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Review

# Traces of *Drosophila* Memory

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**Studies using functional cellular imaging of living flies have identified six memory traces that form in the olfactory nervous system after conditioning with odors. These traces occur in distinct nodes of the olfactory nervous system, form and disappear across different windows of time, and are detected in the imaged neurons as increased calcium influx or synaptic release in response to the conditioned odor. Three traces form at or near acquisition and coexist with short-term behavioral memory. One trace forms with a delay after learning and coexists with intermediate-term behavioral memory. Two traces form many hours after acquisition and coexist with long-term behavioral memory. The transient memory traces may support behavior across the time windows of their existence. The experimental approaches for dissecting memory formation in the fly, ranging from the molecular to the systems, make it an ideal system for elucidating the logic by which the nervous system organizes and stores different temporal forms of memory.**

## Introduction

Memories are formed, stored, retrieved, and lost by a mysterious interplay between sensory cues and the functioning nervous system. The formation of memories occurs through a set of changes within neurons that encode the relevant sensory information. These changes, or cellular memory traces, can in principle be any change in the activity of the cell that is induced by learning, which subsequently alters the processing and response of the nervous system to the sensory information. For instance, changes can occur in the expression or function of ion channels that cause neurons to be more or less excitable and therefore more or less capable of conducting action potentials or other electrical signals. Learning may mobilize neuronal growth processes that establish new connections or neurite retraction to remove existing connections. The changes may include cell signaling adaptations that alter the neuron's overall ability to integrate inputs from different types of cues, and morphological or functional changes in synapses that increase or decrease the neuron's ability to stimulate its synaptic partners. These cellular memory traces, which arise from underlying molecular changes, altogether comprise the overall behavioral memory trace, or memory engram (Dudai, 2002; Squire, 1987), that guides behavior in response to sensory information. A major goal in neuroscience is to understand the nature of cellular memory traces, the mechanisms by which they form, their duration, the neurons in which they develop, and how the complete set of cellular memory traces within different areas of the nervous system underlie the memory engram.

The traces that underlie behavioral memory are currently being probed in numerous organisms using a variety of methodologies. Although many cellular changes have been discovered that occur due to learning, the experimental evidence tying these changes to behavior to ensure that they are relevant to behavior, and not just an inconsequential byproduct of the training, has been difficult to obtain. Thus, for the vast majority of putative cellular memory traces that have been discovered, the evidence implicating them in behavioral memory is largely correlative. For instance, numerous changes occur in the structure of mammalian synapses, such as in the density of dendritic spines, in response to experience or authentic learning (Xu et al., 2009; Yang et al., 2009; Roberts et al., 2010; reviewed by Hübener and Bonhoeffer, 2010). Indeed, there is now much evidence to support the conclusion that learning alters the connectivity in the brain. Although important, correlations such as this are just the beginning—one needs experimental support showing that the altered connectivity underlies memory storage or is related to memory in some other way. For this, disruptive experiments are needed to ''bump'' the system—to block, for instance, the changes in spine density that occur with experience and ask whether memory is disrupted in parallel.

Progress in the study of *Drosophila* olfactory learning has recently afforded the opportunity to peer into the brain of the living fly and visualize cellular memory traces. In addition, numerous mutants and other disruptive strategies are available and have been used whenever possible to probe the relevance of the newly discovered, experience-dependent plasticity to behavioral memory. Beyond establishing the relevance of a cellular memory trace to behavioral memory, some of the more global and broader questions that have driven this research include the following: (1) for any given behavior, such as olfactory classical conditioning in which an organism learns to avoid or respond to an odor previously paired with an unconditioned stimulus (Roman and Davis, 2001; Davis, 2005; Busto et al., 2010), how many different cellular memory traces comprise the overall engram that guides behavior at the time of retrieval? (2) In which neurons do the cellular memory traces form? (3) Is there but one class of neurons that forms cellular memory traces that guide behavior at retrieval, or do memory traces form in a distributed way across many neuronal types in the brain? (4) How long does each cellular memory trace persist? A singular cellular memory trace, in principle, could persist across the time course over which behavioral memory is stored. Alternatively, different memory traces might exist for different periods of time after training, such that behavioral memory is represented not only by distinct cellular memory traces in many different neurons but also by the different life spans for various

