shown that mutant flies incapable of forming LTM generate interesting results using a spaced training protocol. The more the flies were trained, the less they could remember[69]. In other words, it appeared that spaced training extinguished ARM. This lead the researchers to conclude that ARM may act as a gating mechanism for LTM, ensuring its formation only after repeated and spaced training.

Extinction and attention. Memory extinction and attention in flies are two additional exciting yet poorly understood areas of learning and memory research. Memory decay occurs naturally and some researchers believe that this is a result of improper maintenance or breakdown of the mechanisms which store the memory[70]. However, memory can also be extinguished by repeated presentation of the conditioned stimulus without reinforcement[37]. When *uas- Shi^{ts}* is used to block transmission from the MBs during extinction training extinction behavior is still seen. However, blocking transmission to the MBs during training causes the loss of extinction[71]. This demonstrates that it is likely the same MB neurons that are involved in forming the memory are also involved in extinguishing them.

Progress has also been achieved studying fruit fly attention. In order for an animal to learn an event they must be capable of focusing their attention on the events for a long enough period of time to determine their meaning. This is particularly relevant as more and more children around the world are diagnosed with attention deficit hyperactivity disorder causing them to have difficulties in school (reviewed in [72]). Visual choice behavior in fruit flies is correlated with local field potential (LFP) activity in the brain centered around 20 to 30 Hz. This activity

is transiently increased in amplitude by classical conditioning and is suppressed during sleep[73]. Examining LFP activity in STM mutants displayed attenuated and delayed brain responses to visual objects, as compared to wild-type flies[74].

All of the information presented in this section adequately demonstrates that fruit flies are capable of a rich repertoire of behavior which we can characterize and quantify. A better understanding of the underlying circuits and cell biology responsible for this behavior is critical. In the following sections we will look at brain structures and genes which have been implicated in learning and memory behavior.

Neuronal Structures Critical for Learning Behavior

Every neuron appears to possess many characteristics required for learning as demonstrated by single synapse preparations in *Aplysia*[75]. However, the power of neuronal circuits is required for most of the advanced forms of learning behavior demonstrated by organisms. The fruit fly nervous system consists of the brain and ventral nerve cord (CNS), stomatogastric nervous system (SNS) and a multitude of peripheral motor and sensory neurons (PNS). Although the PNS and SNS likely do play roles in learning behavior, in flies the CNS has received the most attention determining relevant neuronal structures for learning. Also, learning behavior in flies is frequently studied based on sensory input through one modality, i.e. olfaction or vision. Therefore we will introduce structures of the CNS which have been shown relevant to learning based on the modality they are associated with then briefly discuss what is known about the role of PNS and

SNS neurons.

CNS Structures of Olfactory Learning

Because olfactory associative learning is the most widely studied learning behavior of fruit flies, much time has been invested elucidating the exact nature of odor processing within the CNS. Chemical odorants are first detected by olfactory sensory neurons (OSN) residing in sensilla located on the third antennal segment and maxillary palps. These neurons typically express 1 of 63 distinct ligand binding olfactory receptor (OR) proteins and a coreceptor[76,77]. OSNs project to structures called the antennal lobes (AL) located antero-medially within the brain which are composed of about 40-50 smaller glial bound partitions known as glumeruli. ORNs expressing the same OR converge on one or only a few distinct glomeruli[77,78]. Local interneurons also connect the distinct glomeruli to each other. Presentation of different odorants can excite or inhibit different ORNs creating a combinatorial code of odor representation within the AL[79]. Cholinergic projection neurons synapsing at the AL send this information downstream for higher order processing to the lateral protocerebrum (See chapter 1) and the dendritic region of another distinct set of brain structures called the mushroom bodies (MB)[80-82]. A good deal of time has been spent characterizing the MBs and their role in olfactory memory processing so we will focus on these structures.

Mushroom bodies. Mushroom bodies are lobed structures composed of long parallel axons originating from clusters of dorso-anterior cells. Structures with these morphological properties are found in many marine annelids and almost all

the arthropod groups, except crustaceans. [83] Fruit fly MB's are composed of 2500-3000 intrinsic neurons referred to as Kenyon cells (KCs) whose perikarya are situated in the extreme dorso-caudal region of the brain[82,84]. While not all arthropod Kenyon cells have a specific dendritic region, those that do have two regions of common dendritic projections in either hemisphere called calyces which are fused in the case of fruit flies[83]. Their axons converge under each calyx to form stalk-like structures know as the pedunculi, which extend rosto- ventrally forming distinct lobes. In fruit flies five distinct lobes (figure 2-2) have been characterized based on birth order, gene expression and axonal projection patterns[85-87]. The α and α' lobes project vertically while the β, β' and γ lobes project toward the midline. Lobe sub compartmentalization has been hypothesized based on Immunocytological, golgi, and GAL4 characterization of Kenyon cell organization in the calyces and lobes which suggested as many as nine distinct neuronal subsets[88].

It is unclear what neurotransmitters are released by intrinsic Kenyon cells. While glutamate expression has been shown in a population of MB cells[88] its role in neurotransmission cannot be confirmed because vesicular glutamate transporter protein does not appear to express in Kenyon cells. However, short peptide products from the *short neuropeptide F precursor* (*snpf*) gene may act as cotransmitters[89].

Mushroom bodies are involved in olfactory learning behavior. A large body of evidence supports MB involvment in learning and memory behavior. Gene mutations affecting MB structural integrity disrupt olfactory learning behavior[90-92]

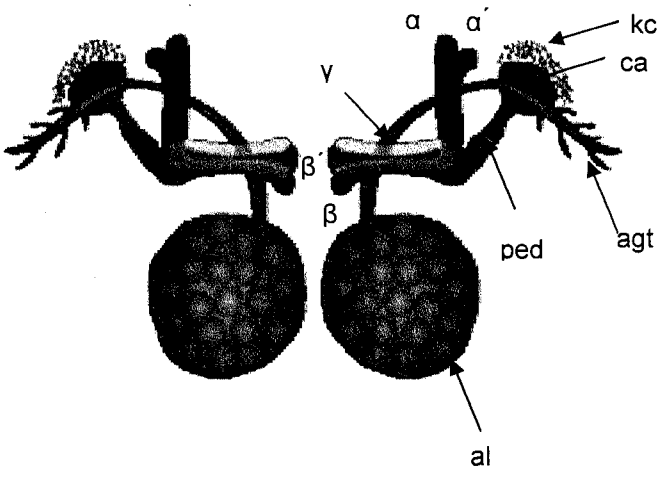

Figure 2-2. Diagramatic representation of the *Drosophila* mushroom bodies. . The mushroom bodies are composed of 2500-3000 intrinsic neurons referred to as Kenyon cells which send axonal projections forward in the brain through the peduncle finally forming five distinct lobes. The dendritic region known as the calyx is immediately anterior to the cell bodies. kc = Kenyon cell bodies, ca = calyx, ped = pedunculus, α , α' β, β', and γ represent the lobes, sp = the spur, agt = antenno-glomular tract, al = antennal lobe.

and many genes shown to be involved in olfactory learning and memory are preferentially expressed in the MBs[86]. Also, precise MB chemical ablation by hydroxyurea abolished olfactory learning[93] while disruption of Kenyon cell neurotransmission with a temperature sensitive *shibire* allele (*uas-shi^{ts}*) demonstrated that MB signaling was essential for olfactory memory formation and retrieval[94-96]. Furthermore, temporal and spatial rescue of genes in the MBs can rescue olfactory learning defects[97,98] while RNAi silencing of the *Notch* gene in the MBs disrupted long term olfactory memory[99].

It was first proposed and then demonstrated that repeated activation of a postsynaptic cell by a presynaptic cell can change the firing properties of both cells upon later activation[100,101]. Memories are formed and stored by changes that occur in the nervous system due to the convergence of signals on a "coincidence detector". These changes, which are collectively known as memory traces, include any molecular, biophysical, or cellular change induced by learning, which subsequently alters the processing and response of the nervous system to sensory information. A cellular assembly can act as a coincidence detector because the convergence of two or more signals onto a single neuron can change the properties of the neurons retaining the "memory trace" of that event as cellular changes either transiently or permanently. If MB Kenyon cells fill the role of coincidence detectors they must demonstrate certain properties associated with this role. Behaviorally we can demonstrate the association between olfactory cues and other stimuli such as electric shock punishment or appetitive rewards[37,38,68,102]. In order to act as coincidence detectors for these

behaviors input signals for all three stimuli must be demonstrated as well as changed cellular properties caused by relevant behavioral input.

Olfactory input to the MBs through the antennal lobe has already been established but clear evidence for electroshock and appetitive information flow to the MBs has been more elusive. However, over the last few years evidence has accumulated establishing that MBs do receive electroshock and appetitive signals.

Many believe dopaminergic neurons deliver the aversive information while octopaminergic neurons deliver appetitive information to the MBs. Dopaminergic neurons extensively innervate the MBs but have minimal contact with the antennal lobes (AL)[103]. Blockage of dopamine biosynthesis by a *uas-shi^{ts}* driven by*TH-GAL4* carrying GAL4 under the control of the regulatory region of the dopamine biosynthesis gene *tyrosine hydroxylase* (*TH*) shows that dopamine release is necessary for aversive conditioning. Calcium (Ca^{++}) imaging studies were used to examine MB neuronal reactivity to shock and odor presentation. When an action potential arrives at the nerve terminal, voltage-gated Ca^{++} channels open, causing a sudden influx of Ca^{++} ions. This pulse of intracellular Ca^{++} results in membrane fusion between the pre-synaptic terminal and release-ready vesicles. Ca^{++} influx has therefore been used as an indicator of neurotransmission. Dopaminergic neurons are responsive to electroshock stimuli while weakly stimulated by odor presentation[103]. Studies in fruit fly larvae also supported a role in aversive learning for dopamine. Light induced dopamine release can substitute for aversive stimuli during olfactory

training in larvae[104]. Octopaminergic neurons extensively innervate both the MBs and AL[105]. Octopamine biosynthetic mutants are unaffected in aversive training but impaired in appetitive training[38] It was also shown that Light induced octopamine release can substitute for an appetitive stimuli in appetitive olfactory training in larvae[104]. A growing compilation of observations provide strong support for the roles of dopamine in aversive and octopamine in appetitive olfactory learning. Muddying the water a little, it was shown that a dopamine receptor in the MBs is necessary for both appetitive and aversive learning[39]. While the exact role of these neurotransmitters in information signaling to the MBs still needs to be elucidated, a preponderance of information supports the idea that MBs do receive both appetitive and aversive information.

Finally, to meet the criterion established for a coincidence detector, MB neurons must exhibit cellular plasticity in response to training. Ca^{++} imaging studies of MB cells provide evidence for physiological changes correlated with training. A subset of MB cells demonstrate significant Ca^{++} amplitude changes in response to a previously trained odor compared to an untrained odor shortly after training[106]. A different subset of MB cells have an increased intracellular Ca^{++} amplitude in response to a previously trained odor which appears around 3 to 9 hours after training and can still be detected 24 hrs later[107].

The fact that we can demonstrate appropriate input channels for the necessary behavioral information and that MB cells exhibit physiological changes associated with training implicate the MBs as coincidence detectors capable of modifying behavior.

Mushroom bodies generalize to multiple forms of learning. Another important question is whether MBs are involved in all forms of conditioning or specific types. A few key experiments help to address this question. Experiments looking at olfactory startle habituation used two methods, hydroxyurea ablation of the MBs and Gal4 driven MB expression of tetanus toxin which interferes with neurotransmitter release, to examine what role the MBs play in habituation[10]. Using both methods of MB inactivation the normal habituation response was reduced in magnitude but not abolished. In a somewhat conflicting result, it was shown that habituation to mild electroshock with MBs disrupted through GAL4 directed tetanus toxin expression occurred much more rapidly than in wild type animals leading the researchers to conclude MBs are involved in preventing premature habituation[14]. The differences seen may be caused by examination of different reflexes however both indicate that the MBs do play a role in nonassociative conditioning protocols such as habituation. Examining the affects of MBs on instrumental conditioning is more difficult particularly because there currently are no olfactory based operant learning paradigms being used in fruit flies. However, for several visual based operant protocols, which will be discussed in greater detail in the next section, MBs have been shown unnecessary for normal instrumental behavior [55,97,108]. This does not rule out the possibility that MBs are required for all forms of olfactory conditioning.

Mushroom bodies are implicated in learning and memory in other insects. Mushroom bodies are common neuronal assemblies in most invertebrates. They have been characterized in a number of insects and have been implicated in

learning and memory behavior in a variety of them including honeybees[109,110], blowflies[111], and cockroaches[112]. While mounting evidence supports a role for MBs in learning and memory behavior across species the exact nature of that role may not be the same in all invertebrates. This may be due to a curious difference in afferents to the MBs. While several species have demonstrated a variety of multimodal input to the MBs including visual information[82,113] no such visual input can be demonstrated for fruit flies and MBs are unnecessary for visual learning in flies[108].

Many elements of the olfactory pathway exhibit memory traces. A lot of attention has been placed on the MBs and their role in learning and memory behavior however, other structures within the olfactory pathway and some not previously associated with olfaction have proven to be more important role than simply relaying information to the MBs. For example both the projection neurons from the antennal lobes to the MBs and the dopaminergic neurons innervating the MBs have demonstrated significant cellular changes after olfactory conditioning. Projection neurons which previously were not activated by odor presentation are briefly recruited as part of the odor representation[114] while dopaminergic neurons demonstrate prolonged Ca^{++} responses after training[103]. These facts could speak to redundancy built into the system or imply that the MBs are involved in some higher order processing step beyond the memory traces generated by these first order neurons.

The discovery of two distinct cells, known as dorsal paired medial (DPM) cells based on their location within the brain, which synapse upon the MB lobes has

provided additional insight into the olfactory learning and memory pathway. These cells were initially discovered based on their expression of the *amnesiac (amn)* gene previously associated with middle term memory processing[115]. GAL4 driven expression of *amn* exclusively in the DPM cells rescued the learning defects caused by this mutation. *UAS-shi^{ts}* was used to block DPM transmission at specific time points during and after training revealing that DPM transmission was only critical during a window of time consistent with the MTM phase[116]. Ca++ and PH monitoring of the DPM cells indicates that they respond to both odor and shock presentation and appear to be both pre and post synaptic to the MBs[117]. Projections from the DPM cells to the vertical lobes of the MBs have an increased response to conditioned odors. It has been speculated that a DPM to MB loop is required to stabilize memory formation in a more permanent fashion[96].

Recent experiments using *RNAi* silencing of critical learning and memory genes and *shi^{ts}* silencing of neuronal output indicated the ellipsoid body is critical in olfactory LTM processing[118]. The ellipsoid body is one of four subunits which comprise the central complex. This structure is located between the pedunculi and immediately behind the β lobes of the mushroom bodies and just above the esophagus. The central complex forms intricate connections to a variety of brain centers, may mediate communication between the two hemispheres and is believed to be a control center for many different behavioral outputs[119,120]. This is the first example of systems level processing of memory in flies involving the transfer of memory from one major brain region (MBs) to another (central complex). This transfer has been noted in vertebrates already with some

differences. Though LTM may eventually recruit the ellipsoid body, the mushroom body appears to be crucial for both memory consolidation and retrieval in flies[94-96]. In contrast, the hippocampus is required for consolidation, but not for retrieval, of long-lasting memories transferred to cortical systems in vertebrates[121,122].

CNS Structures of Visual Learning

The fly visual system is composed of the retina and four optic ganglia known as the lamina, the medulla, the lobula and the lobula plate. The retina is composed of about 750 unit eyes called ommatidia which house eight photoreceptor neurons. These neurons contain different rhodopsins (opsin protein plus retinal chromophore) which are sensitive to particular wavelengths of light. These photoreceptor neurons send information to the four optic ganglia which have distinct functions attributed to them. The lamina is responsible for motion detection, the medulla is involved in color processing, while the lobula and lobulla plate are believed to be involved in higher processing of these features[123,124]. Visual projection neurons (VPNs) then send characteristic information from the optic lobe ganglia to central brain regions. For example, electrophysiological and Ca^{2+} dynamics imaging analyses of the brains of the butterfly *Papilio aegeus*, moth *Manduca sexta*, house fly *Musca domestica*, and the blowfly *Calliphora erythrocephala* showed that certain VPNs deriving from the medulla and the lobula plate respond only to motion of distinct angles[125-127]. Very little is known about the locations these VPNs project to. Several sites within the protocerebrum have been identified as targets but as yet it is unknown what

these regions are involved in[127].

Many behavioral studies have been conducted exploring visual learning and memory however; none of the known optic lobe structures have been implicated in this behavior to date. Because many central brain regions remain uncharacterized both behaviorally and anatomically in fruit flies, researchers have tried to determine if any of the previously identified structures implicated in olfactory memory are involved in visual memory as well. While visual input to the mushroom bodies has been shown in the housefly[82], it has not been demonstrated in fruit flies. Ablation studies have shown that the MBs are dispensable for visual learning[108]. The only neuronal structure implicated in visual learning to date is the central complex. The central complex is composed of four subunits (ellipsoid body, fan shaped body, protocerebral bridge and noduli) and forms intricate connections to a variety of brain centers[119,120]. As noted earlier, the ellipsoid body of the central complex was implicated in LTM processing. Another of the four subunits appears to be important for visual learning tasks. Gal4 driven expression of tetanus toxin light chain disrupted neurotransmission in the central complex and interfered with visual pattern memory[128]. This was localized even further when two visual learning mutants were rescued by Gal4 driven expression exclusively in the fan shaped body[128,129]. In the olfactory pathway many of the primary sensory neurons such as the antennal lobe and its projection neurons display some evidence of a memory trace. As the visual system is studied more it is likely the same will be true for elements of the optic lobe and their projection neurons.

Roles of PNS and SNS in Learning Behavior

The stomatogastric nervous system (SNS) consists of several peripheral ganglia that receive input from the brain; these ganglia in turn innervate muscles, pharynx, and gut[130]. Very little is known about this systems contribution to behavior in fruit flies as it has primarily been studied in terms of nervous system development only. However, inferences about its possible roles can be made based on research in other species. The SNS is common to most invertebrates and has been extensively studied in crustaceans such as crabs for over a hundred years in regards to its function in foregut motor pattern generation[131]. In the American cockroach, SNS neurons involved in salivation have been classically conditioned in a protocol remarkably similar to the ones utilized in conditioning Pavlov's dogs[132]. It is likely this system is involved in many aspects of fruit fly behavior including learning and is open for future investigation.

The peripheral nervous system (PNS) has been used to investigate neuronal plasticity in both structural and electrophysiological studies. Neuronal plasticity is believed to be an underlying factor behind behavioral changes such as learning. Most of the studies of PNS plasticity involve the larval neuromuscular junction (NMJ) and focus on the abdominal segments because they form a predictable array of accessible overlapping fibers which is repeated in each hemisegment[133]. In order to understand the relationships between synaptic plasticity and behavioral plasticity several mutations of genes implicated in learning and memory behavior (discussed in greater detail in the next section) have been examined at the NMJ revealing a variety of relevant defects. In some cases

structural changes can be detected. For example flies mutant for the phosphodiesterase coding *dunce* gene develop increased numbers of motor neuron boutons and branches when compared to wildtype[134]. Neuromuscular transmission can also be altered. Mutations in *dunce* and an adenylate cyclase coding gene *rutabaga* disrupt both synaptic facilitation and potentiation[135]. While there are still a lot of unanswered questions, the NMJ has proven to be a useful location to look for connections between neuronal changes and altered behavior.

Genes Critical for Learning Behavior

Fruit flies have proven to be one of the most genetically tractable model organisms over the years and a great deal of effort has been placed in mutant discovery and generation. An ever increasing number of new genes have been linked with learning and memory behavior over the last 30+ years due to mutant screens. Six candidate strains showed abnormal learning or memory in the first screen. Of those, three were later shown to be components of the cAMP second messenger system[66,136,137]. This section will try to highlight what types of molecules and molecular pathways have been implicated in learning and memory to date. New gene discovery is an important step to understand any behavior. One approach has been to find mutants which affect the development or integrity of neuronal structures known to be associated with behaviors of interest, then test these mutant strains for defects in learning and memory consolidation. The second approach has been to find mutants in behavioral screens for learning or memory defects then determine what enzymes or

molecular pathways they may affect.

Brain Structure Mutants

A screen for structural defects in the brain has the advantage of being faster than behavioral screening plus links can later be drawn between specific brain structures or regions and specific behavioral defects. Several mutants with defects in the MBs including *mushroom body miniature* (*mbm*) and *mushroom bodies deranged* (*mbd*) were identified in screens of this type and later testing revealed impaired olfactory learning[90]. A thorough inspection of the literature revealed at least 19 genes (Table 2-1.) which affect MB and CCX development. Although many of these are developmental genes and do not affect learning and memory molecular pathways, they do affect structures important to learning and memory and therefore are important to behavioral research. Some of these mutants such as *alpha lobes absent* (*ala*) have helped us to more clearly define the roles of specific structures within the brain. A mutant strain of *ala,* which has variable phenotypes including the loss of all vertical MB lobes or the loss of the medial projecting β/β´ lobes, was used to examine if different lobes within the MBs perform different functions. Results from behavioral studies with *ala* indicate that only the alpha lobes are necessary for LTM formation[92]. Other structural mutants such as *minibrain* (*mnb*) have proven to be important genes in human disease research. In *Drosophila mnb* flies have a markedly reduced brain volume[138]. The *mnb* gene codes for a serine/threonine protein kinase which has been implicated in the mental retardation affects caused by trisome 21 or Down syndrome in humans. A human homologue of *mnb* was mapped to the Down

syndrome critical region and extra copies of the gene transgenically placed in mice caused learning defects[139,140]. Overall, this has proven to be a very valuable method of critical gene discovery. Many of the genes derived from structural screens in the 1980's have yet to be fully characterized. Further exploration of these mutant strains could lead to many valuable discoveries.

