2006-08-22

Genetic Dissection of the Neural Circuitry Underlying Memory Stability in Drosophila: A Dissertation

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A Dissertation Presented

By

Alex Carl Keene

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Science,
Worcester, MA 01605

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 22, 2006

Biomedical Sciences

Program in Neuroscience
GENETIC DISSECTION OF THE NEURAL CIRCUITRY UNDERLYING MEMORY STABILITY IN DROSOPHILA

A Dissertation Presented

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August 22nd, 2006
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Portions of this thesis appeared in:

Krashes MJ, Keene AC, Leung BL, Armstrong JD, and Waddell S. Stable
Drosophila odor memory requires the sequential involvement of specific
mushroom body neurons. Submitted.

Keene AC and Waddell S. Drosophila olfactory memory: from single genes to

Keene AC, Krashes MJ, Leung BL, Bernard J, and Waddell S.
Drosophila Dorsal Paired Medial neurons provide a general mechanism

Keene AC and Waddell S. Drosophila memory: Dopamine signals

Yu D, Keene AC, Srivatsan A, Waddell S, and Davis RL. Drosophila DPM
neurons form a delayed and branch-specific memory trace after olfactory

Keene AC, Stratmann M, Keller A, Perrat PN, Vosshall LB, and Waddell, S.
Diverse odor-conditioned memories require uniquely timed dorsal paired
I am greatly indebted to many people for supporting me through the first quarter century of my life. Scientifically, the community at UMass Medical, and in the Department of Neurobiology could not have provided a better environment for my training. I first came to Scott’s laboratory in search of an environment where I could ask open-ended questions, rather than perform the specific aims of a grant. What I ended up with was an advisor whose door was always open, consistently gave (and sometimes received) constructive criticism, and pushed me to think, write, and perform my experiments stringently. I thank Vivian Budnik for chairing all three of my committees at UMass. Her eye for detail and honest advice has been an invaluable part of my graduate education. I have also been lucky enough to have many informal advisors on the floor. In particular, Marc Freeman, Marc Alkema, David Weaver and Patrick Emery, and Tzumin Lee always welcomed my questions and provided thoughtful experimental and career advice. Finally, I am greatly indebted to Steven Reppert for creating an environment that is as close to an ideal of scientific pursuit as any that I have ever been a part of or could have imagined. On the 7th floor of the Lazare Research Building there is little of the petty bickering and egoism that is often associated with science. Instead an open atmosphere where information, ideas, and materials are
shared on a regular basis and questions are pursued in an aggressive and collegial fashion has replaced these aspects.

The work presented in this dissertation was made possible by the atmosphere and my colleagues in the Waddell laboratory. This is in part due to boss’s attitude that science should be intense and enjoyable, but is also due to the workers. Just about every idea I had over the past 4 years (be it scientifically related or not) was bounced off Richard, Shamik, Ben, Paola, Ruth, or Mike. This openness of ideas at times led to many ridiculous (and often inappropriate!) conversations, but more importantly led to many scientific collaborations. This is evidenced by the numerous experiments in this dissertation that were the result of intralab collaborations. I am especially grateful for Ruth’s patience and expertise and collaborative work performed with Mike Krashes, which have led to numerous publications.

There are innumerable people whose love and support have been invaluable. My mother and father have taught me from an early age to think critically, compassionately, challenge dogma, and move through life with an ambitious fire of heart and mind. Danya, my big sister (age-wise), has set a bar unreachably high both academically and athletically and I am constantly trying to live up to her example. My little brother’s (age and size!) clever wit has made life more enjoyable, and without him, there would be no one to make fun of Danya and our parents. Over the past 5
years I have also had the privilege of spending time with my little brother Cory. Together we have seen the Red Sox, the Everglades, and the Basketball Hall of Fame. My interactions with Cory and the Hampshire county big brother’s big sister’s program have been some of the most meaningful and growth promoting in my life.

My residence in Worcester over the past 4 years has been greatly aided by frequent escapes to Boston, New York, and Florida and I greatly thank my aunts, uncles, and grandparents for allowing me to use their residence as a free hotel. I owe great thanks to my cousin Isaac for not acting too bitterly towards me after the Yankees stunning defeat at the hands of the Red Sox in the 2004 ALCS. After three late night phone calls following Yankees victories, I did not hear from him for quite some time. There are many other members of my extended family who I cannot thank by name, but am greatly indebted to.

Throughout my life I have also been blessed by a large support network of friends that have often gone to great lengths and made many personal sacrifices for my means. In particular Chris, Debbie, Audrey, and Lily Gould took me into their family in my final years of high school. Not only did they see me through the most turbulent years of my life, but also they let me into one of the most caring families I have ever known. My high school running mates, Eric, Matt, Jon, John, Taylor, and Thushan have remained my closest friends throughout the last decade of my life.
Their loyalty has shown no limits as was made clear during my first year of graduate school when Matt drove for hours on Christmas Eve to pick me up while I was stranded at the Natick Plaza.
ABSTRACT

Understanding how memory is formed requires looking beyond the genes involved to the neural circuitry and temporal aspects of memory. In this dissertation I have focused my investigation on Dorsal Paired Medial (DPM) neurons, two modulatory neurons essential for memory in *Drosophila*. DPM neurons highly express the *amnesiac* (*amn*) gene, which encodes for a putative pre-pro-neuropeptide. *amn* function in DPM neurons is required for memory. Here I provide evidence that DPM neurons are cholinergic and that acetylcholine (ACh) and AMN act as co-transmitters essential for DPM function. In order to investigate the temporal requirements of DPM output I blocked transmitter release during discrete intervals in the memory process using *shibire*<sup>ts1</sup> and tested flies for shock and sugar-reinforced memory. These experiments demonstrated that stable memory requires persistent transmitter release from DPM neurons. Furthermore these results suggest AMN and DPM neurons act as general stabilizers of mushroom body dependent memory. To further investigate the neural circuitry underlying DPM function I disrupted DPM projections onto the mushroom body lobes by ectopically expressing *DScam17-2::GFP* in DPM neurons. Flies with DPM neurons that predominantly project to the mushroom body α´/β´ lobes exhibit normal memory, and blocking transmitter release from the mushroom
body prime lobes neurons themselves abolishes memory indicating DPM neuron-mushroom body $\alpha' / \beta'$ neuron interaction that are critical for memory. Taken together, the experimental evidence presented here are used to provide a rudimentary model of the neural circuitry involved in memory stability, where DPM neurons form a recurrent feedback loop with the mushroom body $\alpha' / \beta'$ lobe neurons and act to stabilize odor-specific conditioned memories at Kenyon cell synapses.
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LIST OF ABBREVIATIONS

ACh: acetylcholine
AChR: Acetylcholine receptor
ACT: Antennocerebral tract
amn: amnesiac
BA: Benzaldehyde
ChAT/cha: Choline acetyltransferase
CS+: Paired conditioned stimulus
CS: Unpaired conditioned stimulus
DA: Dopamine/dopaminergic
DScam: Down syndrome cell adhesion molecule
DPM: Dorsal Paired Medial
GR: Gustatory receptor
GRN: Gustatory receptor neuron
LH: Lateral horn
MCH: methylcyclohexanol
PN: Olfactory projection neuron
OA: Octopamine/octopaminergic
OR: Olfactory receptor
OCT: Octanol
OSN: Olfactory sensory neuron
PACAP: Pituitary adenylyl cyclase-activating peptide
PKA: cAMP dependent protein kinase
PKC: Protein kinase C
SOG: Subesophageal ganglion
RISC: RNAi induced silencing complex
TβH-Tyrosine beta hydroxylase
VUMmx1: Ventral unpaired median neuron

**Note of Clarification:** This document discusses both “learning” and “memory”. Standard protocols in *Drosophila* olfactory memory research refer to memory tested at 3 minutes (as fast as is possible post-training) as “learning”. Learning is consistent with a putative Short-Term Memory phase which is not protein synthesis independent and primarily anesthesia sensitive. “Memory” in this document refers primarily to middle-term memory, which is protein synthesis independent and largely anesthesia-resistant form of memory that exists from approximately 30 minutes to 5 hours following training. Long-term memory is composed of protein-synthesis dependent and resistant-resistant memory (for review see Tully et al., 1996).
CHAPTER I: INTRODUCTION

Our ability to remember past experience allows us to learn from our mistakes. Memories can often be conceptualized to the pairing of a mild stimulus, such as a perfume, with an emotionally salient event, like enduring love or a painful breakup. Consequently, a waft of familiar perfume can evoke fond memories in one person, and can be utterly repugnant or completely meaningless to another. This ability to pair certain neutral stimuli with meaningful events is conserved across phyla. This introduction will discuss the genes and neural circuitry that bestow the fruit fly with the capacity to form associative memories between odors and punitive or rewarding stimuli. In this dissertation I make efforts to experimentally dissect the genes and neural circuits involved in memory stability in order to further our understanding of how memories are formed and maintained.

A central goal of neural science is identifying the molecules and neural circuits that drive behavior. To date, the ease of forward genetics in the fruit fly Drosophila melanogaster has led to a focus on the genes that regulate behavior. More recent genetic advances have allowed for dissection of the neural circuits involved in behavior. Drosophila is an excellent model organism to study the molecular, cellular and neural circuit basis of memory. Drosophila can be taught to pair a number of
neutral conditioned stimuli with salient unconditioned stimuli in several different learning assays (Duerr and Quinn, 1982; Mery and Kawecki, 2002; Tully and Quinn, 1985; Wolf et al., 1998). However, many investigators teach flies to associate an odor conditioned stimulus (CS) with either a punitive shock or a rewarding sugar unconditioned stimulus (US). Following such training, fly memory is observable as a preferential avoidance of, or attraction to, the reinforced odor (CS). Fruit fly memory persists for hours or days, depending on the training protocol (Mery and Kawecki, 2005; Tully et al., 1994). Until recently much of the study of \textit{Drosophila} memory has focused on the search for genes. The assumption has often been made that the identified “memory” genes function in a single cascade. With such a focus, the study of the network properties of memory has often been lost. This introductory chapter serves to provide a historical background of the field, followed by a detailed analysis of the current knowledge of how neural circuits encode for olfactory memories.

The genetic basis of olfactory learning and memory has been studied in \textit{Drosophila} for over 30 years. Considerable effort has been spent screening for memory defective flies and the field now has a large collection of mutants. The proteins normally encoded by the mutated genes have implicated several well-studied cellular processes and signaling cascades in memory (Reviewed in Margulies et al., 2005;
However, very little attention has been directed to determine exactly how these proteins work in the context of memory and in precisely which cells and relevant circuitry they function. Many researchers have created single neuron models into which all previously identified memory genes are lumped together (Dubnau et al., 2003; McGuire et al., 2005; Waddell and Quinn, 2001b). This precocious logic assumes that all “memory neurons” are homogeneous and completely ignores a role for neural circuitry in memory. Truthfully, it is not know if these learning-related proteins interact with each other, or even if they are expressed in the same neurons!

**The Power of Drosophila Genetics**

*Drosophila* has a great advantage as a model system because genetic manipulation is fast and technological development is frequent and impressive (Venken and Bellen, 2005). These rapidly evolving tools provide drosophilists with an unparalleled ability to dissect the specific roles of identifiable neurons and genes in memory. In the fruit fly, transgenes can be expressed in a tissue specific manner with great ease using the GAL4/UAS system, allowing for genetic analysis of neural subsets (Brand and Perrimon, 1993). Recently live optical imaging of specific neurons expressing genetically-encoded reporters of neural activity and electrophysiological recording from single neurons in the adult brain has been added to the growing arsenal (Gu and O'Dowd, 2006;
Wilson and Laurent, 2005; Yu et al., 2005). Together these technologies allow for the identification of key brain events involved in memory formation, stability and retrieval.

One of the obvious strengths of working with *Drosophila* as a model system is rapid mutant analysis. Mining of the genome for memory mutants has provided a foundation for dissecting the cellular basis of plasticity. Furthermore, *Drosophila* mutants have provided seminal insights into the molecular pathways that modulate synaptic plasticity, which appear to be shared across phyla (Mayford and Kandel, 1999). However, perhaps unsurprisingly, behavioral screens for memory mutants are much more arduous than screens for anatomical defects and it is a gigantic leap in understanding how a single gene mutation impairs behavioral memory. Nevertheless, mutants are the building stones for any serious genetic analysis and the field now possesses an impressive array of mutants. Some of these have been heavily analyzed while most require greater inspection.

**Fly memory mutants**

The first *Drosophila* learning mutant to be isolated, *dunce*, is a mutation in cAMP phosphodiesterase (Chen et al., 1986; Dudai et al., 1976; Qiu and Davis, 1993). Since this original discovery, many other learning mutants have been identified which implicate the cAMP signaling cascade in learning and memory including *rutabaga* (Levin et al.,
1992) (*rut*, a mutation in adenylyl cyclase) and *DCO* (Skoulakis et al., 1993), the protein kinase A (PKA) catalytic subunit. Of course, many of these mutants have been indirectly tied into this pathway, such as is the case for the cell adhesion molecules *volado* and *fasciclin-II* (Cheng et al., 2001; Grotewiel et al., 1998). Undoubtedly, the focus on cAMP signaling in the study fly memory has been misleading. In both invertebrates and mammals other signaling cascades such as MAP kinase and protein kinase C (PKC) have been implicated in memory (Abeliovich et al., 1993; Kuzirian et al., 2006). In fact, in *Drosophila*, PKC has been found to be involved in both olfactory memory and courtship conditioning, yet the particular role of PKC in these memories remains elusive (Kane et al., 1997; Mihalek et al., 1997). Nevertheless, involvement of the cAMP pathway in learning and memory extends across phyla and has been studied extensively in *Aplysia* and mice (Mayford and Kandel, 1999).

A number of different approaches have been used to screen for memory defective flies. The first large scale screens in the laboratory of Seymour Benzer, and later Chip Quinn used chemical mutagenesis. More recent screens have taken advantage of advances in genetic technology for more a targeted approach. While chemical mutagenesis has the advantage of being non-biased, many of the identified mutants have proven difficult to map behaviorally. For example, the mutant *radish (rsh)*, has been heavily studied (Chiang et al., 2004; Folkers et al., 1993; Isabel
et al., 2004), but the gene responsible for the memory defect still remains controversial (Margulies et al., 2005; McGuire et al., 2005). Furthermore, these early screens were limited to the X-chromosome, and therefore only targeted a small percentage of the genome. A later large-scale screen in the laboratory of Ron Davis assayed fly lines with P-element enhancer traps that highly expressed in the mushroom bodies, a neural locus associated with memory (Han et al., 1996b; Skoulakis and Davis, 1996). While this approach has the advantage of increasing the probability of hits through pre-selection based on expression pattern, it also is based on the assumption that learning and memory genes function exclusively in brain regions already identified as memory centers.

**Mapping Circuits**

The simple nature of the associative learning assay leads to the fairly straightforward assumption that the circuitry of the fly brain is able to integrate olfactory information with that of punitive or rewarding stimuli. Using this framework I will describe the field’s rapidly evolving understanding of the conditioned stimulus pathway – olfactory coding in *Drosophila* - followed by our current understanding of the unconditioned stimulus pathways- transmitting either electric-shock or gustatory information. I will finish by reviewing our understanding of the neural and molecular mechanism of CS and US stimulus association. In this dissertation I will present further evidence that memories formed by
differing unconditioned stimuli rely on both shared and divergent mechanisms.

Current models predict that memory is formed at specific synapses within neurons in the CS pathway (Reviewed in Gerber et al., 2004b). Memory specificity is therefore determined by CS pathway activity and it is reinforced by modulatory neurons driven by the US pathway (Schwaerzel et al., 2003). Current understanding of olfactory CS processing in the brain is considerable and our knowledge of gustatory US processing is developing. The anatomy and coding principles of the olfactory system provide possible loci of coincidence detection and presumably memory storage.

**Processing the olfactory conditioned stimulus in the fly brain.**

**Peripheral olfactory coding**

Flies primarily sense odors through the antennae and maxillary palps on the front of the head (Figure 1.1). The fly antennae house approximately 1200 olfactory sensory neurons (OSNs) and the maxillary palps about 120 (Stocker, 2001). The identification of a family of ~62 *Drosophila* olfactory receptor (OR) genes heralded a detailed anatomical and functional analysis of olfactory receptors and olfactory sensory neurons (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). It has long been assumed that *Drosophila* ORs, like mammalian and nematode ORs, signal through GPCRs. However, elegant work in the
laboratory of Leslie Voshall has demonstrated that *Drosophila* ORs exhibit membrane topology opposite from mammalian homologs, suggesting that these receptors do not signal through GPCRs (Benton et al., 2006). Thus, while the mechanism of OR signaling remains elusive, much is known of the manner in which neurons harboring OR subtypes respond to odor.

ORs are expressed in OSNs that are housed in three different kinds of sensory hairs, on the antennae and maxillary palps (Clyne et al., 1999; Vosshall et al., 1999). The development, organization and coding have been recently reviewed by others (Hallem and Carlson, 2004; Jefferis et al., 2005; Vosshall, 2000).

The functional differences between the antennae and maxillary palps and the relative organization of their odor responses are not currently understood. Nevertheless, in both the antennae and palps it appears that for the most part, individual OSNs exhibit a response profile determined by the presence of a single OR. Application of a very elegant technique has determined that the baseline and olfactory response parameters of an individual OSN are governed by the OR type that OSN expresses. Expressing other OR genes in an ‘empty neuron’ (ab3A) that lacks its cognate receptors (OR22a and ORR22b), confers the olfactory response of the neuron in which the particular OR is usually expressed onto the ab3A neuron (Dobritsa et al., 2003). Thus, the OR determines
the odor response specificity and remarkably it also confers whether the neural response to a given odor is excitatory or inhibitory as well as the response dynamics. The only parameter the OR does not seem to determine is the spike amplitude (de Bruyne et al., 1999; de Bruyne et al., 2001; Hallem and Carlson, 2006; Hallem et al., 2004).

*Drosophila* have about 1400 OSNs expressing 1 of 62 ORs and these OSNs converge on approximately 43-50 glomeruli (Couto et al., 2005; Fishilevich and Vosshall, 2005; Laissue et al., 1999; Shanbhag et al., 1995; Stocker, 1994; Stocker, 2001). Exhaustive studies have determined that OSNs expressing each receptor subtype target a specific glomerulus in the antennal lobe (AL) (Couto et al., 2005; Fishilevich and Vosshall, 2005). Within that glomerulus OSNs make synapses with at least two classes of neurons - Projection Neurons (PNs) and Lateral Neurons (LNs) (Jefferis et al., 2001; Ng et al., 2002).

Adult flies have about 150 PNs and therefore there are thought to be 3-5 PNs innervating each AL glomerulus (Stocker et al., 1997; Tissot et al., 1997). Most PN dendrites innervate a single glomerulus and therefore one might assume they relay information from a single class of OSN and therefore a single type of OR. Imaging studies expressing genetically encoded reporters of neural activity in OSNs or PNs support the hypothesis that information is transferred directly from OSNs to PNs (Ng et al., 2002). Imaging PN activity in glomeruli reveals that PN
dendrites, as well as recurrent PN connections, respond selectively to the same odor known to activate the OSNs innervating the same glomerulus (Ng et al., 2002; Wang et al., 2003a; Wong et al., 2002; Yu et al., 2004). However, whole-cell electrophysiological recordings from PNs suggest a greater level of complexity in coding odor information. Some PNs respond to specific odors but many PNs are more broadly tuned and are activated, or inhibited, by almost all odors (Wilson et al., 2004). Most importantly, this study implies that PNs are more broadly tuned than their afferent OSNs. How might that happen? OSNs form synapses with PNs and GABAergic inhibitory LNs, some of which ramify throughout the AL. Therefore it is possible that activity in OSNs and PNs “spreads” inhibition throughout the AL and thereby modulates the PN responses. This type of LN activity could explain why several PNs were found to be inhibited by many odors. It has been proposed that LN inhibitory input amplifies differences in PN responses to varying odor stimuli (Wilson and Laurent, 2005). In addition to LN mediated inhibition, PNs also form recurrent connections within glomeruli. They therefore may also mediate direct effects on neighboring PNs. In order to understand how odor information is represented in the deeper brain, it will be essential to understand how the information is handled at the level of the AL. Unfortunately, current imaging technology is not yet fast or sensitive enough to provide the temporal detail afforded by direct electrophysiological recording.
Therefore although imaging may indicate high threshold slow events that reveal the fundamentals of connectivity, combining optical imaging and whole-cell, perhaps multi-unit, recording will be required to resolve the coding controversy.

In some insects, plasticity in the antennal lobes is involved in memory (Meller and Davis, 1996; Meller et al., 1997; Menzel, 2001). Live imaging the *Drosophila* AL with synaptopHluorin, a pH-sensitive reporter of synaptic vesicle release, before and after training has uncovered short-term plasticity in the odor-evoked response of PNs (Yu et al., 2004). Another study of the antennal lobes has demonstrated odor-induced changes in Ca\(^{2+}\)/calmodulin-dependent protein kinase II synthesis following a long-term memory training protocol (Ashraf et al., 2006). However, in both these cases, it remains to be determined if the plasticity in odor representation contributes to memory.

**PN connections – into the deep**

PNs receive olfactory information in the AL and project it to two locations in the brain – the mushroom bodies and lateral horn (Heimbeck et al., 2001; Marin et al., 2002). The PN projections are organized into three different neural tracts - the inner (i), medial (m) and outer (o) antennocerebral tract (ACT). PNs of the iACT, form synapses in the mushroom body calyx and lateral horn whereas PNs in the mACT and oACT bypass the mushroom body calyx and go straight to the lateral
The mACT PNs innervate multiple glomeruli and therefore by connectivity alone, mACT PNs would be expected to be activated (or inhibited) by many, or all odors (Marin et al., 2002; Wong et al., 2002).

The PN projections to the mushroom bodies and lateral horn represent an obvious bifurcation in odor processing at this anatomical level and the organization of projections appears to be complex. Recent elegant studies labeling single PNs have provided a first glance of PN connectivity within the lateral horn and mushroom body calyx. Projection neurons from the same glomerulus have similar projection patterns in the lateral horn implying connectivity stratification in the lateral horn (Marin et al., 2002; Wong et al., 2002). One report has detailed a collection of intrinsic lateral horn neurons that arborize in discrete lateral horn strata overlapping with projection neuron input zones. These lateral horn neurons project to discrete areas of the protocerebrum and this organization suggests that particular lateral horn neurons receive information from restricted populations of projection neurons and therefore perhaps a restricted odor repertoire (Tanaka et al., 2004). The authors proposed that the lateral horn has limited integrative capacity compared to the mushroom body calyx. PNs innervate wide zones in the mushroom body calyx and mushroom body dendrites are widely distributed across zones consistent with the idea that mushroom body neurons can integrate information across odors (Tanaka et al., 2004).
Why do olfactory projection neurons transmit olfactory information from the antennal lobe to the mushroom body and lateral horn? It is thought that the lateral horn represents an experience-independent pathway and the mushroom bodies provide the fly with a neural network to encode olfactory experience. Pharmacological ablation of mushroom bodies impairs higher-order processing of olfactory information (e.g., learning and memory). However, mushroom body ablated animals retain the ability to avoid certain concentrations of odors and therefore mushroom bodies are not essential for naïve odor avoidance (de Belle and Heisenberg, 1994). Naïve avoidance is apparently driven by the lateral horn because blocking projection neuron synaptic transmission with transgene-encoded tetanus toxin impairs odor avoidance behavior (Heimbeck et al., 2001). One study suggested that attractive and repulsive olfactory information is differentially represented between the lateral horn and mushroom bodies. Blocking mushroom body output perturbed odor driven attraction but not repulsion (Wang et al., 2003b). Multiglomerular projection neurons in the medial antennocerebral tract may respond to high (and possibly noxious) concentrations of odors and thereby drive avoidance behavior through their exclusive lateral horn projections.


**Odor representation in the Mushroom Bodies**

The mushroom bodies are comprised of about 2,500 neurons – also called Kenyon cells - per side of the brain and the intrinsic mushroom body neurons can be roughly categorized into five subdivisions based on their projection pattern in the axonal domain – called the mushroom bodies lobes (Crittenden et al., 1998; Lee et al., 1999). However, splitting mushroom bodies into these five subdivisions ($\alpha, \alpha', \beta, \beta', \gamma$) appears to be unsatisfactorily low resolution because there are clearly further subdomains within the identified lobes. Enhancer-trap studies reveal that some genes are expressed in subsets of mushroom body neurons within a lobe set (Yang et al., 1995) suggesting higher resolution lobe subdivision is likely. Indeed, Strausfeld and colleagues (Strausfeld et al., 2003) defined further lobe subdivisions ($\alpha c, \beta c$ and $\beta''$) based on immunostaining for the putative neurotransmitters, aspartate, glutamate and taurine which label small portions of the mushroom bodies.

Nevertheless, major subtypes of mushroom body neurons appear to be developmentally related and synapse onto similar regions, suggesting that perhaps subtypes of mushroom body neurons have similar functions.

The organization of projection neuron-mushroom body connectivity is not well understood. This is exemplified by the fact that mushroom body neurons are named based on their axonal projection domain rather
than by their dendritic fields in the calyx. The importance of the connectivity somewhat depends on whether odor information is transformed, or not, in the antennal lobe. If activity in projection neurons constitutes ‘labeled lines’ representing specific odors then precise wiring to mushroom body neurons could in turn transfer that labeled line information to mushroom body neurons. This would mean that particular odors are reproducibly handled by a similar array of mushroom body neurons between animals. If however, the odor code is more dynamic, the connectivity could prove less meaningful. *Drosophila* larvae have a greatly reduced olfactory system, having only 21 OSNs that project to 21 antennal lobe glomeruli (Tissot et al., 1997). Remarkably, the larval mushroom body calyces also exhibit a clear glomerular structure and each of the approximately 28 glomeruli is innervated by one or two PNs (Ramaekers et al., 2005). This organization suggests a similar structure may exist in the adult mushroom body calyx that is obscured by the increased number of projection neurons and mushroom body neurons. In fact, beautiful anatomical work has described large glomerulus-like arrangements of cholinergic PN terminals in the adult mushroom body calyx intermingled with GABAergic and occasional peptidergic and monoaminergic terminals (Yasuyama et al., 2002). There are at least three clear types of larval mushroom body neurons based on dendritic architecture. Kenyon cells are either uniglomerular, biglomerular or their
dendrites arborize more diffusely in larger domains of the calyx (Zhu et al., 2003). Similar types of adult Kenyon cells have also been described and these different classes suggest olfactory information may be processed in parallel ways in the larval and adult mushroom bodies (Zhu et al., 2003). Each odor could be represented individually as well as in an integrated manner with other odor representations. This could conceivably aid flies in distinguishing individual odors within complex mixtures.