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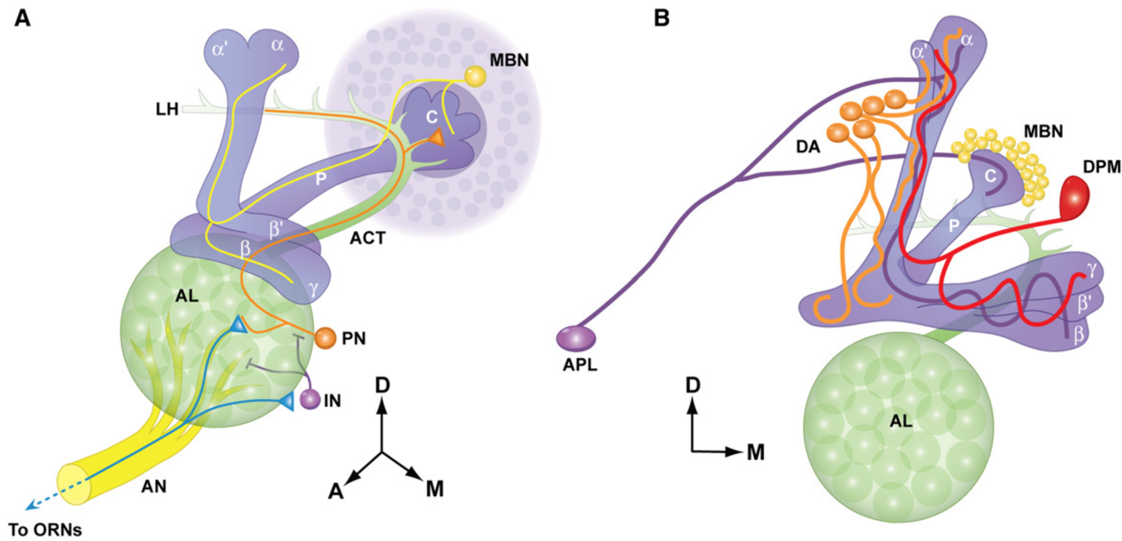


**Figure 1. Anatomical Organization of the Olfactory Nervous System in *Drosophila***

(A) Olfactory nervous system viewed from the left-front and slightly dorsal position of the fly. Olfactory information is transmitted from olfactory receptor neurons (ORNs) located on the antennae (not shown) via the antennal nerve (AN) to the antennal lobe (AL), where the axons of ORNs synapse on two types of secondary olfactory neurons, the projection neurons (PN) and the AL interneurons (IN). The INs are known to be either excitatory or inhibitory. PNs send their axons via a nerve known as the antennal cerebral tract (ACT) to the mushroom body neurons (MBN) and to the lateral horn (LH). The PNs synapse with MBNs in a neuropil region known as the calyx (C). Three classes of MBNs have been described according to their axonal collaterals (α/β, α′/β′, and γ). The axons extended by MBNs follow the pedunculus (P) to reach the MB lobes (α, α′, β, β′, and γ). For simplicity, only one ORN axon (green), one PN (orange), one IN (purple), and one α/β MB neuron (yellow) have been superimposed on a schematic of one hemisphere of the fly brain. Axis arrows: A = anterior, D = dorsal, M = medial. Adapted from Busto et al. (2010); used with permission.

(B) Frontal perspective of neurons that are extrinsic to the MBs in one hemisphere showing the dorsal paired medial (DPM) neuron, anterior paired lateral (APL) neuron, and dopaminergic (DA) neurons. The DPM neuron (red) extends a single neurite which bifurcates to innervate the vertical lobes (α and α′) and the horizontal (β, β′, and γ) lobes of the MBs. Only five of the DA neurons (DA, orange) in the PPL1 cluster are illustrated. These neurons innervate distinct zones of the MB vertical lobes. The APL neuron (magenta) broadly innervates the calyx and the MB lobes. Axis arrows: D = dorsal, M = medial.

traces. (5) Do different types of conditioning induce different types of memory traces, either qualitatively or quantitatively? For instance, does long-term memory (LTM) induced by multiple and spaced conditioning trials produce a cellular memory trace that is different from the cellular memory trace that is induced by only a single training trial? Or is the nature of the cellular memory trace independent of the conditioning protocol used to train the animal?

To date, at least six different cellular memory traces produced by olfactory classical conditioning have been described in *Drosophila*. These memory traces differ from one another in the neurons in which they are formed, their duration, and the type of conditioning required to produce the memory traces.