Molecular Pathway Mutants

The first mutants shown to affect learning in fruit flies impaired cell signaling pathways. This makes perfect sense because cell signaling leads to physiological and structural changes in the neuron, which is believed to underlie behavioral modification. A review of the literature reveals a wide variety of genes which affect learning and memory behavior in *Drosophila* (Table 2-2). Many affect different cell signaling pathways but others have a less clear explanation about their affect on behavior.

Cell signaling pathways involve the binding of extracellular signaling molecules (often neurotransmitters) to cell-surface receptors and trigger events inside the cell. In eukaryotic cells, most intracellular proteins activated by a ligand/receptor interaction possess an enzymatic activity. These enzymes include tyrosine kinase, heterotrimeric G proteins, small GTPases, various serine/threoine protein kinases, phosphatases, lipid kinases, and hydrolases. Some receptor-stimulated enzymes create specific second messengers including cyclic nucleotides, such as cyclic AMP (cAMP) and cyclic GMP (cGMP), Phosphatidylinositol derivatives, such as Phosphatidylinositol-triphosphate (PIP_3), Diacylglycerol (DAG) and

Mutant	Product	Structure	Conditioning Paradigm	Behavioral deficit	reference
alpha lobes absent (ala)		MB	POC	LTM	Boquet[141]
calyx buldging (cxb)		MB	ARC	learning	Heisenberg[90]
central body defect (cbd)		CCX	ARC	learning	Heisenberg[90]
central brain deranged (ceb)		MB,CCX			de Belle[91]
central complex broad (ccb)		CCX	ARC	learning	Heisenberg[90]
central complex deranged (ccd)		CCX	ARC		Heisenberg[90]
ellipsoid body open (ebo)					Heisenberg[90]
fused mushroom bodies (fum)			POC	30 min mem	de Belle[91]
latheo (lat)	component of origin recognition complex	MB, larval NMJ	POC	Learning	Boynton[142]
linotte (lio)	receptor tyrosine kinase (*drl*)	MB, CCX	POC	Learning	Dura[143]
no bridge (nob)		CCX	ARC/ PPC/ PHC	Learning	Heisenberg[90]
minibrain (mnb)	Ser/Thr Protein kinase	Whole brain	POC	learning	Heisenberg[90]
mushroom body defect (mud)			POC	30 min mem	Heisenberg[84]
mushroom bodies deranged (mbd)		MB	POC/ OOC/ ARC	30 min mem	Heisenberg[84]
mushroom body miniature (mbm)	transcription factor	MB	ARC	learning ♀only	Heisenberg[90]
mushroom body miniature B (mbmB)	*importin α 2*	MB	POC/ARC	Learning, LTM	Heisenberg[90]
mushroom body miniature C (mbmC)					Heisenberg[90]
mushroom bodies reduced (mbr)		MB		?	de Belle[91]
small mushroom bodies (smu)		MB	POC	Learning	de Belle[91]

Table 2-1. Structural genes affecting learning and memory. ARC, Arena Reward Conditioning;OOC, Operant Olfactory Conditioning; PHC, PER Habituation conditioning; POC, Pavlovian Olfactory Conditioning; PPC, Pavlovian PER Conditioning; CCX, Central Complex; MB, Mushroom Bodies; NMJ, NeuroMuscular Junction; ARM, Anesthesia Sensitive Memory; LTM, Long Term Memory.

Inositol-triphosphate (IP_3), IP_3, controlling the release of intracellular calcium stores into the cytoplasm. Neurotransmitters and their receptors are the triggers which cause cell signaling cascades to begin, therefore genes necessary for neurotransmitter biosynthesis enzymes and receptors are likely critical for learning and memory behavior.

Neurotransmitter biosynthesis and receptors. Recently evidence has pointed in the direction of dopamine and octopamine as promising candidates critical for learning behavior in fruit flies [38,103,144]. The enzyme Tyrosine Hydroxylase (TH) is critical for dopamine production. Inhibition of TH-positive neurons using *shibire ts* disrupts appetitive learning. Mutants in the Tyramine β Hydroxylase (TβH) encoding gene can't produce octopamine and have disrupted aversive memory[38]. Receptors for dopamine and octopamine have been examined as well. Several have been reported as preferentially expressing in the MBs[144-146] and a dopamine receptor mutant *dumb1* demonstrated reduced aversive and appetitive learning[39]. A third neurotransmitter glutamate was implicated when the dNR1 mutant gene coding an N-methyl-D-aspartic acid (NMDR) receptor revealed reduced learning and LTM[147]. As expected, disruption of neurotransmitter biosynthesis and reception can impair learning and memory. Downstream components of these cascades are also implicated.

Cell signaling cascades. The cAMP pathway is likely the most extensively studied signaling cascade involved in learning and memory in all model organisms. It was first implicated in the sea hare *Aplysia californica* [174,175] and mutant studies in fruit flies revealed enzymes in the pathway affected learning

Gene Name	Product	Pathway	Conditioning Paradigm	Behavioral deficit	REF
dunce (dnc)	cAMP phosphodiesterase	cAMP	POC/OOC/CC	Learning	Dudai[136]
rutabaga (rut)	calcium/calmodulin-activated adenyl cyclase	cAMP	POC/OOC/CC	STM	Livingstone[63]
amnesiac (amn)	neuropeptide related to PACAP	cAMP	POC/OOC/CC	MTM	Quinn[66]
DCO	catalytic subunit of PKA	cAMP	POC	STM	Skoulakis[148]
PKA-R1	regulatory subunit of PKA	cAMP	POC /CC	STM	Buchner[149]
Gαs	guanosine-triphosphate binding protein α subunit	cAMP	POC	Learning	Connelly[150]
CREB	cAMP response element binding protein	cAMP	POC	LTM	Yin[151]
PKC	protein kinase K	PKC	CC	Learning	Kane[61]
αPKM	atypical PKM	PKC	CC	ARM	Drier[152]
turnip(tur)	?	PKC?	POC/OOC/CC	nonassociative	Choi[153]
ignorant (ign)	ribosomal S6 kinase	ERK/MAPK	OOC/ HBC	learning	Brembs[55]
leonardo (leo)	14-3-3	Ras/Raf/MAPK ?	POC	learning	Skoulakis[154]
Neurofibromin (NF1)	Ras-GTPase activating protein		POC	learning	Guo[155]
CamKII	Cam Kinase II	CamKII	CC	learning	Joiner[156]
Notch (N)	Notch	Notch	POC/ CC	LTM	Presente[99]
volado (Vol)	α - integrin	cell adhesion	POC	STM	Grotewiel[157]
fasciclin II (fas II)	fasciclin II	cell adhesion	POC	STM	Cheng[158]
pumullio (pum)		RNA transport	POC	LTM	Dubnau[159]
staufen (stau)		RNA transport	POC	LTM	Dubnau[159]
oskar (osk)		RNA transport	POC	LTM	Dubnau[159]
krasavietz (elf – 5C)		RNA transport	POC	LTM	Dubnau[159]
synapsins (syn)	synapsins		HBC, POC, CC	Learning	Godenschwege[160]
TH/ dopamine	dopamine producing enzyme	neurotransmitter biosynthesis	POC	Learning	Schwaerzel[38]
TβH/ octopamine	octopamine producing enzyme	neurotransmitter biosynthesis	POC	Learning	Schwaerzel[38]
damb	dopamine receptor	receptor			Han[145]
dDA1/DMDOP1(dumb)	dopamine receptor	receptor	POC	learning	Kim[39]
oamb	octopamine receptor	receptor			Han[146]
		Continued.....			

Gene Name	Product	Pathway	Conditioning Paradigm	Behavioral deficit	REF
radish (rsh)	Novel protein with 23 predicted cyclic-AMP-dependent protein kinase (PKA) phosphorylation sequences	?	POC/OOC/CC	ARM	Folkers[161]
nalyot (nal)	Myb relaterd Adf1 transcription factor	?	POC	LTM	Dezazzo[162]
crammer (cre)	cystein protease inhibitor	?	POC	LTM	Comas[163]
murashka (mur)	?	?	POC	LTM	Dubnau[159]
dNR1	NMDA receptor	?	POC	Learning	Xia[147]
Dfmr1	ortholog of human fmr1		CC	Learning	McBride[164]
nebula(neb)	ortholog of human DSCR1 inhibitor of calcineurin		POC	Learning, LTM	Chang[165]
armitage (armi)	RNA-induced silencing complex		POC	LTM	Ashraf[166]
tequila (teq)	Neurotrypsin		POC	LTM	Didelot[167]
cabbage (cab)			OLC	Learining	Booker[168]
foraging (for)	cyclic guanosine-3',5'-monophosphate (cGMP)-dependent protein kinase (PKG)		OVL	Learning	Wang[129]
resistance to dieldrin (Rdl)	GABAA receptor		POC	Learning	Liu[169]
yu	A-kinase anchoring protein (AKAP)		POC	LTM	Lu[170]
presenilin (psn)			LPOV	Learning	Knight[171]
chi	tyrosine phosphatase		POC	LTM	Qian[172]
nemy			CC, POC	Learning, STM	Kamyshev[173]

Table 2-2. Biochemical pathway genes affecting learning and memory. ARC, Arena Reward Conditioning;CC, Conditioned Courtship; LVOC Larval Pavlovial Olfactory Learning; OOC, Operant Olfactory Conditioning; Operant Leg Conditioning; Operant Visual Learning; PHC, PER Habituation conditioning; POC, Pavlovian Olfactory Conditioning; PPC, Pavlovian PER Conditioning; CCX, Central Complex; MB, Mushroom Bodies; NMJ, NeuroMuscular Junction; ARM, Anesthesia Sensitive Memory; LTM, Long Term Memory.

and memory behavior[66,136,137]. It also has proven to be an important signaling cascade in multiple forms of learning including habituation, sensitization, classical, and operant learning and multiple sensory modalities including visual and olfactory[7,37,176]. Mutant genes at different levels of this cascade also affect learning and multiple stages of memory. While genes involved early in the pathway such as *dunce* (cAMP phosphodiesterase II) affects learning[136], others such as *PKA-R1* (protein kinase A catalytic subunit) affects STM[177], *amnesiac* (neuropeptide related to PCAP) affects MTM[66], and *dCREB2* (cAMP response element binding protein) affects LTM[151]. This preponderance of evidence clearly implicates that cAMP signaling cascades play an integral role in learning and memory consolidation. However, further mutant studies reveal it is not the only one.

Several other signaling cascades are implicated in learning in memory including the phosphoinositol cascade through the implication of protein kinase C (PKC).Typical PKC is activated by the second messengers IP_3 (inositol 1,4,5 triphosphate) and DAG (Diacylglycerol) and leads to a variety of different actions in different isoforms and cell types[178]. PKC was first implicated in learning and memory based on the discovery of the *turnip* (*tur*) mutant. This strain exhibited reduced PKC activity and was initially described as a learning mutant[153]. Unfortunately, it was later shown that *tur* failed some necessary sensory acuity controls and therefore it is difficult to assess PKCs role in learning using tur^9. One experiment using temporally restricted expression of a selective PKC inhibitor in the brain, exhibited transgenic flies that failed to show depression of behavior

immediately after associative male courtship suppression training but did show suppression one hour later[61]. The generation of more mutants affecting PKC and other elements of the pathway are necessary to better interpret these results. Other signaling molecules implicated in learning such as Cam kinase II and a 14-3-3 protein *leonardo* (*leo*) potentially involved in the Ras/Raf/MAPK signaling pathway highlight the fact that we only know a small amount of the signaling pathways involved in learning and even less about how they interact. Many of the signaling cascade molecules discussed here have been shown to interact for some processes such as cell proliferation (for review see[179]) so it is possible they do interact in a variety of complex ways to contribute to behavioral modification.

Cell adhesion molecules. Another class of molecules implicated in learning is cell adhesion molecules such as the proteins encoded by the *volado* (*vol*) and *fasciclin II* (*fasII*) genes. The gene *vol* codes for an α-integrin and it is not known how it influences behavior. One possibility is engagement of the Ras/Raf/MAPK pathway which has been demonstrated for α-integrins in other types of cell to cell interactions[180]. Fas II is a member of the immunoglobulin superfamily which is related to vertebrate neural cell-adhesion molecule (NCAM) in both structure and sequence[181]. Rapid reduction of FasII at the larval NMJ coincides with MAPK activation[182]. These and other cell adhesion molecules may influence behavior through activation of signaling cascades.

Translation and RNA transport mutants. Strains in this group all originated in screens for LTM mutants. Previous experiments have shown that LTM is protein synthesis dependant, therefore it makes sense these types of genes would be

isolated in a defective LTM screen[64]. Proteins involved in translational control include the translational repressor Pumillio (*milord*) and the transcription initiation factor eIF-5c (*krasavietz*) [159]. Proteins involved in cellular mRNA transport such as the product of *staufen* (*stau*) also have been implicated[159]. This supports the hypothesis that memory formation is dependent on the translation of preexisting mRNA as was previously indicated in *aplysia* cultured neurons[183].

The diversity of genes with well-established roles in learning and memory is likely only the tip of the iceberg. Mutagenesis screen have one primary drawback. Many of the genes that will prove to be most critical for learning may also be critical for survival and may never by seen as viable mutants. However, researchers today are trying many different approaches to get around this problem including DNA microarray experiments looking for changes in gene expression after conditioning[159] and temporally controlled RNA interference to determine the effects in adulthood of larval lethal genes[99]. Likely the list of genes known to affect learning and memory will continue to expand exponentially in the coming years.

Conclusions

As discussed in this chapter a great deal of information pertaining to how learning and memory is processed has been gained through examination of the fruit fly *Drosophila melanogaster*. A plethora of behavioral conditioning procedures are available to train flies by isolating particular sensory modalities

and precisely controlling stimuli presentation. Behavioral results have been correlated with environmental, pharmacological and genetic manipulation of neuronal structures and biochemical pathways to expand our understanding of the biological underpinnings of behavior. Learning and memory research by the biological sciences has been conducted for over 100 years resulting in many important discoveries. Furthering our understanding of the learning and memory mechanisms of the fruit fly will continue will continue to provide new insight into the mechanisms which underlie our own learning and memory abilities.

Bibliography

1. Thorpe, W. *Learning and instinct in animals* (Methuen, London, 1956).
2. Miller, N. in *The Nuerosciences: A Study Program* (eds. Quarton, G., Melnechuk, T. & Schmitt, F.) (University Press, New York, 1967).
3. Bush, R. & Mosteller, F. *Stochastic Models for Learning* (Wiley, New York, 1955).
4. Harris, J. Habituation response decrement in the intact organism. *Psychological Bulletin* **40**, 385-422. (1943).
5. Kimble, G. *Hilgard and Marquis' Conditioning and Learning* (Appleton-Century-Crofts, New York, NY, 1961).
6. Razran, G. *Mind in Evolution: And East-West Synthesis of Learned Behavior and Cognition* (Houghton Mifflin, Boston, 1971).
7. Duerr, J. S. & Quinn, W. G. Three Drosophila mutations that block associative learning also affect habituation and sensitization. *Proc Natl Acad Sci U S A* **79**, 3646-50 (1982).
8. Scheiner, R., Sokolowski, M. B. & Erber, J. Activity of cGMP-Dependent Protein Kinase (PKG) Affects Sucrose Responsiveness and Habituation in Drosophila melanogaster. *Learn. Mem.* **11**, 303-311 (2004).
9. Mihalek, R. M., Jones, C. J. & Tully, T. The Drosophila mutation turnip has pleiotropic behavioral effects and does not specifically affect learning. *Learn Mem* **3**, 425-44 (1997).

10. Cho, W., Heberlein, U. & Wolf, F. W. Habituation of an odorant-induced startle response in Drosophila. *Genes, Brain and Behavior* **3**, 127-137 (2004).

11. Wolf, F. W., Eddison, M., Lee, S., Cho, W. & Heberlein, U. GSK-3/Shaggy regulates olfactory habituation in *Drosophila*. *Proceedings of the National Academy of Sciences* **104**, 4653-4657 (2007).

12. Asztalos, Z., Arora, N. & Tully, T. Olfactory Jump Reflex Habituation in *Drosophila* and Effects of Classical Conditioning Mutations. *Journal of Neurogenetics* **21**, 1 - 18 (2007).

13. Asztalos, Z., Baba, K., Yamamoto, D. & Tully, T. The *fickle* Mutation of a Cytoplasmic Tyrosine Kinase Effects Sensitization But Not Dishabituation in *Drosophila Melanogaster*. *Journal of Neurogenetics* **21**, 59 - 71 (2007).

14. Acevedo, S. F., Froudarakis, E. I., Kanellopoulos, A. & Skoulakis, E. M. C. Protection from premature habituation requires functional mushroom bodies in *Drosophila*. *Learn. Mem.* **14**, 376-384 (2007).

15. Corfas, G. & Dudai, Y. Habituation and dishabituation of a cleaning reflex in normal and mutant Drosophila. *J Neurosci* **9**, 56-62 (1989).

16. Jin, P., Griffith, L. C. & Murphey, R. K. Presynaptic calcium/calmodulin-dependent protein kinase II regulates habituation of a simple reflex in adult Drosophila. *J Neurosci* **18**, 8955-64 (1998).

17. Asztalos, Z. et al. Protein phosphatase 1-deficient mutant Drosophila is affected in habituation and associative learning. *J Neurosci* **13**, 924-30 (1993).

17. Asztalos, Z. et al. Protein phosphatase 1-deficient mutant Drosophila is affected in habituation and associative learning. *J Neurosci* **13**, 924-30 (1993).

18. Rees, C. T. & Spatz, H.-C. o. Habituation of the Landing Response of *Drosophila* Wild-Type and Mutants Defective in Olfactory Learning. *Journal of Neurogenetics* **5**, 105 - 118 (1989).

19. Engel, J. E. & Wu, C.-F. Genetic Dissection of Functional Contributions of Specific Potassium Channel Subunits in Habituation of an Escape Circuit in Drosophila. *The Journal of Neuroscience* **18**, 2254-2267 (1998).

20. Engel, J. E. & Wu, C. F. Altered habituation of an identified escape circuit in Drosophila memory mutants. *J Neurosci* **16**, 3486-99 (1996).

21. Engel, J. E., Xie, X.-J., Sokolowski, M. B. & Wu, C.-F. A cGMP-Dependent Protein Kinase Gene, *foraging*, Modifies Habituation-Like Response Decrement of the Giant Fiber Escape Circuit in Drosophila. *Learning and Memory* **7**, 341-352 (2000).

22. Sanes, J. & Ison, J. Habituation and sensitization of components of the human eyeblink reflex. *Behavioral Neuroscience* **5**, 833-836 (1983).

23. Leussis, M. P. & Bolivar, V. J. Habituation in rodents: A review of behavior, neurobiology, and genetics. *Neuroscience & Biobehavioral Reviews* **30**, 1045-1064 (2006).

24. Bao, S. C., Lu; Thompson, Richard F. Classical eyeblink conditioning in two strains of mice: Conditioned responses, sensitization, and spontaneous eyeblinks. *Behavioral Neuroscience* **112**, 714-718 (1998).

25. Peckham, G. W. & Peckham, E. G. Some observations on the Mental Powers of Spiders. *j. Morphol.* **1**, 383-419 (1887).

26. Palka, J. & Babu, K. Toward the physiological analysis of defensive responses in scorpions. *Z. vergl. Physiologie* **55**, 286-298 (1967).

27. Pinsker, H., Kupfermann, I., Castellucci, V. & Kandel, E. Habituation and dishabituation of the gill-withdrawal reflex in Aplysia. *Science* **167**, 1740-2 (1970).

28. Castellucci, V. F. & Kandel, E. R. A quantal analysis of the synaptic depression underlying habituation of the gill-withdrawal reflex in Aplysia. *Proc Natl Acad Sci U S A* **71**, 5004-8 (1974).

29. Carew, T. J., Pinsker, H. M. & Kandel, E. R. Long-term habituation of a defensive withdrawal reflex in Aplysia. *Science* **175**, 451-454 (1972).

30. Carew, T. J., Castellucci, V. F. & Kandel, E. R. An analysis of dishabituation and sensitization of the gill-withdrawal reflex in Aplysia. *Int J Neurosci* **2**, 79-98 (1971).

31. Thompson, R. F. & Spencer, W. A. Habituation: a model phenomenon for the study of neuronal substrates of behavior. *Psychol Rev* **73**, 16-43 (1966).