Due to the abundance of OSNs in comparison to olfactory projection neurons, neural connectivity therefore predicts that odors are sparsely represented in the mushroom bodies and optical imaging studies support this notion. The first *Drosophila* live imaging study using the genetically-encoded Ca$^{2+}$ indicator cameleon suggested that odors elicit stereotyped activation of projection neurons in distinct regions of the mushroom body calyx (Fiala et al., 2002). Similar odor-stereotyped Ca$^{2+}$ influx patterns have been imaged using the Ca$^{2+}$ sensor G-CaMP in the cell bodies of mushroom body neurons (Wang et al., 2004b), suggesting odors do in fact activate specific subsets of Kenyon cells. These studies imply that regardless of how the information gets there, individual odors could be represented as sparsely labeled lines in the mushroom bodies and this belief is central to current models of odor memory. However, it is worth noting that although odors may evoke activity in a sparse array of
mushroom body neuron cell bodies, and perhaps dendrites (Wang et al., 2004b), it is not clear how the information is represented in the mushroom body lobes because the extent of mushroom body neuron interconnection by gap junctions and/or chemical synapses is unknown. Mushroom body neurons that have diverse dendritic arborization patterns distributed across calycal zones appear to contribute to all the mushroom body lobes which suggests that odors will also be distributed across the lobe subdivisions (Zhu et al., 2003).

Projection neurons are predominantly cholinergic and therefore the physiology of mushroom bodies neurons likely respond to activation of ACh receptors in mushroom body dendrites (Yasuyama et al., 1996; Yasuyama et al., 2002). Imaging neural Ca\(^{2+}\) activity with camgaroo revealed that ACh application to the mushroom body calyx induces a rapid depolarization throughout the mushroom bodies that can be blocked by application of nicotinic receptor antagonists (Yu et al., 2003).

Furthermore, in a recent and impressive study (Gu and O'Dowd, 2006) directly recorded from individual Kenyon cells in a dissected brain and established that mushroom bodies are part of a spontaneously active circuit that is driven by cholinergic (presumably projection neuron) input.

**What is the US pathway?**

The olfactory learning paradigms most often employ either a punitive electric-shock or rewarding sucrose as the US. Although the
sensory mechanism for electric shock punishment is a mystery, the mechanism of gustatory sensation has followed olfaction with the identification of a family of gustatory receptors (Clyne et al., 2000; Scott et al., 2001). Shock may be sensed by a relatively non-specific activation of multiple sensory neurons – presumably in the legs because the flies are standing on the grids. Alternatively it seems possible that the shock-sensing mechanism is not peripheral and instead is manifest as a fairly non-specific depolarization of a group of internal neurons. Sugar stimuli in contrast are sensed by gustatory receptor neurons on the tarsae and proboscis. These neurons co-express groups of gustatory receptors that are either responsive to sweet, bitter, sour or salty stimuli (Reviewed by Scott, 2005). Gustatory receptor neurons project this information to the subesophageal ganglion where they appear to ramify in discrete strata (Chyb et al., 2003; Thorne et al., 2004; Wang et al., 2004c). Direct aversive or appetitive drive is abolished in flies if the relevant sensory neurons expressing the appropriate receptor are ablated, and ectopic activation of the sweet sensing or bitter sensing neurons is sufficient to drive appetitive or aversive behavior respectively (Marella et al., 2006).

By direct analogy to other organisms, the US activated sensory pathways are believed to stimulate monoaminergic modulatory neurons. The best evidence for a role of specific monoamines in Drosophila memory comes from a single landmark study. In this study it was shown
that dopaminergic (DA) neurons are required exclusively for shock-reinforced odor memory whereas octopamine (OA) is required for sugar-reinforced odor memory. Using the uas-\textit{shibire}^{ts1} (uas-\textit{sh}^{ts1}) transgene to inactivate neural subsets, it was determined that synaptic transmission from dopaminergic neurons is required for negatively reinforced memory but is not required for positively reinforced memory (Schwaerzel et al., 2003). The exact identity of the relevant dopamine and octopamine releasing neurons is not known but imaging studies have provided a tantalizing glimpse (Riemensperger et al., 2005). Dopaminergic neurons innervate wide areas of the fly brain including the mushroom bodies (Friggi-Grelin et al., 2003). Riemensperger and colleagues (2005) used the ratiometric Ca$^{2+}$ reporter cameleon to optically record activity in DA neuron projections in the vicinity of the mushroom body lobes. These DA projections respond weakly to odor, but strongly to shock confirming the notion that DA neurons are activated by electrical shock. Followia training session pairing odor and shock, the previously shock paired odor evoked a prolonged activation of the DA neurons suggesting that DA release onto mushroom bodies might be predictive of shock during memory retrieval. However, selectively blocking DA neurons during retrieval does not impair memory performance (Schwaerzel et al., 2003) and therefore DA neurons are unlikely to provide predictive value during retrieval of a negatively reinforced odor memory. These studies suggest that although DA
neurons are clearly involved in aversive odor memory, their role is likely to be complex. It will be interesting to test if DA neurons generally represent aversive stimuli or whether they are specific to shock-reinforcement. In the cricket, pharmacologically blocking DA receptors impairs aversive conditioning with high salt as a reinforcer (Unoki et al., 2005). Finally, DA neurons ramify throughout the brain and a requirement for the subset that project onto the mushroom bodies has not been demonstrated.

Much less is known about OA function in the fly brain. Octopamine deficient TβH mutant flies are defective in sugar-reinforced olfactory memory but have wild-type levels of shock-reinforced memory. The sugar-reinforced memory defect was rescued by inducing a heat shock-TβH transgene in adult flies, or by octopamine feeding prior to training suggesting an acute role for OA in memory (Schwaerzel et al., 2003). Unfortunately, although the location of OA cell bodies is known, the arborization of OA neurons in the brain has not been described. A great candidate for the relevant OA neurons would be those resembling the honeybee octopaminergic Ventral unpaired median (VUMmx1) neuron. VUMmx1 has a cell body that resides in the Suboesophageal ganglion (SOG) where it could be directly driven by sugar responsive Gustatory receptor neurons (GRNs). VUMmx1 in the bee projects bilaterally to the antennal lobes, mushroom body and lateral protocerebrum. In a classic study VUMmx1 was shown to be activated by sugar reward and electrical
stimulation of VUMmx1 substituted for sugar presentation in the honeybee proboscis extension reflex model of associative conditioning (Hammer, 1993). It will be interesting to see if the cluster of OA cell bodies in the fly SOG contains VUMmx1-like neurons.

Understanding of monoamine function in memory will be greatly aided through the study of receptors for these transmitters. Kenyon cells express both DA and OA receptors (Han et al., 1998; Han et al., 1996a) but their detailed localization in the mushroom bodies has not been reported. An important question in the field is how different types of memory are represented within the mushroom bodies. It is possible that specific subsets of Kenyon cells are responsive to different monoamine reinforcers. Alternatively, DA and OA neurons may activate different synapses on the same Kenyon cells (Gerber et al., 2004b; Schwaerzel et al., 2003). It is interesting to note that DA neurons do not evenly innervate the entire mushroom bodies. They more strongly innervate the α lobe stalk, the tip of the β lobes and the heel region (Riemensperger et al., 2005). This may indicate underlying organization of negatively reinforced memory.

**Mushroom bodies as coincidence detecting memory centers**

Current dogma assumes that the fly nervous system likely stores associative memories within neurons that receive both the conditioned stimulus odor information and the unconditioned stimulus of shock
punishment or sugar reward. Although it is by no means a closed book, the current favorite locale that appears to fit these criteria is the mushroom bodies. The mushroom bodies were first suggested to be the center of insect intelligence in 1850 by Dujardin (Dujardin, 1850). Several observations in other insects have led to the belief that mushroom bodies are required for learning. The first evidence for a role of the *Drosophila* mushroom bodies in olfactory learning and memory came from a collection of ‘anatomical’ brain mutants by Heisenberg and colleagues (Heisenberg et al., 1985). Mushroom body defective flies could sense odors and shock but could not associate the two cues. Although these anatomical defects were not necessarily exclusive to mushroom bodies, learning defects correlated with morphologically defective mushroom bodies and not with lesions in other brain regions, e.g., the central complex. Following up on these morphological studies, pharmacological ablation of the mushroom bodies and a few antennal lobe neurons led deBelle and Heisenberg to the same conclusion (de Belle and Heisenberg, 1994).

In rather nice coincidence with anatomical studies, the first attempts to localize memory-relevant gene products (*dunce, rutabaga* and *DCO*) labeled the intrinsic cells of the mushroom bodies – the Kenyon cells (Crittenden et al., 1998). It is important to note that most of these memory relevant gene products express at low levels throughout the
brain, and therefore further study is needed to address the neural cites in which these molecules function. It remains to be determined whether these molecules, that are assumed to represent a linear signal transduction cascade, are expressed in exactly the same mushroom body neurons, overlapping or distinct sets of mushroom body neurons. It is fair to say that most studies investigating the role of the mushroom bodies do not acknowledge the apparent complexity, or our naïveté of the extent of the complexity.

In the case of *rut*, functional studies have confirmed that mushroom body expression is critical for memory. Selectively expressing *rut* cDNA in the mushroom bodies rescues the olfactory memory defect of *rut* mutant flies, indicating that Kenyon cells encompass a critical location of *rut* function in memory (Zars et al., 2000a; Zars et al., 2000b). Selectively expression of *rut* cDNA in the $\gamma$ lobes of a *rut* mutant rescues short-term memory, suggesting that a functioning cAMP cascade in the $\gamma$ lobes is sufficient for short-term memory, while expression with an $\alpha/\beta$ lobe driver had no effect (Zars et al., 2000a). While these results add powerful support for $\gamma$ lobe involvement in short-term memory, they have been misinterpreted as implying that short-term memory exclusively resides in the $\gamma$ lobes. It is possible that other lobes are involved in short-term memory, but not in a *rut* dependent fashion. Furthermore, it is important to mention that the mushroom body GAL4 drivers used do not
express in all Kenyon cells. Therefore it is entirely possible that the subset of $\alpha/\beta$ Kenyon cells studied is not sufficient for $nut$-dependent memory, but a different or larger subset is sufficient.

Examining neural circuitry involved in memory has been greatly aided by an ability to block neurotransmission in discrete subsets of neurons and examine the effects of this manipulation on memory. These studies have almost exclusively utilized targeted expression of the transgene $\text{shibire}^{ts1}$ ($\text{shi}^{ts1}$), and have allowed selective inhibition of Kenyon cell subsets. Selectively blocking transmitter release from the $\alpha/\beta$ or $\gamma$ lobes has only a minor effect on middle-term memory. When flies are anaesthetized 1 hour following training, so that only anesthesia resistant memory remains, blocking output from $\gamma$ lobes has no effect, whereas anesthesia resistant memory is completely abolished when output from $\alpha/\beta$ lobes is abolished (Isabel et al., 2004). These data suggest that middle term memory involves the $\alpha/\beta/\gamma$ lobes, but that anesthesia resistant memory is selectively localized to the $\alpha/\beta$ lobes.

As discussed above, the mushroom bodies receive olfactory input from cholinergic projection neurons. Therefore, it seems likely that odors signal the mushroom bodies through activation of Acetylcholine Receptors (AChRs). This idea is supported by elegant neuroanatomical and physiological studies (Yasuyama et al., 2002). $\text{Ca}^{2+}$ imaging reveals that ACh application to the calyces induces a rapid depolarization
throughout the mushroom bodies and this can be blocked by application of nicotinic receptor antagonists (Yu et al., 2003). Furthermore, electrophysiological studies using dissociated Kenyon cells of *Drosophila* larvae or adult cricket show nAChR mediated excitatory post-synaptic currents and GABA receptor mediated inhibitory post-synaptic currents. More recently, recordings from the intact adult brain also support a role for ACh-mediated fast acting synaptic transmission (Cayre et al., 1999; Gu and O'Dowd, 2006; Su and O'Dowd, 2003). Taken together these results indicate that Kenyon cells are responsive to both ACh and GABA, and further work is required to determine if the mushroom body lobes, as well as dendrites, respond to these transmitters.

In the study of mammalian memory, much attention has been paid to glutamate as a critical transmitter in synaptic plasticity. A favored and long held model of Hebbian plasticity posits that glutamate binding to NMDA receptors is permissive to memory (Abbott and Nelson, 2000). Activation of NMDA receptors requires both glutamate binding and a depolarizing stimulus. The opening of NMDA receptors leads to Ca$^{2+}$ influx and second messenger activation. While there is evidence of NMDA receptor involvement in *Drosophila* memory (Lin, 2005; Xia et al., 2005), glutamatergic and NMDA receptor expressing neurons are sparsely distributed in the adult CNS (Strausfeld et al., 2003; Xia et al., 2005). It therefore seems unlikely that glutamatergic signaling is widely
involved in memory. It is more likely that ACh acts as the fast acting transmitter, providing the cue of odor specificity to Kenyon cells. Activation of Ca$^{2+}$ signaling and second messenger cascades are likely due to monoaminergic neurons, and this will be discussed in detail.

Current evidence suggests that mushroom body circuits may act as a coincidence detector for memory. Blocking olfactory input to the mushroom bodies following training does not affect memory, suggesting that olfactory cues from second order neurons are only critical during acquisition and retrieval. (Schwaerzel et al., 2002). Mushroom body output is dispensable during training, but also required during testing (Dubnau et al., 2001; McGuire et al., 2001). While it is difficult to test the location in which a memory is stored we know that transmitter signaling to the mushroom body lobes and olfactory projection neuron signaling to the calyces are critical for memory. Taken together, this supports the notion that the mushroom body lobes act as a coincidence detector of projection neurons signaling the CS and extrinsic neurons signaling the US.

**Mushroom body-associated neurons – DPM neurons and memory stability**

It is most parsimonious that olfactory memories involve circuitry that is directly and/or indirectly driven by olfactory cues. Indeed, current published data suggest that olfactory memory requires neural circuits that
involve intrinsic mushroom body neurons and mushroom body-associated neurons.

The mushroom bodies are not obviously connected to one particular region of the brain, e.g. pre-motor areas. Instead, mushroom bodies send information to many of the surrounding, poorly defined neuropil areas. Several types of mushroom body-associated neurons that project to surrounding neuropil were identified using Golgi-impregnation and enhancer trapping (Ito et al., 1998). The lack of projection to pre-motor centers led Ito et al (1998) to suggest that mushroom bodies are a pre-processor rather than a memory center that directly gates descending pathways. There is currently no evidence for another ‘memory center’ receiving olfactory input from mushroom bodies. However, some aspects of this idea are testable. If identified mushroom body-associated neurons link mushroom bodies with another memory center, one would predict that disrupting the connection during training (and perhaps retrieval) would perturb memory performance. If however, the mushroom body-associated neurons gate motor output then only blocking these neurons specifically during memory retrieval should abolish memory performance. The availability of fairly specific GAL4 lines expressing in these neurons makes it possible to temporally inhibit synaptic transmission from these neurons and functionally test their role in memory. Such experiments
have been performed to analyze two of these mushroom body-associated neurons, termed Dorsal Paired Medial (DPM) neurons.

DPM neurons were identified as a site of expression of the putative neuropeptide encoded by the *amnesiac* gene. Expressing *amn* in DPM neurons of an otherwise mutant *amn* fly, restores olfactory memory suggesting that DPM are a critical site of *amn* action in the brain (Tamura et al., 2003; Waddell et al., 2000). Furthermore, blocking synaptic transmission from DPM neurons causes an *amn*-like memory phenotype (Waddell et al., 2000). The necessity of DPM neurons appears to be exclusive to middle-term memory because blocking neurotransmission from these neurons does not affect learning (Waddell et al., 2000). DPM neuron processes lie exclusively throughout the mushroom body lobes and base of the peduncle. A synaptobrevin-GFP marker of presynaptic terminals labels all the mushroom body lobes indicating that DPM neurons transmit information to the mushroom bodies (Ito et al., 1998; Tamura et al., 2003).

Imaging DPM neural activity with the Ca$^{2+}$ reporter G-CaMP and with synaptophysin, a pH-sensitive reporter of synaptic vesicle release has greatly added to our understanding of DPM function. Both odors and shock evoke responses in DPM neurons suggesting they could report conditioned activity. In fact, following a training-session of paired odor and shock, the odor-evoked response in DPM neurons is elevated 30 minutes
after training – paralleling the temporal requirement for DPM neurons in memory stability (presented here). Strikingly this conditioned response is specific to the previously shock-paired odor and is only evident in DPM neuron projections to the vertical $\alpha/\alpha'$ lobes of the mushroom bodies (Yu et al., 2005). In addition, the conditioned response does not form in $amn$ mutants, and can be restored by selectively expressing the $amn$ transgene in DPM neurons suggesting that release of the putative AMN peptide onto the mushroom bodies may be required for the development of the conditioned response (Yu et al., 2005).

The apparent localization of DPM neuron plasticity to the vertical lobes suggests that memories may require changes in DPM function in projections to the $\alpha/\alpha'$ lobes. Because DPM neurons are thought to signal the mushroom bodies these results imply the vertical lobes are critical for middle-term memory. $\alpha$ lobes absent ($ala$) flies are missing $\alpha$ lobes, and display normal middle-term memory suggesting the vertical lobes dispensable. The differing conclusion of these two studies highlights the need to examine the relationship between mushroom body-associated neurons and Kenyon cells. The relevance of plasticity in DPM neurons to behavioral plasticity remains unclear, while studies using $ala$ mutant flies are inherently flawed because they rely on a severe morphological mutant to infer function of mushroom body sub-structure in the wild-type fly (Pascual and Preat, 2001). Furthermore, these studies
rely on post-testing brain dissection to isolate the small population of flies with the desired defect making these experiments difficult to replicate, and even more difficult to interpret. In this dissertation I have relied upon the powerful ability to temporally inactivate small subsets of neurons in order to investigate DPM neuron-mushroom body connectivity.

In the future many more mushroom body inputs and outputs will undoubtedly be identified. Chiang et al., (2004) have identified as many as 8 extrinsic subsets of mushroom body-interacting neurons through clonal analysis of the GAL4 line c133. c133{GAL4} represents an insertion in phospholipase 2A, which disputably is thought to encode for the gene mutated in the memory mutant radish (Chiang et al., 2004). While these subsets of neurons represent interesting candidates for neurons that signal to the mushroom bodies in memory, no specific role has been identified for any of these subsets. Ito et al (1998) have also identified 3 subsets of neurons that project onto the mushroom bodies. While the authors used this study to suggest involvement of the protocerebrum in memory, they failed to provide functional evidence that these neurons directly receive input from the mushroom bodies or are involved in memory. Using the tools currently available for tissue-specific expression, cell ablation, and neural manipulation, we should be able to elucidate a role for more mushroom body-interacting neurons in memory.
Because DPM neurons represent the best studied mushroom-body associated neurons, I have chosen to focus my graduate study on the role of these neurons in memory. Identifying neurons and neural circuits essential for memory will only serve to reveal the base-coat of a much larger masterpiece. It is also necessary to identify the mechanism by which neurons in these circuits interact. Most obviously, identifying the temporal requirements of transmitter release and the transmitters and signaling cascades through which these neurons communicate, is essential for understanding memory. Here, I use DPM neurons and the mushroom bodies as a model, for investigating the neural basis of memory.
Portions of this chapter have been submitted for publication:

CHAPTER II: NEURAL MECHANISMS OF DPM NEURON FUNCTION

Introduction

Previous work has identified amnesiac as being essential for middle-term memory, but dispensable for learning. Selectively rescuing amn function in DPM neurons in an amn mutant fly is sufficient for wild-type levels of memory, and blocking transmitter release from DPM neurons abolishes memory indicating that function of these neurons is essential for memory (Waddell et al., 2000). AMN peptide exhibits sequence similarity with the mammalian neuropeptide PACAP (Feany and Quinn, 1995). In the murine brain, PACAP has a clear role in modulating plasticity (Matsuyama et al., 2003) and this has led to speculation that AMN release from DPM neurons acts to modulate the cAMP cascade in the mushroom bodies (Waddell and Quinn, 2001b). This chapter takes a number of approaches to investigate the role of amn function in DPM neurons, as well as its functional similarity to PACAP.

Many neurons contain multiple transmitters. While a transmitter is generally defined as a substance that is packaged into vesicles, secreted transmitters can be further broken down into classical neurotransmitters and neuropeptides. Classical neurotransmitters are small molecules that are packaged into small, lucent vesicles that associate with active zones. Examples of classical neurotransmitters include glutamate, acetylcholine...
(ACh), dopamine (DA), and octopamine (OA). Neuropeptides, on the other hand, are composed of short polymers of amino acids. These are packaged into large dense-core vesicles, which often contain both classic neurotransmitter, as well as neuropeptide(s). Neurotransmitters and neuropeptides also differ in the means of synthesis. While neurotransmitters are synthesized from precursors and packaged into vesicles in the synaptic bouton, neuropeptides are packaged into vesicles in the cell body and transported to the synaptic bouton.

The necessity of amn in DPM neurons for normal memory has been well documented (Tamura et al., 2003; Waddell et al., 2000). However, it is possible that AMN functions in conjunction with a classical neurotransmitter. Interaction between mammalian neuropeptides of the VIP/glucagon/growth hormone-releasing hormone (GHRH)/secretin superfamily and ACh signaling have been studied in detail. Most notably, PACAP and vasoactive intestinal peptide (VIP), AMN’s two closest mammalian homologs, have been shown to potentiate both nicotinic (ion channel gated) and muscarinic (G-protein coupled) ACh receptors (AChRs) (Kawatani et al., 1985a; Liu et al., 2000). Furthermore, the modulation of AChRs by VIP is dependent on cAMP signaling (Gurantz et al., 1994; Margiotta and Pardi, 1995). There is also evidence that this interaction occurs in a cellular correlate of mammalian memory. Application of PACAP facilitates long-term potentiation in a hippocampal
culture system, and this facilitation is blocked by the co-administration of atropine, a muscarinic receptor blocker (Roberto and Brunelli, 2000). These results raise the possibility that AMN, like PACAP, may act through modulation of AChR function.

In this chapter I identify DPM neurons as being cholinergic and examine the role of ACh release from DPM neurons in memory. I also transgenically expressed mPACAP in the DPM neurons of amn mutant flies to examine the putative functional similarity between AMN and PACAP peptides.

**Materials and Methods**

**Fly stocks and maintenance**

Fly stocks were raised on standard cornmeal food at 25°C and 40%–50% relative humidity. The wild-type *Drosophila* strain used in this study is Canton-S and originated from W.G. Quinn’s lab (Massachusetts Institute of Technology).

The *amn*\(^1\), *amn*\(^{ss1}\), and *amn*\(^{X8}\) alleles were described previously (Moore et al., 1998; Quinn et al., 1979; Waddell et al., 2000). *amn*\(^{X8}\) is a *amn* null allele generated by imprecise excision of the *amn*\(^{28A}\) P-elements (DeZazzo et al., 1999) It has been reported that *amn*\(^{X8}\) lacks the entire *amn* open reading frame (ORF).
New deletion alleles of the amn ORF were generated by imprecise excision of the amn\textsuperscript{c651} P[w+] element were generated for the experiments presented here. Briefly, amn\textsuperscript{c651} females were crossed to transposase-bearing Sb(Δ2-3)/TM3Ser males. Dysgenic amn\textsuperscript{c651}, Sb(Δ2-3) males were crossed to FM7a females, and excision chromosomes were selected by the absence of the P[w+] element. In the next generation, putative amn\textsuperscript{ex} males were isolated and genomic DNA was prepared. Fifty of these putative excisions were analyzed for the integrity of the amn locus by PCR and sequence analysis. Two of these lines—amn\textsuperscript{ex1} and amn\textsuperscript{ex39}—contained nearly complete deletion of the amn ORF. amn\textsuperscript{ex1} deletes a region of DNA extending from -661 nucleotides upstream of the ATG to position +369 within the amn ORF. amn\textsuperscript{ex39} deletes a region of DNA extending from -785 nucleotides upstream of the ATG to position +477 within the amn ORF. amn\textsuperscript{ex39} leaves only a small C-terminal fragment that is not expected to have function (Figure 2.1E).

The uas-cd8::GFP flies are described in Lee and Luo (1999). The uas-\textit{shbire}\textsuperscript{ts1} flies were those previously used in assaying DPM neurons (Waddell et al., 2000) and described by Kitamoto (2001). The DPM neuron restricted c316{GAL4} and the uas-\textit{amn} flies have been previously described (Waddell et al., 2000). The uas-\textit{amn} flies are those previously denoted as "uas-amn#1." Mz717{GAL4} flies were described (Ito et al., 1998). uas-mPACAP flies were generated in the lab by Ruth Brain.
c772{GAL4} was generated by Douglas Armstrong (University of Edinburgh), and has been previously described (Zars et al., 2000a). The MB247{GAL4} promoter-fusion line was generated by the fusion of a 247bp segment of the Dmef regulatory sequence and drives expression of uas-controlled transgenes in the mushroom bodies (McGuire et al., 2001). cha{GAL4} and cha{GAL80} promoter fusions have been previously described (Kitamoto, 2002; Salvaterra and Kitamoto, 2001).

ACh{RNAi} lines were generated in the Waddell laboratory as described in the text. 12 transformant lines were generated and 2 lines, 40A and 38A (later termed ACh{RNAi}$^\#1$ and ACh{RNAi}$^\#2$) were chosen for further study based on being mapped to the 2$^{nd}$ and 3$^{rd}$ chromosomes respectively. ACh{RNAi}$^\#1$ is a lethal insertion due to insertion site, and therefore, crosses using this line were always sorted from balancers following behavioral testing.

**Histochemistry**

Adult brains were removed from the head capsule and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (1.86 mM NaH$_2$PO$_4$, 8.41 mM Na$_2$HPO$_4$, 175 mM NaCl) for 15 minutes and rinsed in PBS-T (PBS containing 0.25% Triton X-100). For immunohistochemistry experiments, brains were incubated in primary antibody overnight at 4°C unless otherwise noted. Anti-FAS-II 1D4 (Iowa Hybridoma Bank) was used at 1:2, and anti-PACAP38 (Penninsula Labs) and used at 1:200.
Anti-Choline Acetyl Transferase (ChAT) 4B1 (Iowa Hybridoma Bank) was used at 1:50 and brains were incubated at room temperature for 4 hours. For all experiments, following incubation in primary antibody brains were washed 3x15 minutes in PBS-T, and incubated for 2 hours in 1:200 secondary antibody conjugated with FITC or Texas-Red (Jackson Immuno Research). Brains were again washed in PBS-T for 3x15 minutes and mounted in Vectashield. Confocal analysis was performed on a Leica TCS-SP laser scanning confocal microscope. All brains were scanned in one µm stacks extending through the region of interest. Images were analyzed with Image Analysis and Processing in Java (ImageJ, NIH). Figures represent a z-stack of 1µM slices merged at maximum intensity, unless otherwise noted.

**Behavioral Analysis**

For learning and memory experiments, training, testing and statistical analysis were performed as previously described (Tully and Quinn, 1985). Briefly, flies were exposed to the odor octanol (OCT) for 60 seconds while receiving a foot shock every 5 seconds. Flies were then given 30 seconds of fresh air, followed by the odor methylcyclohexanol (MCH) for 60 seconds in the absence of foot shock. Flies were tested for their odor memory in a T-maze apparatus where they chose between the two odors used in training. Half-scores were calculated as \( \frac{(# \text{ choosing } \text{MCH} + # \text{ choosing } \text{OCT})}{2} \).
unreinforced odor - # choosing reinforced odor)/ (Total flies). In the example above, if 75 percent of the flies choose methylcyclohexanol, the half score would be 0.50. The reinforced odor was switched and a new set of flies was trained and tested to reduce the likelihood of odor bias and non-associative effects. This reduces the likelihood of odor bias and non-associative effects. The performance index (the final measure of memory) is the mean of the two half-scores.