## *Drosophila* Olfactory Nervous System

The anatomical organization of the insect olfactory nervous system shares many fundamental similarities to that of mammals, suggesting that the mechanisms for olfactory perception, discrimination, and learning are shared (reviewed in Davis, 2004). The study of olfactory memory traces in flies thus offers reassurance that the principles established may be conserved to other organisms. Such design homology is much more difficult to discern for other sensory systems, such as the visual or somatosensory systems when comparing representative organisms between the two classes. Below is a brief account of principal cell types in the olfactory nervous system and their connections.

The neurons representing the interface between the environment and the nervous system are the olfactory receptor neurons (ORNs, first order olfactory neurons), which reside in the antennae and maxillary palps of the fly. About 1300 ORNs are distributed between the antenna and maxillary palp on each side of the head and project axons to the antennal lobe (AL) where they terminate in ∼43 morphologically discrete and synapse-dense processing modules known as glomeruli (Figure 1A). The projection patterns of the ORNs are stereotyped between animals; ORNs that express the same olfactory receptor gene, although distributed across the surface of the antenna and maxillary palps, project their axons to the same glomerular target in the AL. There, they are thought to form excitatory synapses with at least two classes of second order neurons, the local interneurons (INs) and the projection neurons (PNs). Many of the INs are axonless and are GABAergic inhibitory neurons, with broad, multiglomerular ramifications within the AL. A unique feature of the circuitry within the insect AL is the existence of reciprocal dendro-dendritic connections between the PNs and the INs. PNs, like the mitral cells that populate the vertebrate olfactory bulb, have both presynaptic and postsynaptic specializations on the neurites that innervate the glomeruli, providing the opportunity of visualizing synaptic release by using fluorescent reporters of synaptic transmission (see below). PNs are generally uniglomerular, with an average of 4–5 PNs innervating each individual glomerulus, and convey the processed olfactory information to the third order olfactory neurons

(Figure 1A) which includes the mushroom body neurons (MBNs) and neurons in a brain area named the lateral horn (LH). The MBNs receive information through their dendrites in the calyx and fall into three different classes. Each $\alpha/\beta$ MB neuron sends a single axon toward the anterior face of the brain to a location just dorsal to the AL known as the heel. The axon divides at the heel into a vertically oriented $\alpha$ branch, and a horizontally oriented $\beta$ branch. The neuropil that houses the $\alpha$ and $\beta$ branches of the $\alpha/\beta$ MBNs are referred to as the $\alpha$ and $\beta$ lobes. The $\alpha'/\beta'$ MBNs exhibit a parallel organization with the $\alpha/\beta$ MBNs. The $\gamma$ MBNs do not have a branched axon. Their axons extend along the same path as the axons from other MBNs but turn medially at the heel to form the $\gamma$ lobe. The neuroanatomy thus suggests that distinct odors are first represented by the stimulation of distinct sets of ORNs; second, by spatial patterns of synaptic (glomerulus) activation within the AL; and third, by a distinct set of synaptic fields activated in the MBs and the lateral horn.

Three classes of neurons that are extrinsic to the MBs are relevant to the discussion of memory traces (Figure 1B). There exist two dorsal paired medial (DPM) neurons in the brain, each with a large cell body residing in the dorsal and medial aspect of each brain hemisphere. They have no obvious dendritic field and extend a single neurite in an anterior direction toward the MB lobes. The neurite from each DPM neuron splits, with one branch broadly innervating the vertical lobes and the other innervating the horizontal lobes. A GABAergic neuron that probably provides input to the MBs through a $GABA_A$ receptor (Rdl, resistance to dieldrin) on the MBNs is named the anterior paired lateral (APL) neuron. It resides in each brain hemisphere near the LH (ventrolateral to the MB calyces) and separately innervates the calyces and the MB lobes through two branches of a single APL neurite (Liu and Davis, 2009). The dopaminergic neurons (DA) that innervate various areas of the fly brain and in particular the MBs have recently been mapped using tyrosine hydroxylase (*TH-GAL4*) expression as a surrogate for the neurons along with anti-TH immunoreactivity (Mao and Davis, 2009). Three clusters of DA neurons innervate the MB neuropil. The PAM (protocerebral anterior medial) neurons project to a medial zone of the horizontal lobes; the PPL1 (protocerebral anterior lateral) neurons project to the vertical lobes and associated neuropil; and PPL2ab neurons project to the calyx. The PPL1 neurons can be further divided into five distinct classes based on their targets: the tip of $\alpha$ lobe, the tip of $\alpha'$ lobe, the upper stalk, the lower stalk/heel area, and the spur/distal peduncle. Since there are 12 neurons within each PPL1 cluster, there must be 2–3 neurons each within the PPL1 cluster that project to these five distinct areas of neuropil.