32. Jennings, H. Studies on reactions to stimuli in unicellular organisms. IX. On the behavior of fixed infusoria (*Stentor* and *Vorticella*) with special reference to modifiability of protozoan reactions. *American Journal of Physiology* **8**, 23-60. (1901).

33. Applewhite, P. Temperature and habituation in protozoan. *Nature* **219**, 91-92. (1968).

34. Moore, B. R. The evolution of learning. *Biological Reviews* **79**, 301-335 (2004).

35. Revusky, S. & Garcia, J. *Learned associations over long delays* (ed. Bower, G. H.) (Academic Press, New York, 1970).

36. Thorndike, E. L. Animal Intelligence: An Experimental Study of the Associative Processes of Animals. *Psychological Review* **2**, 1-109 (1898).

37. Tully, T. & Quinn, W. G. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J Comp Physiol [A]* **157**, 263-77 (1985).

38. Schwaerzel M, M. M., Scholz H, Friggi-Grelin F, Birman S, Heisenberg M. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. *J Neurosci* **23**, 10495-502 (2003).

39. Kim, Y.-C., Lee, H.-G. & Han, K.-A. D1 Dopamine Receptor dDA1 Is Required in the Mushroom Body Neurons for Aversive and Appetitive Learning in Drosophila. *J. Neurosci.* **27**, 7640-7647 (2007).

40. Holliday, M. & Hirsch, J. Excitatory conditioning of individual Drosophila melanogaster. *J Exp Psychol Anim Behav Process* **12**, 131-42 (1986).

41. Mehren, J. E., Ejima, A. & Griffith, L. C. Unconventional sex: fresh approaches to courtship learning. *Current Opinion in Neurobiology* **14**, 745-750 (2004).

42. Brembs, B. & Heisenberg, M. The operant and the classical in conditioned orientation of Drosophila melanogaster at the flight simulator. *Learn Mem* **7**, 104-15 (2000).

43. Scherer S, S. R., Gerber B. Olfactory learning in individually assayed Drosophila larvae. *Learn Mem* **10**, 217-25 (2003).

44. Gerber, B. et al. Visual learning in individually assayed Drosophila larvae. *J Exp Biol* **207**, 179-188 (2004).

45. Brembs B, H. M. Conditioning with compound stimuli in Drosophila melanogaster in the flight simulator. *J Exp Biol* **204**, 2849-2859 (2001).

46. Tabone, C. & de Belle, J. S. in *12TH European Drosophila Neurobiology Conference* (Würzburg, Germany, 2008).

47. Bitterman, M. E., Menzel, R., Fietz, A. & Schäfer, S. Classical conditioning of proboscis extension in honeybees (Apis mellifera). *Journal of Comparative Psychology* **97**, 107-119 (1983).

48. Daly, K. & Smith, B. Associative olfactory learning in the moth Manduca sexta. *J Exp Biol* **203**, 2025-2038 (2000).

49. Wustmann, G. & Heisenberg, M. Behavioral manipulation of retrieval in a spatial memory task for Drosophila melanogaster. *Learn Mem* **4**, 328-336 (1997).

50. Wolf, R. & Heisenberg, M. Basic organization of operant behavior as revealed in Drosophila flight orientation. *J Comp Physiol [A]* **169**, 699-705 (1991).

51. Liu, L., Wolf, R., Ernst, R. & Heisenberg, M. Context generalization in Drosophila visual learning requires the mushroom bodies [see comments]. *Nature* **400**, 753-6 (1999).

52. Peng, Y., Xi, W., Zhang, W., Zhang, K. & Guo, A. Experience Improves Feature Extraction in *Drosophila*. *J. Neurosci.* **27**, 5139-5145 (2007).

53. Yarali, A., Mayerle, M., Nawroth, C. & Gerber, B. No evidence for visual context-dependency of olfactory learning in Drosophila. *Naturwissenschaften* **95**, 767-774 (2008).

54. Tang, S. & Guo, A. Choice Behavior of *Drosophila* Facing Contradictory Visual Cues. *Science* **294**, 1543-1547 (2001).

55. Brembs, B. Operant conditioning in invertebrates. *Current Opinion in Neurobiology* **13**, 710-717 (2003).

56. Erber, J., Pribbenow, B., Kisch, J. & Faensen, D. Operant conditioning of antennal muscle activity in the honey bee (Apis mellifera L.). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **186**, 557-565 (2000).

57. Dyer, A. G., Neumeyer, C. & Chittka, L. Honeybee (Apis mellifera) vision can discriminate between and recognise images of human faces. *J Exp Biol* **208**, 4709-4714 (2005).

58. D'Hooge, R. & De Deyn, P. P. Applications of the Morris water maze in the study of learning and memory. *Brain Research Reviews* **36**, 60-90 (2001).

59. Goodall, J. *In The Shadow of Man* (Collins, London, UK, 1971).

60. Varga, M., Pavlova, O. & Mats, V. Role of the parietal associative area of the cortex for counting behavior in dogs. *Neuroscience and Behavioral Physiology* **37**, 791-797 (2007).

61. Kane, N. S., Robichon, A., Dickinson, J. A. & Greenspan, R. J. Learning without Performance in PKC-Deficient Drosophila. *Neuron* **18**, 307-314 (1997).

62. Byers, D. A review of the behavior and biochemistry of dunce, a mutation of learning in Drosophila. *Basic Life Sci* **16**, 467-74 (1980).

63. Livingstone, M. S. Genetic dissection of Drosophila adenylate cyclase. *Proc Natl Acad Sci U S A* **82**, 5992-6 (1985).

64. Tully, T., Preat, T., Boynton, S. C. & Del Vecchio, M. Genetic dissection of consolidated memory in Drosophila. *Cell* **79**, 35-47 (1994).

65. Margulies, C., Tully, T. & Dubnau, J. Deconstructing Memory in Drosophila. *Current Biology* **15**, R700-R713 (2005).

66. Quinn, W. G., Sziber, P. P. & Booker, R. The Drosophila memory mutant amnesiac. *Nature* **277**, 212-4 (1979).

67. Quinn, W. G. & Dudai, Y. Memory phases in Drosophila. *Nature* **262**, 576-7 (1976).

68. Tempel, B. L., Bonini, N., Dawson, D. R. & Quinn, W. G. Reward learning in normal and mutant Drosophila. *Proc Natl Acad Sci U S A* **80**, 1482-6 (1983).

69. Isabel, G., Pascual, A. & Preat, T. Exclusive consolidated memory phases in Drosophila. *Science* **304**, 1024-7 (2004).

70. Waddell, S. Forgetting those painful moments. *Neuron* **35**, 815-7 (2002).

71. Schwaerzel M, H. M., Zars T. Extinction antagonizes olfactory memory at the subcellular level. *Neuron* **35**, 951-960 (2002).

72. Mayes, R., Bagwell, C. & Erkulwater, J. ADHD and the Rise in Stimulant Use Among Children. *Harvard Review of Psychiatry* **16**, 151 - 166 (2008).

73. van Swinderen, B. & Greenspan, R. J. Salience modulates 20-30 Hz brain activity in Drosophila. *Nature Neuroscience* **6**, 579-586 (2003).

74. van Swinderen, B. Attention-Like Processes in Drosophila Require Short-Term Memory Genes. *Science* **315**, 1590-1593 (2007).

75. Walters, E. T. & Byrne, J. H. Associative conditioning of single sensory neurons suggests a cellular mechanism for learning. *Science* **219**, 405-8 (1983).

76. Benton, R., Sachse, S., Michnick, S. W. & Vosshall, L. B. Atypical Membrane Topology and Heteromeric Function of Drosophila Odorant Receptors In Vivo. *PLoS Biology* **4**, e20 (2006).

77. Vosshall, L. B. W., A. M.; Axel, R. An Olfactory Sensory Map in the Fly Brain. *Cell* **102**, 147-159 (2000).

78. Fishilevich, E. & Vosshall, L. B. Genetic and Functional Subdivision of the Drosophila Antennal Lobe. *Current Biology* **15**, 1548-1553 (2005).

79. Vosshall, L. B. & Stocker, R. F. Molecular Architecture of Smell and Taste in Drosophila. *Annual Review of Neuroscience* **30**, 505-533 (2007).

80. Stocker, R. F., Heimbeck, G., Gendre, N. & de Belle, J. S. Neuroblast ablation in Drosophila P[GAL4] lines reveals origins of olfactory interneurons. *J Neurobiol* **32**, 443-56 (1997).

81. Stocker, R. F., Lienhard, M. C., Borst, A. & Fischbach, K. F. Neuronal architecture of the antennal lobe in Drosophila melanogaster. *Cell Tissue Res* **262**, 9-34 (1990).

82. Strausfeld, N. J. *Atlas Of An Insect Brain* (Springer-Verlag, Heidelberg, Germany, 1976).

83. Strausfeld NJ, H. L., Li Y, Gomez RS, Ito K. Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn Mem* **5**, 11-37 (1998).

84. Heisenberg, M. in *Development and Neurobiology of Drosophila* (eds. Siddiqi, O., Babu, P., Hall, L. M. & Hall, J. C.) 373-390 (Plenum Press, New York, 1980).

85. Armstrong, J. D., de Belle, J. S., Wang, Z. & Kaiser, K. Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in Drosophila. *Learn Mem* **5**, 102-14 (1998).

86. Crittenden, J. R., Skoulakis, E. M., Han, K. A., Kalderon, D. & Davis, R. L. Tripartite mushroom body architecture revealed by antigenic markers. *Learn Mem* **5**, 38-51 (1998).

87. Heisenberg, M. Mushroom body memoir: from maps to models. *Nat Rev Neurosci* **4**, 266-75 (2003).

88. Strausfeld, N. J., Sinakevitch, I. & Vilinsky, I. The mushroom bodies of Drosophila melanogaster: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes. *Microsc Res Tech* **62**, 151-69. (2003).

89. Johard, H. A. D. et al. Intrinsic neurons of *Drosophila* mushroom bodies express short neuropeptide F: Relations to extrinsic neurons expressing different neurotransmitters. *The Journal of Comparative Neurology* **507**, 1479-1496 (2008).

90. Heisenberg, M., Borst, A., Wagner, S. & Byers, D. Drosophila mushroom body mutants are deficient in olfactory learning. *J Neurogenet* **2**, 1-30 (1985).

91. de Belle, J. S. & Heisenberg, M. Expression of Drosophila mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). *Proc Natl Acad Sci U S A* **93**, 9875-80 (1996).

92. Pascual, A. P., Thomas. Localization of Long-Term Memory Within the Drosophila Mushroom Body. *Science* **294**, 1115-1117 (2001).

93. de Belle, J. S. & Heisenberg, M. Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. *Science* **263**, 692-5 (1994).

94. Dubnau, J., Grady, L., Kitamoto, T. & Tully, T. Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. *Nature* **411**, 433-4 (2001).

95. McGuire, S. E., Le, P. T. & Davis, R. L. The role of Drosophila mushroom body signaling in olfactory memory. *Science* **293**, 1330-3 (2001).

96. Krashes, M. J., Keene, A. C., Leung, B., Armstrong, J. D. & Waddell, S. Sequential Use of Mushroom Body Neuron Subsets during Drosophila Odor Memory Processing. *Neuron* **53**, 103-115 (2007).

97. Zars, T., Fischer, M., Schulz, R. & Heisenberg, M. Localization of a short-term memory in Drosophila. *Science* **288**, 672-5 (2000).

98. McGuire SE, L. P., Osborn AJ, Matsumoto K, Davis RL. Spatiotemporal rescue of memory dysfunction in Drosophila. *Science* **302**, 1765-68 (2003).

99. Presente, A., Boyles, R. S., Serway, C. N., de Belle, J. S. & Andres, A. J. Notch is required for long-term memory in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 1764-1768 (2004).

100. Kandel, E. The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**, 1030-8 (2001).

101. Hebb, D. *The Organization of behavior* (John Wiley, New York, 1949).

102. Quinn, W. G., Harris, W. A. & Benzer, S. Conditioned behavior in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **71**, 708-12 (1974).

103. Riemensperger, T., Völler, T., Stock, P., Buchner, E. & Fiala, A. Punishment Prediction by Dopaminergic Neurons in Drosophila. *Current Biology* **15**, 1953-1960 (2005).

104. Schroll, C. et al. Light-Induced Activation of Distinct Modulatory Neurons Triggers Appetitive or Aversive Learning in Drosophila Larvae. *Current Biology* **16**, 1741-1747 (2006).

105. Sinakevitch, I. & Strausfeld, N. J. Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. *The Journal of Comparative Neurology* **494**, 460-475 (2006).

106. Wang, Y., Mamiya, A., Chiang, A.-s. & Zhong, Y. Imaging of an Early Memory Trace in the Drosophila Mushroom Body. *J. Neurosci.* **28**, 4368-4376 (2008).

107. Yu, D., Akalal, D.-B. G. & Davis, R. L. Drosophila [alpha]/[beta] Mushroom Body Neurons Form a Branch-Specific, Long-Term Cellular Memory Trace after Spaced Olfactory Conditioning. *Neuron* **52**, 845-855 (2006).

108. Wolf, R. et al. Drosophila mushroom bodies are dispensable for visual, tactile, and motor learning. *Learn Mem* **5**, 166-78 (1998).

109. Menzel, R. Memory dynamics in the honeybee. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **185**, 323-340 (1999).

110. Malun D, P. N., Giurfa M, Moseleit AD, Muller U. Hydroxyurea-induced partial mushroom body ablation in the honeybee Apis mellifera: volumetric analysis and quantitative protein determination. *J Neurobiol* **50**, 31-44 (2002).

111. Ikeda, K., Numata, H. & Shiga, S. Roles of the mushroom bodies in olfactory learning and photoperiodism in the blow fly Protophormia

terraenovae. *Journal of Insect Physiology Diapause and Biological Clocks* **51**, 669-680 (2005).

112. Mizunami, M., Weibrecht, J. M. & Strausfeld, N. J. Mushroom bodies of the cockroach: their participation in place memory. *J Comp Neurol* **402**, 520-37 (1998).

113. Gronenberg, W. Subdivisions of hymenopteran mushroom body calyces by their afferent supply. *J Comp Neurol* **435**, 474-89 (2001).

114. Yu, D., Ponomarev, a. & Davis, R. Altered Representation of the Spatial Code for Odors after Olfactory Classical Conditioning: Memory Trace Formation by Synaptic Recruitment. *Neuron* **42**, 437-449 (2004).

115. Waddell, S., Armstrong, J. D., Kitamoto, T., Kaiser, K. & Quinn, W. G. The *amnesiac* Gene Product Is Expressed in Two Neurons in the *Drosophila* Brain that Are Critical for Memory. *Cell* **103**, 805-813 (2000).

116. Keene, A. et al. Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output. *Neuron* **44**, 521-33 (2004).

117. Yu, D., Keene, A. C., Srivatsan, A., Waddell, S. & Davis, R. L. Drosophila DPM Neurons Form a Delayed and Branch-Specific Memory Trace after Olfactory Classical Conditioning. *Cell* **123**, 945-957 (2005).

118. Wu, C.-L. et al. Specific requirement of NMDA receptors for long-term memory consolidation in Drosophila ellipsoid body. **10**, 1578-1586 (2007).

119. Hanesch, U., Fischbach, K.-F. & Heisenberg, M. Neuronal architecture of the central complex in Drosophila melanogaster. *Cell Tissue Res.* **257**, 343-366 (1989).

120. Strauss, R. & Heisenberg, M. A higher control center of locomotor behavior in the Drosophila brain. *J Neurosci* **13**, 1852-61 (1993).

121. Dudai, Y. The Neurobiology of Consolidations, Or, How Stable is the Engram? *Annual Review of Psychology* **55**, 51-86 (2004).

122. Wiltgen, B. J., Brown, R. A. M., Talton, L. E. & Silva, A. J. New Circuits for Old Memories: The Role of the Neocortex in Consolidation. *Neuron* **44**, 101-108 (2004).

123. Morante, J. & Desplan, C. Building a projection map for photoreceptor neurons in the Drosophila optic lobes. *Seminars in Cell & Developmental Biology Protein Misfolding and Human Disease and Developmental Biology of the Retina* **15**, 137-143 (2004).

124. Ting, C.-Y. & Lee, C.-H. Visual circuit development in Drosophila. *Current Opinion in Neurobiology Development* **17**, 65-72 (2007).

125. Ibbotson, M. R., Maddess, T. & DuBois, R. A system of insect neurons sensitive to horizontal and vertical image motion connect the medulla and midbrain. *J Comp Physiol [A]* **169**, 355-367 (1991).

126. Gilbert, C. & Strausfeld, N. Small-field neurons associated with oculomotor and optomotor control in muscoid flies: functional organization. *J Comp Neurol* **316**, 72-86 (1992).

127. Otsuna, H. & Ito, K. Systematic analysis of the visual projection neurons of Drosophila melanogaster. I. Lobula-specific pathways. *The Journal of Comparative Neurology* **497**, 928-958 (2006).

128. Liu, G. et al. Distinct memory traces for two visual features in the Drosophila brain. **439**, 551-556 (2006).

129. Wang, Z. et al. Visual pattern memory requires foraging function in the central complex of *Drosophila*. *Learn. Mem.* **15**, 133-142 (2008).

130. Forjanic, J. P., Chen, C.-K., Jäckle, H. & Gaitán, M. G. Genetic Analysis of Stomatogastric Nervous System Development inDrosophilaUsing Enhancer Trap Lines. *Developmental Biology* **186**, 139-154 (1997).

131. Ayali, A. The Insect Frontal Ganglion and Stomatogastric Pattern Generator Networks. *Neurosignals* **13**, 20-36 (2004).

132. Watanabe, H. & Mizunami, M. Pavlov's Cockroach: Classical Conditioning of Salivation in an Insect. *PLoS ONE* **2**, e529 (2007).

133. Keshishian, H., Broadie, K., Chiba, A. & Bate, M. The Drosophila Neuromuscular Junction: A Model System for Studying Synaptic Development and Function. *Annual Review of Neuroscience* **19**, 545-575 (1996).

134. Zhong, Y., Budnik, V. & Wu, C. F. Synaptic plasticity in Drosophila memory and hyperexcitable mutants: role of cAMP cascade. *J Neurosci* **12**, 644-51 (1992).

135. Zhong, Y. & Wu, C. F. Altered synaptic plasticity in Drosophila memory mutants with a defective cyclic AMP cascade. *Science* **251**, 198-201 (1991).

136. Dudai, Y., Jan, Y. N., Byers, D., Quinn, W. G. & Benzer, S. dunce, a mutant of Drosophila deficient in learning. *Proc Natl Acad Sci U S A* **73**, 1684-8 (1976).

137. Aceves-Pina, E. O. et al. Learning and memory in Drosophila, studied with mutants. *Cold Spring Harb Symp Quant Biol* **48 Pt 2**, 831-40 (1983).

138. Tejedor, F. et al. minibrain: A new protein kinase family involved in postembryonic neurogenesis in Drosophila. *Neuron* **14**, 287-301 (1995).

139. Guimera, J. et al. A human homologue of Drosophila minibrain (MNB) is expressed in the neuronal regions affected in Down syndrome and maps to the critical region. *Hum Mol Genet* **5**, 1305-10 (1996).

140. Smith, D. J. et al. Functional screening of 2 Mb of human chromosome 21q22.2 in transgenic mice implicates minibrain in learning defects associated with Down syndrome [see comments]. *Nat Genet* **16**, 28-36 (1997).

141. Boquet, I. H., R; Dumas, M; Chaminade, M; Preat, T. Central brain postembryonic development in Drosophila: implication of genes expressed at the interhemispheric junction. *J Neurobiol* **42**, 33-48 (2000).

142. Boynton, S. & Tully, T. latheo, a new gene involved in associative learning and memory in Drosophila melanogaster, identified from P element mutagenesis [published erratum appears in Genetics 1992 Sep;132(1):289]. *Genetics* **131**, 655-72 (1992).

143. Dura, J. M., Preat, T. & Tully, T. Identification of linotte, a new gene affecting learning and memory in Drosophila melanogaster. *J Neurogenet* **9**, 1-14 (1993).

144. Kim, Y., Lee, H., Seong, C. & Han, K. Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of Drosophila melanogaster. *Gene expression patterns* **3**, 237-45 (2003).

145. Han, K. A., Millar, N. S., Grotewiel, M. S. & Davis, R. L. DAMB, a novel dopamine receptor expressed specifically in Drosophila mushroom bodies. *Neuron* **16**, 1127-35 (1996).

146. Han, K. A., Millar, N. S. & Davis, R. L. A novel octopamine receptor with preferential expression in Drosophila mushroom bodies. *J Neurosci* **18**, 3650-8 (1998).

147. Xia, S. et al. NMDA receptors mediate olfactory learning and memory in Drosophila. *Curr Biol* **15**, 603-15 (2005).

148. Skoulakis, E. M., Kalderon, D. & Davis, R. L. Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. *Neuron* **11**, 197-208 (1993).

149. Buchner, E. Genes expressed in the adult brain of Drosophila and effects of their mutations on behavior: a survey of transmitter- and second messenger-related genes. *J Neurogenet* **7**, 152-92 (1991).

150. Connolly, J. B. et al. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. *Science* **274**, 2104-7 (1996).

151. Yin, J. C. et al. Induction of a dominant negative CREB transgene specifically blocks long-term memory in Drosophila. *Cell* **79**, 49-58 (1994).