**Statistical Analysis**

Statistical analyses were performed using KaleidaGraph (Synergy Software, Reading, PA). Overall analyses of variance (ANOVA) were followed by planned pair-wise comparisons between the relevant groups with a Tukey HSD post-hoc test. Significance was set as p<0.05.

**Acknowledged contributions**

David Gorczyca helped with initial brain dissection and confocal microscopy examining DPM morphology in \( \text{amn}^{X} \) mutant flies. Paola Perrat and Scott Waddell generated and molecularly characterized new \( \text{amn}^{ex} \) lines. Ruth Brain designed and generated \( \text{uas}-\text{mPACAP} \) flies and generated the \( \text{uas}-\text{ACh}\text{(RNAi)} \) lines used here.

**Results**

**Is \( \text{amn} \) essential for DPM development?**

Expression of \( \text{amn} \) in DPM neurons is required for memory (Tamura et al., 2003; Waddell et al., 2000), yet, it is unclear if \( \text{amn} \) acts
acutely in memory formation, or if it is essential for proper development of DPM neurons. To address this issue DPM neurons were labeled with the membrane-tethered fluorescent marker, CD8::GFP. Confocal microscopy was used to analyze DPM morphology in *amn* mutant fly brains by driving a uas-cd8::GFP transgene with the DPM neuron driver c316{GAL4} (Figure 2.1A). Examination of DPM morphology in the most commonly used behavioral null, *amn*<sup>X8</sup>, was not possible because these flies exhibit GAL4 activity in the mushroom bodies without a driver (Figure 2.1D). This fly line was originally isolated from imprecise excisions of the P{GAWB} mutant *amn*<sup>28a</sup>. It appears that a portion of the P-element encoding for {GAL4} has hopped into a genomic region where surrounding promoters drive mushroom body expression, yet this has not been identified in previous publications using this allele (Dezzazo et al., 1999; Moore et al., 1998; Rosay et al., 2001; Waddell et al., 2000). For these experiments *amn*<sup>1</sup>, a strong behavioral allele that has not been molecularly characterized in detail, as well as two new *amn* alleles, *amn*<sup>ex1</sup> and *amn*<sup>ex39</sup>, generated here by imprecise excision of the single P-element in the *amn*<sup>651</sup> mutant (Waddell et al., 2000) were used for histological analysis. The *amn*<sup>ex1</sup> and *amn*<sup>ex39</sup> are not predicted to produce any functional AMN peptide (Figure 2.1E).

DPM neurons are present in *amn* mutants (n > 10 per genotype; Figure 2.1B shows a typical *amn*<sup>ex1</sup> brain). In both wild-type and *amn*
mutant fly brains, each DPM neuron sends a single large-diameter neurite toward the mushroom body lobes. The neurite splits and projects to the vertical and horizontal mushroom body lobes. These neurites further divide and extend toward the vertically arranged $\alpha$ and $\alpha'$ lobes and the horizontally arranged $\beta$, $\beta'$, and $\gamma$ lobes (Figure 2.1C). The processes form a network of fibers and synaptic boutons throughout all of the lobes and into the spur and anterior region of the peduncle. These data indicate that \textit{amn} is not essential for DPM targeting to the mushroom bodies during development. Furthermore, these results imply that the memory defect in \textit{amn} mutant flies is not due to absence or gross maldevelopment of DPM neurons, and therefore the mnemonic phenotype may result from dysfunction of AMN peptide in adult flies.

\textbf{The role of classical transmitter function in DPM neurons}

To identify cholinergic neurons in the fly brain, \textit{choline acetyltransferase (cha)} expressing neurons were labeled. ChAT is an essential enzyme for ACh production and is a commonly used marker for cholinergic neurons (Salvaterra and McCaman, 1985). cha\{GAL80\} and cha\{GAL4\} are promoter-fusion transgenics containing \textit{GAL80} or \textit{GAL4} downstream of the 5' flanking region of the \textit{ChAT} open reading frame. cha\{GAL80\} has been reported to suppress GAL4 induced transcription in all cholinergic neurons, while cha\{GAL4\} drives transgene expression in these neurons (Kitamoto, 2002). To confirm the ability of cha\{GAL80\} to
block GAL4 driven transgene expression, GFP labeling was examined in cha{GAL4};uas-cd8::GFP flies with and without the cha{GAL80} transgene. GAL80 is an extremely potent suppressor of GAL4, and consequently GAL4 driven transcription (Lee and Luo, 1999; Ma and Ptashne, 1987) and therefore the addition of the cha{GAL80} transgene should abolish all cha{GAL4} driven activity. Confocal imaging of cha{GAL4};uas-cd8::GFP brains revealed GFP expressing neurons scattered throughout the brain, in agreement with previously published results (Figure 2.2A; Salvaterra and Kitamoto, 2001). Addition of the cha{GAL80} transgene suppressed almost all GFP reporter-gene expression (Figure 2.2B), indicating that it effectively acts to suppress GAL4 in cholinergic neurons. A small handful of neurons remained labeled in cha{GAL4};uas-cd8::GFP;cha{Gal80} brains. It is unclear if this is the result of cha{GAL4} driving transgene expression in non-cholinergic neurons, or an inability of cha{GAL80} to suppress GAL4 activity in a small subset of cholinergic neurons.

To confirm that cha{GAL4} drives expression in cholinergic neurons, cha{GAL4};uas-cd8::GFP brains were immunostained with anti-ChAT. High-resolution, single slice confocal images of neurons of the protocerebrum taken with a 63x objective show colocalization of GFP and anti-ChAT signal, indicating that cha{GAL4}GFP properly recapitulates endogenous expression of ChAT, and therefore serves to mark
cholinergic neurons (Figure 2.3 C-E). It would be valuable to employ a similar experiment to follow cha(GAL80)-mediated suppression, however, the GAL80 product cannot be assessed as the transgene is currently designed.

Next, I used the cha(GAL80) transgene to investigate the classical transmitter expressed in DPM neurons. If DPM neurons are cholinergic, cha(GAL80) should suppress transgene expression in these neurons (Figure 2.3A). Indeed, expression of uas-cd8::GFP under c316(GAL4) control clearly labels DPM neurons (Figure 2.3B), while the addition of the cha(GAL80) driver suppresses all GAL4 expression in DPM neurons, indicating that DPM neurons are cholinergic (Figure 2.3C).

One possibility is that leaky, non-specific GAL80 expression results in general suppression of GAL4-driven transcription throughout the brain. Overexposure of c316(GAL4);uas-cd8::GFP;cha(GAL80) brains revealed air sac autofluorescence and a few glial cells in the protocerebrum remained labeled (not shown), suggesting these results were not due to general suppression by cha(GAL80). To confirm that cha(GAL80) does not suppress GAL4 activity throughout the brain, I examined GFP labeling in MB247(GAL4);uas-cd8::GFP flies with and without the cha(GAL80) transgene. Mushroom body GFP expression in double transgenic MB247(GAL4);uas-cd8::GFP flies is indistinguishable from triple transgenic flies containing cha(GAL80) (Figure 2.3D-E). These results
differ slightly from a previous report indicating that cha\{GAL80\} blocked
GAL4-driven transgene expression in the $\gamma$ lobes, while leaving
expression in the remaining lobes intact (Kitamoto, 2002). Nevertheless,
taken together these results strongly support the notion that DPM neurons
are cholinergic.

A more direct way to examine whether DPM neurons are
cholinergic is to examine the localization of ChAT. This approach is not
without problems. In *Drosophila*, cholinergic neurons are in extreme
abundance throughout the brain (Gorczyca and Hall, 1987) and this broad
expression may obscure the identification of specific subsets of neurons.
Nevertheless, this approach was used to confirm the results garnered
with the cha\{GAL80\} transgene. Brains of transgenic flies with uas-
cd8::GFP under c316\{GAL4\} control were dissected. Immunostaining
with anti-ChAT revealed colocalization of GFP labeling in DPM neurons
and immunoreactive ChAT signal (Figure 2.4A-C). High-magnification
analysis of an individual section confirms the colocalization observed with
the projection (Figure 2.4 D-F). Of course, these results must be
interpreted with great caution. Synaptic resolution is beyond the
limitations of confocal microscopy. Because DPM neurons ramify
throughout the dense neuropil of the mushroom body lobes, it is possible
that neurons surrounding DPM terminal are cholinergic, resulting in the
appearance of immunofluorescence in DPM neurons. ChAT signal was
absent from DPM cell bodies (data not shown), however, ACh is synthesized in pre-synaptic regions and therefore, this could be the result of sub-cellular localization.

The finding that DPM neurons are cholinergic raises the possibility that ACh release from DPM neurons is involved in memory. While cholinergic neurons are likely involved in many aspects of behavior, the functional study of ACh in *Drosophila* has been hampered by a lack of viable mutant alleles. The vast majority of mutations impairing ACh function are lethal and affect the entire fly. Temperature sensitive *ChAT* mutants were created to address this problem (Salvaterra and McCaman, 1985; Yasuyama et al., 1996). While this approach appears to be effective in diminishing ACh function in adult flies, it causes general behavioral defects and lacks the spatial specificity required to address the role of cholinergic neurons in memory.

To circumvent the non-specific effects of blocking ACh function in the entire brain, I designed a GAL4-controlled RNAi transgene to target ACh synthesis machinery (Figure 2.5). Briefly, a cDNA-derived construct containing an inverted repeat that is predicted to form double stranded RNA was subcloned downstream of a UAS-promoter, permitting GAL4 control (Lee and Carthew, 2003). Following transcription of the transgene, double stranded RNA is converted into small interfering RNAs (siRNAs) that consist of 21-22 nucleotide double stranded fragments.
These siRNAs can target their complementary mRNA for degradation (Elbashir et al., 2001). In *Drosophila*, this technique is remarkably effective and specific. RNAi has been shown to nearly abolish transcripts to which it has been targeted, creating a 50-100 fold decrease in protein levels (Kalidas and Smith, 2002). Most importantly, these RNAi transgenes can be expressed with region-specificity using the GAL4/UAS system.

The ACh{RNAi} construct consists of a P-element backbone harboring an inverted repeat of the ChAT/Vesicular Acetylcholine Transporter (VACht) locus cloned downstream of the GAL4 UAS-promoter. The inverted repeat is a 568 base pair fragment that is common to the ChAT and VACht transcripts (Figure 2.5). ChAT (choline acetyltransferase) is necessary for ACh synthesis, while VACht is essential for transporting ACh into synaptic vesicles. Therefore, this ACh{RNAi} construct is predicted to disrupt synthesis of both of these proteins and thereby impair ACh function.

To examine whether ACh release is essential for DPM function, we generated flies expressing ACh{RNAi} transgenes in DPM neurons and tested flies for 1 hour memory (Figure 2.6). This time point was selected because DPM output is essential for 1 hour memory (Waddell et al., 2000). Two different ACh{RNAi} insertions, ACh{RNAi}\#1 and ACh{RNAi}\#2, were expressed in DPM neurons with c316{GAL4}. 
Performance of wild-type flies was statistically indistinguishable from those harboring an undriven or DPM driven copy of ACh{RNAi}$^{#1}$ (p>0.978), or ACh{RNAi}$^{#2}$ (p>0.977) transgenes. Furthermore, performance of flies harboring both RNAi transgenes was indistinguishable from wild-type (p>0.931). Driving expression of both RNAi transgenes in DPM neurons, however, impaired 1 hour memory (Figure 2.6A). c316{GAL4};ACh{RNAi}$^{#1}$, ACh{RNAi}$^{#2}$ flies performed significantly worse than wild type flies (p<0.028). To further inhibit ACh function I over-expressed dicer2 (dcr2) in combination with ACh{RNAi}. DCR2 is a component of the RNAi Induced Silencing Complex (RISC) and over-expression of dcr2 has been reported to increase the efficacy of RNAi (Bernstein et al., 2001; G.Dietzl and B.J. Dickson, personal communication). Flies expressing both uas-dcr2 and ACh{RNAi}$^{#2}$ had memory defects, while there was no effect of expressing dcr2 alone (Figure 2.6B). c316{GAL4};uas-dcr2; ACh{RNAi}$^{#2}$ performed worse than wild-type(p<0.022), while flies expressing c316{GAL4};uas-dcr2 flies performed at wild-type levels(P=0.80). These results indicate that dcr2 increases the efficacy of RNAi, and that ACh release from DPM neurons is likely critical for middle-term memory.

To investigate the possibility that results obtained with the c316{GAL4} driver were due to ACh{RNAi} expression in neurons other than DPMs, I expressed the ACh{RNAi} constructs with Mz717{GAL4}. 
This driver expresses in DPM neurons, and a small number of other neurons in the brain including Kenyon cells (Ito et al., 1998) Figure 1.3C). To my surprise, there was no effect of driving ACh{RNAi} transgenes with Mz717{GAL4} (PIs: wild-type=0.30+/−0.09 and Mz717{GAL4};ACh{RNAi}$^1$:ACh{RNAi}$^2$=0.51+/−0.08, ANOVA,p>0.17). There are two primary possibilities for the discrepancies in results with the c316{GAL4} and Mz717{GAL4} drivers. It is possible that c316{GAL4} is a “stronger” driver, thereby leading to higher levels of dsRNA synthesis in DPM neurons. From these data obtained, it is apparent that the effectiveness of the ACh{RNAi} transgenes is dose-dependent, because memory is only impaired when two copies of the transgene are present. An alternate possibility is that the memory defect in flies expressing ACh{RNAi} under c316{GAL4} control, is due to expression in non-DPM neurons in which the c316{GAL4} driver expresses. Further experimentation using multiple copies of the Mz717{GAL4} driver, the combination of MZ717{GAL4} and uas-dcr, or an additional genetic system to increase the specificity of the c316{GAL4} driver will help to address this issue.

Because evidence suggests that DPM neurons are cholinergic, and expression of amn cDNA in DPM neurons rescues the memory defect of the amn mutant flies, the extended logic indicates that expression of amn cDNA in cholinergic neurons should rescue the
memory defect of amn mutant flies. To test this prediction, drove uas-amn with cha{GAL4} in the background of the amn\textsuperscript{x8} mutant. The resultant flies, amn\textsuperscript{x8};uas-amn;cha{GAL4}, should express amn only in cholinergic neurons. Surprisingly, these flies exhibited 1 hour memory defects (Figure 2.7). Memory of amn\textsuperscript{x8};uas-amn;cha{GAL4} was impaired compared to wild-type flies (p<0.04) and did not differ from amn\textsuperscript{x8} mutants(p>0.99). There are many possible explanations for these results. The first is that ectopic expression of amn may impair memory. This is unlikely because memory in amn\textsuperscript{x8}/+; uas-amn;cha{GAL4} heterozygotes is equal to wild type(p>0.23). A second, and more likely possibility is that the cha{GAL4} driver does not express in all cholinergic neurons and fails to drive DPM expression. To address this question I have immunostained cha{GAL4}GFP with anti-AMN to look for colocalization. Unfortunately, I was unable to attain specific staining using the currently available AMN antibody with immunofluorescence procedures. I have also combined cheapdate, a P{LacW} insertion in the amn locus encoding for a nuclear βgal marker (Moore et al., 1998), with cha{GAL4}uas-cd8::GFP. Examination of cheapdate;cha{GAL4};uas-cd8::GFP brains revealed that the mutant exhibited altered GAL4 mediated expression, likely due to changes in genetic background caused by combining of transgenes (data not shown). With these results it has proven difficult to further examine the reason behind the negative
behavioral results. Generation of a DPM neuron enhancer-reporter gene fusion would help to determine if cha\{GAL4\} drives expression in DPM neurons.

**Is mPACAP able to substitute for AMN?**

Because of sequence similarity between AMN and mammalian PACAP a model has arisen where AMN neuropeptide (Figure 2.8) is released from DPM neurons and activates the cAMP cascade in the mushroom bodies enabling for cAMP dependent synaptic plasticity (Feany and Quinn, 1995; Waddell and Quinn, 2001a). While this model is certainly attractive, many components of it have not been tested. For example, to date, there is little evidence indicating that AMN functions as a neuropeptide. The majority of secreted peptides are amidated (Kolhekar et al., 1997) indicating that perhaps the amidated sequence is the active fragment of *amn* is involved in memory. However, it is worth noting that the predicted amidated fragment of AMN shows little sequence similarity to the active PACAP fragment (Figure 2.8).

To investigate the functional similarity between *amn* and PACAP I tested the ability of mouse PACAP (mPACAP) to rescue the memory defects in *amn* mutant flies. It is not unprecedented for a mammalian transgene to modulate *Drosophila* physiology (Bilen and Bonini, 2005). Furthermore, application of mammalian PACAP to the fly neuromuscular junction was reported to cause an immediate depolarization followed by
an enhancement of K+ current, suggesting that mPACAP may indeed have functional activity in the fly (Zhong and Pena, 1995). However, these results do not directly test whether PACAP is capable of replacing amn function in the fly. In order to examine a possible functional similarity between mPACAP and AMN, I tested the ability of mPACAP to rescue the memory defect of amn mutant flies.

mPACAP cDNA was subcloned into a pUAST vector so that it could be expressed under GAL4 control (Figure 2.9A). Fly embryos were injected with cDNA according to standard procedure. To examine whether the uas-mPACAP transgene could be expressed in the adult brain, mPACAP was driven in either the mushroom bodies with MB247{GAL4}, or DPM neurons with c316{GAL4}. To verify expression, brains were stained brains with antibody targeted to PACAP38, a 38 amino-acid active fragment of mPACAP. c316{GAL4};uas-mPACAP;uas-mcd8::GFP flies expressed mPACAP in DPM cell bodies and in projections to the mushroom body lobes suggesting that the transgene is expressed, and its product is transported to synapses (Figure 2.9 B-G). Similar results were obtained with MB247{GAL4};uas-mPACAP;uas-mcd8::GFP flies (data not shown).

Selectively expressing amn in DPM neurons rescues the memory defect of amn null flies (Tamura et al., 2003; Waddell et al., 2000). To determine if mPACAP is able to substitute for amn function, I ectopically
expressed mPACAP in DPM neurons of \( amn^x8 \) flies. Virgin female \( amn^x8; c316\{GAL4\} \) flies were crossed to uas-mPACAP flies and the F\(_1\) generation was tested for 1 hour memory. Expression on mPACAP in DPM neurons was not able to rescue the \( amn \) memory defect (Figure 2.10), indicating that PACAP is not able to functionally substitute for AMN. \( amn^x8; uas\text{-}mPACAP; c316\{GAL4\} \) flies displayed poorer memory than wild-type control flies \((p<0.02)\) and were not significantly different from \( amn^x8 \) or \( amn^x8; c316\{GAL4\} \) flies \((p>0.91, p>0.99)\). It is unlikely that the inability of mPACAP to rescue \( amn^x8 \) is due to detrimental effects of mPACAP on DPM function because \( amn^x8 \) heterozygous females with DPM expression of mPACAP display wild-type memory \((p>0.87)\).

**Discussion**

**amn** and DPM Development

Genetically labeling DPM neurons of \( amn \) mutant flies reveals that the gross morphology of DPM neurons is not affected by deletion of the \( amn \) locus. Of course, examining adult morphology is only suggestive of an adult role for \( amn \) and does not definitively rule out a developmental role in physiology or ultra-structural morphology that cannot be observed with confocal microscopy. The ideal experiment to differentiate between an adult versus acute role for \( amn \) in memory is to rescue the \( amn \) memory defect through adult or development specific expression of \( amn \)
cDNA in DPM neurons. Multiple genetic systems, including TARGET, gene-switch, and GAL4/Tetracycline-off (McGuire et al., 2004a; Mehren and Griffith, 2004; Roman et al., 2001), have been used for adult-specific control of tissue expression. I have attempted to use TARGET to specifically rescue \textit{amn} function in DPM neurons of adult flies, however these experiments have proven difficult. I found that incubating flies at high temperatures during adulthood did not rescue the memory defect of \textit{amn} \textsuperscript{x8} flies with DPM neuron restricted expression of \textit{amn} (c316\{GAL4\};\textit{uas-amn}) in the presence of the GAL80\textsuperscript{ts} transgene, and flies were unhealthy when grown at a non-permissive temperature throughout development (Figure 2.11). It is possible that these techniques only serve to partially modulate GAL4 activity. With some drivers, this may be sufficient to temporally regulate gene expression. However, with other drivers, such as c316\{GAL4\}, this system may not work. More careful histochemical or biochemical analysis of the effectiveness of GAL80\textsuperscript{TS} in manipulating \textit{amn} expression may be informative. Future study using different transgenes, or different methods of temporal control will help to address this aspect of DPM function.

\textbf{Preliminary findings suggest ACh release from DPM neurons is critical for memory}

Disruption of ACh function in c316\{GAL4\} expressing neurons impairs memory. These findings provide functional evidence that ACh
release from DPM neurons is involved in memory. However, they do not distinguish whether ACh function in DPM neurons is involved in development, adulthood, or both. Further complicating the interpretation of these results is GAL4 variation throughout development. I found that the c316(GAL4) expresses in a large number of Kenyon cells during puparium, yet very few in adulthood (Figure 2.12). Therefore, it is possible that the results observed are due to mushroom body maldevelopment. Indeed, ACh has been implicated in Drosophila development (Yang and Kunes, 2004). Distinguishing between a developmental versus acute role for ACh function in DPM neurons will require spatio-temporal regulation of the ACh{RNAi} transgene.

The lack of effectiveness of the ACh{RNAi} transgenes when under control of the Mz717(GAL4) driver confirms that further study is needed to determine whether the memory defects observed with the ACh{RNAi} transgenes are due to expression in DPM neurons. The discrepancies in results between Mz717(GAL4) and c316(GAL4) are likely due to differences in the strength or the expression pattern of these drivers. Performing experiments with two copies of Mz717(GAL4) may help to address the issue of driver strength, while performing experiments with more DPM-expressing drivers (e.g. 169y(GAL4), amr^{651}(GAL4), and amr^{28A}(GAL4); DeZazzo et al., 1999; Waddell et al., 2000) will help to address differences due to expression pattern.
Further characterization of the ACh{RNAi} constructs is required to confirm that they specifically disrupt ACh function. Expressing ACh{RNAi} with the pan-neuronal driver does not cause the lethality observed in cha null mutants, indicating that the ACh{RNAi} construct does not abolish ACh function. In the future, it will be useful to use quantitative biochemical analysis such as Western blots of head extracts to determine ChAT levels. Single-cell quantitative PCR techniques may help to confirm that DPM neurons are cholinergic and that ACh{RNAi} impairs ChAT/VACHT levels in these neurons (Dulac and Axel, 1995; Tietjen et al, 2003)

The effect observed by expressing ACh{RNAi} in DPM neurons raises the possibility that AChRs localize to the mushroom body lobes. The *Drosophila* genome encodes for multiple subtypes of nicotinic acetylcholine receptors and muscarinic receptors (www.flybase.org). Canonically, muscarinic receptors signal through phospholipase C-IP3 to increase intracellular Ca\(^{2+}\) levels while nicotinic receptors signal through gated cation channels (Raymond-Delpech, 2004; Schafer et al, 2002). In both cases, signaling through these receptors could provide a possible mechanism for synaptic plasticity. According to this model, DPM neurons form pre-synaptic contacts with the mushroom body lobes that modulate plasticity. Both Ca\(^{2+}\) imaging and single cell recordings indicate that Kenyon cells are responsive to ACh (Gu and O'Dowd, 2006; Yu et al.,
2003), thus indicating that Kenyon cells express AChRs or that they are part of a circuit that is responsible to ACh. To date, the localization of AChRs within the fly brain is unclear. Muscarinic AChRs are highly expressed in the antennal lobe (Blake et al., 1993), while nicotinic AChRs are broadly expressed. Interestingly, nicotinic AChRs seemed to be enriched in the horizontal lobes of the mushroom bodies, and absent from the vertical lobes (Schuster et al., 1993). Current evidence supports a model where projection neurons signal through ACh receptors to activate Kenyon cells and this has been used to infer that Kenyon cells express ACh receptors. A better understanding of the neuroanatomical and intracellular localization of ACh receptor subtypes within the mushroom bodies will be greatly aided by detailed immunohistochemical study.

**Relationship of AMN to mPACAP**

Pharmacological application of mPACAP to the *Drosophila* NMJ alters synaptic output through modulation of cAMP signaling (Zhong, 1995; Zhong and Pena, 1995). The cloning of *amn* provided the tantalizing possibility that PACAP activates cAMP signaling at the NMJ through binding to an unidentified AMN receptor. A currently favored hypothesis for the mechanism of AMN function in memory is that AMN acts as a neuropeptide, and when released from DPM neurons, serves to activate the cAMP cascade in Kenyon cells (Feany and Quinn, 1995; Waddell and Quinn, 2001a). Much of the reasoning behind this model is
due to sequence similarity between AMN and mPACAP peptides. I have attempted to address potential functional similarities by assaying if mPACAP can substitute for AMN. These studies revealed that expression of mPACAP in DPM neurons does not rescue the memory defect of amn mutant flies. Therefore, these findings do not support the notion that mPACAP is functionally homologous to AMN. Of course these results by no mean rule out the possibility that AMN functions as a neuropeptide to enhance the cAMP levels. It is also possible that amn is a true PACAP homolog, but that divergence between receptors for the two signaling peptides does not allow for function when PACAP is substituted for AMN. These experiments were originally motivated by a separate project in the laboratory that aimed at identifying the AMN receptor. The approach used was to target genes with high levels of similarity to mPACAP receptors. This approach assumes close functional and sequence similarity between AMN and mPACAP receptors. These results presented here suggest this approach may not be a productive means of assigning AMN a receptor.

The current relationship between amn and cAMP signaling also remains unclear. It seems that the view of AMN acting in a PACAP-like manner has been championed by many because it strongly supports the dogma of neurotransmitter mediated cAMP activation in memory. The linking of amn to cAMP activation has been primarily based on three
pieces of evidence. Perhaps the strongest link between AMN and PACAP is their sequence similarity, and this is not particularly convincing (Figure 2.8). Secondly, amn was reported to rescue dnc infertility indicating that the fertility defects caused by hyperactive cAMP signaling are compensated for in the amn mutant flies (Feany and Quinn, 1995), however, this result has not been replicated (Waddell S., personal communication). Finally, the ethanol sensitivity defect of amn mutants was reported to be rescued by the cAMP agonist forskolin (Moore et al., 1998). Unfortunately, these results have not been confirmed during the past eight years following these initial findings. Furthermore, the sequence comparison of AMN and mPACAP brings into question their functional similarity. The majority of secreted peptides are amidated indicating that perhaps the amidated sequence is the active fragment of AMN involved in signaling. However, it is worth noting that the amidated fragment of AMN does not exhibit sequence similarity to the active PACAP fragment, suggesting that perhaps the active AMN fragment has very little sequence similarity to PACAP38. Taken together, it is clear that the assumption that AMN functions as a neuropeptide to activate the cAMP cascade is a tenuous one, at best.