## Early Forming Cellular Memory Traces

*Drosophila* can develop a robust association between an odor, the conditioned stimulus (CS), and electric shock, the unconditioned stimulus (US), if the CS and the US are presented together (Tully and Quinn, 1985; Roman and Davis, 2001; Busto et al., 2010). Flies display their memory of this association by avoiding the odor CS during a test after CS/US pairing. A single training cycle that usually consists of a 1 min presentation of the CS odor along with 12 electric shock pulses rapidly generates

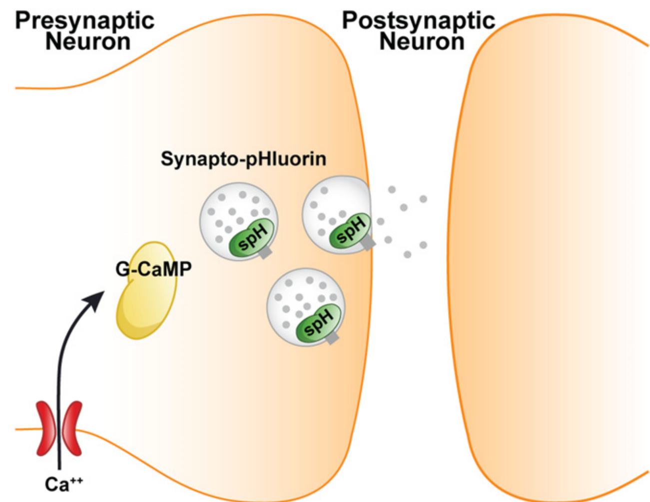


**Figure 2. Reporter Molecules Used to Identify Memory Traces in *Drosophila* by Functional Optical Imaging**
These reporter molecules are supplied to the organism using transgenic techniques and are expressed in a defined and limited set of neurons using the GAL4/*UAS* system (Brand and Perrimon, 1993). Synapto-pHluorin (spH) is a pH-sensitive GFP molecule that is trafficked to the lumen side of the synaptic vesicle by virtue of its fusion with the synaptic vesicle protein, VAMP (Miesenböck et al., 1998). Fusion of the synaptic vesicle with the plasma membrane upon neurotransmitter release alters the pH environment around synapto-pHluorin such that its fluorescence is increased until vesicles are internalized and reacidified. G-CaMP is a circularly permuted EGFP fused to the M13 fragment of myosin light chain kinase at one end and the calcium binding site of calmodulin on the other (Nakai et al., 2001). Upon influx of calcium through voltage-dependent calcium channels, calmodulin interacts with the M13 fragment eliciting a conformational change in EGFP that increases its fluorescence. Improved versions of G-CaMP, including G-CaMP1.6, G-CaMP2, and G-CaMP3.0, are now available (Ohkura et al., 2005; Díez-García et al., 2005; Tian et al., 2009).

conditioned behavior that consists of both short-term memory (STM) and intermediate-term memory (ITM), with all performance gains decaying to near zero within 24 hr after training. Long-term olfactory memory (LTM) lasts 4–7 days and is produced by spaced conditioning, in which the trained animals receive 5–10 training trials with a rest of usually 15 min between each training trial (Tully et al., 1994; Pascual and Préat, 2001; Yu et al., 2006). Robust LTM, often assayed at one day after conditioning is dependent on normal protein synthesis at the time of training and on the activity of the transcription factor, CREB (Tully et al., 1994; Yin et al., 1994).

The search for cellular memory traces in the olfactory nervous system using functional optical imaging requires molecules that can report relevant physiological events with high fidelity. Two such GFP-based reporter molecules, supplied on transgenes with expression driven in specific neurons using the GAL4/*UAS* system, have been used extensively. Synapto-pHluorin (Figure 2) is a pH-sensitive GFP molecule that is localized to the synaptic vesicle. It provides an optical assay for synaptic transmission due to the change in pH environment between its vesicular localization and synaptic localization that occurs upon neurotransmitter release. G-CaMP (Figure 2) is an EGFP fused to a calcium binding domain and designed in a way that increases in intracellular calcium lead to increased fluorescence. The