152. Drier EA, T. M., Cowan M, Wu P, Blace N, Sacktor TC, Yin JC. Memory enhancement and formation by atypical PKM activity in Drosophila melanogaster. *Nature Neuroscience* **5**, 289-90 (2002).

153. Choi, K. W., Smith, R. F., Buratowski, R. M. & Quinn, W. G. Deficient protein kinase C activity in turnip, a Drosophila learning mutant. *J Biol Chem* **266**, 15999-606 (1991).

154. Skoulakis, E. M. & Davis, R. L. Olfactory learning deficits in mutants for leonardo, a Drosophila gene encoding a 14-3-3 protein. *Neuron* **17**, 931-44 (1996).

155. Guo, H. F., Tong, J., Hannan, F., Luo, L. & Zhong, Y. A neurofibromatosis-1-regulated pathway is required for learning in Drosophila. *Nature* **403**, 895-8 (2000).

156. Joiner, M. A. & Griffith, L. C. Visual input regulates circuit configuration in courtship conditioning of Drosophila melanogaster. *Learn Mem* **7**, 32-42 (2000).

157. Grotewiel, M. S., Beck, C. D., Wu, K. H., Zhu, X. R. & Davis, R. L. Integrin-mediated short-term memory in Drosophila. *Nature* **391**, 455-60 (1998).

158. Cheng Y, E. K., Wu K, Rodan AR, Heberlein U, Davis RL. Drosophila fasciclinII is required for the formation of odor memories and for normal sensitivity to alcohol. *Cell* **105**, 757-68 (2001).

159. Dubnau, J. et al. in *Curr Biol* 286-296 (2003).

160. Godenschwege, T. A. R., Dietmar; Diegelmann,Sören; Eberle,Kai; Funk,Natalja; Heisenberg,Martin; Hoppe,Viviane; Hoppe,Jürgen; Klagges,Bert R. E.; Martin, Jean-René; Nikitina,Ekaterina A.; Putz,Gabi; Reifegerste,Rita; Reisch,Natascha; Rister,Jens; Schaupp,Michael; Scholz,Henrike; Schwärzel,Martin; Werner, Ursula; Zars, Troy; Buchner, D.Sigrid; Buchner,Erich. Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. *European Journal of Neuroscience* **20**, 611-622 (2004).

161. Folkers, E., Drain, P. & Quinn, W. G. Radish, a Drosophila mutant deficient in consolidated memory. *Proc Natl Acad Sci U S A* **90**, 8123-7 (1993).

162. DeZazzo, J. et al. nalyot, a Mutation of the Drosophila Myb-Related Adf1 Transcription Factor, disrupts Synapse Formation and Olfactory Memory. *Neuron* **27**, 145-158 (2000).

163. Comas, D., Petit, F. & Preat, T. Drosophila long-term memory formation involves regulation of cathepsin activity. *Nature* **430**, 460-63 (2004).

164. McBride, S. M. J. et al. Pharmacological Rescue of Synaptic Plasticity, Courtship Behavior, and Mushroom Body Defects in a Drosophila Model of Fragile X Syndrome. *Neuron* **45**, 753-764 (2005).

165. Chang, K. T., Shi, Y.-J. & Min, K.-T. The Drosophila homolog of Down's syndrome critical region 1 gene regulates learning: Implications for mental retardation. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 15794-15799 (2003).

166. Ashraf, S. I., McLoon, A. L., Sclarsic, S. M. & Kunes, S. Synaptic Protein Synthesis Associated with Memory Is Regulated by the RISC Pathway in Drosophila. *Cell* **124**, 191-205 (2006).

167. Didelot, G. et al. Tequila, a Neurotrypsin Ortholog, Regulates Long-Term Memory Formation in Drosophila. *Science* **313**, 851-853 (2006).

168. Booker, R. & Quinn, W. G. Conditioning of leg position in normal and mutant Drosophila. *Proc Natl Acad Sci U S A* **78**, 3940-4 (1981).

169. Liu, X., Krause, W. C. & Davis, R. L. GABAA Receptor RDL Inhibits Drosophila Olfactory Associative Learning. *Neuron* **56**, 1090-1102 (2007).

170. Lu, Y. et al. The AKAP Yu is required for olfactory long-term memory formation in Drosophila. *Proceedings of the National Academy of Sciences* **104**, 13792-13797 (2007).

171. Knight, D., Iliadi, K., Charlton, M. P., Atwood, H. L. & Boulianne, G. L. Presynaptic plasticity and associative learning are impaired in a *Drosophila presenilin* null mutant. *Developmental Neurobiology* **67**, 1598-1613 (2007).

172. Qian, M. et al. Receptor-Like Tyrosine Phosphatase PTP10D Is Required for Long-Term Memory in Drosophila. *J. Neurosci.* **27**, 4396-4402 (2007).

173. Kamyshev, N. et al. Novel memory mutants in Drosophila: behavioral characteristics of the mutant nemyP153. *BMC Neurosci* **3**, 9 (2002).

174. Castellucci, V. F. et al. Intracellular injection of t he catalytic subunit of cyclic AMP- dependent protein kinase simulates facilitation of transmitter

release underlying behavioral sensitization in Aplysia. *Proc Natl Acad Sci U S A* **77**, 7492-6 (1980).

175. Klein, M. & Kandel, E. R. Mechanism of calcium current modulation underlying presynaptic facilitation and behavioral sensitization in Aplysia. *Proc Natl Acad Sci U S A* **77**, 6912-6 (1980).

176. Gong Z, X. S., Liu L, Feng C, Guo A. Operant visual learning and memory in Drosophila mutants dunce, amnesiac and radish. *J Insect Physiol* **44**, 1149-1158 (1998).

177. Drain, P., Folkers, E. & Quinn, W. G. cAmp-dependent protein kinase and the disruption of learning in transgenic flies. *Neuron* **6**, 71-82 (1991).

178. Cameron, A. J. et al. Protein kinases, from B to C. *Biochemical Society Transactions* **035**, 1013-1017 (2007).

179. Fimia, G. M. & Sassone-Corsi, P. Cyclic AMP signalling. *J Cell Sci* **114**, 1971-1972 (2001).

180. Ruoslahti, E. Fibronectin and its integrin receptors in cancer. *Advanced Cancer Research* **76**, 1-20 (1999).

181. Cunningham, B. A. et al. Neural Cell Adhesion Molecule: Structure, Immunoglobulin-Like Domains, Cell Surface Modulation, and Alternative RNA Splicing. *Science* **236**, 799-806.

182. Hoeffer, C. A., Sanyal, S. & Ramaswami, M. Acute Induction of Conserved Synaptic Signaling Pathways in Drosophila Melanogaster. *J. Neurosci.* **23**, 6362-6372 (2003).

183. Martin, K. C. et al. Synapse-Specific, Long-Term Facilitation of Aplysia Sensory to Motor Synapses: A Function for Local Protein Synthesis in Memory Storage. *Cell* **91**, 927-938 (1997).

CHAPTER 3

DEVELOPMENTAL AND BEHAVIORAL DEFECTS CAUSED BY THREE *DROSOPHILA MUTATIONS*

One of the many approaches to studying learning and memory is to screen for gene mutations which affect the structural integrity of the brain in regions critical for learning and memory such as the MBs[1,2] and CCX[3,4]. Almost 30 years ago, researchers in the Heisenberg lab examined flies obtained from two ethane methyl sulfonate (EMS) mutagenesis screens and discovered a large number of mutations which affected these structures[5]. Many of those mutations are still poorly characterized to this day. Three of the mutant genes discovered in that study, *mushroom body miniature B* (*mbmB*), *mushroom bodies reduced* (*mbr*), and *small mushroom bodies* (*smu*) will be further characterized in this Dissertation.

The *Drosophila* mutant allele of *mbmB* was discovered in a collection of 1400 stocks of unknown genetic origin carrying EMS treated 2nd chromosomes (courtesy of J. Nüsslein- Volhard)[6,7]. Serial paraffin histology analysis revealed a reduced MB calyx volume (30% of WT), as well as a slim peduncle and lobes. Minor central complex defects were also noted in the original genetic background which diminished upon outcrossing to (*CS*)[7]. It was described as female sterile and exhibited reduced learning scores in an appetitive olfactory arena paradigm[6].

Recent work has also demonstrated a reduced viability and growth rate and that loss of *mbmB* function does not affect the early development of MB neuroectoderm and neuroblasts up to stage thirteen[8].

The mutant alleles of *mbr* and *smu* were found in a similar serial paraffin histology screen of EMS treated *X*-chromosomes[6,7]. In their original genetic background they displayed very similar phenotypes. Both have calyx volumes reduced to about 25% of wildtype, CCX defects including open ellipsoid bodies and misshapen noduli, both are male sterile and semi-lethal. When outcrossed to Canton S, *mbr* only exhibited minor changes however, all CCX defects were no longer evident in *smu* flies[7]. In our current investigations we have not found any *mbr* homozygous females and their presence is a rare event in *smu* flies as well. Until now, neither mutation has been characterized for behavioral defects.

The further characterization of these mutant lines will serve two important purposes. First, each of these mutations affects MB development and by examining defects resulting from them we will improve our understanding of this process. Also, as an ongoing project in our lab we are characterizing MB structural mutants to build a catalog of genes preferentially affecting particular lobes; which will then be correlated with their behavior to determine any patterns that may arise. The analysis of these three mutations is the start of this larger project. An expanding body of evidence supports the theory that deferent lobes perform distinct functions.

Drosophila MBs develop sequentially. Two distinct cell fate switches occur during mid-third instar and the pupal stage allowing the linear generation of first

the γ lobe neurons followed by α'/β' neurons and finally α/β neurons respectively from only 4 neuroblasts[9]. Spatial rescue experiments of the mutant adenylate cyclase gene *rut* specifically implicated the γ lobe in STM formation[10], *Shibire ts* disruption of neurotransmission and calcium imaging studies support a role for α'/β' in STM and MTM[11,12], and mutant analysis of the *ala* gene and calcium imaging studies implicate the α lobes in LTM formation[13,14]. These studies, demonstrate that our approach is a viable way to examine structure function relationships in MB lobes.

In this chapter I will establish that lobe structure is unaffected in wandering third instar *mbmB, smu* and *mbr* larvae. All three mutations have disrupted lobe formation in adult flies while *mbmB* and *smu* preferentially disrupt certain lobes, leaving others intact. I show that MB cell number is unaffected until late 3rd instar larval development in *mbmB* and *smu* flies. Two of the three mutations (*mbmB* and *smu*) have olfactory learning and memory defects. Finally, both odor and shock avoidance defects were revealed in *mbr* flies. These results expand our understanding of how these mutations affect MB development and what functions the MB lobes perform in learning and memory behavior.

Materials and Methods

Fly care

We cultured flies (20 males and 50 females) for behavior at equal density in plastic bottles with cotton plugs on 40 ml of standard Drosophila cornmeal and molasses medium at 24° while flies for microscopy were raised in a similar

manner in vials containing 8 ml of food. For all behavioral experiments we used *Canton S* (*CS*) as a control line for the mutant *mbmB, smu* and *mbr* strains. All mutations had been previously outcrossed to *CS* to ensure as little genetic background effects as possible.

All flies used for confocal laser scanning microscopy are listed in Table 3-1. We generated wild type heterozygous GFP-expressing flies by utilizing the GAL4-UAS system[15]. Flies carrying either a membrane bound or nuclear localized GFP reporter construct were crossed with five different GAL4 strains in which expression was reported in distinct subsets of MB neurons. Membrane-targeted GFP expression was examined in GAL4/ mCD8::GFP. Nuclear-localized GFP expression in GAL4/GFP:lacz nls was used to count KC nuclei. All five of these GAL4 elements were crossed into the mutant genetic backgrounds in order to visually compare them with wild type. Elements located on the second chromosome (Table 3-1) required recombination with *mbmB* carrying chromosomes. All other elements resided on different chromosomes than the mutant genes and required no recombination. Samples of the crossing schemes can be found in Figure 3-1. Since changes in genetic background have previously been shown to affect these brain phenotypes[7], paraffin mass histology[5] was used to verify that there was no change in calyx volume by these crosses.

Behavioral experiments

Associative odor learning, memory and sensory acuity controls were assayed using a Pavlovian conditioning T-maze paradigm as described

Name	Expression	Chromosome	reference
Genes			
mushroom body miniature B (mbmB)		*2nd*	[6]
small mushroom bodies (smu)		*X*	[7]
mushroom bodies reduced (mbr)		*X*	[7]
UAS-Reporter constructs			
P[UAS-mCD8::GFP.L]LL6 (mCD8::GFP)	membrane	*3rd*	[17]
P{UAS-GFP::lacZ.nls}30.1 (GFP::lacz nls)	nuclear	*2nd*	[18]
GAL4- constructs			
P[GAL4]201Y] (201Y)	γ and α/β lobes	*2nd*	[19]
P[GAL4]C739](C739)	α/β lobes	*2nd*	[19]
P[GAL4]C772] (C772)	γ, α′/β′ and α/β lobes	*2nd*	[19]
P[Mef2-GAL4.247] (247)	γ, α′/β′ and α/β lobes	*3rd*	[20]
P[GAL4]OK107] (OK107)	γ, α′/β′ and α/β lobes	*4th*	[21]

Table 3-1. Fly strains used to generate confocal images. Column 1 lists the genes, reporter constructs, and GAL4 constructs used in the anatomical analysis of the three mutant strains. Column 2 lists the locations of GFP and GAL4 expression of the reporter and GAL4 construct flies. Column 3 lists the chromosome the particular gene or construct is located. Column 4 lists the first reference for each gene or construct.

mbr & *smu* WITH CHROMOSOME-2 TRANSPOSON COMBINATIONS

G_1 *w/Y; al Bl/SM5 × FM7a/FM7a*

↙ ↘

G_2 *w/Y; ▽/▽ × w/FM7a; al Bl/2*$^+$ & *FM7a/Y; SM5/2*$^+$ *× w mbr/FM7a*

↘ ↙

G_3 *FM7a/Y; ▽/al Bl × w mbr/FM7a; SM5/2*$^+$

↙ ↘

G_4 *FM7a/Y; ▽/SM5 × w mbr/FM7a; ▽/SM5*

↙ ↘

G_5	*w mbr/Y; ▽/▽ & FM7a/Y; ▽/▽ × w mbr/FM7a; ▽/▽*

mbr & *smu* WITH CHROMOSOME-3 TRANSPOSON COMBINATIONS

G_1 *w/Y; TM3/TM6b × FM7a/FM7a*

↙ ↘

G_2 *w/Y; ▽/▽ × w/FM7a; TM3/3*$^+$ & *FM7a/Y; TM6b/3*$^+$ *× w mbr/FM7a*

↘ ↙

G_3 *FM7a/Y; ▽/TM3 × w mbr/FM7a; TM6b/3*$^+$

↙ ↘

G_4 *FM7a/Y; ▽/TM6b × w mbr/FM7a; ▽/TM6b*

↙ ↘

G_5	*w mbr/Y; ▽/▽ & FM7a/Y; ▽/▽ × w mbr/FM7a; ▽/▽*

continued...

mbr & *smu* WITH CHROMOSOME-*4* TRANSPOSON COMBINATIONS

G_1 *w/Y; ▽/▽ × FM7a/FM7a*

↙

G_2 *FM7a/Y; ▽/4$^+$ × w mbr/FM7a*

↙ ↘

G_3 *FM7a/Y; ▽/4$^+$ × w mbr/FM7a; ▽/4$^+$*

↙ ↘

G_4	*FM7a/Y; ▽/▽ × w mbr/FM7a; ▽/▽*

STOCK: USE ONLY REDDEST EYE FLIES (*▽/▽* NOT *▽/4$^+$*)

mbmB WITH CHROMOSOME-*2* TRANSPOSON COMBINATIONS

G_1 *w/Y; ▽/▽ × w/w; mbmB/SM5*

↘

G_2 *w/Y; mbmB/SM5 × w/w; ▽/mbmB*

↙

G_3 ^{n}[*w/Y; ▽ mbmB/mbmB* Γ× *w/w; mbmB/SM5* EE], 1/n ~ distance

↙ ↘

G_4 SECTION 1 COLLAR RED *Cy*$^+$ FLIES/LINE

[*w/Y; ▽ mbmB/SM5 × w/w; ▽ mbmB/SM5*] STOCKS: KEEP RED *Cy* FLIES/LINE

continued...

mbmB WITH CHROMOSOME-*3* TRANSPOSON COMBINATIONS

G_1 *w/Y; TM3/TM6b × w/w; al Bl/SM5*

↙ ↘

G_2 *w/Y; al Bl/2$^+$; TM6b/3$^+$ × w/w; mbmB/SM5* & *w/Y; SM5/2$^+$; TM3/3$^+$ × w/w; ▽/▽*

↘ ↙

G_3 *w/Y; mbmB/al Bl; TM6b/3$^+$ × w/w; SM5/2$^+$; ▽/TM3*

↙ ↘

G_4 *w/Y; mbmB/SM5; ▽/TM6b × w/w; mbmB/SM5; ▽/TM6b*

↙ ↘

G_5 *w/Y; mbmB/SM5; ▽/▽ × w/w; mbmB/SM5; ▽/▽*

mbmB WITH CHROMOSOME-*4* TRANSPOSON COMBINATIONS

G_1 *w/Y; ▽/▽ × w/w; al Bl/SM5*

↙ ↘

G_2 *w/Y; al Bl/2$^+$; ▽/4$^+$ × w/w; mbmB/SM5*

↙ ↘

G_3 *w/Y; mbmB/al Bl; ▽/4$^+$ × w/w; al Bl/SM5; ▽/4$^+$*

↙ ↘

G_4 *w/Y; mbmB/SM5; ▽/▽ × w/w; mbmB/SM5; ▽/▽*

STOCK: USE ONLY REDDEST EYE FLIES (▽/▽ NOT ▽/4$^+$)

Figure 3-1. Crossing Schemes used to Create Flies for Microscopy. FM7a is a first chromosome balancer. alBl/SM5 are second Chromosome balancers. TM3/TM6b are thirs chromosome balancers. ▽= P element line.

previously[1,7,16]. Groups of approximately 100 3-6 day-old flies were aspirated into Training tube embedded with an internal double-wound electrifiable copper grid. To assay short term odor learning and memory, flies were exposed to an air current (750 ml/min) bubbled through one odor [1.4 X 10^{-3} dilution of 4-methyl cyclohexanol (MCH) (Sigma) or 8 X 10^{-4} Benzaldehyde (Benz) (Sigma) in heavy mineral oil (Sigma)] paired temporally with 1.25 second pulses of 120V dc electric shock delivered every 5 sec for 1 min. They were then exposed to an air current bubbled through a second odor without electric shock for an additional 1 min. We assessed learning and memory by presenting trained flies with both odors in converging air currents for 2 min. Performance was measured as a function of shock-paired odor avoidance at a variety of time points ranging from 1 min (giving an approximation of learning at the earliest testable time in the T-maze) to 6 hr after training. A second group of flies were trained in a reciprocal manner and tested. Scores from both tests were averaged to account for odor preferences among different populations of flies. Long-term memory was assessed using both spaced and massed protocals[22]. In a spaced protocol the short-term protocol listed above is repeated 10 times with 15 min rest intervals between each cycle. The massed protocol also repeats the short-term protocol 10 times but with no intervening rest periods. The flies are initially placed in the apparatus for 150 min before training to ensure an equal time in the machine for both protocols. In electric shock avoidance controls, one arm of the T-maze was electrified with 120 V dc for 2 min. In odor-avoidance controls, flies were exposed to 1.4 X 10^{-3} dilutions of MCH or 8 X 10^{-4} Benz versus air for 2 min. A

performance index represents the average normalized percent avoidance of the shock-paired odor (learning, memory) or individual stimulus (sensory acuity).

Histology and anatomy

Paraffin mass histology was used to process flies for neuroanatomical analysis as described previously[1,5,7]. Three – four day old *Drosophila* adults were cold-anaesthetized and placed in collars. They were then fixed in Carnoy's solution [6 parts EtOH (95 %), 3 parts chloroform, 1 part acetic acid; made fresh daily], dehydrated in ethanol, embedded in paraffin, cut in 7 μm serial frontal sections, and photographed under a fluorescence microscope with an AXIOCAM digital camera (Zeiss). Calyx volumes were derived from planimetric measurements of serially-sectioned brains[1,7] using AXIOVISION software (Zeiss). The mean of both calyces was used for each fly. To examine GFP expression in whole mounted fly brains, heads were dissected in 1X phosphate buffered saline (PBS) (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4 in 1L distilled H_2O at PH 7.4) and washed in FOCUS-CLEAR (Pacgen) for 15 min. They were then mounted and viewed under a fluorescence microscope with a far blue (FITC) filter. Z-series confocal images were collected (Zeiss LSM510) covering the whole MB for viewing structure (1.5 μm virtual sections), or perikarya clusters (1 μm virtual sections) for counting cells. GFP-labeled KC nuclei in brains were counted manually in every 7th section with the assistance of IMAGE-J software[23], ensuring that all perikarya (diameters, 5-6 μm) in each of these sections would each be counted only once. Larval brains were prepared in the same manor only they were not washed in FOCUS-CLEAR

before mounting. Because *mbmB* flies have a reduced growth rate during development[8] staging of all larvae was based on mouth hook structure[24].