Portions of this chapter have been published:

CHAPTER III: TEMPORAL REQUIREMENTS OF DPM NEURONS IN SHOCK-REINFORCED MEMORY

Introduction

Perhaps the most neglected aspect in the search for the mechanisms underlying memory is the temporal requirements of transmitter release. Hebb addressed the notion that the timing of neural activation may be critical in behavioral plasticity. In these seminal speculations he suggested that neurons that are activated at the same time undergo modification of output either via morphological or metabolic changes (Hebb, 1949), providing critical groundwork for models on the mechanism of neural plasticity and the modulation of entire neural circuits. While the temporal basis of memory coding has received a fair amount of theoretical consideration, it has proven difficult to study experimentally. Recently developed genetic technology in *Drosophila* allows for the manipulation of transmitter release in a temporally regulated manner (Kitamoto, 2001). In the experiments presented in this chapter, I have used these technological advances to dissect the temporal requirements for DPM neuron output in memory.

The role of specific neural structures in various behaviors can be examined through selective expression of temperature sensitive *sh*^1^,
which was first identified in a screen for temperature sensitive paralytic mutants (Grigliatti et al, 1973). $sh^{ts1}$ encodes a temperature sensitive dynamin-GTPase that, at non-permissive temperatures acts dominantly to block synaptic vesicle recycling and consequently synaptic release (Koenig and Ikeda, 1989). Later, a transgenic fly was created with this mutated gene under uas-control allowing for manipulation of neural output in specific subsets of neurons. Targeted expression of uas-$sh^{ts1}$ is a particularly powerful tool because it blocks neuronal release within minutes of placement at a non-permissive temperature and function is returned soon after flies are returned to a permissive temperature (Kitamoto, 2001). Furthermore, the manipulation of neural output occurs in the adult and consequently minimizes the possibility of developmental abnormalities.

Tissue-specific expression of $sh^{ts1}$ has been employed to investigate neural sites involved in many behaviors including courtship (Kitamoto, 2001; Stockinger et al., 2005; Villella et al., 2005), attention (van Swinderen and Greenspan, 2003), sleep (Pitman et al., 2006) and memory (Dubnau et al., 2001; McGuire et al., 2001; Waddell et al., 2000).

One model for the different temporal requirements of neural subsets in memory holds that there are three different periods within a memory: Acquisition, stability/consolidation, and retrieval. The role of a given neural structures in these three phases can be tested by selectively
blocking output during training, between training and testing, or during testing. Previous studies have found mushroom body output is dispensable during the acquisition and stability/consolidation phases, but is essential for retrieval (Dubnau et al., 2001; McGuire et al., 2001).

Little is known of the temporal requirements of mushroom body-associated neurons. Unlike the mushroom bodies, DPM neurons are dispensable for learning (Waddell et al., 2000), indicating that neurotransmission from DPM neurons may be required at time-points other than memory retrieval. To examine the temporal requirements of DPM neurons, we manipulated DPM function at discrete intervals in the memory process. Here, we show that DPM neuron output is only required during consolidation of middle-term odor memory and is dispensable during acquisition and recall when flies are trained with the odors OCT and MCH. We also found that the odor benzaldehyde (BA) is sensed in a non-canonical fashion. This led us to investigate DPM involvement in BA memories.

The vast majority of the memory mutants were isolated using a single odor pair—3-octanol (OCT) and 4-methylcyclohexanol (MCH) (Boynton and Tully, 1992; DeZazzo et al., 1999; Dubnau et al., 2001; Dudai et al., 1976; Folkers et al., 1993; Livingstone et al., 1984; Quinn et al., 1979). From a selection of 40 odors, (Quinn et al., 1974) concluded that "not all odors work." OCT and MCH were chosen because they
consistently produced good memory scores. It is not known why these odors are salient to the fruit fly and why they are potent conditioning stimuli. To our knowledge, no large-scale screen has asked whether odor-conditioned memories are relatively generic and can be formed with a variety of odors or whether pathways and genes that are required for memories are odor specific. BA is used by some groups in a BA-OCT combination (Cheng et al., 2001; Grotewiel et al., 1998; Schwaerzel et al., 2002; Skoulakis and Davis, 1996) or BA-MCH (Guo et al., 2000; Zars et al., 2000a), and recently ethylacetate paired with isoamylacetate has been successfully employed to teach wild-type flies (Schwaerzel et al., 2003). However, it remains to be determined whether the existing memory mutants have a general odor memory defect or differentially affect the coding of individual odors.

Published memory experiments with amn mutants have used OCT and MCH as odors (DeZazzo et al., 1999; Quinn et al., 1979; Tamura et al., 2003; Tully and Gergen, 1986; Waddell et al., 2000). It has previously been demonstrated that blocking DPM neurons did not affect learning for these odors but abolished later memory (Waddell et al., 2000). Here, we show that prolonged DPM output is required for persistent OCT and MCH memory, consistent with a role for DPM neurons in the consolidation of odor memory. We found a different result with BA—an odor that we show is sensed by the classical olfactory pathway and also by a noncanonical
route. *amn* flies have a short-term memory defect with BA. Strikingly, this BA memory defect can be mimicked in wild-type flies by blocking DPM output during acquisition, suggesting that DPM neurons have an additional function in acquiring BA memory.

**Materials and Methods**

Flies expressing *sh*lts1 in DPM cells were generated by crossing homozygous w,uas-*shi*lts1;uas-*shi*lts1 females to homozygous w;c316{GAL4} males. All progeny from this cross carry two uas-*shi*lts1 transgenes and one c316{GAL4}. Heterozygous w;c316{GAL4} and w,uas-*shi*lts1;uas-*shi*lts1 flies were generated by crossing homozygote females to Cantonized *w*-males. A mixed population of sexes was tested in the olfactory conditioning paradigm.

For rescue of the *amn*X8 memory defect, we crossed *amn*X8;c316{GAL4} and *amn*X8;uas-*amn* flies. All progeny from these crosses were homozygous for *amn*X8 and heterozygous for c316{GAL4} and uas-*amn*. Mixed sex populations were tested.

The olfactory avoidance paradigm was performed according to (Tully and Quinn, 1985) except that odors were delivered by bubbling air through 15 ml scintillation vials containing odor dilutions in 10 ml of mineral oil. The performance index (PI) was calculated as previously described. The number of flies avoiding the CS+ minus the number of
flies avoiding the CS- divided by the total number of flies. A single PI value is usually the average score from flies of the identical genotype tested with each odor. In experiments highlighting odor-specific effects, individual odor scores were calculated separately. For experiments involving uas- shi\textsuperscript{ts1}, two behavior rooms with different temperatures (25°C or 31°C) were used, and flies were transported between rooms so that temperature shifts could be tightly regulated.

For T-maze experiments with olfactory organ-less flies, the antennae and maxillary palps were removed from several hundred wild-type flies. Olfactory organ-less flies were mixed with a 5-fold excess of Cantonized \( w^- \) flies to obtain optimal numbers of flies for the experiments. Scores were calculated independently after sorting \( \text{white}^- \) from \( \text{white}^+ \) (olfactory organ-less) flies.

To test olfactory acuity, untrained flies were given 2 minutes to choose between a diluted odor (1:80 dilution in mineral oil of OCT, 1:107 of MCH, 1:210 of BA) as used in conditioning and air was bubbled through mineral oil into the T maze. Electroshock avoidance was performed and calculated similarly. Untrained flies chose between a tube containing an electrified grid and a tube containing a non-electrified grid. Percent avoidance was calculated according to Tully and Quinn (1985). To assess relative odor avoidance, we gave untrained (or previously
electric-shocked) flies 2 minutes to choose between two diluted odors as used in conditioning in the T-maze.

Odor avoidance was also tested in an arena by measuring the distance of single freely moving flies from an odor source. Odorants were placed on a piece of filter paper at the wall of a petri dish (8.5 cm diameter, 1.3 cm height). The position of the fly was tracked at 6 Hz using a video camera and Ethovision tracking software (Noldus). The fly's average position relative to the stimulus was determined over 3 min. Avoidance was calculated by subtracting the average distance of a fly from an odorless filter paper from the value measured in the different experimental conditions. A zero avoidance value indicates that the flies behave like there is no odor stimulus.

Statistical analyses were performed using KaleidaGraph (Synergy Software). Overall analyses of variance (ANOVA) were followed by planned comparisons among the relevant groups with a Tukey HSD post hoc test. Unless stated otherwise, all experiments are \( n \geq 8 \), and all data points denoted as "statistically significant" are \( p < 0.05 \).

**Acknowledged contributions**

The peculiarity of BA was discovered by Markus Strattman (SW’s laboratory) and independently by Andreas Keller (LV’s laboratory). Markus Strattman collected the data presented in Figure 3.7B, and a portion of the data in 3.7A. Experiments examining olfactory response to
BA were done in collaboration with Andreas Keller and Leslie Vosshall. Andreas Keller performed experiments presented in Figure 3.6A-B.

Results

DPM neuron output is required during consolidation for 3 hour OCT-MCH memory

In this study, we use the electric-shock reinforced olfactory conditioning paradigm of (Tully and Quinn, 1985), because it produces a robust memory that allows a detailed analysis of specific memory phases. In this olfactory training protocol, a population of flies is exposed to one odor with an electric shock reinforcement followed by another odor without punishment. The flies are then tested for memory in a T-maze, where they choose between the two odors used in training. Normal flies learn to avoid the shock-paired odor in a single training trial. Memory performance is calculated as the number of flies that avoid the shock-paired odor minus the number that avoid the non-shock-paired odor divided by the total number of flies. This memory score is a "half score" because normally a single performance index (PI) data point represents the average score of two experiments. In the second experiment, a new population of flies is taught to associate the other odor with shock. Score averaging eliminates odor bias; therefore, averaging half scores may obscure whether one odor is forgotten more quickly than the other. Later
in this study (Figure 3.7, Figure 3.8, Figure 3.9 and Figure 3.10), we present half scores to highlight odor-specific effects. Until then, all data presented for OCT and MCH memory are average scores from reciprocal of shock with odors. We used OCT with MCH or OCT with BA, and we denote the odor pair used as either OCT-MCH or OCT-BA.

We used the GAL4-UAS system (Brand and Perrimon, 1993) to silence synaptic transmission in DPM neurons. We expressed the dominant temperature-sensitive shibirets1 transgene, uas-shits1 (Kitamoto, 2001), in DPM neurons using the c316(GAL4) or Mz717(GAL4) DPM drivers (Ito et al., 1998; Waddell et al., 2000). The shi gene encodes a dynamin that is essential for endocytosis and synaptic vesicle recycling (van der Bliek and Meyerowitz 1991 and Chen et al. 1991). The shits1 allele has a vesicle recycling defect at temperatures above 29°C that results in a rapid cessation of synaptic transmission (Koenig and Ikeda, 1989). High-temperature inactivation of shits1 is reversible and allows for temporal control of neuron output by simply shifting flies between permissive and restrictive temperatures. Importantly, this allows us to test the role of DPM neurons in memory independent of amn mutation and therefore without confounding developmental defects that might arise from studying a non-conditional amn mutant.

It has previously been shown that blocking DPM output throughout an entire operant olfactory conditioning experiment did not affect learning
(3 minute memory) but abolished 1 hour OCT-MCH memory (Waddell et al., 2000). In this study, we first determined whether blocking DPM output caused a comparable memory defect in the classical conditioning paradigm of (Tully and Quinn, 1985). We conducted entire 3 hour memory experiments at 25°C (at which temperature we expected the neurons to function normally) and 31°C (under which conditions the shl151-expressing neurons were expected to be synaptically silent). In each experiment, we compared the performance of c316(GAL4);uas-shl151 double transgenic flies to wild-type and single transgenic c316(GAL4) and uas-shl151 control flies. uas-shl151 flies at 31°C are a very appropriate control, because these flies often show a modest but significant reduction in performance at 31°C when compared to wild-type flies. We also included amnX8 flies to illustrate the effect of a null amn allele on 3 hour memory. At the permissive temperature of 25°C, both immediate (3 min) memory (wild-type = 0.64 ± 0.02; c316(GAL4);uas-shl151 = 0.63 ± 0.04; uas-shl151 = 0.63 ± 0.03) and 3 hour memory of c316(GAL4);uas-shl151 flies were statistically indistinguishable (p > 0.7) from wild-type, c316(GAL4), and uas-shl151 control flies, while all groups showed greater memory than amnX8 mutant flies (p < 0.02) (Figure 3.1A). At the restrictive temperature of 31°C, immediate (3 min) memory of c316(GAL4);uas-shl151 flies (0.67 ± 0.04) was statistically indistinguishable (p > 0.7) from wild-type (0.69 ± 0.02), and uas-shl151 flies (0.66 ± 0.04). However, 3 hour memory was
statistically lower (p < 0.01 for all groups) than wild-type, c316(GAL4), and uas-*sh*ts1 flies and statistically indistinguishable (p > 0.7) from that of amnX8 mutant flies (Figure 3.1B). These results are consistent with a previously reported finding (Waddell et al., 2000) and demonstrate that DPM output is required for 3 hour, but not for immediate, OCT-MCH memory.

We next used the reversibility of uas-*sh*ts1 to test whether DPM output during training or testing was required for memory. To block DPM neuron output during training, we incubated c316(GAL4);uas-*sh*ts1 flies and all control flies at 31°C for 15 min prior to and during training. Flies were returned to 25°C immediately following training, and 3 hour memory was tested at 25°C (Figure 3.2A). Blocking DPM output during training did not affect memory. The memory of c316(GAL4);uas-*sh*ts1 flies was indistinguishable (p > 0.9) from uas-*sh*ts1 control flies that were trained at the restrictive temperature. Therefore, memory acquisition does not require output from DPM neurons.

We similarly tested whether DPM output was required during memory recall (Figure 3.2B). We trained flies at 25°C, and 15 min before testing 3 hour memory we inactivated DPM neurons by shifting the flies to the restrictive temperature of 31°C. The 3 hour memory of c316(GAL4);uas-*sh*ts1 flies was again indistinguishable (p > 0.8) from the
uas-\textit{shi}^{ts1} transgene control flies, suggesting that DPM output is not required for memory recall.

I also tested whether blocking DPM output during both training and testing (Figure 3.2C) affected memory. We placed flies at 31°C 15 min prior to training and returned them to 25°C immediately after. Fifteen minutes before testing, we shifted them to 31°C again and tested olfactory memory. Strikingly, memory following this manipulation was no worse than that of flies receiving either manipulation alone and was indistinguishable (p > 0.5) from the memory of uas-\textit{shi}^{ts1} control flies. Therefore, DPM output is not essential during training and testing for 3 hour OCT-MCH memory.

Next, I tested whether DPM output was required in the period between training and testing (Figures 3.3, 3.4, and 3.5). This is the expected window of time in which memories become consolidated (Folkers et al., 1993; Quinn and Dudai, 1976; Tully et al., 1994). Flies were trained at 25°C, and shifted immediately following training to 31°C for 2 hours. Flies were then returned to 25°C and tested 1 hour later for 3 hour memory. Blocking DPM output between training and testing produced a dramatic loss of memory to levels statistically indistinguishable (p = 1) from that of \textit{amn}^{Xo} flies (Figure 3.4A). Therefore, DPM output is required between training and testing for 3 hour memory. I next tested whether blocking DPM output at later time points disrupted 3
hour memory. The 2 hour DPM blockade was delayed by 30 minutes into the middle of the experiment (Figure 3.4B). Blocking DPM output 30 minutes after training for 2 hour produced the same memory impairment as blocking output immediately after training (p<0.01).

To control for a nonspecific memory deficit produced by blocking DPM output for 2 hour at any point in the experiment, we incubated flies at 31°C for 2 hour, then returned them to 25°C and trained them 15 minutes later. This manipulation had no effect on 3 hour memory (wild-type flies= 0.33 ± 0.02; c316(GAL4);uas-\textit{shi}\textsuperscript{ts1} flies = 0.29 ± 0.06; P = 0.4). Therefore the intermediate 2 hour block likely causes a specific disruption of memory. None of the temperature manipulations that were used significantly impaired odor or shock acuity (Table 1). In conclusion, these data suggest that prolonged DPM output lasting at least 30 minutes immediately after training is required for wild-type 3 hour OCT-MCH memory, consistent with the idea that DPM neurons are involved in memory consolidation.

To identify the minimum time in which blocking DPM output disrupts memory, I trained flies at the permissive temperatures and blocked DPM output for 0,15, 30, or 60 minutes immediately following training. Following incubation at 31°C, flies were returned to 25°C in order to restore DPM function, and were tested at 3 hours following training (Figure 3.4). As previously reported (Figure 3.1), expression of \textit{shi}\textsuperscript{ts1} in
DPM neurons did not affect memory when flies were maintained at 25°C throughout the training/testing process (p>0.46). Similarly blocking DPM output for 15 minutes following training did not affect memory (p>0.52). Disrupting DPM function for 30 or 60 minutes following training abolished memory, indicating that DPM output is required for at least 30 minutes following training. For 30 and 60 minute inactivation respectively, c316{GAL4};uas-\textit{shi}\textsuperscript{ts1} flies exhibited memory defects compared to wild-type (p<0.001)(p<0.01), and uas-\textit{shi}\textsuperscript{ts1}flies (p<0.02)(p<0.01), and were statistically indistinguishable from \textit{amn}\textsuperscript{ts8} (p>0.50)(p>0.99).

To investigate if DPM output is persistently required between training and testing, c316{GAL4};\textit{shi}\textsuperscript{ts1} flies were incubated at 31°C for 30 minutes at either 30-60 minutes or 150-180 minutes following training and were tested for 3 hour memory (Figure 3.5). Flies in which DPM output was disrupted from 30-60 minutes following training displayed impaired memory. Three hour memory scores of c316{GAL4};uas-\textit{shi}\textsuperscript{ts1} were worse than wild-type (p<0.001) and uas-\textit{shi}\textsuperscript{ts1}flies (p<0.03) and indistinguishable from \textit{amn}\textsuperscript{ts8} (p>0.70). Blocking DPM output 150-180 minutes following training did not affect memory. Memory of c316{GAL4};\textit{shi}\textsuperscript{ts1} was indistinguishable from wild-type (p>0.26) and uas-\textit{shi}\textsuperscript{ts1} (p>0.37) flies, and superior to \textit{amn}\textsuperscript{ts8} (p<0.015). These results indicate that DPM dependence diminishes with time following memory acquisition, and that by 150 minutes following training, DPM neurons are dispensable. This is
consistent with the temporal requirements for conversion of memories to an anesthesia-resistant state (Quinn and Dudai, 1976).

**BA is sensed by the olfactory apparatus and a noncanonical pathway**

*Drosophila* olfactory memory experiments typically involve a single odor pair. However, it is not known if results obtained with a single odor pair are representative of other odors. Some investigators use BA (the odor of bitter almond) instead of OCT or MCH. We discovered that BA is sensed by the classical olfactory route and a non-classical route. We tested whether BA avoidance behavior was dependent on the classical olfactory apparatus—the antennae and maxillary palps. We surgically removed the antennae and palps from wild-type flies and tested avoidance of OCT, MCH, and BA in two different assays—the arena situation (Figures 3.6A and 3.6B) and the T-maze used for olfactory learning. Strikingly, significant BA responses were measured in both behavioral paradigms in the absence of olfactory organs, but OCT and MCH avoidance was abolished. These results suggests that OCT and MCH are classical odor stimuli sensed solely by the olfactory organs, but BA is also sensed by an entirely different mechanism that could be gustatory and/or somatosensory in nature.

To further define the nonolfactory BA-sensitive cells, we ablated other sites of chemosensation genetically or surgically. In homozygous
pox-neuro (poxn) mutant flies, the chemosensory bristles on the wings, legs, and labelum are transformed into mechanosensory bristles (Awasaki and Kimura, 1997). poxn flies with an intact olfactory system show intermediate BA avoidance, whereas surgical removal of olfactory organs from poxn mutants abolishes BA avoidance (Figure 3.6C). Therefore poxn-affected neurons are responsible for the nonolfactory BA response.

We removed wings from flies to test whether poxn-expressing wing neurons mediate BA avoidance. Wing removal in flies lacking olfactory organs did not alter BA avoidance, suggesting that tarsal or labelar poxn-expressing neurons are more likely involved. We therefore independently ablated two subpopulations of labelar gustatory neurons by ectopically expressing a diphtheria toxin transgene. Ablating sweet-sensitive gustatory neurons (Gr5a-driven ablation) did not affect the response to BA, whereas ablating bitter-sensitive gustatory neurons (Gr66a-driven ablation) significantly reduced BA avoidance (Figure 3.6B). However, ablating bitter gustatory neurons does not decrease the BA avoidance of flies lacking olfactory organs and is therefore not equivalent to poxn mutation. Taken together, these experiments demonstrate that BA is perceived by olfactory sensory neurons on the antennae and maxillary palps and by poxn-positive gustatory neurons located elsewhere. Some but not all of the poxn neurons are Gr66a-expressing labelar neurons.
The additional neurons may reside in the pharynx, the mouthparts, or the legs. Since these BA-sensitive organs are unlikely to project to the antennal lobe (Thorne et al., 2004; Wang et al., 2004c), BA information must be processed in parallel by multiple brain structures.

Following demonstration that BA avoidance was partially independent of the antennae and maxillary palps (Figures 3.6A, 3.6B, and 3.6C), we tested whether these organs were required for BA learning. We surgically removed antennae and maxillary palps from approximately 400 flies and tested their ability to associate OCT and BA with electric shock punishment (Figure 3.6D). Unlike naïve avoidance behavior, the ability to associate BA with electric shock requires the antennae and maxillary palps. Flies lacking these structures do not learn with OCT or BA. However, it should be noted that the learning experiment without olfactory organs is not ideal, because flies lacking olfactory organs cannot sense OCT and therefore should only be able to partially sense one of the odors used in training and testing—BA.

*amn* mutant flies learn poorly with BA, and the defect is partially DPM dependent

The finding that BA is sensed differently to OCT and MCH raised the question of whether BA odor memory was acquired differently. We therefore tested wild-type and *amn* mutant fly learning with OCT-BA. We noticed a dramatic asymmetry in the learning scores (Figure 3.7A). The
half score data revealed that, whereas wild-type flies learned well with OCT and BA, OCT learning of amnX8 flies was indistinguishable (p = 1) from wild-type flies but BA learning was greatly reduced (p < 0.01).

Expressing amn in DPM neurons with c316{GAL4} rescues the OCT-MCH memory defect of amn mutant flies (Waddell et al., 2000). We therefore tested if DPM expression of amn restored BA immediate memory to amn mutant flies. In these experiments, we also used the Mz717 driver to increase the confidence that rescue could be ascribed to DPM neurons. We generated amnX8;c316{GAL4}/uas-amn and amnX8;Mz717{GAL4}/uas-amn flies and tested BA and OCT immediate memory (Figure 3.7A). The amnX8;c316{GAL4}/uas-amn and amnX8;Mz717/uas-amn flies learned to avoid BA significantly better than amnX8 flies (p < 0.01 for both), but their performance was still significantly worse than that of wild-type flies (p < 0.01 for both). Thus, expressing amn principally in DPM neurons partially restored BA immediate memory.

In contrast, OCT immediate memory of amnX8 flies was indistinguishable from wild-type flies and amnX8;c316{GAL4}/uas-amn or amnX8;Mz717{GAL4}/uas-amn flies. This result implies that DPM neurons are involved in BA learning.

It has previously been reported that amn1 mutant flies have altered olfactory acuity following electric shock (Preat, 1998). It was therefore conceivable that our observed BA effect resulted from a selective loss of
BA acuity or an increase in OCT acuity following electric shock. We tested relative odor acuity in amn\textsuperscript{x8} mutant flies both before and after electric shock.

Prior to conducting a learning experiment, the odors are balanced so that naïve flies distribute evenly between the odors. Wild-type flies and amn\textsuperscript{x8} mutant flies distributed evenly between BA and OCT prior to shock (Figure 3.7B). We assayed the effect of shock on relative olfactory acuity by shocking flies in the absence of odor for 1 min (one shock every 5 seconds, total of 12 shocks as in the regular olfactory training protocol) and then allowing them to choose between OCT and BA. Shock did not change the distribution and hence did not change the relative odor acuity of wild-type or amn\textsuperscript{x8} mutant flies (p>0.5). Therefore, the BA learning defect of amn\textsuperscript{x8} flies cannot be explained by a change in relative odor acuity.

We also tested whether amn affected the alternate noncanonical pathway for sensing BA. We removed the antennae and palps from wild-type and amn\textsuperscript{x8} mutant flies and tested avoidance of BA, MCH, and OCT (Figure 3.7C). amn\textsuperscript{x8} flies without olfactory organs displayed BA avoidance that was indistinguishable from wild-type flies lacking olfactory organs (p > 0.3). These data suggest that amn does not affect BA sensation by the classical olfactory or the noncanonical route and instead is likely to affect neurons that are involved in processing BA information.
**Blocking DPM Output Impairs BA Learning**

We tested if directly blocking DPM output impaired BA learning. We expressed *uas-shi* in DPM neurons with c316{GAL4}. We used the BA-OCT odor pair and tested immediate memory at both the permissive (25°C) and the restrictive temperature (31°C). At 25°C, the BA learning scores of all genotypes, except *amnX8*, were not statistically different (p > 0.1) (Figures 3.8A and 3.8B). However, blocking DPM output with c316{GAL4};*uas-shi* specifically reduced BA immediate memory (p < 0.01) (Figure 3.8B) and left OCT immediate memory intact (p > 0.2) (Figure 3.8A). Crucially, the *uas-shi* control flies do not have a defect with BA or OCT at 31°C (Figures 3.8A and 3.8B). For comparison, we also tested whether blocking DPM output impaired OCT and MCH immediate memory (Figures 3.8C and 3.8D). The OCT and MCH performance of c316{GAL4};*uas-shi* flies is unaffected by temperature and is indistinguishable from the memory of wild-type flies (p > 0.5 for both odors). These data imply that DPM output is required to learn BA but not OCT or MCH. It is notable that *amnX8* mutant flies also have a significant MCH immediate memory defect. However, this defect is not reproduced when DPM neurons are inactivated. Therefore, the MCH immediate memory defect is DPM independent and likely resides in other neurons that are affected by *amn* mutation.
Blocking DPM output during acquisition impairs BA but not OCT memory

Transmitter release from DPM neurons is required to stabilize OCT-MCH memory but is not required during acquisition of these odor memories (Figures 3.3-3.5). Having observed a significant BA learning defect when we blocked DPM output (Figure 3.7B), we tested whether DPM output was required during acquisition of BA memory (Figure 3.9). We blocked DPM neuron output 15 min before training by incubating c316{GAL4};uas-\textit{sh}^{ts1} and Mz717{GAL4};uas-\textit{sh}^{ts1} flies at 31°C. We trained the flies with BA-OCT at 31°C and immediately returned the flies to 25°C to restore DPM neuron function. We tested olfactory memory 1 hour later and again analyzed individual odor half scores separately.