Immunocytochemistry

Intact adult brains were dissected under PBS, fixed in 4% paraformaldehyde at 4°C for 3hrs. and washed 3 X 30min in PBT (PBS pH 7.4 with .2% Triton X-100). Brains were blocked 1hr with PBSBT (PBS pH 7.4, .2% Triton X-100, 1%BSA) and then incubated overnight at 4°C with primary antibody [mouse anti-FAS II (mAB1D4)[25] (1:5) (Developmental Studies Hybridoma Bank)]. Next the brains were washed 4 X 30min in PBSBT and placed in secondary antibody [goat anti mouse Alexa Fluor 568 (1:1000) (Molecular Probes)] for 4hrs at room temperature. Finally, they were washed 3 X30min in PBT and transferred to slides for viewing.

Results

Analysis of larval lobe structure

GAL4 expression elements have been used in a variety of studies to assess normal neuronal morphology in *Drosophila*[26-29]. We have crossed several GAL4 elements (listed in table 3-1) into the three mutant genetic backgrounds in this study for use in assessing MB structural changes in finer detail than is allowed by paraffin mass histology. Previous experiments have shown that mutants with structural effects are sensitive to genetic background manipulation often resulting in changed phenotypes[7]. Therefore after crossing these elements into the mutant strains, we used paraffin mass histology to determine if any change in calyx

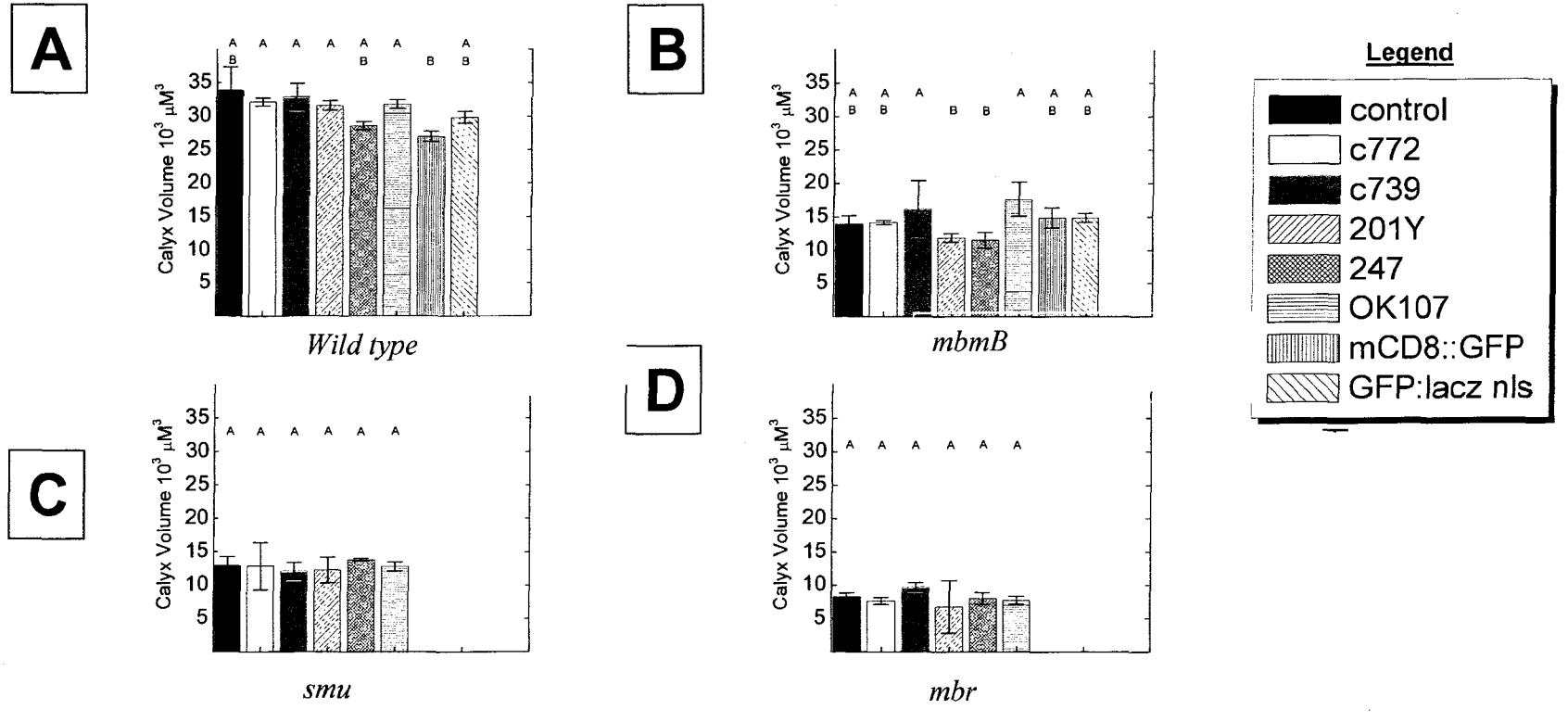

Figure 3-2. Calyx volumes of wild type and mutant flies combined with Gal4 elements. . A) calyx volumes of wild type strains used in this study. B) Calyx volumes of *mbmB* flies with GAL4 inserts. C) Calyx volumes of *smu* flies with GAL4 inserts. D) Calyx volumes of *mbr* flies with GAL4 inserts. Bars are mean ± SE calyx volume, groups indicated by different letters are significantly different (SNK;α=.05).

volume compared to the original mutant lines was detectable. Some minor significant ($F_{[7,66]}$ = 2.485, P = .025) volume differences can be seen between the largest volumes and smallest volumes among the wild type GAL4 lines (figure 3-2 A) but none are significantly different from the control flies (SNK α =.05). As with the wild type control flies there was a significant difference seen between the largest volumes (*mbmB* C739 and *mbmB* OK107) and the smallest volume (*mbmB* 201Y) ($F_{[7,56]}$ = 4.586, P = .0001) but none of the lines are significantly different from the *mbmB* control line as seen in figure 3-2 B (SNK α =.05). The *smu* and *mbr* lines appear quite stable (*smu* $F_{[5,45]}$ = .305, P = .907; *mbr* $F_{[5,35]}$ = .587, P = .710) with no significant differences (figure 3-2 C and D).

During fly metamorphosis there is a large scale rearrangement of MB morphology were γ axons are pruned and re-grown medially[9,30]. For this reason it is important to look at how the mutations affect MB development in larvae as well as in adults. Drosophila larval MBs are predominantly composed of γ neurons which bifurcate into vertical and medial branches Membrane-targeted GFP expression was driven with the *OK107* driver in wandering 3rd instar larvae to examine MB structure (figure 3-3). Larval MBs in all three mutants have normal morphology compared to wild type as vertical and medial projections are apparent. There were no significant differences in thickness between the *mbmB* or *smu* mutant flies and their wild type counterparts. However, *mbr* flies appear much thinner than wild type controls suggesting a reduced cell number.

Assessment of Adult MB lobe structure

Next we crossed the mutant/GAL4 lines with a membrane bound GFP to

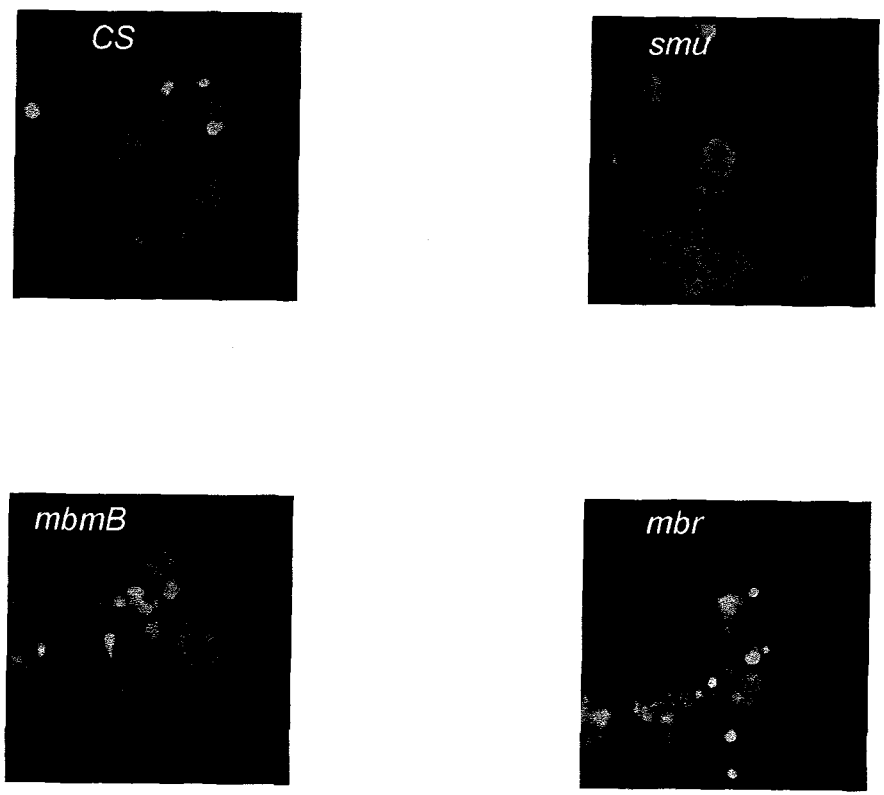

Figure 3-3. Analysis of mutant larval lobe structure. Membrane-targeted GFP expression patterns driven by *OK107* expressing elements in whole mount brains of 3RD instar larval flies viewed with a laser scanning confocal microscope.

assess MB lobe structural integrity in adults. Ten images where obtained for all five lines for a total of 50 per mutant (25 male and 25 female for *mbmB* and all males for *smu, mbr*). The *mbmB*-GAL4 flies were crossed with *mbmB*/mCD8::GFP flies to obtain homozygous *mbmB* flies with GAL4 and GFP elements. For *smu* and *mbr*, both *X*-chromosome mutations, mutant /GAL4 flies were crossed with control mCD8::GFP flies and only males were examined as there are not homozygous female viable. Wild type control images were obtained for each GAL4 line (figure 3-4 A-E) for comparison with mutant images. An ideal MB illustration is presented in figure 3-3F showing all five lobes in their proper configuration.

The *mbmB* mutation causes the mildest phenotypic changes in the MBs (figure 3-4 G-K, illustration in L). All lobes appear to be present but the vertical lobes (α and α') are much thinner than wild type flies. Also, the β lobes cross the midline and appear to fuse (indicated by white arrows figure 3-4 G-K). This phenotype was 100% penetrant in 50 samples. Although this phenotype was not anticipated as it was not previously detected by paraffin mass histology, it has been seen before in mutant strains exhibiting learning defects[31,32]. The γ lobe appears to be unaffected at this level of analysis.

The *smu* mutation caused a much more severe phenotypic change. The γ lobe looks unaffected in *smu* flies however all other lobes appear severely reduced (figure 3-4 M-R, illustration in R). White arrows in figure 3-4 M,N, P and Q indicate a thin vertical lobe. It is not possible to distinguish between α and α' neurons in these images. The red arrow in figure 3-4N illustrates what can be

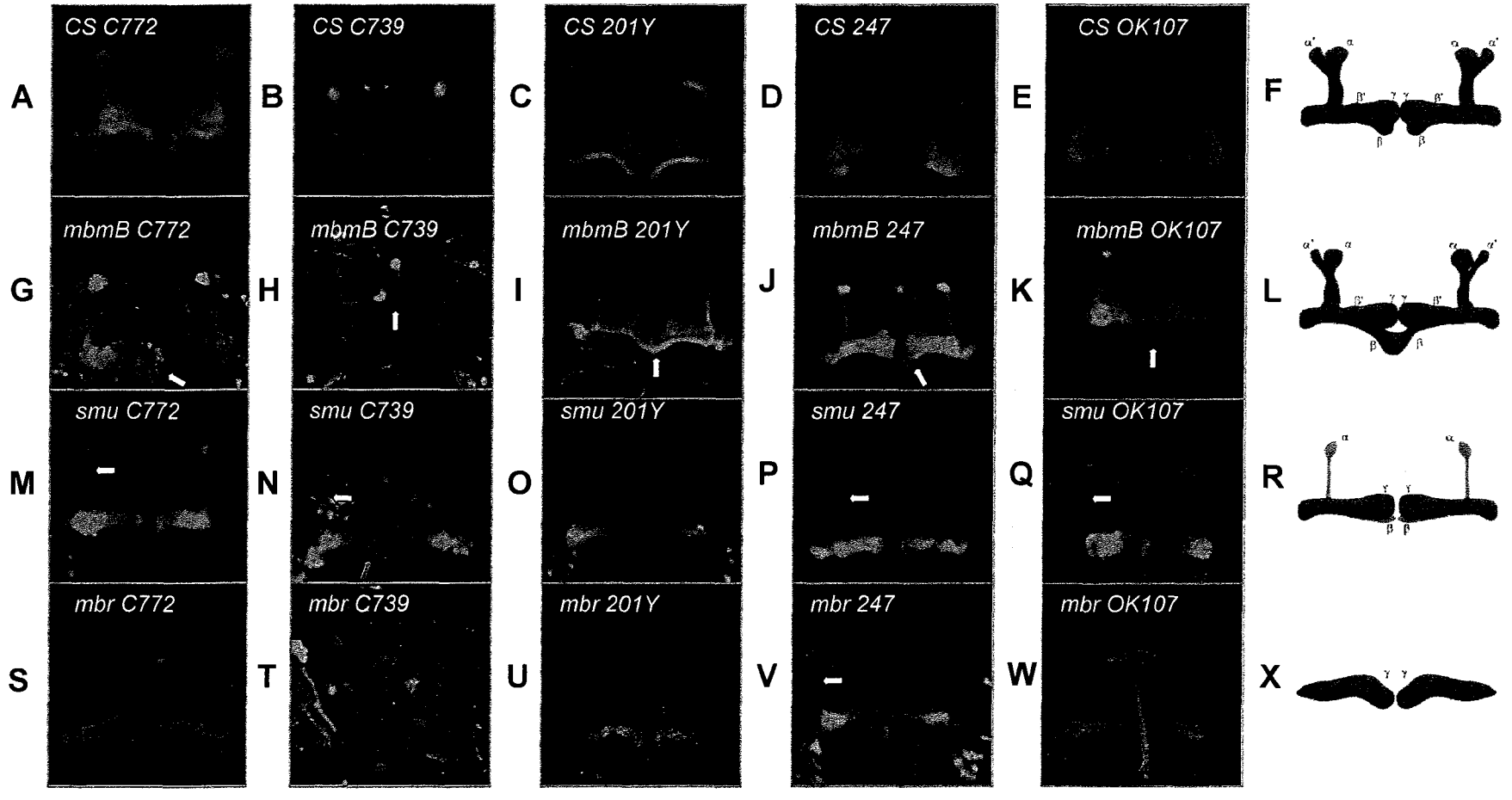

Figure 3-4. Analysis of lobe structure in mutant strains. . A-E) Membrane-targeted GFP expression patterns driven by different GAL4 expressing elements in whole mount brains of wildtype flies viewed with a laser scanning confocal microscope. F) Illustration of the proper organization of mushroom body lobes. G-L) Membrane-targeted expression patterns of the same GAL4 elements in *mbmB* homozygous flies.. White arrows in the images indicate a fusion of the β lobes not seen in wild type. M-R) Expression of GAL4 elements in *smu* homozygous flies. S-X) Expression of GAL4 elements in mbr homozyguous flies. L, R, X) Illustration highlighting main phenotypes seen in *mbmB, smu, and mbr* flies compared with F.

seen of the β lobe in this fly. It appears thin consistent with α. The most severe defects were observed in the *mbr* flies. The γ lobe is still prominently seen but appears reduced in thickness compared to wild type (figure 3-3 S-W, illustration in X). Another striking feature is the near complete absence of the α/α' lobes. The yellow arrow in figure 3-4 V illustrates the one of two whisper thin vertical lobes seen in any of the 50 images (4%) of *mbr* flies . GAL4 c739 has strong expression in the α/β lobes. Figure 3-4 T is a representative image of *mbr*; *C739* in which no MB neurons are detectable.

One of the drawbacks to using GAL4 expression to visualize the MBs is that only a subset of MB cells is represented[33]. At best only half of the present MB neurons can be visualized with any given line. This is particularly important for *smu* and *mbr* characterization where it is impossible to differentiate between α and α′ neurons in the vertical lobe of *smu* and *mbr* appears to have few if any α/β/α′ neurons. In order to more clearly distinguish how each of the three developmental lobe sets are affected, I counterstained mutant/*C793* or *C772* flies with mouse anti-FASII, which preferentially stains the α/β and γ lobes (figure 3-5). This permitted a more clear differentiation between α and α' neurons. Also, staining of all α/β neurons through immunohistochemistry may reveal more vertically projecting neurons than GAL4 expression has indicated. The first row of images in figure 3-5 (A-C) is wild type flies for comparison with the mutant phenotypes. The next rows are representative images of the three mutants [*mbmB* (D-F); *smu* (G-I); and *mbr* (J-L)]. Column 1 is *C739* compressed Z stack GFP expression images with anti-FASII counterstaining while column 2 are

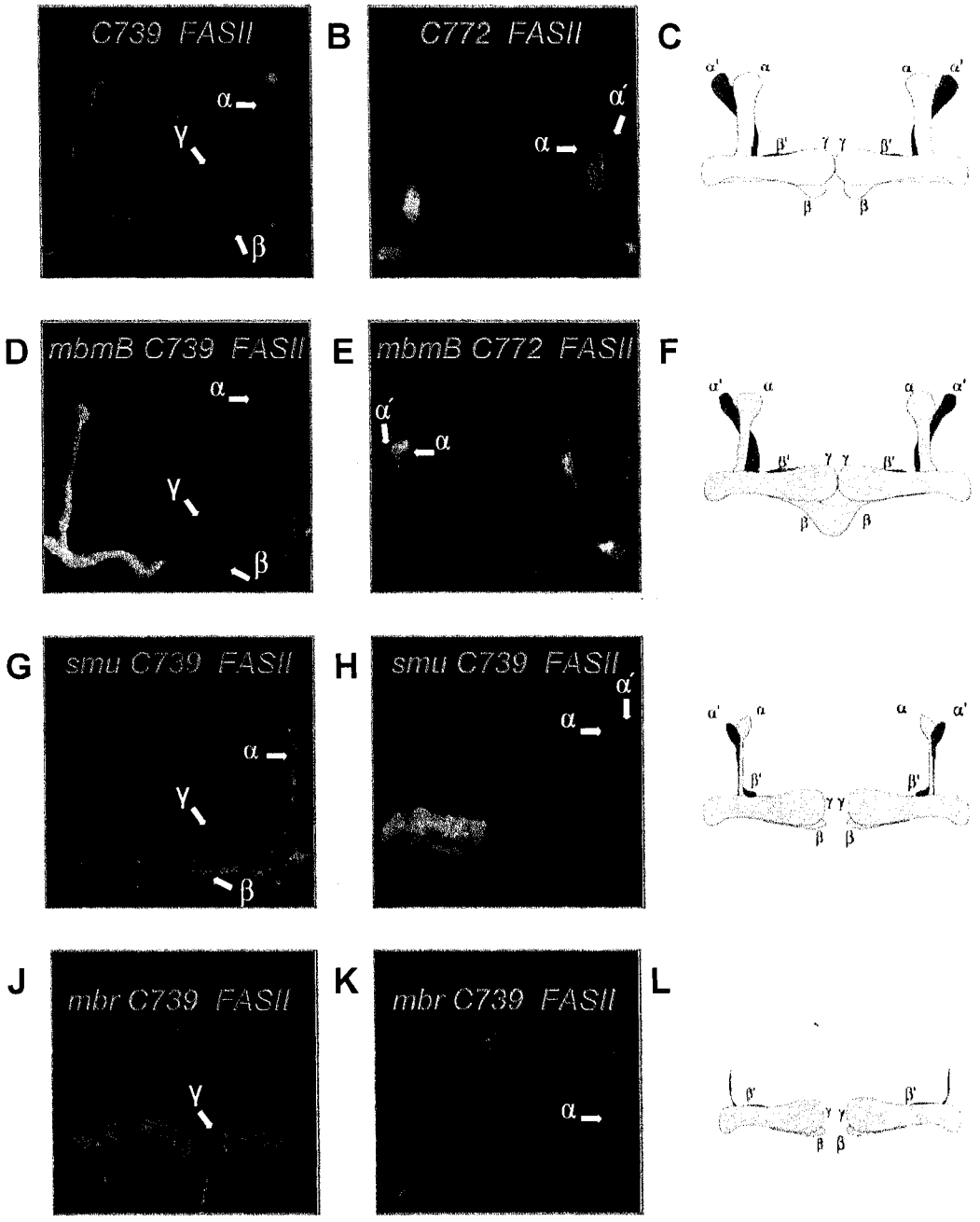

Figure 3-5. FAS II staining of mutant/GAL4 flies Column 1 consists of compressed z stack images of fly brains with *c739* GFP expression in green, co-stained with anti-FASII in red. C739 expresses in α/β lobe only while FASII expresses in α/β and γ lobes. Column 2 consists of one slice from a z-stack of images at an appropriate level to examine the prime lobes using *C772* GFP expression co-stained with anti-FASII. *C772* expresses in all five lobes. A-B). Wild type flies. D-E). *mbmB* fly brains. G-H). *smu* fly brains. J-K). *mbr* fly brains. C, F, I, L). Illustration representing phenotypes seen in the various lines. All white arrows indicate what lobes are present in the individual brain.

individual slices from *C772* confocal Z-stacks with anti-FASII counterstaining highlighting all lobe subsets. Column 3 illustrations indicate which lobes are present based on our images. All lobes can be seen in *mbmB* flies which are indicated by white arrows in (D) and (E). Counterstaining was particularly useful for the *smu* and *mbr* flies. White arrows in (G) indicate the γ and β medially projecting lobes can be distinguished while arrows in (H) indicate both vertical lobes. Based on these results it appears that all three lobe sets are represented in *smu* flies. FASII expression in all α neurons as in (G) still reveals a severely thin lobe. Expression in *mbr* fly medial lobes (J) does not clearly differentiate the lobes. However, in the slice indicating vertical lobes (K) a thin strand with only green expression (α′) and a thin strand with only red expression (α) can be seen. The majority of the neurons visible with *C772* expression are γ neurons.

Mutant flies exhibit reduced cell number in late larval and adult stages

I hypothesized that the thin lobes we described in all three mutant flies were caused by a cell proliferation defect resulting in less Kenyon cell birth during development. A previous finding that *mbmB* flies have no significant difference in *dachshund* expressing cells in the MB neuroectoderm up to stage 13 of embryogenesis[8] made it important to determine the developmental stage when cell number is affected. In order to answer this question nuclear-targeted GFP was expressed in whole mount brains at three developmental stages to count cells and determine when there was a significant difference. Previous studies have reported that MB Kenyon cells develop sequentially. Neurons projecting into the γ lobe of the adult MB are born first, prior to the mid-3rd instar larval

stage. Neurons projecting into the α' and β' lobes are born between the mid-3rd instar larval stage and puparium formation. Finally, neurons projecting into the α and β lobes are born after puparium formation[9]. Therefore we counted cells of larvae in 3rd instar pre-wandering stage, 3rd instar wandering stage and adult flies to determine when cell number became significantly different from wild type controls. Consistent with the previous result indicating early cell number was unaffected in *mbmB* embryos, we found no significant difference in *OK107* expressing cells between *mbmB* and wild type control flies in pre-wandering 3rd instar larvae (figure 3-6A). However, there was a significant difference in *OK107* expressing cells in both wandering 3rd instar and adult *mbmB* mutant flies (SNK α=.05). A 62% reduction in *OK107* expressing cells was seen in adult *mbmB* flies compared to *CS*. These results are consistent with the fact that we observed a γ lobe of normal thickness in adult *mbmB* flies while all other lobes appeared much thinner. We obtained very similar results with *smu* flies (figure 3-6B). There was no significant difference in *OK107* expressing cells in pre-wandering 3rd instar larvae but a significant difference at both later time points (SNK α=.05). A 74% reduction in *OK107* expressing cells was seen in adult s*mu* flies compared to *CS*. We attempted a similar experiment with *mbr* flies; however, we found that expression of *OK107* in *mbr* larvae was particularly faint and difficult to distinguish from background noise. Counts were obtained for adult *mbr* flies (figure 3-6C). *mbr* adult flies showed the biggest reduction with 84% lost *OK107* expressing cells. Based on the fact that larval and adult lobes appear thinner than wild type controls we believe cell number is likely reduced at a very early

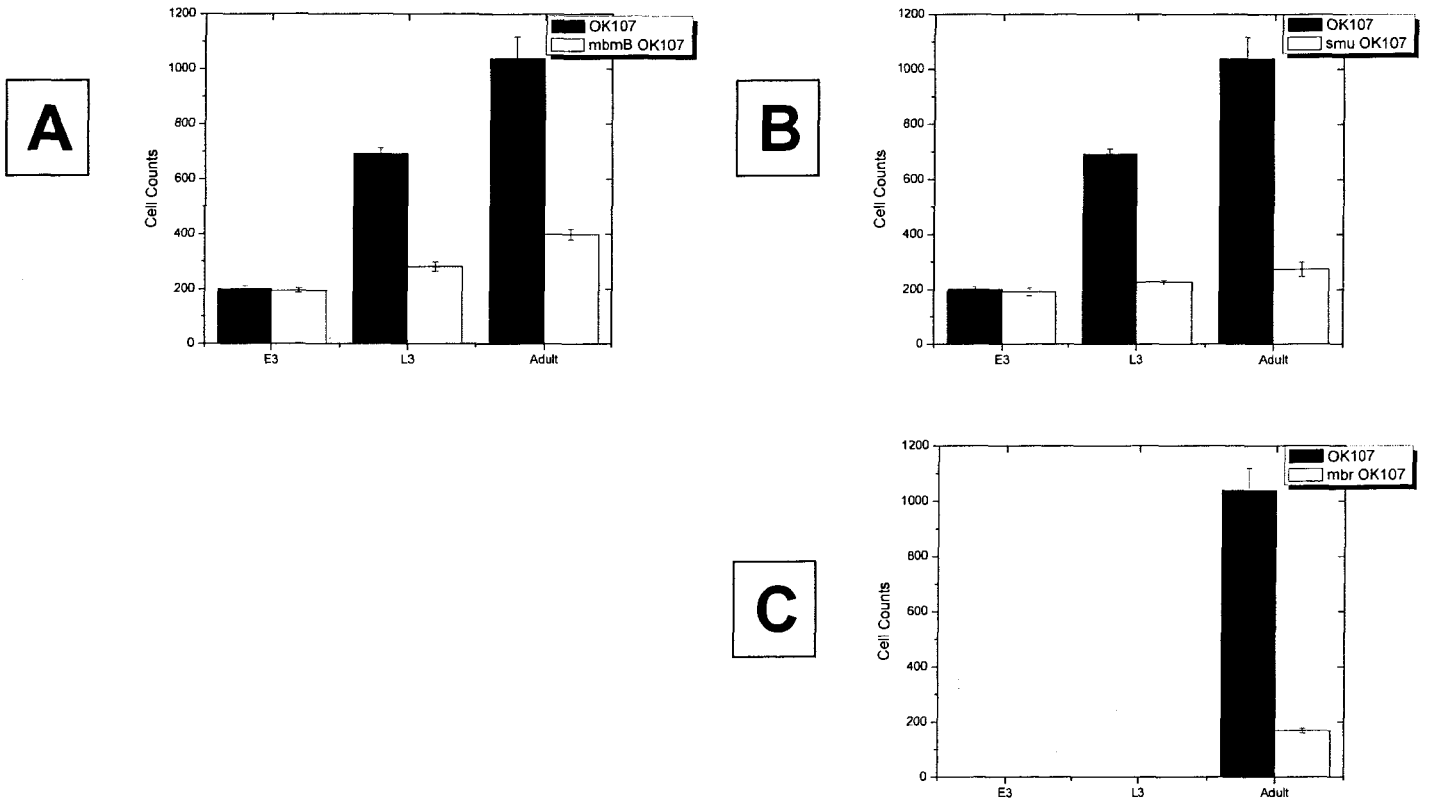

Figure 3-6. Kenyon cell counts in larval and adult mutant flies . A) Nuclear-targeted GFP expression patterns driven by different GAL4 expressing elements in whole mount brains of wildtype larval and adult flies viewed with a laser scanning confocal microscope A&B) Cell counts at three developmental time points. E3 is early 3rd instar, L3 is Late (wandering) third instar and A is 3 day old adults. N=9 for all time points. A) Cell counts for wild type and *mbmB* flies. $F_{5,72}$=85.76, P<.0001) groups indicated by different letters are significantly different (SNK;α=.05). Significant differences in cell number occur later in development starting at late 3rd instar. B) Cell counts for wild type and *smu* flies. $F_{5,72}$=92.45, P<.0001) groups indicated by different letters are significantly different (SNK;α=.05). Significant differences in cell number occur later in development starting at late 3rd instar. C) Adult cell counts for wild type and *mbr* flies.

time in development in *mbr* flies.

Influence of mutant genes on learning and memory behavior

Both *mbmB* and *smu* were isolated because of aberrant MB structure. Since MBs are a secondary olfactory neuropil essential for mediating associative odor learning and memory in *Drosophila*[34,35], we examined the behavior of *mbmB* and *smu* flies using three different paradigms. The first was a Pavlovian conditioning assay which assesses learning and short term memory using a single training bout[1,7,16]. Learning of odors paired with electric shock was profoundly reduced in homozygous *mbmB* (20%) and male *smu* (58%) flies relative to *cs* flies (figure 3-7 A and C) while *mbmB* heterozygotes were unaffected. There appears to be no significant impact on short term memory consolidation in *mbmB* since the ANOVA genotype X time interaction component was not significant (*mbmB* $F_{[8,104]}$ = .46, P = .876) However *smu* flies do have a significant short term memory defect (genotype X time $F_{[3,64]}$ = 257.214, P = .026). Similar olfactory conditioning defects and decreased rates of memory decay have been described for several *Drosophila* mutants[36,37], including those with observed reductions in MB anatomy[7,34,38].

We also employed spaced and massed training protocols to assess LTM and ARM retention. The spaced protocol has been shown to produce LTM lasting weeks while massed training generates ARM which lasts for about 3 days[22]. At both 12 and 24 hrs there is no significant difference between *mbmB* heterozygotes and *CS* for massed or spaced training (SNK α = .05). However, *mbmB* flies are impaired in both forms of long term memory (genotype

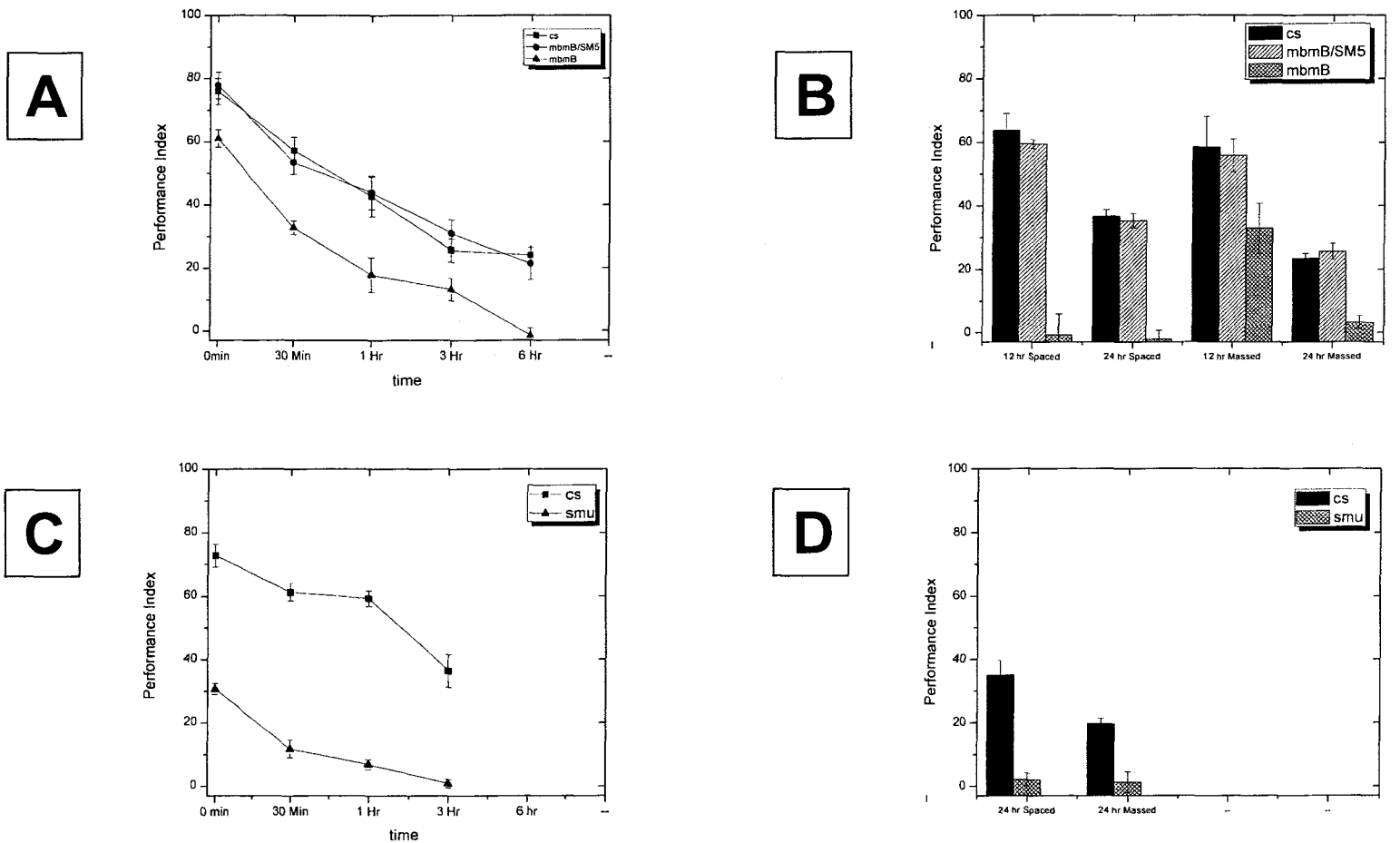

Figure 3-7. Learning and memory in *mbmB* and *smu* flies. *A)* Olfactory learning is strongly reduced in *mbmB* flies. B)Spaced training was used to assess long term memory (LTM) and massed training was used to assess anesthesia resistant memory (ARM).The *mbmB* flies were unable to form LTM however, ARM was present at 12hrs at a reduced level and absent by 24hrs. C) The *smu* mutation causes a more severe learning reduction than the *mbmB* mutation and a small but significant short term memory defect ($F_{[3,64]}$ = 257.214, P = .026). D) LTM and ARM were examined for *smu* at 24hrs and no appreciable levels of memory are retained at this time point. N=9 for all PI. All *mbmB* PI include mixed population of males and females while *smu* is exclusively male due to the extremely low percentage of homozygous females. Bars are mean ± SE.

$F_{[2,60]}$=132.190, P < .0001; time $F_{[3,60]}$ = 49.077, P < .0001; Treatment X time $F_{[6,60]}$ = 8.027, P < .0001). There is a striking difference between the spaced training results and those for massed. ARM generated by massed training is reduced (38%) at 12hrs and is completely abolished by 24 hrs. LTM generated by spaced training is not observed at either 12 or 24hrs and appears not to form at all (figure 3-7 B). For *smu* flies ARM and LTM were assessed at 24 hrs only and both are abolished (genotype $F_{[1,32]}$ = 67.05, P < .0001) at that time (figure 3-7D).

Assessment of sensory acuity

In order to determine if defects observed in a pavlovian olfactory conditioning paradigm can be attributed to defective learning and memory mechanisms it is essential to first determine that the animal's sensory acuity is not impaired and therefore mimicking a learning phenotype. We examined the ability of the three mutants to avoid electric shock and odor (Figure 3-8). Mutant flies of the *mbmB* and *smu* genes did not have sensory acuity defects in control tests relevant to our conditioning paradigm. They avoided 120 V dc shock pulses normally compared to control (*CS*) flies. Similarly, both showed normal avoidance of both benzaldyhyde (8×10^{-4}) and 4- methylcyclohexanol (1.4×10^{-3}) odorants (*mbmB* $F_{[2, 45]}$ = 87.515 P = .129; *smu* $F_{[1, 30]}$ = .234 P = .632) at the dilutions used in classical conditioning (figure 3-8A-B).

The case is quite different with the *mbr* mutant flies. They are significantly impaired for avoidance of shock and both benzaldehyde and 4-methylcyclohexanol at testing concentrations (figure 3-8 C) ($F_{[1, 30]}$ =117.348 P

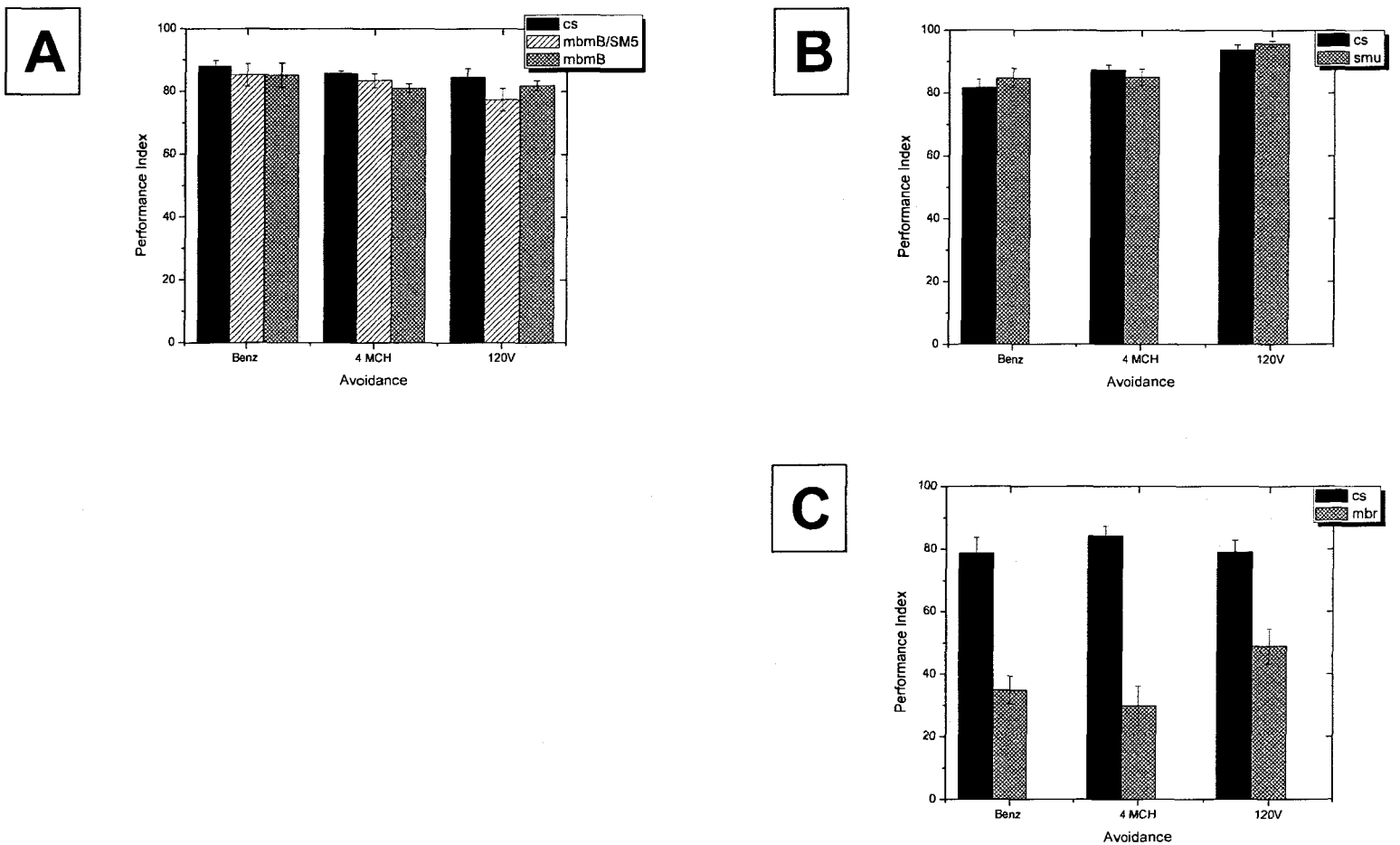

Figure 3-8. Sensory acuity in *mbmB*, *smu* and *mbr* flies. Odor avoidance was tested for Benzaldehyde [8 X 10^{-4} (4µl in 5ml)] and 4 Methyl cyclohexanol [1.4 X 10^{-3} (7µl in 5ml)] while shock avoidance was tested at 120v. There were no significant differences in odor or shock avoidance for *mbmB* or *smu* flies (*mbmB* $F_{[2, 45]}$ = 87.515 P = .129; *smu* $F_{[1, 30]}$ = .234 P = .632). Odor and shock avoidance were both significantly impaired for *mbr* flies ($F_{[1, 30]}$ =117.348 P = .0001). N=6 for all PI. All *mbmB* PI include mixed population of males and females while *smu* and mbr are exclusively male due to the extremely low percentage of homozygous females. Bars are mean ± SE.

=.0001). For this reason *mbr* was not tested for learning or memory.