Blocking DPM output during acquisition did not effect 1 hour OCT memory: c316{GAL4};uas-\textit{sh}^{ts1} and Mz717{GAL4};uas-\textit{sh}^{ts1} fly memory was indistinguishable (p > 0.8 for both genotypes) from wild-type (Figure 3.9A). However, DPM blockade in c316{GAL4};uas-\textit{sh}^{ts1} and Mz717{GAL4};uas-\textit{sh}^{ts1} flies severely impaired 1 hour BA memory (p < 0.05 for both) (Figure 3.9B). In contrast, blocking transmitter release from DPM neurons during acquisition did not significantly affect memory with OCT-MCH (Figures 3.2 and 3.9). Furthermore, olfactory acuity and the response to electric shock of c316{GAL4};uas-\textit{sh}^{ts1} and Mz717{GAL4};uas-\textit{sh}^{ts1} flies were unaffected by temperature (Table 1).
Therefore, neurons expressing GAL4 in c316(GAL4) or Mz717(GAL4) flies are not directly involved in sensing and avoiding BA. Instead, these data imply that DPM output is required during acquisition of BA memory but not for OCT and MCH memory.

Discussion

DPM Neurons and Consolidation of OCT-MCH Memory

Assaying MCH and OCT olfactory memory, DPM neurons—large putative modulatory neurons that innervate the mushroom bodies—were previously identified as being the critical site of amn function in olfactory memory (Waddell et al., 2000). Using uas-shlts1 (Kitamoto, 2001) as a temperature-sensitive blocker of DPM neuron function, it was shown that DPM output was not required for OCT-MCH learning but was required for extended (up to 1 hour) memory (Waddell et al., 2000). These results, demonstrates that DPM output is dispensable during training and recall for 3 hour OCT-MCH memory (Figures 3.3, 3.4, and 3.5). Strikingly, DPM output is required at least 60 minutes into the period between training and testing. This timing is consistent with the idea that DPM function and AMN neuropeptide is involved in memory consolidation.

It is noteworthy that the requirement for DPM output in memory differs from the reported requirements of mushroom body neuron output. Mushroom body output has been reported to be dispensable during
acquisition and during storage but is required for memory recall (Dubnau et al., 2001; McGuire et al., 2001). DPM neuron output is required during storage but is dispensable during acquisition and recall (Figures 3.3, 3.3, and 3.4). Furthermore, the requirement of DPM neuron output is gone by 150 minutes following training. Taken together, these results suggest that with canonically sensed odors, such as OCT and MCH, DPM neurons are persistently activated following a learned event, and their activity is only diminished at a time when memory becomes consolidated (Figure 3.10).

**How is AMN Involved?**

Blocking DPM output with the uas-\(sh^{ts1}\) transgene phenocopies mutation of the *amnesiac* gene (Waddell et al., 2000). Blocking DPM neurons does not affect immediate memory but abolishes later memory. With some variability between alleles, *amn* mutant flies have a near wild-type immediate memory but a pronounced later memory defect (DeZazzo et al., 1999; Quinn et al., 1979; Tully and Gergen, 1986). These data are consistent with the possibility that AMN peptides contribute to the consolidation process.

Although blocking DPM neuron output with uas-\(sh^{ts1}\) produces an *amn* mutant-like memory defect (Waddell et al., 2000 and this study), it is not known if the \(sh^{ts1}\)-encoded dynamin blocks release of peptide-containing dense core vesicles (DCVs). DCVs, unlike typical synaptic vesicles, are derived from the trans-Golgi network. Dynamin is involved in
endocytosis (Chen et al., 1991) and vesicle budding from the Golgi (for review, see Allan et al., 2002), but whether it is involved in DCV release is unclear. The amnesic effect of blocking DPM output suggests that uas-\textit{sh}^{hs1} blocks AMN release and/or blocks release of an essential cotransmitter. The role of neurons expressing Neuropeptide F (NPF), a well-studied neuropeptide, have been studied using \textit{sh}^{hs1}. Expression of \textit{sh}^{hs1} in NPF neurons causes an NPF null-like phenotype (Wen et al., 2005), supporting the notion that \textit{sh}^{hs1} disrupts neuropeptide function at non-permissive temperatures.

It is plausible that AMN peptides are coreleased from DPM synapses with a classical fast-acting transmitter. Glutamate is used in the CNS of \textit{Drosophila}, but it is not the predominant transmitter (Strausfeld et al., 2003). Instead, this role appears to be taken by acetylcholine (Gorczyca and Hall, 1987). We showed that a DPM neuron marker is coexpressed with a cholinergic neuron-specific marker (Figure 2.2), suggesting that a DPM cotransmitter is ACh. Assuming that DPM neurons corelease ACh and AMN transmitters, DPM neuron release may trigger a postsynaptic response in receptive mushroom body neurons that involves ACh receptors and AMN receptors. Genetic and pharmacological experiments suggest that the \textit{amn} mutants are deficient in cAMP synthesis (Feany and Quinn, 1995; Moore et al., 1998), and it has previously been posited that memory stabilization may depend on
prolonged cAMP cascade stimulation by AMN peptide (Waddell et al., 2000). Perhaps the role of ACh versus AMN peptides in DPM-dependent memory will rely on the evoked firing pattern of DPM neurons, with repetitive activity being required to release AMN (Zhong and Pena, 1995).

In mammals, the putative AMN homolog PACAP and the related vasoactive intestinal peptide (VIP) can be coreleased with ACh. In several neural systems, PACAP and VIP can potentiate both muscarinic and nicotinic ACh-evoked currents by a cAMP-dependent mechanism (Gurantz et al., 1994; Kawatani et al., 1985b; Margiotta and Pardi, 1995). In a hippocampal slice preparation, PACAP38 enhances excitatory CA3-CA1 synaptic transmission, and the facilitation can be blocked by inhibition of muscarinic receptors (Roberto and Brunelli, 2000; Roberto et al., 2001). Perhaps AMN and ACh peptides are persistently co-released following ‘memory events’ to fulfill a similar function in fly memory.

**DPM Neuron Output and Acquisition of BA Memory**

We discovered that DPM output is required during acquisition to associate BA with electric shock. Blocking DPM output during acquisition blocks BA memory but not memory for OCT or MCH (Figure 3.9). Therefore, the temporal requirements for DPM output show some odor specificity. Furthermore, these finding implies that DPM neurons may be differentially required for odor memory.

**Why Is BA Different?**
Our learning experiments suggested that BA might be unique for flies. Importantly, our data (Figures 3.6B and 3.6C) and a previous study (Charro and Alcorta, 1994) demonstrate that flies respond to BA independently of the antennae and maxillary palps. We found that the bitter almond smell of BA is sensed by the olfactory system, bitter-sensitive gustatory neurons, and poxn-affected neurons that are likely on the legs or mouthparts of the fly. This implies that Drosophila can use multiple neural pathways to sense some odors. In vertebrates, both the olfactory and a somatosensory system called the trigeminal system respond to most odorous chemicals. The free nerve endings of the trigeminal system are sensitive to thermal and mechanical stimuli as well as to very high and potentially harmful concentrations of chemicals. Trigeminal stimulation induces a reflex that stops inspiration to prevent inhalation of hazardous substances. Our finding that BA is a particularly potent somatosensory stimulus is consistent with the fact that BA is a highly effective insecticide (Dettner et al., 1992) and also a potent trigeminal stimulus in humans (Doty et al., 1978). Therefore, these data suggest that Drosophila possess additional odor detecting systems that are perhaps analogous to the trigeminal system in vertebrates to detect potentially harmful chemicals.
What type of sensory neuron outside of the classical olfactory system is likely to detect BA?

The fact that BA is a volatile stimulus would argue that the nonantennal/palp neurons are olfactory in nature, while our poxn results argue that they are gustatory. We feel that this apparent contradiction is purely semantic, because the strict division of sensory systems into olfactory and gustatory modalities is becoming increasingly blurred by new molecular and functional information. For instance, receptors from the same subclass function as odor receptors for amino acids in fish olfactory neurons (Speca et al., 1999), putative pheromone receptors in the vertebrate vomeronasal system (Dulac and Axel, 1995; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997), and taste receptors tuned to sweet and umami substances in the vertebrate tongue (Nelson et al., 2002). Similarly, several fly gustatory receptor genes are expressed selectively in olfactory neurons in the fly (Clyne et al., 2000; Couto et al., 2005; Suh et al., 2004) and a prominent member of the insect odorant receptor gene family is expressed in the mosquito proboscis (Pitts et al., 2004), classically defined as a gustatory organ. Therefore, neither the class of molecular receptor expressed in a given sensory system nor the sensory organ itself is necessarily a clear indication of whether a given neuron is tasting or smelling a stimulus. In the nematode, chemosensory neurons have been divided into those responding to volatile stimuli and
nonvolatile stimuli, corresponding to olfactory and gustatory senses, respectively (Bargmann et al., 1993; Bargmann and Horvitz, 1991). This division based on the stimulus type seems most relevant for the biology of terrestrial animals, and we favor the interpretation that chemosensory neurons of the olfactory class but lying outside of the classical olfactory system are tuned to BA. Future work will be aimed at characterizing these atypical sensory neurons and mapping their circuitry in the brain.

It is plausible that associative learning of BA involves signal integration of the electric shock pathway with BA information from all the systems that detect BA—an antennae/palp pathway, a bitter-sensitive pathway on the labelum, and poxn-affected neurons located elsewhere. This multimodal BA information would be initially processed by distinct brain regions. Antennal and palp input projects to the antennal lobe (Couto et al., 2005; Fishilevich and Vosshall, 2005), but labelar gustatory neurons project to the subesophageal ganglion (Thorne et al., 2004; Wang et al., 2004c). We assume that tarsal chemosensory neurons will project to the ventral ganglion. We speculate that this unique and potentially integrative circuit specificity accounts for the different requirement of DPM neuron involvement in learning BA versus the memory of OCT and MCH. Alternatively, it is possible that DPM neurons differently process BA information that comes through the antennal and
maxillary palp pathway. Future work will determine the importance of BA input through the noncanonical pathway in BA memory.

Previous studies have indicated that *Drosophila* process BA differently to other odors. Flies with a mutation in the *acj*\(^6\) gene have a reduced olfactory jump response and a reduced electrophysiological response in the antennae and maxillary palps to all odors tested except BA (Ayer and Carlson, 1992). In contrast, mutation of the *ptg* gene produces a near reciprocal result to *acj*\(^6\). *ptg*\(^-\) mutant flies are defective in their response to BA but normal with other odors tested (Helfand and Carlson, 1989). In addition, disrupting olfactory receptor neuron expression of the \(G_{\alpha_q}\) heterotrimeric G protein subunit gene with region-restricted RNA interference abolished behavioral responses to isoamylacetate but not BA (Kalidas and Smith, 2002).

Is there any reason BA may have inherent meaning to an insect? BA is the odor of bitter almond. Many plants (including almond), when damaged, produce hydrogen cyanide and BA from a cyanogenic glycoside. This cyanogenesis is believed to protect against predation from herbivores (Gleadow and Woodrow, 2002). Perhaps it would be profitable for an organism that might otherwise lay its eggs on the fruits of a cyanogenic plant to be primed to associate the smell/taste of BA with the possibility of cyanide release. The detrimental effect of hydrogen cyanide is unquestioned—it causes a near universal respiratory arrest. BA, on the
other hand, is considered more of a general irritant. In addition to plants, some insects use hydrogen cyanide and/or BA as a defensive/alert signal (Nahrstedt, 1988). For example, some millipede species release hydrogen cyanide and BA as defensive emissions (Conner et al., 1977). Perhaps more interesting, harvester ants release BA when agitated, and conditioned air suffused with this emission elicits an avoidance behavior in non-agitated naïve ants (Blum et al., 1969). However, we have no evidence that BA is a constituent of a similar emission in *Drosophila*.

**DPM Neurons involvement in Odor Memories**

In conclusion, the results presented here demonstrate that DPM neuron output is differentially involved in odor memory. DPM activity lasting at least 30 min after training is required for normal OCT and MCH memory, supportive of a role for DPM neuron function (and presumably AMN peptide) in consolidation of OCT and MCH memory. The dependence of DPM neurons appears to diminish with time following an association, and DPM neurons are dispensable at 150 minutes following training. In contrast, DPM output is required during acquisition of BA memory. Taken with our finding that BA is sensed by both olfactory and nonolfactory routes, we speculate that DPM neurons are uniquely involved in the learning and memory of odors that require multisensory integration.
Portions of this chapter have been published:


CHAPTER IV: THE ROLE OF DPM NEURONS IN SUCROSE-REINFORCED ODOR MEMORY

Introduction

It is widely believed that memory is encoded as changes in synaptic efficacy between neurons in a network. This concept of synaptic plasticity predicts that it will be possible to localize memory to discrete synapses in neural networks in the brain. The relatively small brains of insects are well suited to this endeavor and genetic manipulation in the fruit fly *Drosophila* has greatly aided neural circuit mapping of odor memory. Flies can be taught to associate an odor conditioned stimulus (CS) with either a punitive electric shock (Dudai et al., 1976; Tully and Quinn, 1985) or a gustatory-sugar reward (Schwaerzel et al., 2003; Tempel et al., 1983) unconditioned stimulus (US). Strikingly, learning and memory with these opposing unconditioned stimuli requires differential transmitter involvement: sugar-reinforced odor memory is dependent on intact octopamine signaling while shock-punished (aversive) odor memory is dependent on dopamine signaling (Schwaerzel et al., 2003). However, despite the differential requirement for these monoamine transmitters, blocking mushroom body output during retrieval impairs both aversive and rewarded odor memories (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2003) implying that these memories rely on
overlapping brain regions. We therefore tested whether the circuitry involving DPM neurons was involved in the stability of sucrose-reinforced odor memory.

Beyond strictly examining the role of DPM neurons in reward-mediated olfactory memory, there are many advantages to using an assay that requires gustatory cues as opposed to electric shock (see Chapter I). Briefly, gustatory receptors have been identified based on sequence and studies have begun to assign ligands to these receptors (Clyne et al., 2000; Scott et al., 2001). Perhaps more importantly, neural circuitry governing the attractive or inhibitory response to different gustatory stimuli has been identified (Wang et al., 2004c). On the other hand, the mechanism of electric shock sensation remains elusive. Imaging studies in the fly indicate many neuropil regions respond to electric shock (Riemensperger et al., 2005; Yu et al., 2004; Yu et al., 2005), and the receptors that modulate this response are completely unknown. Therefore, because a major goal in the study of memory is to identify the neural pathways that encode for the unconditioned stimulus, using gustatory cues may prove more fruitful.

Larvae and adult flies can be taught to pair gustatory cues with odor. Larval studies have used sucrose as a rewarding reinforcer, and quinine as a punishing reinforcer (Gerber et al., 2004a; Honjo and Furukubo-Tokunaga, 2005; Scherer et al., 2003). Flies trained to pair a
neutral stimulus with a gustatory reinforcer must be food deprived, indicating a motivational component to this type of learning that does not exist when electric shock is used as a reinforcer (Tempel et al., 1983). This could simply be attributed to flies not receiving the unconditioned stimulus when they are well-fed. Fly express taste receptors on feet and wings as well as their proboscis, however, it is not clear if tasting a chemical on the feet is sufficient for mediating memory. Therefore a fly fed ad libitum may not place its proboscis on food, and may therefore never learn. Alternatively, flies may learn to pair the odor with a cue, but do not exhibit the memory unless they are hungry. This phenomena, termed latent learning, or learning without performance, has been demonstrated in Drosophila courtship memory (Kane et al., 1997).

Despite great effort, I have been unable to teach flies to associate olfactory cues with quinine punishment. I have used many different training paradigms in both the T-maze and the older Quinn-Harris-Benzer paradigm. Because memories of a punishing gustatory reinforcer seem to be less robust (data not shown), we have focused on sucrose-rewarded odor memory for the experiments presented here. These studies show that DPM function is essential for sucrose-reinforced olfactory memory, indicating that DPM neurons are generally involved in stabilizing mushroom body dependent memories.
Materials and Methods

The amn1, and amnX8 null alleles were described previously (Moore et al., 1998; Quinn et al., 1979). The uas-cd8::GFP flies are described (Lee and Luo, 1999). The uas-shi^ts1 flies were those previously used by (Waddell et al., 2000) and first described by Kitamoto (2001). The DPM neuron-restricted c316{GAL4} and the uas-amn flies have been described (Waddell et al., 2000).

Flies expressing shi^ts1 in DPM neurons were generated by crossing homozygous w,uas-shi^ts1;uas-shi^ts1 females to homozygous w;c316{GAL4} males. All progeny from this cross carry two uas- shi^ts1 transgenes and one c316{GAL4}. Heterozygous w,uas-shi^ts1;uas- shi^ts1 flies were generated by crossing homozygote females to w- males. A mixed population of sexes was tested in the olfactory conditioning paradigm.

For rescue of the amnX8 and amn1 memory defect I crossed amn1;c316{GAL4} females with amnX8;uas-amn males. Male progeny from these crosses are hemizygous for amn1, and heterozygous for c316{GAL4} and uas-amn. Female progeny from these crosses are transheterozygote amnX8/amn1 and heterozygous for c316{GAL4} and
uas-\textit{amn}. Males, females, and \textit{amn}^{Xs} controls were trained and tested together and sorted after testing and before counting.

We adapted a previously described protocol for olfactory conditioning with sugar reward. Flies were starved for 16-20 hours before conditioning. A conditioning tube (CS+) was made by spreading saturated sucrose (allowed to dry before use) onto a filter paper that covered the entire training tube. Another tube representing the CS- was prepared containing a filter paper soaked in water (and allowed to dry).

Approximately 100 starved flies were loaded into the elevator section of a T-maze, and trained as follows: Flies were transferred to the CS- tube and exposed to an odor for 2 minutes. Following 30 seconds of clean air stream they were transferred back into the elevator and into the sugar reward (CS+) tube where they were exposed to another odor for 2 minutes. We tested olfactory memory 3, 60, 180, and 360 minutes after training. Flies were stored in empty food vials containing damp filter paper between training and testing.

We previously determined that the \textit{amn}$^1$, \textit{amn}^{Xs}, c316(GAL4), uas-\textit{amn}, uas-\textit{shi}^{s1}, and c316(GAL4); uas-\textit{shi}^{s1} strains tested in this study have normal odor and electric-shock acuity (Table 1 and (Waddell et al., 2000).

\textbf{Acknowledged contributions}
Jessica Bernard assisted in optimizing the protocol for teaching flies with sucrose reinforcer. All behavioral experiments were performed in collaboration with Michael Krashes.

**Results:**

*amn* mutants are defective for sucrose-reinforced memory

*amn* mutant flies were first confirmed to have a memory defect when conditioned with odors and sugar reward. A modification protocols used previously (Schwaerzel et al., 2003; Tempel et al., 1983) was employed, that more closely resembles the odor-shock conditioning protocol and that produces robust memory that lasts for more than 6 hours (Figure 4.1). Briefly, approximately 100 starved flies were exposed to an odor for 2 minutes in the absence of sugar, followed by a clean air stream for 30 seconds and a second odor with sugar reward for 2 minutes. Olfactory memory was tested at 3, 60, 180, and 360 minutes after training. Flies homozygous for the strong *amn* alleles- *amn*¹ or *amn*²⁸ - learn to associate the appropriate odor with sugar reward but they forget this association within 60 minutes of training. *amn*¹ and *amn*²⁸ mutants have small, but significant learning defects compared to wild-type flies (p<0.05) and memory is abolished at later time-points. These data are consistent with the earlier report that *amn*¹ flies have defective sucrose-reinforced odor memory (Tempel et al., 1983).
Selective expression of *amn* in DPM neurons rescues the memory defect of *amn* mutant flies

Since *amn* mutant flies forget quickly when trained with either a punitive-shock or a gustatory-reward US, we wondered if similar neural circuitry was involved in both types of memory. Expressing the *amn* gene in DPM neurons restores aversive odor memory performance to *amn* mutant flies (Tamura et al., 2003; Waddell et al., 2000). We therefore tested whether restoring *amn* expression in DPM neurons of *amn* mutant flies would rescue the sucrose-reinforced odor memory defect. The c316{GAL4} line was used to transgenically express the *amn* gene in DPM neurons of *amn* mutant flies. Three hour memory of *amn^{Xa}/amn^{1};c316{GAL4}/uas-*amn*, and *amn^{1};c316{GAL4}/uas-*amn* flies was similar to wild-type (p>0.99, P=1) flies and was statistically different from the memory of *amn^{Xa}* (p<0.05, p<0.05) and *amn^{1}; uas-*amn* mutant flies (p<0.05, p<0.05) (Fig. 4.2). These data demonstrate that *amn* expression in DPM neurons is sufficient to restore sucrose-reinforced odor memory to *amn* mutant flies and suggest that DPM neurons are generally critical for olfactory memories.

**DPM output is required for stability of sucrose-reinforced memory**

We next directly tested the role of DPM neurons in sugar-reinforced odor
memory by temporally blocking their output during the course of the experiment (Figure 4.3). The temperature sensitive $sh^{ts1}$ transgene (Kitamoto, 2001) was expressed in DPM neurons and flies were tested in the sugar rewarded conditioning paradigm at either the permissive ($25^\circ C$) or the restrictive temperature ($31^\circ C$). It is presumed that at the restrictive temperature, $sh^{ts1}$ either blocks release of an essential AMN cotransmitter (likely ACh) or that it directly, or indirectly, compromises AMN peptide release. At $25^\circ C$ sucrose-reinforced odor memory of $c316\{GAL4\};uas-sh^{ts1}$ flies was comparable to memory of wild-type and $uas-sh^{ts1}$ flies ($p>0.2$) (Figure 4.3A). However, at $31^\circ C$, $c316\{GAL4\};uas-sh^{ts1}$ flies displayed worse memory than wild-type and $uas-sh^{ts1}$ flies ($p<0.001$) (Figure 4.3B). Therefore, DPM synaptic release is necessary for stable sucrose-reinforced odor memory as it is with aversive odor memory (Waddell et al., 2000 and Figure 3.1).

Stable shock-reinforced aversive odor memory requires prolonged DPM output between training and testing and DPM output is dispensable during training and retrieval with the odors used here (see Chapter III). We therefore tested if transmitter release from DPM neurons was similarly required for sucrose-reinforced odor memory. We again blocked DPM output with $uas-sh^{ts1}$ but this time inactivation was restricted to either the training, testing or storage period. Blocking output from DPM neurons during acquisition did not produce memory loss (Figure 4.4A).
Memory of c316(GAL4); uas-shi\textsuperscript{fs\text{\textdagger}} flies was comparable to wild-type flies (p>0.1) and uas-shi\textsuperscript{fs\text{\textdagger}} controls (p>0.9). Similarly, DPM neuron output was not required during memory retrieval (Figure 4.4B). Memory of c316(GAL4); uas-shi\textsuperscript{fs\text{\textdagger}} flies was comparable to wild-type (p>0.2) and uas-shi\textsuperscript{fs\text{\textdagger}} flies (p>0.2). However, blocking DPM output for 30 min after training significantly reduced sucrose-reinforced odor memory (Figure 4.4C). Memory of c316(GAL4); uas-shi\textsuperscript{fs\text{\textdagger}} flies was severely reduced and is statistically different from wild-type (p<0.01) and uas-shi\textsuperscript{fs\text{\textdagger}} flies (p<0.01). These data parallel previous results with aversive odor memory (Figure 3.6) and suggest that there is a similar requirement for DPM neuron output to stabilize both aversive and rewarding odor memory. We further analyzed the temporal involvement of DPM neurons in sucrose-reinforced odor memory by blocking their output from 30-60 min after training. I previously found that blocking DPM output from 30-60 min after training compromised shock-reinforced aversive odor memory similar to a 0-30 min block (Figures 3.4 and 3.5A). Disrupting DPM neuron output from 30-60 min appeared to compromise sucrose-reinforced odor memory, although the effect did not reach statistical significance (P>0.05) (Figure 4.4D). These data suggest that DPM output is persistently required for sucrose-reinforced olfactory memory.

To confirm that the effect observed by blocking output with c316(GAL4) is due to expression in DPM neurons, I expressed shi\textsuperscript{fs\text{\textdagger}}
under control of Mz717(GAL4). Mz717(GAL4) drives expression in DPM neurons, and a small number of Kenyon cells (Ito et al., 1998; Figure 1.3C). At 25°C, Mz717(GAL4);uas-shi-ts1 flies showed memory indistinguishable from wild-type or those harboring the uas-shi-ts1 transgene (Data not shown). As previously observed with the c316(GAL4) driver, blocking DPM output during the window associated with consolidation also cause memory defects. Placing flies at 31°C for 60 minutes following training also caused memory impairments in Mz717(GAL4);uas-shi-ts1 flies when compared to wild-type (p<0.02) and uas-shi-ts1 controls (p<0.001) (Figure 4.5). Attaining the same results with a second GAL4 driver that also expresses in DPM neurons provides further evidence that output from DPM neurons is essential for multiple forms of olfactory memory. Taken together these results indicate that synaptic activity of DPM neurons is essential for sucrose-reinforced olfactory memory. Further the amn and temporal requirements of DPM neurons output appears to be similar for sugar and shock-reinforced olfactory memory.

Discussion

Mechanisms of DPM involvement in memory stability

In the mammalian brain, reward and fear trigger different monoamine transmitter system networks. Reward is tightly coupled to the
dopaminergic system whereas fear and anxiety is attributed to noradrenergic/adrenergic signaling (Tanaka et al., 2000; Wise, 2005). These transmitter systems are apparently required for animals to learn whether a stimulus predicts a reward or a punishment (Hyman et al., 2006). In *Drosophila* there is a striking dissociation of monoamine transmitters for reward and punishment. Dopamine is required for aversive odor memory formation whereas octopamine is necessary for rewarded odor memory (Schwaerzel et al., 2003). Octopaminergic and dopaminergic neurons are found throughout the brain (Schwaerzel et al., 2003). Although it is not known if the mushroom body arborization of these monoaminergic neurons is required for odor memories, mushroom body output is required to retrieve both aversive and rewarded odor memory (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2003). Therefore, it is possible that both types of odor memory are stored in the mushroom bodies. DPM neurons ramify throughout the mushroom body lobes and provide a general stabilizing mechanism for both aversive and attractive forms of odor memory.

A possible mechanism for synaptic plasticity is that DPM neurons act presynaptically to modulate DA or OA output onto the mushroom bodies. However, the temporal requirements for monoaminergic projections and DPM neurons make it unlikely that these sets of neurons directly interact. Output from DA neurons is required only during
acquisition (Schwarzel et al., 2003), while DPM output is dispensable during this period (Figure 3.4). Therefore, a fitting model is for Kenyon cells to act as a coincidence detector for odor specific activation from cholinergic projection neurons and monaminergic input onto the mushroom body lobes. DPM neurons are likely to act distinctly to stabilize memory. This notion is supported by lack of learning defects in DPM-inactivated and amn mutant flies (Quinn et al., 1979; Waddell et al., 2000), as well as the dispensability of DPM neurons during training (Figures 3.2 and 4.4A). Chapter V addresses possible mechanisms of DPM-mushroom body interactions in memory stability.