Discussion

In this study I have examined anatomical and behavioral defects in the mutant strains *mbmB* and *smu*. The anatomical defects caused by a third mutation *mbr* were also examined. Using the GAL4-UAS system I was able to characterize MB gross anatomical defects caused by the three mutations. Lobe anatomy of wandering third instar larvae in *mbmB* and *smu* flies appear essentially wildtype while *mbr* larval MBs look proper in terms of projections but were much thinner than wildtype. All lobes are detectable in adult *mbmB* flies but there is a β lobe fusion across the brain midline and the α/β and α'/β' lobes are thinner than wild type. The γ lobes are not affected. In adult *smu* flies all lobes can again be shown but the α/β and α'/β'lobes are severely thin even when compared to *mbmB* flies. The γ lobes are not affected in *smu* flies as well. The most severe adult phenotypes were seen in *mbr* flies. Only minor traces of α/β and α'/β' neurons can be seen. There are still γ neurons present but not at wild type levels. Cell counts of *OK107* expressing Kenyon cell bodies at three different developmental time points reveal that *mbmB* and *smu* cell number is not reduced until the mid-third instar. Adult cell number is also reduced in *mbr* flies. Significant reductions in odor and shock avoidance were shown for *mbr* flies. While *mbmB* and *smu* flies had normal odor and shock avoidance initial learning was impaired in *mbmB* and *smu* flies. The learning impairment was more severe in *smu* flies and was accompanied by a significant STM defect. Both spaced and

massed training protocols were used to assess LTM and ARM. At 24 hrs both LTM and ARM are abolished in *mbmB* and *smu* flies. At 12 hrs after training *mbmB* flies had reduced but still detectable ARM levels however there was no significant LTM remaining.

β lobe fusion in mbmB flies

In wild-type *Drosophila melanogaster*, axon fibers in the medially projecting lobes typically terminate near the midline but do not cross it[39]. In our studies we found that in *mbmB* mutant flies, β lobe axons cross the midline and appear to fuse the left and right β lobes together. This β lobe fusion in *mbmB* flies was an unexpected phenotype because it was not seen in paraffin sections of adult brains. However, similar phenotyes have been noted before in at least thirteen different genes which cause MB structural defects[40-48] three of which have documented learning or memory defects[31,48-50]. Flies mutant for the gene *fused mushroom bodies (fum)* were the first to be reported with a β lobe fusion phenotype[48] as well as 30 min memory defects[34]. β lobe fusion does occasionally occur in wild type flies but with a very low penetrance (1-7%)[31,32] and moderate expressivity when compared to the mutant fly phenotypes.

Flies with β lobe fusions have appeared in human disease models as well. Two in particular stand out. Fragile X syndrome, a very common form of inherited mental retardation in humans[51] is caused by mutation of the *fragile-X mental retardation 1* (*Fmr1*) gene. Fragile X patients have cognitive deficits, with visuospatial skills more impaired than language[52]. The *Drosophila fragile x mental retardation gene (dFmr1)* is a fly ortholog which revealed a β lobe fusion

upon early characterization of the brain anatomy[32]. Another important human disease gene homologue is the *fused lobes (fdl)*[41] gene which encodes an *N*-Acetylglucosaminidase [45]. This enzyme hydrolyzes glycosides of N-acetylglucosamine producing alcohol and N-acetylglucosamine. A deficiency of this enzyme results in mucopolysaccharidosis III B which is characterised by progressive mental retardation, heparitin sulfate in the urine, mild dwarfism, and other skeletal disorders in humans.

Other mutations which cause a β lobe fusion phenotype include a variety of different genes such as *derailed* (drl^{lio}) a receptor tyrosine kinase involved in learning[53,54], *Ciboulot (cib)* encoding actin binding proteins[43], the *retained/dead ringer* (*retn*) gene which affects courtship behavior[46] and unknown genes that came out of an MB ablation microarray screen[47].

It is not clear what causes the β lobe fusion phenotype but a ring of glial cells only present during development has been implicated. Specifically, A transcient interhemispheric fibrous ring (TIFR) appears during early larval stages in the region of the brain later occupied by the CCX and then disappears around 72hrs after pupariation[40]. It was shown that β-lobe neurons cross the midline and that CCX cells tend to converge on the position of the TIFR which itself appears disrupted in *drl* mutant brains. This led to the conclusion that the TIFR aided in proper brain formation by acting as a scaffold for the CCX and releasing repulsive signals preventing midline crossing. If this hypothesis is true then mutant alleles of genes causing a β lobe fusion phenotype will play some role in either allowing β neurons to sense the repulsive signals or disrupt the TIFR cells

and the repulsive signal they produce. One of the ongoing projects of our lab is to molecularly characterize the *mbmB* gene allowing us to determine its expression pattern. When we have detailed images of MbmB protein expression it will help us see how it fits into this proposed model.

Although there are quite a few mutants known to induce a β lobe fusion phenotype, the behavioral outcome of this has not been well characterized. Two studies have tried to address this question. First, molecular work on the *dFmr1* gene (which displays a beta fusion) has shown that excess glutamate signaling is involved in fragile x syndrome behavior defects. Treatment with glutamate receptor agonists during development rescued courtship behavior and the β midline crossing phenotype[50]. However, glutamate receptor agonist treatment in adults rescued courtship behavior defects but not the midline crossing. This suggested that the lobe fusion was not causal for the noted behavior defects.

The second study involved the *retn* gene which is also involved in courtship behavior[46]. They found that all of the mutant females they tested showed an increased resistance to male courting while only about 1/3 of the flies dissected for anatomical analysis showed the midline crossing phenotype. This led them to conclude that the β lobe fusion phenotype was not causal to the behavioral defects.

While it has been clearly established that the MBs are involved in several types of behavior including learning[55], walking[56] and sleeping[57] there is no documented role for the β lobes in these behaviors. One of the goals of our project as well as that of many other researchers is to determine functional roles

for the individual MB lobes. In a later section I will discuss in greater detail what is known about the individual roles of lobes in learning behavior. I believe that *mbmB* flies will prove to be useful tools to help us better understand the functional role of these lobes.

Neuroblast proliferation defects in Drosophila

The reduced calyx volume phenotypes initially seen in our three mutants may be explained by any one of three different scenarios including improper neurite growth, reduced cell proliferation or cell death. Analysis of GAL4 driven membrane bound GFP in the MBs of mutant flies revealed thinner lobes for *mbmB* and *smu* but there is no evidence of improper pathfinding or neurite formation (Figure 3-3G-R). In *mbr* mutants, the neurites do appear to have an odd bending in the medially projecting lobes but no evidence that neurite growth is impeded (Figure 3-3V and W). All three mutants have a reduced Kenyon cell number in adult flies (Figure 3-5). Experiments ongoing in our lab will determine if cell death contributes to this cell number reduction or not but my current hypothesis is that cell proliferation defects are the likely cause of this phenotype for all three mutations. This is particularly true for the *mbmB* mutant. Recent experiments in our lab have shown that *mbmB* fails to compliment mutant *pendulin* (*pen*) alleles for calyx volume reduction and sequence analysis has shown that *mbmB* causes a premature stop codon in pen^{58}. Further experiments are being conducted to show that *pen* and *mbmB* fail to complement learning defects as well. This is an interesting finding because *pen* is a homologue of the mammalian *importin α 2* gene which has been shown to be involved in nuclear

transport and cell proliferation[59].

While we know nothing about the molecular nature of the *smu* or *mbr* genes it also appears likely they have a reduced proliferation rate causing the lessened cell number in adults. A large variety of different genes can affect cell proliferation including any housekeeping genes involved in the cell cycle or cell proliferation regulatory genes.

There are many mutations that cause a reduction of cell number throughout the brain[60,61] so why are MB neurons more affected than other cell types in the brain of many mutants? MB development lends itself to amplified affects for a variety of reasons. There are only four MB neuroblasts which divide continuously throughout development[9,62] while most other neurophil develop from a larger number of neuroblasts dividing at specific windows during development[30,62]. This makes MB cells particularly susceptible to developmental[63] and environmental influences[64,65] as there is fewer neuroblasts to compensate for any losses as well as a greater window of time for disturbances to occur during division.

In two of our mutants (*mbmB*, *smu*) I have shown that there was no affect on cell number until roughly midway through the third instar (Figure 3-5). There are three possible explanations why a change occurs at this time. Previous work has shown that MB neurons are derived in a sequential manner with γ neurons appearing from embryogenesis to approximately mid way through the third instar about 3 days after larval hatching[9]. If a mutation in a regulatory gene caused a defective cell fate switch to occur you might see a disruption in cell proliferation at the exact time seen in these mutants. However, the result of such a defect

would likely be no lobes other than γ if cell proliferation stopped or an enlarged γ lobe caused by further proliferation with no fate switch. Both *mbmB* and *smu* flies have neurons of all five lobe types present. It seems unlikely at this time that the cell fate switch cues are affected by these mutations.

BrdU incorporation studies have shown that MB neuroblast proliferation rates steadily increase until they peak in early pupal animals at 3X their initial rate[44]. Flies with a *slender lobe* (*sle*) mutation affect nucleolar organization and fail to increase their cell proliferation rate causing a reduced cell number in adult flies[44]. In fact the *sle* mutant has a remarkably similar lobe structure to *mbmB* including thin α′/β′ and α/β lobes and a β lobe fusion across the midline. This indicates that it may be possible for a fly with a mildly defective MbmB or Smu protein to be functional at a relatively slow proliferation rate but have increasing difficulty as the rate increases resulting in a reduced adult cell number.

Similar BrdU studies with flies carrying a mutant *latheo* (*lat*) gene show a relatively normal cell proliferation until at least the 2nd instar but have reduced levels of cell proliferation in 3rd instar flies[38]. Another common phenotype seen in *lat* flies is reduced imaginal disks. Screens for pupal lethality in *Drosophila* have identified several mutants with missing or degenerating discs like *lat*, and many of these genes appear to be involved with cell proliferation and are maternally contributed, suggesting that embryonic cell proliferation is supported by maternal transcripts[66].The Pen protein is detectable in very early embryos indicating that it was likely maternally contributed[59]. In *mbmB* flies cell proliferation likely continues unaffected until the maternally contributed protein is gone.

At this time it is not possible to determine exactly what is causing the reduced Kenyon cell number in our three mutant flies. Evidence for *mbmB* flies indicates that maternal contributions may allow normal cell proliferation at early stages of development resulting in normal γ lobe development. Because of its suspected role in nuclear transport it is likely both maternal contribution and escalating problems caused by an increased cell proliferation rate affect *mbmB* flies. Since we do not know about the molecular nature of *smu* and *mbr* at this time, BrdU incorporation studies would help clearly determine exactly when during development cell proliferation becomes affected in these flies as well as to determine if maternal contributions or increased cell proliferation rates are influential factors as well.

Effect of lobe disruptions on learning and memory

A primary goal of this dissertation work was to try and characterize the anatomical specificity of MB mutant flies and correlate their anatomy with behavioral defects. Of the three mutants examined *mbmB* and *smu* are promising candidates for further study. Because of their behavioral defects in sensory perception (figure 3-1) *mbr* flies cannot be tested in the T-maze apparatus for olfactory learning. I suspect one of the primary reasons the flies could not avoid shock or odors is a failure to walk properly. It is possible that out crossing to a genotype other than *CS* might produce better behavioral results. However the more likely solution to this problem is to use a different paradigm not dependent on walking. Although primarily used in honeybee research, a few labs have successfully used the Proboscis Extension Reflex (PER) assay to

assess learning in flies[67,68]. In this assay flies are individually tethered and the only movement required is to extend there proboscis in response to stimulus presentation. Incorporating this assay in our lab may provide learning data for *mbr* flies in the future.

There is mounting evidence that the different lobes perform distinct functions in the learning and memory consolidation process. As researchers began to explore gene expression through immunohistochemistry and GAL4 lines showing MB preferential expression it became clear that the MBs did not have a homogeneous array of expression[27,69]. Because of these observations it was hypothesized that the MBs also had functional subdivision. It is not clear which MB structures are the sights of acquisition of new information, but it seems likely that the calyx is involved as this is the dendritic region of the MBs. Any loss of cells through environmental or genetic manipulation can have an adverse affect on learning. Studies of flies that have undergone thermal shock during development reveal a reduced Kenyon cell number and reduced odor learning scores[65] while mutant fly strains with reduced MBs also have impaired odor learning scores[6,70].

In terms of memory consolidation there have been several useful studies linking specific lobes with different memory phases. The (*easily shocked*) eas^{ala} mutation results in three distinct phenotypes: all five lobes are present, βand β' are lacking, or α and α' are lacking[13]. The γ lobe appears to be normal in these mutants. It was shown that short term memory was normal in eas^{ala} flies lacking either vertical (α, α') or median lobes (β, β'). However, long term memory was

abolished in α, α' lacking flies but not in β, β' lacking flies. Two key pieces of information came of this work. First, that γ is important for short term memory. This idea is supported by research on the learning defects of a mutant adenylate cyclase gene *rutabaga* (*rut*) which were rescued by expression of a P[UAS-*rut*$^{+}$] transgene driven by multiple GAL4 lines expressing in γ but not by transgenes expressing in exclusively the α/β lobes or the CCX[10]. Secondly, the *eas*^{ala} research showed that LTM is processed in some way by the vertical lobes (α, α') because their loss impaired LTM while the loss of β,β' lobes left LTM intact. A recent study supported LTM maintenance by the α lobe, reporting an increased calium influx generated by a spaced conditioning protocol, and not by single cycle or massed conditioning. The trace is delayed, forming between 3 and 9 hr after conditioning, and intriguingly is axon branch-specific, forming only in the α axon branch of the α/β MB neurons and not in the β branch[14]. In addition, the data suggest that the memory trace is dependent on protein synthesis at the time of conditioning. While the α lobe has been implicated in LTM maintenance it is not involved in all forms of long term memory. The finding that five hour memory in α, α' lacking flies trained with the short protocol was not impaired indicated that the vertical lobes do not support ARM[71].

A role for the α'/β' lobes has been shown for memory consolidation in the first hour after training by using *shibire ts* to block α'/β' transmission. These lobes were required during training and consolidation phases for normal memory but dispensable during recall[11]. Also, three *in vivo* and *in vitro* calcium imaging studies all showed that Ca^{2+} activities in the axonal branches of α'/β' neurons in

response to a conditioned olfactory stimulus became larger compared with flies that were not conditioned[12]. It has been suggested that a DPM neuron α'/β' neuron loop is involved in consolidating memory which is then stored in the α/β lobe sets.

All of these experiments together describe a learning and memory circuit where γ is involved in initially forming and maintaining a memory in a short term store (STM), and α′/β′ further consolidates and maintains the memory (MTM). In spaced conditioning situations a DPM / α'/β' cycling circuit is involved in strengthening the permanent memory in the α lobe (LTM). Currently no known function has been attributed to the β lobe.

My work supports many aspects of this model. Consistent with the idea that a reduction in Kenyon cell number will lead to a learning defect, both *mbmB* and *smu* flies have reduced odor learning scores (Figure 3-7) as well as reductions in cell number (Figure 3-6). My work indicates a direct correlation between cell number and learning PI, as *smu* displays the most severe cell number reduction and learning impairment, while *mbmB* shows the same pattern, just not with the same intensity. This leads to the interesting question, how many Kenyon cells can be lost before learning defects are detectable. One possible way to answer this question involves partial MB ablation with hydroxyurea (HU). HU is an inhibitor of ribonucleotide reductase that blocks DNA synthesis and kills dividing cells[72]. For the first 8 to 12 hours after *Drosophila* larval hatching only five neuroblasts are proliferating in each hemisphere[73], four of which give rise to the mushroom bodies and the fifth produces local inter and projection neurons within

the antennal lobes[62,74]. When HU is fed to larva within this window only MB neurons of embryonic origin remain and MB ablation appears complete under a light microscope[1]. If we feed HU to several groups of larvae for increasing lengths of time during this window we should get a series of flies with increasingly fewer Kenyon cells. These flies can be tested for learning then sacrificed for cell counts to determine the critical Kenyon cell number for learning to occur.

The *mbmB* data supports the idea that the γ lobe is involved in short term memory. Anatomical analysis by GAL4 driven membrane bound GFP expression in the MBs (Figure 3-3) and Kenyon cell counts through development both show a γ lobe that is unaffected in *mbmB* flies (Figure 3-5). Memory decay slopes of mbmB compared to *CS* indicate no significant short term memory defects. However, anatomical analysis (Figure 3-4) and larval cell counts (Figure 3-6) also indicate *smu* flies have an intact γ lobe but STM is disrupted (Figure 3-7). This conflicting result is confusing, although it could be explained by several possibilities. It is possible that the γ lobe is disrupted in subtle ways our analysis couldn't detect or that the Smu protein is involved in STM maintenance in some capacity confounding our results. However, it may also mean that more lobes than simply γ alone are essential for STM and the severe disruptions in all the other lobes of *smu* flies highlight this fact Further molecular analysis of *smu* will help to support or refute its potential role in STM. Also, the previously mentioned BrdU incorporation studies will more clearly define the defects of the lobes in *smu* flies.

Because both *mbmB* and *smu* exhibit disrupted prime lobes we predicted that they should have impaired MTM because of the evidence linking DPM/ prime lobe (α'/β') cycling with that phase's maintenance[11]. Neither mutant shows any evidence of disrupted MTM which falls around 3 to 6 hours after initial learning. While my data doesn't support a role for the prime lobes in MTM maintenance it doesn't necessarily refute it either. It is possible that this phase has built in redundancy allowing a greater amount of cell loss with no affect. This may be due to the DPM cells playing a primary role in MTM maintenance while only a few prime lobe neurons are necessary for proper cycling and sending signals downstream to α for LTM formation when needed.

My data supports a role for the α lobe in LTM formation. In both mutants we have a severe reduction in α lobe neurons and a concurrent absence of LTM at 24 hrs. The most interesting result however, is that LTM in *mbmB* flies is abolished at 12 hrs while the ARM component is reduced but still present. This argues in favor of previously published reports that ARM and LTM are mechanistically independant[22,71] for aversive olfactory learning. Conflicting results from appetitive assays indicate they are mechanistically dependant[75]. It also raises an interesting question. Are the LTM defects seen a result of the structural abnormalities in the α lobes or pleiotropic affects of *mbmB* interacting with cell signaling pathways. Since *mbmB* and the nuclear transporter *pen* are allelic as discussed in earlier sections then it stands to reason a protein synthesis dependent memory phase such as LTM would be more severely affected by this mutation than a memory phase such as ARM which is thought to be independent

of protein synthesis[22]. Further molecular analysis of *mbmB* will help to clarify this issue.

The only known mutation affecting ARM is the *radish* (*rad*) gene which has a protein product expressed weakly throughout the brain and preferentially in the α/β and γ lobes[76]. Further evidence supports ARM maintenance by the α/β lobes. Researchers used shi^{ts} to selectively block synaptic transmission from all the lobes, the α/β lobes or the γ lobe. To be sure they were only measuring ARM, flies were trained with the short protocol and subjected to a cold shock 1hour after conditioning to eliminate nonconsolidated memories[77]. When all MB lobes were blocked during the experiment, ARM was erased. ARM was similarly decreased by blockage of the α/β lobes alone, but not significantly decreased by blockage of the γ lobes. Thus, ARM is supported by the MBs and appears to rely more heavily on α/β neurons[71]. Since the α/β lobe neuritis are bifurcations of the same neuron it is tempting to speculate they are involved in long term memory store with an established role for the α branch in LTM[13,14] and a speculated role for β in ARM. Given that *mbmB* flies have a β lobe fusion you would expect it to produce a behavioral phenotype. An analysis of ARM using massed training and then testing at several time points from 6 to 24hrs would allow a determination of whether there is a defect in ARM maintenance in *mbmB* flies or merely a decrease in initial learning score and normal decay.

If the β lobe fusion is not found to cause an ARM disruption then we can use *mbmB* to confirm an observation made with flies mutant for the eas^{ala} gene. As discussed above these flies have a variable phenotype and one of them is the

absence of vertical lobes causing an LTM specific disruption[13]. Flies with no vertical lobes were trained using both the short and spaced protocols and then tested for memory at 30 minutes and 5hrs. At 30 minutes performance was similar from both protocols but flies trained with the spaced protocol showed an extremely significant decrease in 5 hour memory[71]. Therefore more training resulted in decreased memory leading to the conclusion that spaced training leads to LTM formation and the elimination of ARM. Since we know LTM is also abolished in *mbmB* flies we should be able to show the same affect. The 12 hr time point is compelling for this reason. If ARM and LTM are independent additive processes, then ARM should still be present at 12 hrs after training even in *mbmB* flies trained using a spaced protocol.. What we see at 12 hrs is a complete loss of memory in *mbmB* flies, which argues in favor of the elimination of ARM by spaced training. Further experiments at earlier time points should be done to further confirm this.

Bibliography

1. de Belle, J. S. & Heisenberg, M. Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. *Science* **263**, 692-5 (1994).
2. Fiala, A. Olfaction and olfactory learning in Drosophila: recent progress. *Current Opinion in Neurobiology Motor systems / Neurobiology of behaviour* **17**, 720-726 (2007).
3. Sitnik, N. A., Tokmacheva, E. V. & Savvateeva-Popova, E. V. The Ability of Drosophila Mutants with Defects in the Central Complex and Mushroom Bodies to Learn and Form Memories. *Neuroscience and Behavioral Physiology* **33**, 67-71 (2003).
4. Wang, Z. et al. Visual pattern memory requires foraging function in the central complex of *Drosophila*. *Learn. Mem.* **15**, 133-142 (2008).
5. Heisenberg, M. & Bohl, K. Isolation of anatomical brain mutants of Drosophila by histological means. *Z. Naturforsch* **34c**, 143-147 (1979).
6. Heisenberg, M., Borst, A., Wagner, S. & Byers, D. Drosophila mushroom body mutants are deficient in olfactory learning. *J Neurogenet* **2**, 1-30 (1985).
7. de Belle, J. S. & Heisenberg, M. Expression of Drosophila mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). *Proc Natl Acad Sci U S A* **93**, 9875-80 (1996).

8. Ginsburg, M. Characterization of the Pleiotropic Effects of the *Drosophila* Gene Mushroom Body Miniature B. *University of Nevada, Las Vegas Master Thesis.* (2002).

9. Lee, T., Lee, A. & Luo, L. Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development* **126**, 4065-4076 (1999).

10. Zars, T., Fischer, M., Schulz, R. & Heisenberg, M. Localization of a short-term memory in Drosophila. *Science* **288**, 672-5 (2000).

11. Krashes, M. J., Keene, A. C., Leung, B., Armstrong, J. D. & Waddell, S. Sequential Use of Mushroom Body Neuron Subsets during Drosophila Odor Memory Processing. *Neuron* **53**, 103-115 (2007).

12. Wang, Y., Mamiya, A., Chiang, A.-s. & Zhong, Y. Imaging of an Early Memory Trace in the Drosophila Mushroom Body. *J. Neurosci.* **28**, 4368-4376 (2008).

13. Pascual, A. P., Thomas. Localization of Long-Term Memory Within the Drosophila Mushroom Body. *Science* **294**, 1115-1117 (2001).

14. Yu, D., Akalal, D.-B. G. & Davis, R. L. Drosophila [alpha]/[beta] Mushroom Body Neurons Form a Branch-Specific, Long-Term Cellular Memory Trace after Spaced Olfactory Conditioning. *Neuron* **52**, 845-855 (2006).

15. Brand, A. H. & Dormand, E. L. The GAL4 system as a tool for unravelling the mysteries of the Drosophila nervous system. *Curr Opin Neurobiol* **5**, 572-8 (1995).

16. Tully, T. & Quinn, W. G. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J Comp Physiol [A]* **157**, 263-77 (1985).

17. Lee, T. & Luo, L. Mosaic analysis with a repressible neurotechnique cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-61 (1999).

18. Shiga, Y., Tanaka-Matakatsu, M., Hayashi, S. A nuclear GFP/ beta-galactosidase fusion protein as a marker for morphogenesis in living Drosophila. *Dev., Growth Diffn* **38**, 99-106 (1996).

19. Armstrong, J. D., de Belle, J. S., Wang, Z. & Kaiser, K. Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in Drosophila. *Learn Mem* **5**, 102-14 (1998).

20. Schulz, R., Chromey, C., Lu, M., Zhao, B. & Olson, E. Expression of the DMEF2 transcription in the Drosophila brain suggests a role in neuronal cell differentiation. *Oncogene* **12**, 1827-1831 (1996).

21. Connolly, J. B. et al. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. *Science* **274**, 2104-7 (1996).

22. Tully, T., Preat, T., Boynton, S. C. & Del Vecchio, M. Genetic dissection of consolidated memory in Drosophila. *Cell* **79**, 35-47 (1994).

23. Abramoff, M. D. M., P.J.; Ram, S.J. Image Processing with ImageJ. *Biophotonics International* **11**, 36-42 (2004).

24. Demerec, M. *Biology of Drosophila* (John Wiley & Sons, New York, NY, 1950).

25. Grenningloh, G., Jay Rehm, E. & Goodman, C. S. Genetic analysis of growth cone guidance in drosophila: Fasciclin II functions as a neuronal recognition molecule. *Cell* **67**, 45-57 (1991).

26. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15 (1993).

27. Yang, M. A., JD; Vilinsky, I; Strausfeld, NJ; Kaiser, K. Subdivision of the Drosophila mushroom bodies by enhancer-trap expression patterns. *Neuron* **15**, 45-54 (1995).

28. Ito, K., Awano, W., Suzuki, K., Hiromi, Y. & Yamamoto, D. The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-71 (1997).

29. Duffy, J. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis* **34**, 1-15 (2002).

30. Truman, J. W. Metamorphosis of the central nervous system of Drosophila. *J Neurobiol* **21**, 1072-84 (1990).

31. Moreau-Fauvarque, C., Taillebourg, E., Boissoneau, E., Mesnard, J. & Dura, J. M. The receptor tyrosine kinase gene linotte is required for neuronal pathway selection in the Drosophila mushroom bodies. *Mech Dev* **78**, 47-61 (1998).

32. Michel, C. K., R; Restifo, LL. Defective neuronal development in the mushroom bodies of Drosophila fragile X mental retardation 1 mutants. *J Neurosci* **24**, 5798-809 (2004).

33. Yang, M. Y., Armstrong, J. D., Vilinsky, I., Strausfeld, N. J. & Kaiser, K. Subdivision of the Drosophila mushroom bodies by enhancer-trap expression patterns [see comments]. *Neuron* **15**, 45-54 (1995).

34. de Belle, J. S. & Kanzaki, R. in *Insect Olfaction* (ed. Hansson, B. S.) 243-281 (Springer-Verlag, New York, 1999).

35. Heisenberg, M. Mushroom body memoir: from maps to models. *Nat Rev Neurosci* **4**, 266-75 (2003).

36. Margulies, C., Tully, T. & Dubnau, J. Deconstructing Memory in Drosophila. *Current Biology* **15**, R700-R713 (2005).

37. McGuire, S. E., Deshazer, M. & Davis, R. L. Thirty years of olfactory learning and memory research in Drosophila melanogaster. *Progress in Neurobiology* **76**, 328-347 (2005).

38. Pinto, S. et al. latheo encodes a subunit of the origin recognition complex and disrupts neuronal proliferation and adult olfactory memory when mutant [see comments]. *Neuron* **23**, 45-54 (1999).

39. Strausfeld, N. J., Sinakevitch, I. & Vilinsky, I. The mushroom bodies of Drosophila melanogaster: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes. *Microsc Res Tech* **62**, 151-69. (2003).

40. Simon, A. F., Boquet, I., Synguelakis, M. & Preat, T. The Drosophila putative kinase linotte (derailed) prevents central brain axons from converging on a newly described interhemispheric ring. *Mech Dev* **76**, 45-55 (1998).

41. Boquet, I. H., R; Dumas, M; Chaminade, M; Preat, T. Central brain postembryonic development in Drosophila: implication of genes expressed at the interhemispheric junction. *J Neurobiol* **42**, 33-48 (2000).

42. Hitier, R. C., M; Preat, T. The Drosophila castor gene is involved in postembryonic brain development. *Mech Dev* **103**, 3-11 (2001).

43. Boquet, I., Boujemaa, R., Carlier, M.-F. & Préat, T. Ciboulot Regulates Actin Assembly during Drosophila Brain Metamorphosis. *Cell* **102**, 797-808 (2000).

44. Orihara-Ono, M. et al. The slender lobes gene, identified by retarded mushroom body development, is required for proper nucleolar organization in Drosophila. *Developmental Biology* **281**, 121-133 (2005).

45. Leonard, R. et al. The Drosophila fused lobes Gene Encodes an N-Acetylglucosaminidase Involved in N-Glycan Processing. *J. Biol. Chem.* **281**, 4867-4875 (2006).

46. Ditch, L. M. et al. Drosophila retained/dead ringer is necessary for neuronal pathfinding, female receptivity and repression of fruitless independent male courtship behaviors. *Development* **132**, 155-164 (2005).

47. Kobayashi, M. et al. Differential microarray analysis of Drosophila mushroom body transcripts using chemical ablation. *Proceedings of the National Academy of Sciences* **103**, 14417-14422 (2006).

48. Heisenberg, M. in *Development and Neurobiology of Drosophila* (eds. Siddiqi, O., Babu, P., Hall, L. M. & Hall, J. C.) 373-390 (Plenum Press, New York, 1980).

49. Moreau-Fauvarque C, T. E., Preat T, Dura JM. Mutation of linotte causes behavioral defects independently of pigeon in Drosophila. *Neuroreport* **13**, 2309-12 (2002).

50. McBride, S. M. J. et al. Pharmacological Rescue of Synaptic Plasticity, Courtship Behavior, and Mushroom Body Defects in a Drosophila Model of Fragile X Syndrome. *Neuron* **45**, 753-764 (2005).

51. Sherman, S. L. et al. Cognitive and behavioral performance among FMR1 high-repeat allele carriers surveyed from special education classes. *American Journal of Medical Genetics* **114**, 458-465 (2002).

52. Bennetto, L. & Pennington, B. *Neuropsychology. In: Fragile X syndrome: diagnosis, treatment, and research, (Hagermann PJ, ed),* (John Hopkins University Press, Baltimore, MD, 2002).

53. Dura, J. M., Preat, T. & Tully, T. Identification of linotte, a new gene affecting learning and memory in Drosophila melanogaster. *J Neurogenet* **9**, 1-14 (1993).

54. Dura, J. M., Taillebourg, E. & Preat, T. The Drosophila learning and memory gene linotte encodes a putative receptor tyrosine kinase

homologous to the human RYK gene product. *FEBS Lett* **370**, 250-4 (1995).

55. Zars, T. Behavioral functions of the insect mushroom bodies. *Curr Opin Neurobiol* **10**, 790-795 (2000).

56. Martin, J. R., Ernst, R. & Heisenberg, M. Mushroom bodies suppress locomotor activity in Drosophila melanogaster. *Learn Mem* **5**, 179-91 (1998).

57. Sehgal, A. et al. Molecular Analysis of Sleep: Wake Cycles in Drosophila. *Cold Spring Harbor Symposia on Quantitative Biology* **72**, 557-564 (2007).

58. Serway, C. N., Nolan, N. W., Freer, S. & de Belle, J. S. in *A. Dros. Res. Conf. 49* (The Genetics Society of America, San Diego, CA, 2008).

59. Kussel, P. & Frasch, M. Pendulin, a Drosophila protein with cell cycle-dependent nuclear localization, is required for normal cell proliferation. *J. Cell Biol.* **129**, 1491-1507 (1995).

60. Tejedor, F. et al. minibrain: A new protein kinase family involved in postembryonic neurogenesis in Drosophila. *Neuron* **14**, 287-301 (1995).

61. Reuter, J. et al. A mosaic genetic screen for genes necessary for Drosophila mushroom body neuronal morphogenesis. *Development* **130**, 1203-1213 (2003).

62. Ito, K. & Hotta, Y. Proliferation pattern of postembryonic neuroblasts in the brain of Drosophila melanogaster. *Dev Biol* **149**, 134-48 (1992).

63. Heisenberg, M., Heusipp, M. & Wanke, C. Structural plasticity in the Drosophila brain. *J Neurosci* **15**, 1951-60 (1995).

64. Technau, G. M. Fiber number in the mushroom bodies of adult Drosophila melanogaster depends on age, sex and experience. *J Neurogenet* **1**, 113-26 (1984).

65. Wang, X., Green, D. S., Roberts, S. P. & de Belle, J. S. Thermal Disruption of Mushroom Body Development and Odor Learning in Drosophila. *PLoS ONE* **2**, e1125 (2007).

66. Gatti, M. & Baker, B. S. Genes controlling essential cell-cycle functions in Drosophila melanogaster. *Genes Dev.* **3**, 438-453 (1989).

67. Vaysse, G. & Hirsch, J. Conditioned inhibition of the proboscis extension reflex in lines of D. melanogaster selectively bred divergently for conditioned excitation: the stability of genetic differences across behavioral measures. *DIS* **68** (1989).

68. Chabaud, M.-A., Devaud, J.-M., Pham-DelÃ¨gue, M.-H., Preat, T. & Kaiser, L. Olfactory conditioning of proboscis activity in Drosophila melanogaster. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **192**, 1335-1348 (2006).

69. Crittenden, J. R., Skoulakis, E. M., Han, K. A., Kalderon, D. & Davis, R. L. Tripartite mushroom body architecture revealed by antigenic markers. *Learn Mem* **5**, 38-51 (1998).

70. Boynton, S. & Tully, T. latheo, a new gene involved in associative learning and memory in Drosophila melanogaster, identified from P element mutagenesis [published erratum appears in Genetics 1992 Sep;132(1):289]. *Genetics* **131**, 655-72 (1992).

71. Isabel, G., Pascual, A. & Preat, T. Exclusive consolidated memory phases in Drosophila. *Science* **304**, 1024-7 (2004).

72. Timson, J. Hydroxyurea. *Mutat Res* **32**, 115-32 (1975).

73. Truman, J. W. & Bate, M. Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. *Dev Biol* **125**, 145-57 (1988).

74. Stocker, R. F., Heimbeck, G., Gendre, N. & de Belle, J. S. Neuroblast ablation in Drosophila P[GAL4] lines reveals origins of olfactory interneurons. *J Neurobiol* **32**, 443-56 (1997).

75. Krashes, M. J. & Waddell, S. Rapid Consolidation to a radish and Protein Synthesis-Dependent Long-Term Memory after Single-Session Appetitive Olfactory Conditioning in Drosophila. *J. Neurosci.* **28**, 3103-3113 (2008).

76. Folkers, E., Waddell, S. & Quinn, W. G. The Drosophila radish gene encodes a protein required for anesthesia-resistant memory. *Proceedings of the National Academy of Sciences* **103**, 17496-17500 (2006).

77. Folkers, E., Drain, P. & Quinn, W. G. Radish, a Drosophila mutant deficient in consolidated memory. *Proc Natl Acad Sci U S A* **90**, 8123-7 (1993).

CHAPTER 4

CONCLUSIONS

In the earlier chapters of this dissertation I have introduced the use of model organisms to study learning and memory behavior and shown that much of the information we learn from them can be carried over to human studies. Also, I went into greater detail about our model organism of choice, the fruit fly *Drosophila melanogaster*, and described what is currently known about their behavior as well as the underlying neuronal structure and gene products implicated in learning and memory. In the last chapter I have discussed experiments which have expanded our understanding of the development of three *Drosophila melanogaster* mutant strains (*mbmB, smu mbr*) which were initially isolated based on structural abnormalities to the mushroom bodies, a critical brain structure for learning and memory behavior. I was also able to further characterize the structural abnormalities of *mbmB, smu* and *mbr* flies showing that each had some lobes which were abnormal in appearance and that each has a reduced number of Kenyon cells at adulthood. Interestingly, cell counts show that as late in development as early third instar larvae cell number is not significantly different from wild type in *mbmB* and *smu* flies indicating that the γ lobe has a full complement of neurons. Two of these mutant flies (*mbmB, smu*) were amenable to behavioral examination using a T-maze apparatus for

olfactory aversive conditioning. Both revealed a learning deficit and no memory retention at 24hrs after training while *mbmB* had reduced memory at 12hrs using massed training but abolished memory using spaced training. This was a particularly interesting discovery because there has been some debate in the literature about the relationship between ARM and LTM. Originally it was felt these two were additive in affect and acted independently of each other[1]. However later research indicated that ARM may acts as a gating mechanism forLTM and upon spaced training ARM was abolished[2]. The finding that *mbmB* has no memory present at all at 12hrs after training supports the hypothesis that spaced training abolished ARM. Based on the discovery that *mbmB* and *pen* are allelic I believe that LTM should be disrupted in these flies because LTM is protein synthesis dependant and *pen* disrupts nuclear transport disturbing new protein synthesis. Massed training proves that at least some ARM should still be retained at 12 hrs but memory is completely abolished at this time in spaced trained flies.

There are likely many caveats to any experiment and the ones conducted for this dissertation are no exception. Many researchers believe it is impossible to localize a memory because it does not reside in a particular place. Karl Lashley spent his whole career trying to localize memories. He would train rats to perform various tasks then perform lesions to their cortex in order to determine sites of memory storage. He found after years of study that the site of the lesion was not important but that the size of the lesion had a greater impact on memory. He concluded that memory was widely distributed around the cortex[3]. However,

Lashley and many others were using complex training procedures such as spatial learning tasks which require many sensory modalities. More simple forms of memory may be easier to isolate in the brain. Richard F. Thompson, sought the engram of memory in the cerebellum instead of the cerebral cortex. Thompson and his colleagues used classical conditioning of the eyelid response in rabbits in their search for an engram. They puffed air upon the cornea of the eye and paired it with a tone. This airpuff normally causes an automatic blinking response. After a number of trials they conditioned the rabbits to blink when they heard the tone even though the airpuff was no longer administered. During the experiment, they monitored several brain cells to try to locate the engram.

One brain region that Thompson's group monitored that they thought was a possible part of the memory engram was the lateral interpositus nucleus (LIP). When chemically deactivated, it resulted in the rabbits, which were previously conditioned to blink when hearing the tone, to act as if the conditioning never took place; however, when researchers re-activated the LIP, they responded to the tone again with an eyeblink. This gives evidence that the LIP is a key element of the engram for this behavioral response[4]. The difference is the complexity of the task. Now the general view in neuroscience is that memory involved in complex tasks is distributed across multiple neural systems. At the same time, certain types of knowledge are processed and contained in specific brain regions[5]. I believe the type of knowledge we are exploring in flies fits into the latter category allowing us to at least find regions of the invertebrate brain important for different aspects of the memories.

It is also important that we not fall into the trap of assuming anatomical defects we can see on a macro scale are the cause of the behavior defects we see. For example, before I knew of the experiments indicating that *mbmB* and *pen* were allelic I had hypothesized that the LTM defect I saw was a result of the α lobe disruption. However, because $mbmB^{pen}$ disrupts nuclear transport and may interfere with protein synthesis necessary for LTM the memory defects may have nothing to do with the anatomical defects. It is important to remember these are correlations only. The power of these types of experiments is compounded when more and more mutant fly strains show the same results. That is why it is important to frame the results of my work in relation to what has previously been correlated in the literature. It will also provide more evidence for future researchers to interpret their results. However, we cannot completely rule out the possibility that unknown affects of the mutant proteins affect learning on a molecular level and the gross anatomical defects are merely side effects.

These new insights and other comments discussed in this dissertation can be added to an ever expanding cache of knowledge we have garnered about how learning and memory behavior is processed in the fruit fly *Drosophila melanogaster*. As I discussed in chapter one, our understanding of how a fly learns is not only important from an ecological point of view but also for comparison and expansion of our knowledge about how our own learning processes operate. Do MBs across species all perform exactly the same functions? Can we find some common threads among all the information we gather from less complicated species to the most complex? Although learning

has yet to be investigated in many insect species the MBs have been implicated in learning behavior for many that have been investigated including fruit flies[6], honeybees[7] and cockroaches[8]. However, evidence does suggest that MBs may perform slightly different functions in different species. This is highlighted by the finding that ablation studies show that MBs are required for odor discrimination tasks in flies but are not required in honeybees[9]. Analysis of learning and memory behavior across a variety of species including fruit flies will lead us to a better understand of our own neural foundations.

Bibliography

1. Tully, T., Preat, T., Boynton, S. C. & Del Vecchio, M. Genetic dissection of consolidated memory in Drosophila. *Cell* **79**, 35-47 (1994).
2. Isabel, G., Pascual, A. & Preat, T. Exclusive consolidated memory phases in Drosophila. *Science* **304**, 1024-7 (2004).
3. Lashley, K. *Brain mechanisms and intelligence: A quantitative study of injuries to the brain* (University of Chicago Press, Chicago, 1929).
4. Christian, K. & Thopson, R. Long-term storage of an associative memory trace in the cerebellum. *Behav Neurosci* **2**, 526-37 (2005).
5. Gerrig, R. & Zimbardo, P. *Psychology and Life* (Allyn & Bacon, Boston, MA, 2004).
6. Heisenberg, M. Mushroom body memoir: from maps to models. *Nat Rev Neurosci* **4**, 266-75 (2003).
7. Menzel, R. et al. in *Neural Basis of Behavioural Adaptations* (eds. Schildberger, K. & Elsner, N.) (Gustav Fischer Verlag, Stuttgart, 1994).
8. Lent, D., Pintér, M. & Strausfeld, N. Learning with half a brain. *Developmental Neurobiology* **67**, 740-751 (2007).
9. Malun, D. et al. Hydroxyurea-induced partial mushroom body ablation does not affect acquisition and retention of olfactory differential conditioning in honeybees. *Journal of Neurobiology* **53**, 343-360 (2002).

VITA

Graduate College
University of Nevada, Las Vegas

Brian S. Dunkelberger

Local Address:
555 Christpher Ct
Las Vegas, NV 89110

Degrees:
Bachelor of Science (Biology), 1995
The Pennsylvania State University

Publications:

Dunkelberger, B. S., Serway, C. N. and Steven de Belle, J. (2008b). A Biological Basis for Animal Model Studies of Learning and Memory. Advances in Psychology. In *Human Learning - Biology, Brain, and Neuroscience*, (ed. J. S. D. B. Aaron S. Benjamin, Bruce Etnyre and Thad A. Polk), pp. 211-225: North-Holland.

Dissertation title: The effects of mushroom body lobe disruption on learning and memory.

Dissertation examination committee:

Chairperson, J. Steven de Belle, Ph.D.
Committee Member, Andrew Andres, Ph.D.
Committee Member, Ralph Greenspan, Ph.D.
Committee Member, Stephen Roberts, Ph.D.
Graduate Faculty Representative, Jefferson Kinney, Ph.D.