**How is the US represented in the mushroom bodies?**

A major question in the effort to expand on this line of research will be how different memory subtypes are represented within the mushroom bodies. It has been proposed that components of both aversive and rewarded odor memory may reside at discrete synapses within the same mushroom body neurons (Gerber et al., 2004b; Schwaerzel et al., 2003). Alternatively, aversive memories for a particular odor may be represented in a subset of the total number of mushroom body neurons activated by an odor and rewarded memories may be stored in a non-overlapping subset (Gerber et al., 2004b; Schwaerzel et al., 2003). It is currently unclear if the monoamine receptors that putatively confer reinforcer specificity are localized to similar or different subsets of Kenyon cells. The
DPM data cannot distinguish between the two models but they provide improved resolution and suggest that aversive and rewarded odor memories are represented, and stabilized, in mushroom body neurons. Furthermore, these data raise the possibility that DPM neurons provide input to the same sets of Kenyon cells that receive DA and OA input. Further morphological characterization of monoaminergic and DPM neuron projections onto the lobe may provide insight into how these mushroom body-associated neurons signal the mushroom bodies.

Identification of DA and OA receptors involved in memory and close examination of their localization within the mushroom bodies will likely shed light on how positively and negatively reinforced memories are represented within the mushroom bodies. The fly genome encodes for 3 putative DA receptors and 3 OA receptors. Both the DA receptor DAMB, and the OA receptor OAMB, are highly expressed in the mushroom bodies (Han et al., 1998; Han et al., 1996a). In the cricket, pharmacological inhibition of all DA receptors impairs negatively reinforced memory, with a minor effect on reward reinforced memory. Conversely, pharmacological blockade of OA receptors impaired reward reinforced memory. However, this study did find overlap between requirements for both memory types and each receptor. It is unclear if this is due to an organismal difference, or a difference in receptor
requirements versus transmitter (Unoki et al., 2005). Future studies in *Drosophila* targeting receptors should address these questions.

**Sucrose-reinforced memory and motivation**

The cues mediating these motivational aspects of memory are of great interest, and were not addressed in the experiments presented here. A strong potential candidate for mediating this behavior is neuropeptide F (NPF), the fly homolog of mammalian neuropeptide Y (NPY). In both *Drosophila* and mammals NPF/NPY is upregulated when animals are food deprived and this neuropeptide is involved in motivational feeding (Levine and Morley, 1984; Wu et al., 2003). Therefore it is possible that NPF, like OA, is involved in sucrose-reward memory, but dispensable for shock.

Portions of this chapter have been published:

CHAPTER V: DPM NEURON-MUSHROOM BODY $\alpha'/\beta'$ NEURON INTERACTIONS IN MEMORY

Introduction

From a minimalist perspective, a major goal in the study of memory is to identify the neural circuits necessary for memory to be formed. In the case of memory stability, the requirements for DPM output give insight into the circuitry involved in this component of memory. Unfortunately, little is known of the neurons that drive DPM activity. Furthermore, it has often been assumed that DPM neurons strictly provide input to the mushroom bodies, but careful analysis reveals that this conclusion is by no means certain.

How do DPM neurons signal the mushroom bodies?

Morphological evidence has been used to suggest DPM neurons drive mushroom body activity. Labeling DPM neurons with markers that express throughout these neurons reveals that a primary neurite exits the cell body and undergoes multiple bifurcations prior to ramification throughout all mushroom body lobes (Figures 1.3 and 2.1). This morphology is consistent with a unipolar characterization. Because these neurons are peptidergic, it has been suggested that DPM neurons release AMN neuropeptide onto the mushroom body lobes. However, the receptor for AMN remains unknown, and therefore, the neurons or
brain regions that receive input from DPM neurons remain elusive. Furthermore, because DPM neurons appear to be unipolar with no clear dendritic region, the neurons and brain regions that drive DPM activity remain unknown. This chapter uses transgenic markers of cell polarity and targeted neuronal silencing to investigate the inputs to, and outputs from, DPM neurons.

Functional imaging studies have allowed some insight into the environmental cues that drive DPM neurons. Imaging of DPM projections onto the mushroom body lobes reveal that these neurons are activated by both shock, and a broad array of odors. Furthermore, following the pairing of odor and shock, DPM neurons show a conditioned response as revealed by increased Ca\textsuperscript{2+} influx, specifically to the odor with which the shock was paired (Yu et al., 2005). Because DPM neurons are interneurons, these results reveal that the olfactory map of neurons that drive DPM neurons is altered following conditioning. Since it has previously been established that Kenyon cells are selectively activated by odors (Wang et al., 2003a), and form putative connections with DPM neurons, it is possible that Kenyon cells drive DPM neurons. According to this model, DPM dendrites are localized within the mushroom body lobes. To address this possibility I have transgenically expressed markers of cell polarity in DPM neurons to examine the localization of DPM dendrites and axons. These studies serendipitously caused
developmental abnormalities in DPM ramifications and behavioral analysis revealed that DPM projections to the $\alpha'/\beta'$ lobes of the mushroom bodies are sufficient for memory. These results prompted a detailed investigation into the role of the mushroom body $\alpha'/\beta'$ lobes in memory.

**Mushroom body lobe specificity and memory**

Little is known of the role of the mushroom body prime lobes in memory. In fact, in many related species including the honeybee (*Apis Melifera*), the prime lobes are apparently absent (Strausfeld, 2002). In *Drosophila*, these lobes develop in the late larval stage and were first identified by immunostaining for proteins previously determined to be involved in memory (Crittenden et al., 1998; Lee et al, 1999). However, no behavioral role has been attributed to these structures. To investigate a possible role for mushroom body $\alpha'/\beta'$ neurons in olfactory memory, we obtained drivers that express in the mushroom body $\alpha'/\beta'$ lobes, but not the $\alpha,\beta, or \gamma$ lobes. By transgenically expressing $\text{shl}^{61}$ in these neurons, we blocked output from these neurons and determined that synaptic release from the prime lobes is essential for olfactory memory. Furthermore, these studies suggested that synaptic release from prime lobe neurons is required during the same window following training as transmitter release from DPM neurons is required. These results support
a model where DPM neurons both provide and receive input from the Kenyon cells that comprise the prime lobes.

Materials and Methods

Fly stocks and histochemistry

The uas-syb:GFP, uas-CD2, and uas-DScam[exon17-1]GFP (here designated as uas-DScam17-1::GFP) and uas-DScam[exon17-2]GFP (here designated as uas-DScam17-2::GFP) have been previously described (Wang et al, 2004). Five GAL4 lines (C68a, c728, c320, c305a) were identified on flytrap (www.flytrap.org) as driving expression selectively in the mushroom body prime lobes and were obtained from Douglas Armstrong (University of Edinburgh). Expression patterns were verified by crossing virgin females carrying a P{GAL4} insertion to males harboring uas-lacZ or uas-cd8::GFP. Brains were dissected and stained with anti-FAS-II 1D4 (Iowa Hybridoma Bank) as previously described. A lack of colocalization between FAS-II and reporter gene revealed that expression was limited to regions outside of the mushroom body α, β, and γ lobes.

Transgenic flies for neuronal inactivation experiments were generated by crossing homozygous w,uas-shi^ts1;uas-shi^ts1 females to homozygous males containing a GAL4 enhancer trap. All insertions were
on autosomes except for c728(GAL4), and in this case were sorted by sex following testing and only female flies were analyzed.

Adult brains expressing transgenic uas-cd8::GFP or uas-DScam17-2::GFP and uas-CD2 were removed from the head capsule and fixed in 4% paraformaldehyde in Phosphate Buffered Saline (PBS), [1.86mM NaH2PO4, 8.41mM Na2HPO4, 175mM NaCl] for 15 min, and rinsed in PBS-T (PBS containing 0.25% Triton X-100). Brains were incubated with (1:1000) anti-CD2 antibody (Caltag Laboratories), 1:2000 anti-βgal (Cappell Laboratories) or (1:4) mAb1D4 anti-FASII antibody (Hybridoma Bank, University of Iowa ) in PBS-T for 8 hours at 4°C and washed 3X in PBS, 0.2% triton X-100. Following incubation in primary antibody brains were washed in PBS-T, 0.2% triton X-100, then incubated with Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Laboratories) or Cy5-conjugated donkey anti-rabbit for 1 hour and washed 3X in PBS, 0.2% triton X-100. Fixed brains and stained brains were mounted in Vectashield (Vector Labs). Confocal analysis was performed on a Zeiss LSM 5 Pascal confocal microscope.
Behavioral Analysis.

Both sucrose and shock reinforced olfactory conditioning were performed as described in previous chapters.

Acknowledged contributions

Following the initial discovery that prime lobe output is required for memory stability, Michael Krashes aided in all behavioral experiments. J. Douglas Armstrong provided the {GAL4} drivers that are prime lobe restricted, and Benjamin Leung assisted in imaging. Figures 5.3 and 5.4 B-C are exclusively the work of BL.

Results

DPM projections to the α´/β´ lobes are sufficient for memory

DPM neurons appear to innervate all the lobes of the mushroom bodies and previous imaging studies suggest that the DPM projections may be both transmissive and receptive (Yu et al., 2005). To gain insight into DPM neuron organization I expressed markers of neuronal polarity in DPM neurons. The markers uas-syb:GFP and DScam17-2::GFP have been reported to label pre-synaptic regions while DScam17-1::GFP has been reported to label dendrites (Wang et al, 2004). To independently label all regions of DPM neurons I co-expressed uas-\textit{lacZ} or uas-\textit{CD2} with many of the polarity markers. Expression of the dendritic marker DScam17-1::GFP revealed expression in DPM ramifications throughout
all lobes of the mushroom bodies (Figure 5.1). If in this case, DScam17-1::GFP is indeed acting as a true marker of dendrites, DPM neurons are likely activated by mushroom bodies output. It has previously been reported that DScam17-1::GFP selectively labels projection neurons and mushroom bodies dendrites. However, evidence gleaned from polarity markers should be interpreted with caution. Each driver likely expresses at different levels, and it is possible that high expression of a driver in a neuron could overwhelm the ability of the neuron to appropriately compartmentalize the reporter’s expression.

Driving the axonal marker syb:GFP in DPM neurons results in expression in all DPM projections, suggesting that DPM neurons also transmit information to the mushroom bodies (Ito et al., 1998; Tamura et al., 2003). However, expression of the marker syb:GFP in the mushroom bodies with MB247{GAL4} resulted in labeling of all regions of the mushroom bodies (data not shown), bringing the utility of this marker into question. Seeking another indicator of polarity I expressed a second axonal marker, DScam17-2::GFP in DPM neurons with c316{GAL4}. DPM morphology in uas-DScam17-2::GFP;c316{GAL4} flies differed compared to uas-CD8::GFP;c316{GAL4} flies. Brains of flies ectopically expressing Dscam17-2::GFP in DPM neurons display dense innervations of the prime lobes, with little innervations of the $\alpha$, $\beta$ and $\gamma$ lobes (Figure
5.2B), compared to cd8::GFP expression in DPM neurons which results in projections to all lobes (Figure 5.2A).

To confirm that ectopic expression of uas-DScam17-2::GFP disrupts DPM projections onto the α, β, and γ lobes, brains of flies expressing uas-DScam17-2::GFP or uas-cd8::GFP were double labeled with the α/β lobe marker anti-FASII and the prime lobe marker anti-TRIO (Figure 5.3F). DPM neurons in uas-cd8::GFP;c316{GAL4} flies innervate anti-FASII and anti-TRIO stained regions (Figure 5.3A-C), while DPM neurons in uas-Dscam:17-2GFP;c316{GAL4} flies appeared to be absent from prime lobe projections in anti-FASII stained brains (Figure 5.3D-E). Coexpressing uas-DScam17-2::GFP and uas-CD2 in DPM neurons reveals that DScam17-2::GFP labels the remaining projections rather than a subset of existing projections (Figures 5.3D-E) and DPM neuron expression of uas-CD2 in the absence of uas-DScam17-2::GFP results in projections to all lobes (Figures 5.2C) indicating that the DScam17-2::GFP transgene selectively disrupts DPM neuron projections onto the α, β and γ lobes, while leaving projections onto the prime lobes intact.

A role for the mushroom body prime lobes in memory has not been reported. Therefore uas-DScam17-2::GFP; c316{GAL4} flies were used to assess the role of DPM neuron projections to the prime lobe subset in shock and sucrose-reinforced odor memory (Figure 5.4). Heterozygote uas-DScam17-2::GFP flies were included in these experiments as a
control, as well as wild-type and *amn*\textsuperscript{X8} flies for comparison. The presence of the uas-DScam17-2::GFP transgene had no effect on aversive odor memory. Remarkably, DPM neurons that lack projections to the α, β and γ lobes (uas-DScam17-2::GFP; c316(GAL4)) retain shock-reinforced memory function (Figure 5.4B). Memory of uas-DScam17-2::GFP; c316(GAL4) flies was similar to memory of uas-DScam17-2::GFP (p>0.70), and wild-type controls (p>0.50) and was significantly higher than that of *amn*\textsuperscript{X8} flies (p<0.0001). Therefore DPM neuron projections to the α, β and γ lobes of the mushroom bodies are dispensable for aversive odor memory. DScam17-2::GFP; c316(GAL4) flies were next tested in the sucrose-reinforced odor memory paradigm. Again, reward memory of DScam17-2::GFP; c316(GAL4) flies was similar to memory of uas-DScam17-2::GFP (p>0.95), and wild type (p>0.27) control flies and was significantly better than that of *amn*\textsuperscript{X8} flies (p<0.003) (Figure 5.4A). These data indicate that the DPM neuron projections to the α, β and γ lobes of the mushroom bodies are also dispensable for sucrose-reinforced odor memory. Furthermore, these results imply that the circuit requirements for the stability of sucrose-reinforced and shock-reinforced odor memory are very similar, and suggest the stabilizing aspects of both types of odor memory could reside in the mushroom body α´/β´ lobes. An alternative possibility is that a transmitter released from DPM neurons, likely AMN, acts as a neurohormone. Because of neuropeptide diffusion, DPM
projections onto specific lobe subsets may be dispensable. While this cannot be ruled out, it seems unlikely because of the elaborate innervation pattern of DPM neurons.

**Output from the mushroom body $\alpha'/\beta'$ is essential for memory**

To investigate the potential role of the $\alpha'/\beta'$ neurons we first searched the FlyTrap collection of enhancer-traps (Douglas Armstrong, University of Bristol, [www.flytrap.org](http://www.flytrap.org)) for P{GAL4} insertions that appeared to drive reporter gene expression selectively in the $\alpha'/\beta'$ neurons of the mushroom bodies. The expression pattern of these lines was verified by crossing flies harboring a {GAL4} insertion to flies harboring the GAL4-responsive promoter driven membrane bound GFP, uas-cd8::GFP or uas-lacZ. Brains were stained with the $\alpha/\beta/\gamma$ marker anti-FASII. Enhancer-traps that selectively drive expression in the mushroom prime lobes exhibited Kenyon cell expression (clearly distinguishable by stereotypical lobe projections) but no colocalization between reporter gene and anti-FASII signal. This analysis provided four drivers, c305a{GAL4}, c68A{GAL4}, c320{GAL4}, c728{GAL4} that selectively express in the mushroom body prime lobes, but not other lobes of the mushroom bodies. It is important to note that each of these drivers express in regions of the brain outside of the mushroom bodies, while Kenyon cell expression is relatively specific to the $\alpha'/\beta'$ lobes.
In the two lines that were extensively characterized behaviorally, c320\{GAL4\} and c305a\{GAL4\}, GAL4 drivers were combined with uas-
\text{cd8::GFP} and brains were also stained with anti-TRIO, to confirm
selective expression in the \(\alpha'/\beta'\) lobes (Figure 5.5). Both drivers resulted
in reporter-gene expression that was largely confined to the mushroom
body \(\alpha'/\beta'\) neurons of the mushroom bodies as well as various other brain
regions. These data indicate that the common region of expression in
these enhancer-trap lines is the mushroom body \(\alpha'/\beta'\) neurons.

The c320, c305, c68a, and c728 GAL4 lines were used to examine
the role of the mushroom body \(\alpha'/\beta'\) neurons in shock-reinforced olfactory
memory. The role of the \(\alpha/\beta\) lobes were also examined with the driver
c739\{GAL4\} (Figure 5.5C). It has previously been reported that output
from the \(\alpha/\beta\) lobes is necessary for memory retrieval, and dispensable for
stability and acquisition (Dubnau et al., 2001; McGuire et al., 2001), and
therefore, this driver was used as a control. Flies were taught to associate
an odor conditioned stimulus (CS) with a punitive electric shock
unconditioned stimulus (US) using the standard protocol (Tully and Quinn,
1985). The role of mushroom body \(\alpha'/\beta'\) neurons in memory was first
examined by temporally blocking their output throughout the entire
olfactory conditioning experiment (Figure 5.6). The dominant negative
temperature-sensitive \textit{shibire}^{ts1} transgene (Kitamoto, 2001) was
expressed in mushroom body \(\alpha'/\beta'\) neurons and memory experiments
were performed at either the permissive (25˚C) or the restrictive
temperature (31˚C). At the restrictive temperature, shibire^{ts1} blocks
vesicle recycling and thereby blocks synaptic vesicle release. At 25˚C
odor memory of flies with c320(GAL4), c305a(GAL4), and c739(GAL4)
flies driving uas-shi^{ts1} flies was comparable to memory of wild-type and
uas-shi^{ts1} flies (p>0.5). Flies with c68a or c728 driving uas-shi^{ts1} appeared
sick and exhibited severe memory defects at the permissive temperature
(data not shown) and therefore were not used in any subsequent
experiments. Next, output from subsets of the mushroom bodies was
blocked by incubating flies at 31˚C for 15 minutes prior to training and
maintaining them at this temperature through testing (Figure 5.6B).
Memory of c320(GAL4); uas-shi^{ts1} c305a(GAL4) uas-shi^{ts1} as well as
c739(GAL4) uas-shi^{ts1} flies was severely reduced and was statistically
different from wild-type(p<0.001) (p<0.01) (p<0.001) and uas-shi^{ts1} flies
(p<0.001) (p<0.001) (p<0.001). Therefore, mushroom body output from
α’/β’ and α/β neuron is necessary for odor memory.

**Mushroom body α’/β’ lobes are involved in memory stability**

Two previous reports concluded that mushroom body output was
dispensable during memory storage but was required during memory
retrieval (Dubnau et al., 2001; McGuire et al., 2001). These studies
primarily relied on the c747 and c739 GAL4 drivers, which mostly drive
transgene expression in α/β and γ mushroom body neurons. Because the
role of output from the mushroom body prime lobes had yet to be examined, I wondered if mushroom body output from the $\alpha'/\beta'$ neurons were required during memory acquisition, consolidation or retrieval. Again, output from the mushroom body $\alpha'/\beta'$ neurons was blocked through transgenic expression of with uas-$shl^{ts1}$ but this time we restricted the inactivation to either the training, or testing periods. Blocking mushroom body $\alpha'/\beta'$ neuron output during acquisition produced memory defects (Figure 5.7A). c320{GAL4}; uas-$shl^{ts1}$ and c305a{GAL4} uas-$shl^{ts1}$ performed worse than wild-type ($p<0.001$) ($p<0.01$) and uas-$shl^{ts1}$ flies ($p<0.002$) ($p<0.02$). In agreement with a previously published report (McGuire et al., 2001), blocking $\alpha/\beta$ lobe output did not impair 3 hour memory. c739{GAL4};uas-$shl^{ts1}$ flies displayed memory comparable to that of wild-type ($p>0.99$) and uas-$shl^{ts1}$ controls ($p>0.96$). Therefore, unlike mushroom body $\alpha/\beta$ and $\gamma$ neurons, output from mushroom body $\alpha'/\beta'$ neurons is required for acquisition.

To examine the role of $\alpha'/\beta'$ neurons in memory retrieval, flies were tested for 3 hour memory and incubated at the non-permissive temperature of $31^\circ$C for 15 minutes prior to training. In contrast to acquisition, these experiments revealed that mushroom body $\alpha'/\beta'$ neuron output is dispensable for memory retrieval. Performance of c320{GAL4}; uas-$shl^{ts1}$ and c305a{GAL4}; uas-$shl^{ts1}$ flies did not differ from wild-type ($p>0.78$) ($p>0.74$) and uas-$shl^{ts1}$ flies ($p>0.39$) ($p>0.34$). In
agreement with previously published results (Dubnau et al., 2001; McGuire et al., 2001) blocking output from the mushroom body $\alpha/\beta$ lobes abolished memory. $c739(GAL4); uas-shh^{ts1}$ performed worse than wild-type ($p<0.002$) and $uas-shh^{ts1}$ controls ($p<0.001$). Taken together, these results suggest dynamic, lobe-specific involvement in memory. While neurotransmission from the $\alpha'/\beta'$ is required for the formation of a memory, $\alpha/\beta$ output is required for retrieval.

To test whether mushroom body $\alpha'/\beta'$ neuron output is required during memory storage, flies were trained at the permissive temperature and immediately after incubated for 60 minutes at $31^\circ C$, to block output in $shh^{ts1}$ expressing neurons. Flies were returned to the permissive temperature and memory was assayed two hours later. These experiments also included $c739(GAL4); uas-shh^{ts1}$ flies to confirm previous reports that mushroom body $\alpha/\beta$ neuron output was not required during memory storage. Strikingly this manipulation severely impaired memory if $\alpha'/\beta'$ neuron output was blocked but did not affect performance if mushroom body $\alpha/\beta$ neurons were blocked (Figure 5.8). $c320(GAL4); uas-shh^{ts1}$ and $c305a(GAL4); uas-shh^{ts1}$ flies displayed memory defects compared to wild-type ($p<0.001$) ($p<0.001$) and $uas-shh^{ts1}$ controls ($p<0.001$) ($P<0.001$). $c739(GAL4); uas-shh^{ts1}$ displayed memory comparable to that of wild-type ($p>0.62$) and $uas-shh^{ts1}$ ($p>0.85$) controls. The lack of effect with a mushroom body $\alpha/\beta$ lobe driver is consistent with
a previous report (McGuire et al., 2001). These data suggest that output from the α´/β´ mushroom body neurons is required for memory stability, and for the first time indicate that mushroom body output is required between training and testing. Furthermore these data are consistent with the notion that mushroom body neuron subgroups have different temporal roles in memory processing.

A previous report has suggested that shock-reinforced and sucrose-reinforced memories both rely on the mushroom bodies (Schwaerzel et al., 2003). Furthermore, it is apparent that DPM neurons, which are thought to provide input to the mushroom bodies, are similarly involved in punitive and rewarded memories (Chapters III and IV). Because of this apparent shared circuitry, we wondered if the mushroom body α´/β´ lobes were similarly involved in sucrose-reinforced olfactory memory. At 25°C, flies containing the mushroom body drivers c320{GAL4}, c305a{GAL4} or c739{GAL4} and the uas-shi^{ts1} transgene displayed statistically indistinguishable memory from wild-type and uas-shi^{ts1} flies (p>0.5) (Figure 5.9A). When neural transmission from the mushroom bodies was blocked by incubating flies at 31°C for 60 minutes immediately following training, 3 hour memory of c320{GAL4};uas-shi^{ts1} and c305a{GAL4} uas-shi^{ts1} was severely reduced compared to wild-type (p<0.0001) (p<0.001) and uas-shi^{ts1} (p<0.0001) (p<0.002) (Figure 5.9B). Similar to shock-reinforced memory, blocking α/β output during
consolidation did not impair sucrose-reinforced memory. Memory of c739\{GAL4\};uas-shi\textsuperscript{ts1} was equivalent to wild-type (p>0.96) and uas-shi\textsuperscript{ts1} flies (p>0.99). Therefore, \(\alpha'/\beta'\) is also necessary for consolidation of sucrose-reinforced odor memory.

**Requirements of rut function in the mushroom body \(\alpha'/\beta'\) neurons**

Another way to examine the functional role of a subset of neurons is to rescue gene function with tissue specific expression in the background of the mutant. Function of the cAMP cascade is thought to be essential for synaptic plasticity in memory formation and maintenance, and consequently for behavioral plasticity (Kandel and Mayford, 1999). Therefore, tissue specific rescue of \(\textit{rut}\) cDNA in the background of a \(\textit{rut}\) null fly is thought to restore synaptic plasticity only in the neurons that express the transgene. Many studies have performed such manipulations and determined that \(\textit{rut}\) expression in the mushroom bodies is sufficient for memory (Mao et al., 2004; McGuire et al., 2004b; Zars et al., 2000a; Zars et al., 2000b).

To determine whether synaptic plasticity in the mushroom body prime lobes is sufficient for memory, \(\textit{rut}\) cDNA was selectively expressed in the prime lobes of the \(\textit{rut}\textsuperscript{2080}\) null mutant (Levin et al., 1992). Either c305a\{GAL4\} or c320\{GAL4\} were used to drive prime lobe expression of \(\textit{rut}\) cDNA and flies were tested for 3 hour memory (Figure 5.10A). c320\{GAL4\} driven expression of uas-\(\textit{rut}\) rescued the 3 hour memory
defect of rut mutant flies. $rut^{2080}\text{uas-rut;c320}\{\text{GAL4}\}$ flies displayed memory comparable to wild-type ($P>0.31$), and performed better than $rut^{2080}\text{uas-rut}$ control flies ($P<0.05$). However, $c305a\{\text{GAL4}\};rut^{2080}\text{uas-rut}$ flies were indistinguishable from $rut^{2080}\text{uas-rut}$ flies ($P>0.94$), indicating that not all prime lobe drivers are able to rescue the rut memory defect.

Because these two prime lobe drivers provide different results, these data are difficult to interpret. It is possible that the two mushroom body drivers express in different subsets of prime lobe neurons. Alternatively, we did find that with a cd8::GFP reporter (but not lacZ) $c320\{\text{GAL4}\}$ drives low levels of reporter gene expression in the $\alpha/\beta$ lobes, and expression in these neurons may be responsible for the rescue of the rut phenotype. We also tested these genotypes for learning (3-minute memory) (Figure 5.10B). Both $c305a\{\text{GAL4}\};rut^{2080}\text{uas-rut}$ and $c320a\{\text{GAL4}\};rut^{2080}\text{uas-rut}$ flies performed worse than wild-type indicating that rut expression in 305a and c320{GAL4} expressing neurons is not sufficient for short-term memory. These results suggest that different neural circuits and mushroom body subsets are required for immediate and intermediate memory. Future behavioral analysis using additional lobe specific drivers should further our understanding of the neuroanatomical requirements of cAMP-mediated plasticity in odor memory.
Taken together, the results presented in this chapter indicate that DPM projections onto the $\alpha'/\beta'$ lobes are sufficient for memory, and that output from the $\alpha'/\beta'$ neurons of the mushroom bodies is critical for memory acquisition and stability. Considering the well-documented role of *amn* in DPM neurons, these results suggest a model where AMN peptide is released onto the lobes of the mushroom bodies, and acts to stimulate the cAMP cascade and synaptic plasticity in the mushroom body $\alpha/\beta$ neurons.

**Discussion**

**Effects of DScam17-2 expression on DPM function**

The results presented here indicate that driving DScam17-2::GFP with the DPM driver c316{GAL4} specifically disrupts projections to the $\alpha$, $\beta$, and $\gamma$ lobes, while leaving projections to the prime lobes intact. It is unclear as to why overexpression of the DScam17-2 variant would disrupt a subset of DPM projections but not others, and we have been unable to identify a specific branching defect. Developmental study of the *Drosophila* embryo has revealed many genetic mutants with specific branching defects (Reviewed in (Huber et al., 2003), and more recently the male-specific variant of *fruitless*, FRU$^M$, has been implicated in sexually dimorphic branching of *fru* expresing interneurons (Kimura et al., 2005).
Ectopic expression of DScam has been shown to alter neural projections and pre-mitotic ectopic expression of DScam17-2::GFP causes developmental defects (Wang et al., 2004a; Zhu et al., 2006). Furthermore, DScam mutants display increased neural branching, indicating that perhaps DScam plays a role in suppression of neurite outgrowth and branching (Wang et al., 2002). Therefore, ectopic expression of DScam may act to suppress axonal branching. Identifying the specific branch defect will be critical in understanding the morphological disruption caused by DScam17-2 expression.

The lack of a memory defect in flies expressing DScam17-2::GFP in DPM neurons indicates that only a subset of DPM projections are necessary for normal memory. It remains unclear as to whether projections onto the α, β, and γ lobes alone would also be sufficient for memory. Perhaps, exclusive innervation of the α/β lobes alone is also sufficient for memory. However, it is noteworthy that the prime lobes appear to differ in function from the α/β lobes. While output from α/β neurons appears to be exclusively required for retrieval, output from the prime lobes appears to be important during the acquisition and consolidation phases of memory. Therefore it is possible that memories are initially formed in the prime lobes, and later transferred to the α/β lobes. While the neural mechanism of a dynamic shift in lobe
dependency is unclear, genetic inactivation of specific lobe subsets supports this model.

An alternative explanation to the observed defect in DPM neurons with DScam17-2::GFP is that a small number of Kenyon cells that these drivers label has been disrupted while DPM neurons remain intact. This interpretation would indicate that in the wild-type fly, DPM neurons project primarily to the prime lobes. While this is a drastically different interpretation of the data presented in this chapter, the functional implications remain the same. In effect, this result would mean that the Kenyon cells labeled by c316(GAL4) are not required for memory and that the connections between the DPM projections to the prime lobes and mushroom bodies are important for memory.

**DPM-mushroom body α´/β´ neuron interactions in memory stability**

Imaging from DPM neurons with markers of neural activity has revealed an odor-specific conditioned response in DPM neurons that is dependent on cell autonomous expression of amn (Yu et al., 2005). If DPM neurons are driven by the mushroom bodies, then imaging from DPM neurons may report synaptic strength changes in Kenyon cells neurons. It is possible that DPM neuron feedback onto the relevant Kenyon cells is essential for the development of persistent synaptic change in these Kenyon cells. This model posits that DPM neurons are receptive to Kenyon cells and that is why they show responses to all
odors tested as well as shock. If it is assumed that Kenyon cells carry ‘channel-specific’ odor information, then conditioning the animal with one odor and shock will specifically enhance activity in the odor responsive channels. On future odor challenge, only these channels will exhibit increased activity and will provide enhanced drive to DPM neurons. I think this model is the most parsimonious explanation for the development of odor-specific conditioned responses in DPM neurons.

How do DPM neurons contribute? It is possible that DPM neurons are also transmissive to the mushroom bodies and the behavioral experiments presented here strongly suggest they provide essential memory stabilizing input to mushroom bodies. Since DPM neuron activity is required in a prolonged manner for memory stability and the conditioned response develops after 30 minutes, there is an obvious requirement for ongoing neural activity between the mushroom bodies and DPM neurons during memory storage. This is not altogether supported by published data. Two studies have reported that blocking mushroom body output during storage does not compromise memory (Dubnau et al., 2001; McGuire et al., 2001). However, we believe these studies did not block all of, or the relevant parts of, the mushroom body neural ensemble.

Until this study, mushroom body output was believed to only be required for retrieval of olfactory memory (Davis, 2005) implying that
ongoing circuit activity involving the mushroom body was dispensable for memory stability. Our data presented here strongly suggest that mushroom body output after training is critical to consolidate memory from a labile to a more stable state. Moreover, output specifically from the Kenyon cell $\alpha'/\beta'$ neurons is required for memory stabilization but output from $\alpha/\beta$ neurons (at least those covered by the c739(GAL4) driver) is apparently dispensable, consistent with a previous report (McGuire et al., 2001).

**Role of mushroom body rut function in memory formation**

Preliminary experiments rescuing cAMP signaling with the c320(GAL4) driver was sufficient to rescue memory, but not learning. These results support a model whereby AMN binds to a currently unidentified receptor in the mushroom bodies lobes and activates the cAMP cascade. However, it remains unclear as to why only one prime lobe driver was able to rescue the memory phenotype. Further work will be needed to confirm that the effect of the c320(GAL4) driver is due to expression in Kenyon cells, rather than neurons extrinsic to the mushroom bodies.

Stable aversive and reward odor memory also requires prolonged DPM neuron output between training and testing and DPM output is dispensable during training and retrieval (Figures 3.3, 3.4, 3.5, and 4.4). DPM neurons ramify throughout the mushroom body lobes and evidence
presented here suggests they provide a general stabilizing mechanism for mushroom body-dependent memory. The discovery of an essential role for mushroom body prime lobe neuron output during the first hour after training is consistent with previous findings on the temporal requirements for DPM neurons. I therefore propose that DPM neuron connections to the mushroom body prime lobes may be particularly crucial for memory stability. Indeed, DPM neurons that project mostly to mushroom body prime lobes retain capacity to consolidate both aversive and rewarded odor memory. The data presented here suggest that prime lobe output is required for consolidation and acquisition of middle-term memory. Interestingly, recent live imaging studies suggest that mushroom body prime lobes exhibit a “memory trace” that develops after training and persists for a few hours (Y. Wang and Y. Zhong, personal communication). The data presented here are consistent with the hypothesis that this mushroom body prime lobe memory trace is required to guide middle-term memory behavior. It is possible that memory traces in specific mushroom body subsets persist or develop with different temporal profiles and represent different phases of memory. 

In these studies, I found that expressing rut cDNA under control of c320\{GAL4\} rescues 3-hour memory, but not learning (Figure 5.10). These data support the tantalizing possibility that cAMP mediated synaptic plasticity in the prime lobes is essential and sufficient for middle-
term memory, but dispensable for short-term memory. Because DPM neurons are also required for middle-term memory, these results suggest that perhaps amn acts to activate cAMP signaling in the mushroom bodies during memory formation. This notion is supported by the apparent importance of DPM-prime lobe interactions. However, these results should be interpreted with caution, and further study is needed to confirm a role for rut signaling in the mushroom body prime lobes, and to show phase-specific involvement of the prime lobes in memory formation.

Two previous studies specifically examined the ability of rut expression in specific subsets of mushroom body neurons to rescue learning. One such study suggests that expression of rut specifically in the γ lobes of the mushroom bodies is sufficient for memory (Zars et al., 2000a), however, another group has failed to replicate these results (McGuire et al., 2004b). It is entirely possible that rut is required in different neural structures, during different memory phases. For example, perhaps RUT function is required in the γ lobes for short-term memory, but dispensable in this region for middle-term memory, while the reverse is true for the prime lobes. Such experiments will be greatly aided by loss-of-function experiments. For example, RNAi targeted to rut could be driven in the mushroom body prime lobes. Alternatively, the rut mutant could be rescued with a pan-neuronal or pan-mushroom body driver in the presence of a prime lobe-GAL80 transgene. This would result in wild-
type function in all neurons or mushroom body lobes except the prime lobes.

It is important to note that only one of the two prime lobe drivers tested rescued middle-term memory. There are a few possible explanations for the discrepancies between these drivers. One possibility is that the prime lobe drivers express in different numbers or different subsets of neurons. The c320{GAL4} driver does appear to express weakly in a small number of α/β lobe neurons when mcd8::GFP is used as a reporter. Expression in the α/β lobes is not observed with uas-lacZ and the reason for this discrepancy is unclear. Another possibility is the rescue observed with c320{GAL4} is due to expression of the rut transgene in regions outside of the mushroom bodies. This driver does drive expression in a number of central complex neurons. Future studies using GAL4-drivers that cleanly label the prime lobes will be necessary for localizing rut-dependent plasticity these structures.

Portions of this chapter have been published:

CHAPTER VI: CONCLUSIONS

A main goal in the study of the brain is elucidating the mechanisms of behavioral plasticity. Uncovering the cellular basis of memory requires an understanding of neural circuitry involved in memory and the molecules that function within this circuitry. Here I have functionally dissected specific neural subsets in the fly brain to map the neural circuitry involved in memory stability. This work, taken with the work of others, has allowed us to develop a model where DPM neurons form a positive feedback loop with mushroom body α′/β´ neurons. Persistent output from both these structures is required for memory stabilization.

Mechanisms of AMN function

The interpretation of AMN acting as a neuropeptide should be handled with caution. AMN peptide exhibits weak similarities to mammalian neuropeptides of the VIP/glucagon/growth hormone-releasing hormone (GHRH)/secretin superfamily, and PACAP in particular (Figure 2.8 Feany and Quinn, 1995; Hashimoto et al., 2002). While PACAP has been reported to act as a bioactive molecule in Drosophila (Mertens et al., 2005; Zhong and Pena, 1995), it is unable to functionally substitute for amn in DPM neurons (Figure 2.10). Localizing AMN peptide to the DPM presynaptic region would support the notion that AMN functions as a
neuropeptide. Currently the only report of AMN localization used an antibody targeted to the AMN C-terminus that only stains the cell bodies (Waddell et al., 2000). It is possible that the C-terminus is not part of the AMN active fragment, and therefore it is not transported to the synapse. Further immunohistochemical study of AMN may help to support a role for AMN as a neuropeptide.

DPM neurons co-express amn and a cholinergic neuron-specific marker suggesting that ACh is an AMN cotransmitter (Figures 2.3 and 2.4). Consistent with this idea, expression of ACh{RNAi} in DPM neurons with the c316{GAL4} driver impairs memory (Figure 2.6). Therefore, DPM neurons may functionally depend on ACh as a transmitter. However, further study is needed, both on the putative role of ACh release from DPM neurons, and its potential neural targets. Nicotinic AChRs are expressed throughout the horizontal lobes of the mushroom bodies (Schuster et al., 1993), raising the possibility that these neurons may be modulated by mushroom-body associated neurons. ACh is released onto the mushroom body dendrites by the projection neurons terminals in the calyces and could be released onto the mushroom body lobes by DPM neurons as well. Therefore nicotinic and muscarinic-type ACh receptors, as well as receptors for the putative AMN peptides, may contribute to DPM-dependent plasticity. However, the AMN receptor has not been identified to date, and the precise localization of AChRs remains unclear.
Temporal Requirements of DPMs and BA memory

Proper DPM function and stable memory requires *amn* expression in DPM neurons (Waddell et al., 2000; Tamura et al., 2003; and presented here). In this dissertation I have also shown that *amn* function is necessary for learning with the odor BA. These results also indicated that BA is sensed by a non-canonical pathway, and memory for this odor apparently requires different DPM activity. It is likely that this requirement for *amn* and DPM neurons in learning is specific to the odor BA, or possibly a small handful of odors, and the remainder of this discussion will focus on memory for odors sensed through the canonical pathway.

DPM neurons and phase specificity

DPM neurons are essential for middle-term memory, but dispensable for short-term memory. Both these types of memory are apparently protein synthesis independent. Induction of long-term memory (LTM) requires multiple, spaced training trials and the study of this memory phase has lagged behind other memory phases due to technical difficulties associated with conditioning flies using a spaced training protocol. Therefore, the circuitry of LTM is still unclear. However, it is known that the transcription factor cAMP Response Element Binding Protein (CREB) is essential for LTM (Perazzona et al., 2004; Yin et al., 1994). It is tempting to speculate that persistent DPM activity could result in persistently elevated levels of cAMP and consequently CREB
activation. Furthermore, *amn* mutant flies apparently have a long-term memory defect (Data cited in Tully et al., 1994), suggesting that AMN function in DPM neurons could be an essential component of LTM. Future studies repeating many of the same *amn* rescue and DPM inactivation experiments discussed here, but with a spaced training protocol, may provide important insight into the neural circuitry of LTM.

**DPM neurons and memory stability**

The experimental evidence presented in this dissertation represent a near exhaustive study of the temporal requirements for DPM neuron output in 3 hour memory. It is apparent that DPM neurotransmission is required between training and testing for odor memory formation. These results indicate that the transmitter(s) released from these neurons (likely AMN and/or ACh) are essential for memory. The study of the signaling mechanisms of these transmitters and the neurons that they modulate should provide important insight into the mechanisms by which memory is stabilized. Study of ACh receptors localization is not likely to advance our neuroanatomical understanding of memory, because ACh neurons are pervasive and are functionally pleiotropic (Gorczyca and Hall, 1987; Kitamoto, 2001). However, it is possible that identification of an AMN receptor will be a treasure trove for understanding the neural circuits involved in memory stability. Recently developed tools such as a genome-wide RNAi library (Dietzl and Dickson, unpublished results) and cell
culture-CRE reporter systems (Mertens et al., 2005) should aid in assigning a receptor to AMN. However, it is important to note that often times pleiotropy or promoter constraints lead to broad expression and therefore the expression of a gene may tell little about the neural circuitry in which it acts (Hall, 1994). Such was the case with the much-heralded identification of the PDF receptor. PDF, an intensely studied neuropeptide involved in circadian rhythms is expressed in discrete subsets of CNS neurons. However, the identification of the receptor does not appear to have provided great insights into the circuitry underlying circadian rhythms (Hyun et al., 2005; Mertens et al., 2005)

DPM neurons are not the only example of single neurons essential for memory. In the honeybee, elegant electrophysiological studies from the single octopaminergic neuron termed ventral-unpaired medial (VUMmx1) neuron during sucrose-reinforced odor memory have been particularly revealing. The VUMmx1 neuron ramifies throughout the mushroom body lobes and respond to the US sucrose. Both DPM neurons and VUMmx1 neurons display an odor-specific conditioned response following the odor-reinforcer pairing (Hammer, 1993; Yu et al., 2005). The Pe1 neurons in the honeybee also innervate the mushroom bodies, are activated by odors, and exhibit an altered odor-specific response following the pairing of odor with a sucrose reinforcer (Mauelshagen, 1993; Rybak and Menzel, 1998). These physiological
similarities between mushroom body-associated neurons, despite differences in morphology and signaling peptides, raise the possibility of a conserved mechanism of neural-network plasticity.

In the sea slug, Aplysia californica, serotonin release from modulatory interneurons has been shown to be essential for habituation of the siphon withdrawal reflex (Klein and Kandel, 1978; Pinsker et al., 1973). Similarly, activation of the B51 neuron in Aplysia is essential and sufficient for feeding reflex plasticity (Brembs et al., 2002; Lorenzetti et al., 2006). Perhaps closer to DPM function, elegant work in the pond snail Lymnaea has shown that a single neuron, the RPeD1, is essential for memory consolidation (Sangha et al., 2003; Scheibenstock et al., 2002). However, unlike DPM neurons, RPeD1 neurons are specifically involved in the formation of long-term, protein synthesis dependent memory, and are dispensable for middle-term memory. Nevertheless, it seems that DPM neurons remain a flagship example of neurons involved in memory stability and provide an excellent example of how Drosophila can be utilized to map neural circuits.

**DPM neuron-mushroom body connectivity**

Functional imaging from DPM neurons has provided insight into the environmental cues that can drive these neurons. These studies have revealed that DPM neurons respond to odor and shock, and that a conditioned response is observed in these neurons following the pairing
of these two stimuli (Yu et al., 2005). Our interpretation of these data is that imaging from DPM neurons reports synaptic strength changes in Kenyon cells, and that DPM neuron feedback onto the relevant Kenyon cells is essential for the development of persistent synaptic change in these Kenyon cell (Figure 6.1). We believe that DPM neurons are receptive to Kenyon cells and that is why they show responses to all odors tested as well as shock. If it is assumed that Kenyon cells carry ‘channel-specific’ odor information, then conditioning the animal with one odor and shock will specifically enhance activity in the odor responsive channels. On future odor challenge, only these channels will exhibit increased activity and will provide enhanced drive to DPM neurons. This model seems to be the most parsimonious explanation for the development of odor-specific conditioned responses in DPM neurons (Figure 6.2). In accordance with this model, DPM neurons are also transmissive to mushroom bodies and our behavioral experiments strongly suggest they provide essential memory-stabilizing input to mushroom bodies. Since DPM neuron activity is required in a prolonged manner for memory stability and a conditioned response develops after 30 minutes, there is an obvious requirement for ongoing neural activity between the mushroom bodies and DPM neurons during memory storage.
To investigate DPM-mushroom body interactions, we blocked output from the mushroom body prime lobes during the same time window in which DPM output is essential for memory. These experiments revealed that mushroom body $\alpha'/\beta'$ neurons, but not $\alpha/\beta$ neurons, are essential for stability of shock and sucrose-reinforced memories (Figures 5.8 and 5.9). These experiments provide the first evidence that mushroom body output between training and testing is essential for memory. Taken together with our finding that DPM projections onto the mushroom body $\alpha'/\beta'$ lobes are sufficient for memory (Figure 5.4), we propose that DPM-mushroom body $\alpha'/\beta'$ interactions are critical for memory stability.

Because second order projection neurons do not make contact with DPM neurons, while third order Kenyon cells do, a plausible interpretation is that the mushroom bodies drive DPM neurons. This model is supported by evidence that olfactory representation in the mushroom bodies changes following the pairing of shock and odor (Zhong, Y and Davis, RL personal communications). Because it is also believed that DPM neurons modulate Kenyon cell activity through AMN/ACh release, it is not a giant leap to suggest that DPM neurons both receive and provide information to the mushroom bodies. This is supported by tenuous morphological evidence provided by markers of cell polarity, indicating that DPM neurons project to both dendrites and axons.
into the mushroom body lobes (Tamura et al., 2003 and Figure 5.1). According to this model, blocking output from mushroom body $\alpha'/\beta'$ neurons should abolish the DPM neuron response to odor. This experiment can be performed through physiological imaging of DPM neurons in mushroom body-inactivated flies, and this experiment will be a critical test of the model proposed here.

We have demonstrated that DPM neurons are similarly involved in sucrose-reinforced odor memory and shock-reinforced memory. It is tempting to speculate that the olfactory response of DPM neurons is altered following the pairing of sucrose and odor, just as it is following the pairing of shock and odor. To date, it remains unclear if DPM neurons are activated by sucrose sensation. The similar behavioral requirements for DPM neuron function with shock and sucrose reinforced olfactory memory suggest that DPM neurons likely respond similarly to sucrose reward as they do to electric shock punishment. Much is known of the circuitry underlying sucrose sensation, while shock sensation remains nebulous. Even though a sucrose receptor has not been identified, sucrose activates neurons expressing the gene $GR5a$, and these neurons project to various regions of the subesophageal ganglion in a somatotopic fashion (Scott et al). Because the circuitry mediating gustatory sensation is much better understood than shock sensation, gustatory-mediated memory may prove a fruitful avenue for the circuit mapping of memory.
We have demonstrated that DPM neurons are not specific to shock-reinforced memory and are generally required for memory stability. While OA and DA neurons appear to be differentially involved in reward and punishment reinforced memory (Schwarzel et al., 2003), DPM neuron output during the period between training and testing is critical for stability of both sugar and shock memories. These results suggest that DPM neurons may be involved in stabilizing all mushroom body-dependent memory. Indeed, *amn* flies are deficient in visual memory (Gong et al., 1998) and conditioned courtship suppression (Ackerman and Siegel, 1986; Ejima et al., 2005). Further examination of DPM neuron requirements in contextual visual memory and courtship conditioning will provide an opportunity to add further support to the notion that DPM neurons act as general stabilizers of mushroom body-dependent memory. Study of DPM involvement in courtship conditioning may prove particularly informative. Conditioned suppression of male courtship at time-points associated with DPM-dependent olfactory memory requires the mushroom bodies (McBride et al., 1999). Courtship memory can be modulated by multiple sensory cues including hydrocarbon profile, visual, and olfactory cues. Depending on the sensory stimuli involved, different complexities of memories can be formed (Ejima et al., 2005). Determining the role of DPM neurons in various courtship conditioning
paradigms may tell us if DPM neurons are critical for stability of many types of mushroom body-dependent memory.

Current evidence suggests that the mushroom bodies are a key site of olfactory memory processing. Mushroom bodies receive CS olfactory input from cholinergic olfactory PNs and US input via modulatory monoaminergic neurons. Coincident activity likely triggers cAMP synthesis and synaptic plasticity in the relevant CS pathway neurons. Ongoing activity in these Kenyon cells neurons after training in turn drives DPM neurons that feedback onto the mushroom bodies and stabilize the memory traces in relevant mushroom body neurons. Inactivation of specific subsets of mushroom body neurons, suggest mushroom body lobes may be differentially involved in memory. Like DPM neurons, the $\alpha'/\beta'$ lobes are required for memory stability and dispensable for retrieval (Figures 5.6, 5.7, and 5.8). We interpret these results as suggesting DPM neurons are driven by the $\alpha'/\beta'$ neurons. Neurotransmission from the mushroom body $\alpha/\beta$ neurons, on the other hand, are dispensable during acquisition and consolidation but required for retrieval (McGuire et al., 2001; Figures 5.5, 5.7, and 5.8). These results raise the tantalizing possibility that persistent activity of a DPM-mushroom body $\alpha'/\beta'$ feedback loop results in stabilized plasticity of the mushroom body $\alpha/\beta$ neurons.
I expect there will be further complexity added to this very simple model. For example, the exact relationship between DPM neurons and monoaminergic neurons remains unclear. It is likely that monoaminergic neurons specifically strengthen subsets of Kenyon cell synapses, because DA and OA are differentially required for shock and sugar reinforced olfactory memories. Furthermore, a conditioned memory trace in Dopaminergic projections onto the mushroom bodies indicates that plasticity in these neurons occurs following training (Riemensperger et al., 2005). However, it remains unclear as to how these neurons interact with DPM neurons. It is unlikely that there is a direct interaction because output from DA neurons is essential during training, while DPM output is dispensable during this period (Schwaerzel et al., 2003; Figures 3.3, 3.4, 3.5, and 4.4). This suggests that DA output is involved in initial formation of the memory, while DPM neuron output acts to stabilize it.

The fruit fly likely possess other mushroom body-associated neurons that are important for memory. Many mushroom body-associated neurons were reportedly identified (Chiang et al., 2004; Ito et al., 1998), yet these studies did not look beyond morphology. Study of mushroom body-associated neurons in the honey bee and cricket have provided multiple examples of mushroom body-associated neurons that may be functionally involved in memory. Further study of such neurons
in the fly will likely add to our knowledge of the mushroom body inputs involved in memory.

**DPM Polarity**

Identification of the DPM inputs and outputs will be critical in understanding mechanisms of memory stability. Subcellular markers of cell polarity have provided ambiguous results, and may affect DPM morphology (Figures 5.1, 5.2, and 5.3). A more forceful way to address this question would be to examine the localization of active zones and dense core vesicles in DPM projections onto the mushroom body lobes. Electron microscopy has been used to examine the substructure of neurons within the fly brain (Watts et al., 2004; Yasuyama et al., 2002). Such technological feats have been accomplished in the honeybee by dye-filling neurons and performing electron microscopy (Rybak and Menzel, 1998). A recent study utilizing single-cell recordings in central neurons of the fly brain (Gu and O'Dowd, 2006) suggests that dye-fill/electron microscopy analysis may be an effective means of structural analysis in the fly.

**Relating DPM neuron function to mammalian memory**

In the mammalian brain, hippocampus-dependent memory becomes hippocampus-independent once it is consolidated. This transient involvement of the hippocampus has led to the idea that
consolidation of memory results in the transfer of memory from the hippocampal circuits to the cortex. An alternate view is that aspects of the memory are always in the cortex but they are dependent on the hippocampus because recurrent activity from cortex to hippocampus to cortex is required for consolidation. Hence, disrupting hippocampal activity during consolidation leads to memory loss. In fruit flies, DPM neurons are transiently required to consolidate memory and therefore one might argue that DPM neurons are involved in the storing of memory in the mushroom bodies, in much the same way that the hippocampus is involved in consolidating memories in the mammalian cortex. From a cellular perspective, DPM-mushroom body connectivity appears to be loosely analogous to intra-hippocampal connections. Plasticity in the hippocampus is dependent on two main regions, the CA3, which has excitatory recurrent collaterals, and the CA1 region (Reviewed in Kesner et al., 2004). The CA1 receives external cues both directly, though perforant pathway inputs, and indirectly through CA3 modulation. These inputs, and CA3 induced modulation in particular, induce plasticity of CA1 output. Mushroom body-DPM connections seem to act in a similar fashion. The mushroom bodies, like the CA1 region of the hippocampus, receive environmental cues from projection neurons, as well as modulatory input from DPM neurons. Therefore, both DPM neurons, and neurons in the CA3 appear to be involved in ramping up activity of
neurons that receive memory cues, in this case mushroom body and CA1 neurons. These similarities suggest general circuitry mechanisms involved in memory consolidation may be shared across phyla.

**Locating the engram**

The engram, or the physiological change engendering memory, is thought to reside in the mushroom bodies (Gerber et al., 2004b). The dynamic requirements of the $\alpha'/\beta'$ and $\alpha/\beta$ lobes in memory suggests memories may be compartmentalized within specific mushroom body lobes. DPM neurons with projections that predominantly innervate the $\alpha'$ and $\beta'$ lobes retain function. Taken together with the hypothesized role of AMN peptide in activating the cAMP cascade, these results suggest that AMN may be released from DPM neurons and cause *rut*-dependent plasticity in the mushroom body prime lobes. Rescue of the memory defect in *rut* mutant flies through *rut* expression in c320{GAL4} expressing neurons tentatively supports this model. On the other hand, experiments inactivating different subsets of mushroom body lobes, suggest the $\alpha/\beta$ lobes may be the site of lasting mushroom body plasticity. Output regions in which a memory is stored should be required during retrieval, independently of how the memory is processed prior to recall. Output from the $\alpha'/\beta'$ lobes during retrieval was found to be dispensable for memory, while blocking output from the $\alpha/\beta$ lobes abolished memory. These results suggest the engram may reside in the $\alpha/\beta$ lobes. Future
experiments selectively rescuing *rut* function in specific lobe sets, and perhaps genetic inactivation of neural subsets in combination with Ca\(^{2+}\) imaging should help to resolve this issue.

**Final thoughts**

Identifying the biological mechanism of memory is a central aim of science. Indeed, understanding memory will serve not only to aid in the treatment of many diseases, but also provide valuable insight into what it means to be human. The fruit fly represents an ideal model organism for studying the underlying biological principles of associative memory. Study of olfactory memory in the fly is a particularly attractive model because the mechanisms by which flies sense odors have been studied in depth. In this dissertation I have used tissue-specific transgene expression to identify DPM neurons as an essential component of memory stability. Further study using similar approaches will undoubtedly identify additional components in this process.

Increased application of live imaging techniques to memory research will undoubtedly herald important and unexpected information. Imaging approaches have the obvious benefit of allowing simultaneously recording from large ensembles of neurons and the small fly brain provides the added advantage that one can image almost the entire network at once. Although electrophysiological recording misses the ensemble angle, for some applications like recording from single DPM
neurons, ensemble properties are not so important and the detail
provided by electrical recording may be a distinct advantage. Either way,
functional physiology of the nervous system provides another dimension
that can be brought to bear in complementing our analysis, and it fills the
considerable gap between a dysfunctional gene and behavioral output.

Newly developed genetically-encoded reporters of neural activity
and cell signaling cascades are instantly applicable to *Drosophila* memory
research because it is trivial to express them in defined neural subsets. In
fact, it is now possible to transgenically express one gene (e.g. an optical
reporter) in one set of neurons and a different gene (e.g., an effector) in
another set, in the same fly (Lai and Lee, 2006). This technology permits
us to test functional neural circuit connectivity by stimulating, or switching
off, a set of neurons while imaging activity in other neurons. Furthermore,
new tools allowing for increased specificity in labeling neural subcircuitry
and refining expression patterns of GAL4/UAS mediated expression
provide an ever-increasing precision for functional dissection of behavior.
Clever application of these tools coupled with our large collection of
memory defective mutants should revolutionize our understanding of fly
memory.
REFERENCES


Table 1.
Inactivation of DPM neurons does not impair sensory acuity. To test for sensory acuity, we measured odor and shock avoidance in all strains used in memory experiments. Flies were tested at 25°C, or they were incubated at 31°C for 15 minutes prior to testing and tested at this temperature. Data represents percentage of flies avoiding stimuli, followed by standard error. PD, previously determined in earlier studies (Waddell et al, 2000).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature</th>
<th>OCT Acuity</th>
<th>MCH Acuity</th>
<th>BA Acuity</th>
<th>Shock Avoidance</th>
</tr>
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<tbody>
<tr>
<td>wild-type</td>
<td>25°C</td>
<td>PD</td>
<td>PD</td>
<td>71 ± 6</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>c31f3as-shRm</td>
<td>25°C</td>
<td>PD</td>
<td>PD</td>
<td>81 ± 2</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>Mz717/ues-shRm</td>
<td>25°C</td>
<td>83 ± 3</td>
<td>83 ± 4</td>
<td>81 ± 7</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>ues-shRm</td>
<td>25°C</td>
<td>PD</td>
<td>PD</td>
<td>70 ± 6</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>arrwR</td>
<td>25°C</td>
<td>PD</td>
<td>PD</td>
<td>75 ± 6</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>c31f</td>
<td>25°C</td>
<td>PD</td>
<td>PD</td>
<td>69 ± 9</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>wild-type</td>
<td>31°C</td>
<td>86 ± 3</td>
<td>89 ± 2</td>
<td>91 ± 3</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>c31f3as-shRm</td>
<td>31°C</td>
<td>79 ± 4</td>
<td>89 ± 3</td>
<td>81 ± 4</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Mz717/ues-shRm</td>
<td>31°C</td>
<td>86 ± 6</td>
<td>83 ± 5</td>
<td>89 ± 3</td>
<td>62 ± 2</td>
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<tr>
<td>ues-shRm</td>
<td>31°C</td>
<td>90 ± 6</td>
<td>90 ± 3</td>
<td>85 ± 3</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>arrwR</td>
<td>31°C</td>
<td>69 ± 2</td>
<td>77 ± 7</td>
<td>84 ± 5</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>c31f</td>
<td>31°C</td>
<td>76 ± 11</td>
<td>86 ± 6</td>
<td>85 ± 6</td>
<td>79 ± 3</td>
</tr>
</tbody>
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Figure 1.1  
Schematic depiction of the neuroanatomy involved in olfactory memory. AL: Antennal lobes, AN: Antennal nerve, oACT/iACT: outer/inner antennal nerve, MBc: Mushroom body calyx.
Figure 1.2
DPM projections ramify throughout the mushroom body lobes. Brains of c316;uas-cd8::GFP were imaged whole-mount on a confocal microscope (A) Membrane teathered GFP labels DPM projections. (B) Antibody targeted to FAS-II labels the α, β, and γ lobes of the mushroom bodies. (C) Merge of the two images indicates DPM neuron projections surround all mushroom body lobes. The cell bodies and portions of DPM neurites are not shown in this figure.
Figure 1.3 169y{GAL4} and Mz717{GAL4} drive expression in DPM neurons. (A,B) 169y{GAL4};uas-cd8::GFP brains stained with the mushroom body marker anti-FASII. (A) GAL4 driven GFP is expressed in both DPM neurons that ramify throughout the mushroom body lobes. Two large neurons with cell bodies in the lateral protocerebrum are also labeled. (B) Only one DPM neuron (on the right) is labeled, and two large neurons in the protocerebrum. (C) GFP expression in Mz717{GAL4};uas-cd8::GFP brains. DPM neurons and a small number of Kenyon cells are labeled with this driver.
Figure 2.1
DPM morphology is normal in amn mutants

(A) Morphology of DPM neurons in a wild-type fly brain revealed by driving cd8::GFP with c316{GAL4}. The scale bar is 10 μm. (B) Morphology of DPM neurons in an amn\(^{ex1}\) brain revealed by driving cd8::GFP with c316{GAL4}. (C) Schematic of DPM projections onto mushroom body lobes in wild-type and mutant flies. (D) Mushroom body GAL4 activity in amn\(^{X8}\) mutant flies revealed by crossing amn\(^{X8}\) mutants to flies harboring a uas-cd8::GFP transgene in the absence of a {GAL4} transgene. (E) Schematic of putative peptides produced from the wild-type amn and amn\(^{ex1}\) and amn\(^{ex39}\) mutant loci. amn is predicted to encode a neuropeptide processed into three active peptides, AMN1, AMN2, and AMN3. The remaining amn gene in amn\(^{ex1}\) does not have an in-frame ATG. The remaining amn gene sequence in amn\(^{ex39}\) places an in-frame ATG before a potential 22 amino acid. However, this peptide falls after the putative amidation signal and is not expected to have function.
Figure 2.2
Choline acetyl transferase regulatory region drives expression in cholinergic neurons. (A) cha{GAL4} drives uas-cd8::GFP throughout the brain. (B) The addition of the cha{GAL80} transgene suppresses almost all GFP activity indicating that the cha{GAL80} transgene effectively suppresses GAL4 driven transgenes in cholinergic neurons. (C-E) High magnification, single-slice images of the protocerebrom of a cha{GAL4};uas-cd8::GFP fly brain stained with anti-ChAT. GFP (C) and anti-ChAT (D) label cell bodies. (E) Merge reveals colocalization.
Figure 2.3
Cholinergic GAL80 suppresses GAL4 activity in DPM neurons but not the mushroom bodies. (A) Schematic indicating the genetic mechanism by which cha(GAL80) blocks GAL4 driven reporter gene expression. Whole mount brains were visualized under a confocal microscope. (B) c316{GAL4} selectively drives cd8::GFP reporter gene expression in DPM neurons. (C) Combining the cha(GAL80) transgene with uas-cd8::GFP;c316{GAL4} blocks GAL4 activity in DPM neurons. Remaining labeling is air-sac material.
**Figure 2.4**

Immunostaining with anti-ChAT reveals localization in DPM neurons. (A) Projection of confocal images through the mushroom body lobes in brains of uas-cd8::GFP;c316 flies reveals DPM arborizations. (B) Immunostaining with antibody targeted to ChAT reveals widespread expression. (C) Merge of projections indicate DPM neurons project to cholinergic regions. (D-F) High magnification, single slice image from uas-cd8::GFP, anti-ChAT, and merge respectively. (F) Apparent colocalization between GFP (green) and anti-ChAT (red) reveals that DPM neurons are likely cholinergic. Anti-ChAT signal is not observed in the cell bodies.
Figure 2.5
ACh{RNAi} transgene targets the shared ChAT/VACHT first exon. Both ChAT and VACHT mRNAs share their entire first exon, which comprises of a translated region of ChAT and the 5’ untranslated region of VACHT. The RNAi construct is designed to form an inverted repeat of this common 568bp region.
Figure 2.6
Targeted expression of ACh{RNAi} in DPM neurons impairs 1 hour memory. (A) Wild-type and flies harboring the c316 transgene (black bars), flies harboring ACh{RNAi} tranngene(s) and no driver (grey bars) and flies expressing RNAi transgene(s) in DPM neurons (white bars) were tested for 1 hour memory. Flies expressing two ACh{RNAi} transgenes in DPM neurons performed poorly compared to all other genotypes tested. (B) Coexpression of uas-dcr2 and a single copy of ACh{RNAi} causes memory defects indicating dcr2 increases the efficacy ACh{RNAi}. * denotes p<0.05.
Figure 2.7
cha\{GAL4\} driven expression of \textit{amn} fails to rescue the memory defect of \textit{amn} mutant flies. Flies were tested for 1 hour shock-reinforced olfactory memory. \textit{amn}\textsuperscript{X8};\textit{uas-amn} are memory deficient compared to wild type. \textit{amn}\textsuperscript{X8};\textit{uas-amn};\textit{cha\{GAL4\}} flies express \textit{amn} in cholinergic neurons and are indistinguishable \textit{amn}\textsuperscript{X8};\textit{uas-amn} flies and memory defective compared to controls.
A. Mouse pre-proPACAP

B. Drosophila AMN

Figure 2.8
*Drosophila* AMN peptide displays similarity to mouse pre-pro PACAP. (A) Mouse pre-pro PACAP encodes for at least two peptides: PACAP Related Peptide (PRP) and an amidated PACAP38 fragment. (B) *Drosophila* AMN encodes for 3 putative peptides. One with similarity of mouse PRP, another with similarity to mouse PACAP38, and an amidated novel fragment. Percentage of similar (Green) and identical (Red) amino acids are shown.
Figure 2.9
mPACAP transgene can be expressed with tissue specificity in *Drosophila* (A) PACAP cDNA was cloned downstream of the *hsp70* site in the pUAST vector. (B-G) uas-mPACAP;c316 {GAL4};uas-cd8::GFP brains were stained with anti-mPACAP38. (B) mcd8::GFP labels DPM projections (B) and cell bodies (E). Anti-PACAP signal also reveals expression in the area of the mushroom body lobes (C) and DPM cell bodies (F). Colocalization of GFP and mPACAP in merge confirms accuracy of transgene expression (D and G).
mPACAP expression in DPM neurons does not rescue amn memory defect. Wild-type, amnX8, or amnX8 flies expressing mPACAP in DPM neurons (amnX8;uas-mPACAP;c316) flies were tested for 1 hour memory. amnX8;uas-mPACAP;c316 flies were indistinguishable from amnX8 mutants indicating mPACAP expression in DPM neurons does not rescue the amn memory phenotype. Flies heterozygous for the amn mutation that express mPACAP in DPM neurons (white bars) are not memory deficient.
TARGET controlled adult-specific expression of \textit{amn} is unable to rescue the 3-hour memory defects of \textit{amn}^X8 flies. (A) \textit{amn}^X8 flies harboring the transgenes uas-\textit{amn}, GAL80\textsuperscript{ts}, as well as the c316(GAL4) driver were grown, maintained and tested at 25\textdegree C. uas-\textit{amn} did not rescue the \textit{amn}^X8 memory defect in the presence of the GAL80\textsuperscript{ts} transgene. (B) The same genotypes were grown at 25\textdegree C and shifted to 31\textdegree C for 3 days prior to training and maintained at this temperature through testing. Again, \textit{amn}^X8/uas-\textit{amn};GAL80\textsuperscript{ts};c316 flies were memory deficient.
Figure 2.12
c316{GAL4} driven reporter-gene expression is not exclusive to DPM neurons during development. Brains of c316{GAL4};uas-cd8::GFP pupae were dissected and imaged whole mount on a confocal microscope. Labeled DPM neurons and Kenyon cell projections are present in both early (A) and late (B) pupae.
Figure 3.1
Blocking neurotransmission from DPM neurons abolishes memory.  
(A) Flies expressing shi<sup>asl</sup> in DPM neurons were tested for 3 hour memory with the odors OCT and MCH at 25°C.  uas-shi<sup>asl</sup>;c316 flies performed equally to wild-type indicating that there is no effect of expressing the shi<sup>asl</sup>trangene in DPM neurons at a permissive temperature.  (B) To block output from DPM neurons, flies were incubated at 31°C for 15 minutes prior to training and maintained at this temperature through testing.  With this manipulation, uas-shi<sup>asl</sup>;c316 displayed severe memory defects.
Figure 3.2
DPM output is dispensable during acquisition and retrieval.
Flies expressing $sh^{ts1}$ were incubated at $31^\circ C$ in 15 minute intervals to block output during discrete phases of the memory process. Incubating c316;uas-$sh^{ts}$ flies during training (A), testing (B) or both training and testing (C) did not impair 3-hour memory.
Figure 3.3
Neurotransmission from DPM neurons is required for memory stability. (A) To test for a role of DPM neurons in memory stability, *uas-shit1; c316* flies were incubated at 31°C for 2 hours immediately following training, and returned to 25°C for 1 hour prior to testing. This manipulation resulted in severe memory defects. (B) Incubating flies *uas-shit1; c316* at 31°C for two hours, beginning 30 minutes following training, also caused severe memory defects indicating persistent DPM neuron output is required for memory stability.
Figure 3.4
Blocking DPM output for 30 minutes following training disrupts memory. Flies carrying c316{GAL4} and uas-shi^{ts} were trained and tested for 3 hour memory with OCT and MCH as odorants. Synaptic activity from the DPM neurons was blocked for various intervals after training by shifting the flies from 25°C to 31°C. Following this temperature shift, the flies were returned to 25°C and 3 hour memory was tested. Three hour memory performance was assayed following temperature shifts immediately after training for durations of 15, 30, or 60 min. c316;uas-shi^{ts1} with 0 or 15 minutes incubation at 31°C were indistinguishable from wild-type and uas-shi^{ts1} controls. Incubation at 31°C for 30 or 60 minutes caused severe memory defects in uas-shi^{ts1};c316 flies.
Figure 3.5
DPM dependence is diminished 150 minutes following training. (A) A temperature shift between 30 and 60 minutes following training abolishes 3 hour memory of flies carrying c316(GAL4);uas-shi. (B) A temperature shift between 150 and 180 minutes after training has no effect on 3 hour memory of flies carrying c316(GAL4);uas-shi.
**Figure 3.6**

**BA is a multimodal Stimulus.** (A) BA, unlike MCH and OCT, elicits a substantial avoidance response in flies lacking olfactory organs (n > 10 flies). Intact naive wild-type and naive wild-type flies without olfactory organs were tested for avoidance of BA, MCH, and OCT in the arena paradigm. (B) Genetic ablation and microsurgery identified three types of BA-sensitive neurons. Antennal and maxillary palp neurons were removed by surgery, bitter-sensitive neurons were ablated in Gr66a-GAL4, uas-DTI flies (no bitter taste) and all labelar chemosensory neurons were transformed in poxn70-23/Df(2R)WMG mutant flies (no taste). Sweet-sensitive Gr5a-expressing neurons were ablated in Gr5a-GAL4, UAS-DTI flies (no sweet taste). Black bars represent flies without surgery. White bars are flies with olfactory organs removed. (C) Flies lacking olfactory organs retain BA avoidance in the T maze. Intact wild-type flies and flies without olfactory organs were tested for BA, MCH, and OCT avoidance behavior in the T maze. (D) Olfactory organs are required for olfactory conditioning with OCT and BA. Intact wild-type flies and flies without olfactory organs were tested for OCT and BA olfactory memory.
**Figure 3.7**

**amm** mutant flies have a BA learning defect that is partially DPM neuron dependent (A) Three minute OCT and BA memory. All genotypes shown were treated identically. Flies were trained to associate BA or OCT with shock and were tested for their preference between OCT and BA. Expressing the **amm** gene in DPM neurons (**amm**X8; c316/uas-amm and **amm**X8; Mz717/uas-amm flies) partially rescues the BA memory defect of **amm** mutant flies. (B) Olfactory acuity of wild-type and **amm**X8 mutant flies before and after electric shock. Naive or previously electric-shocked flies were given the choice between OCT and BA in the T maze. (C) **amm**X8 mutant flies without olfactory organs retain BA avoidance. Wild-type flies and **amm**X8 mutant flies with or without olfactory organs were tested for OCT, MCH, and BA avoidance in the arena apparatus.
Figure 3.8.
Blocking DPM output impairs BA learning (A) Blocking DPM output does not reduce OCT learning (3 min memory). All genotypes were trained to associate OCT with shock and tested for preference between OCT and BA. (B) Blocking DPM output reduces BA learning. All genotypes were trained to associate BA with shock and tested for preference between BA and OCT. Asterisks denote significant difference (p < 0.05) from wild-type flies. (C) Blocking DPM output does not reduce OCT learning. All genotypes were trained to associate OCT with shock and tested for preference between OCT and MCH. (D) Blocking DPM output does not reduce MCH learning. All genotypes were trained to associate MCH with shock and tested for preference between MCH and OCT.
Figure 3.9.
Blocking DPM output during acquisition impairs BA memory.

All genotypes were incubated at 31°C for 15 min prior to and during training. Immediately after training, they were returned to 25°C, and they were tested for 1 hour memory at 25°C. (A) Blocking DPM output during acquisition does not affect OCT memory. Flies were trained to associate OCT with shock and tested for preference between OCT and BA. (B) Blocking DPM output during acquisition abolishes BA memory. Flies were trained to associate BA with shock and tested for preference between BA and OCT. (C) Blocking DPM output during acquisition does not affect OCT memory. Flies were trained to associate OCT with shock and tested for preference between OCT and MCH. (D) Blocking DPM output during acquisition does not affect MCH memory. Flies were trained to associate MCH with shock and tested for preference between MCH and OCT.
Figure 3.10
A model for the temporal requirements of DPM neurons in 3 hour memory. DPM neurons are dispensable during training and testing, but necessary for the period in between. Blocking DPM output for 30 minutes (colored bars) impairs memory, and this dependence diminishes with time following training.
**Figure 4.1**

*amn* mutants are defective for sucrose-reinforced olfactory memory. Wild-type, *amn¹*, and *amnX⁸* mutant flies were conditioned with odor and sugar reward and different populations were tested once for odor memory 3, 60, 180, and 360 minutes after training. *amn¹* and *amnX⁸* exhibited small defects at 3 minutes and severe defects at later time points.
Figure 4.2
Specific expression of an amn transgene in DPM neurons restores memory in multiple amn alleles. Wild-type, amn mutant, and amn mutant flies expressing amn in DPM neurons were trained with odor and sugar reward and tested for memory 3 hours after conditioning. Flies with selective amn expression in DPM neurons perform at wild-type levels.
Figure 4.3
DPM neuron output is required for 3 hour sucrose-reinforced odor memory. Temperature shift protocols are shown pictographically above each graph. (A) c316;uas-shl ts1 flies perform normally at the permissive temperature of 25°C. (B) Disrupting DPM output by placing flies at the restrictive temperature of 31°C for 15 minutes prior to training and maintaining flies at this temperature through testing abolishes memory in c316;uas-shl ts1.
Figure 4.4
DPM output is required for stability of sucrose-reinforced odor memory. The temperature shift protocols are shown pictographically above each graph. (A) Blocking DPM output during training, or (B) retrieval does not affect memory. Blocking DPM output for 30 minutes, either (C) 0-30 minutes following training, or (D) 30-60 minutes following training causes memory defects.
Figure 4.5
Blocking DPM output with Mz717{GAL4} driven expression of \( sh^{ls1} \) for 1 hour following training impairs memory. Performance of Mz717;uas-\( sh^{ls1} \) flies was impaired compared to wild-type or uas-\( sh^{ls1} \) flies following incubation at 31°C from 0-60 minutes following training.
The dendritic marker DScam17-1:GFP localizes to DPM ramifications throughout the mushroom body lobes. c316;uas-DScam17-1:GFP;uas-lacZ brains were stained with anti-βGal. Confocal sections through the mushroom body lobes reveals GFP expression (A) and anti-βGal signal (B). A merge of the two (C) reveals apparent co-localization, suggesting DPM dendrites are in the mushroom body lobes.
Figure 5.2 Ectopic expression of Dscam17.2 specifically disrupts DPM projections onto α, β, and γ lobes. Panels A and B show confocal projection views of DPM ramifications through the mushroom body lobes. (A) c316;uas-cd8::GFP brain stained with the marker of the α, β, γ lobes, anti-FASII (Red). GFP labeling DPM neurons is observed on all FAS-II labeled lobes, including the prime lobes, which FAS-II does not label. (B) c316, uas-DScam17-2::GFP brains stained with anti-FASII (Red). GFP is only observed in DPM neurons ramifying throughout the prime lobes, which are not marked by FAS-II.
Figure 5.3
Close analysis of uas-DScam17-2::GFP;c316 flies reveal diminished DPM projections. (A-E) Projection views of DPM neuron ramification throughout all the MB lobes. The optical sections with the DPM cell body have been removed to improve visibility of the mushroom body lobe innervation. (A) Wild-type DPM neuron projections to all the MB lobes visualized by expressing uas-cd8::GFP with c316{GAL4}. (B) The same wild-type DPM neurons shown in (A) but with the mushroom body α and β lobes (red) counterstained with FASII antibody. (C) The same wild-type DPM neurons shown in (A) and (B) but with MB α and β lobes (red) counterstained with FASII antibody and α’ and β’ lobes (blue) stained with TRIO antibody. (D) Expressing a uas-DScam17-2::GFP transgene in DPM neurons with c316{GAL4} results in DPM neurons that predominantly project to the mushroom body α’ and β’ lobes. DPM projections are visualized (green) by coexpressing uas-DScam17-2::GFP and uas-CD2 and immunostaining with a CD2 antibody. (E) The same anti-CD2 labeled uas-DScam17-2::GFP-expressing DPM neurons shown in (D) but with the mushroom body α and β lobes (red) counterstained with FASII antibody. Areas where DPM projections to the mushroom body lobes are greatly reduced or absent are marked with arrowheads for comparison with wild-type DPM neurons shown in (B). (F) Gross anatomy of the mushroom body lobes revealed by FASII (red) and TRIO (blue) immunostaining. In these branched lobes, FASII and TRIO are mutually exclusive. The γ lobe neurons lie along the front of the horizontally projecting lobe subdivision and are labeled by both anti-FASII and anti-TRIO. The mushroom body lobes are symmetrical. Scale bar represents 20 μm.
Figure 5.4 Prime lobe projecting DPM neurons retain function. Wild-type, amnX8, uas-DScam17-2::GFP, and uas-DScam17-2::GFP;c316 flies were conditioned in either the rewarded (A), or punished (B) olfactory paradigms and were tested for 1 hour memory. In both paradigms uas-DScam17-2::GFP;c316 flies performed at wild-type levels indicating that prime-lobe projections are sufficient for memory.
Figure 5.5
Enhancer trap GAL4 lines express in the mushroom bodies. All lines are stained with the $\alpha,\beta,\gamma$ lobe marker, anti-FASII (red) and the $\alpha',\beta'$ maker, anti-TRIO (blue). Confocal images of (A) c320(GAL4);uas-mcd8::GFP and (B) c305a(GAL4) cd8::GFP display GFP in the $\alpha',\beta'$, but $\alpha,\beta,\gamma$ lobes. (C) In contrast, c739(GAL4);uas:cd8::GFP flies express GFP in the $\alpha,\beta,\gamma$, but not the prime lobes.
Prime lobe output is required for middle-term shock-reinforced odor memory. Temperature shift protocols are shown pictographically above each graph. (A) The permissive temperature of 25°C does not affect 3 hour aversive odor memory of mushroom body α'/β’ drivers c320{GAL4};uas-shh<sup>ts1</sup>; c305a{GAL4};uas-shh<sup>ts1</sup> flies, or the α,β driver c739{GAL4};uas-shh<sup>ts1</sup>. All genotypes were trained and tested for 3 hour memory at 25°C. (B) Disrupting mushroom body α'/β’, or α,β neurons disrupts memory. At the restrictive temperature of 31°C c320{GAL4};uas-shh<sup>ts1</sup>; c305a{GAL4};uas-shh<sup>ts1</sup> and c739{GAL4};uas-shh<sup>ts1</sup> exhibit impaired memory compared to wild-type and uas-shh<sup>ts1</sup>flies.
**Figure 5.7**
Mushroom body $\alpha'\beta'$ and $\alpha/\beta$ lobes display are differentially involved in middle-term memory. Temperature shift protocols are shown pictographically above each graph. (A) Flies were incubated at 31°C for 15 minutes prior to training and returned to 25°C immediately following training. Blocking output from the $\alpha'\beta'$, but not the $\alpha/\beta$ lobes, disrupts 3 hour memory during acquisition disrupts 3 hour memory memory at 25°C. (B) To block neural output during retrieval flies were trained at 25°C and incubated at 31°C for 15 minutes prior to testing. Memory was abolished in c739;uas-shi flies indicating that output from the $\alpha,\beta$ neurons is essential for memory retrieval. A significant memory defect was not observed when output from the $\alpha'\beta'$ was blocked indicating that output from these lobes is not essential during retrieval.
Figure 5.8
Mushroom body α'/β', but not α/β, output is required for memory stability. Temperature shift protocol is shown pictographically above the graph. Flies were trained at 25°C, and immediately after training they were shifted to 31°C for 60 minutes. Flies were then returned to 25°C and tested for 3 hour shock-reinforced odor memory at 25°C. Expression of uas-shi ts1 with the α'/β' drivers c305a or c320 abolished memory. There was no effect of uas-shi ts1 with the α/β lobe driver c739.
Figure 5.9
Prime lobe output is required for stability of sucrose-reinforced odor memory. Flies expressing shi^{ls1} in the α'/β' or α/β lobes were trained to associate sugar-reward with odor. The temperature shift protocols are shown pictographically above each graph. (A) At 25°C there is no effect of shi^{ls1} expression on mushroom body function and all genotypes perform at wild-type levels. (B) Blocking output from mushroom body α'/β' neurons for 1 hour following training impairs memory. This temperature shift protocol had no effect with the α/β driver c739{GAL4}. 
rut cDNA expression with c320{GAL4} rescues memory, but not learning in rut
^6080 mutant flies. rut^6080 flies were flies harboring the uas-rut transgene and a prime lobe driver were tested for learning (A) or 3 hour memory (B). rut^6080;c320{GAL4};uas-rut flies displayed normal 3 hour memory.
Odor activates mushroom body neurons via activation of second order projection neurons. These mushroom body neurons are necessary for retrieval of memory. DPM neurons form a feedback loop with the mushroom bodies that is essential for memory stability.