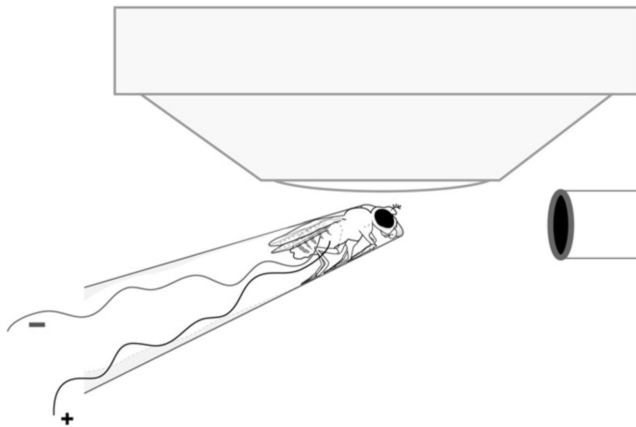


**Figure 3. Schematic Diagram of the Experimental Setup to Visualize the Neuronal Activity in the *Drosophila* Brain**
A small window of cuticle is removed from the dorsal head capsule and the window is then sealed with plastic wrap. The living fly is mounted under the objective of a one-photon or two-photon microscope for visualizing the fluorescence of reporter molecules that are expressed from transgenes (Figure 2). Odors are puffed onto the antennae of the mounted fly from a puffer pipette (cylinder at the right of the figure) to visualize the neuronal responses to the conditioned ($CS^+$) or unconditioned ($CS^-$) odors for "within" animal or "between group" experiments. Electric shock is applied (for aversive learning) to the legs and abdomen of the immobilized fly with conductive wires for "within" animal experiments.

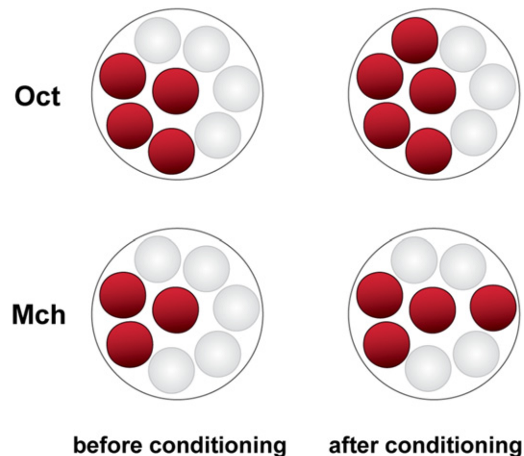


**Figure 4. Olfactory Conditioning Rapidly Alters the Pattern of PN Synaptic Activity in the AL**
The odorant Oct stimulates synaptic activity in four of eight dorsally located glomeruli (circles) in the AL prior to conditioning. Immediately after conditioning, five glomeruli become synaptically activated, indicating that conditioning with Oct recruits at least one additional set of PNs into its neural representation. The odor Mch also recruits an additional set of PNs into its representation after conditioning, but the set of PNs is different than that recruited by Oct. Adapted from Yu et al. (2004).

experimental setup to assay the fluorescence of these molecules in the brain of a living fly is illustrated in Figure 3.

The first memory trace to be discovered by optical imaging was discovered in the AL of the honeybee (Faber et al., 1999). The search for early forming memory traces in *Drosophila* through optical imaging also led to the AL. Yu et al. (2004) expressed the reporter molecule synapto-pHluorin in the PNs of the AL and visualized synaptic release in eight dorsal glomeruli in response to odor and shock stimuli presented to the living fly. This study used a "within animal" experimental design, in which the response properties of the neurons to odor was assessed within each individual animal before and after conditioning. The eight sets of PNs that innervate the eight glomeruli all respond with release of neurotransmitter upon electric shock delivered to the body of the fly, whereas only four and three of the eight sets respond to the odors 3-octanol (Oct) and 4-methylcyclohexanol (Mch), respectively, in naive animals. Most interestingly, an additional set of PNs that innervate the D glomerulus becomes synaptically active in response to Oct as the conditioned odor immediately after conditioning (Figure 4). Conditioning with Mch also recruits an additional set of PNs into the representation of the learned odor—the set that innervates the VA1 glomerulus. Thus, a memory trace forms in the AL immediately after learning and is registered as the recruitment of a new set of PNs into the normal representation of the learned odor. Because only 8 of the ~43 glomeruli were imaged in these experiments, it seems likely that other sets of PNs are also recruited into the representation of the learned odor, but this possibility has not been investigated. In addition, this memory trace is very short lived, with the responses to the $CS^+$ falling to basal levels by 7 min after conditioning. This memory trace appears to be intrinsic to the PNs: tests for the existence of memory traces in neurons presynaptic to PNs (ORNs or INs) were negative. Thus, the increased activity of PNs in response to the $CS^+$ after conditioning does not appear to be the consequence of a memory trace forming in upstream neurons.

Wang et al. (2008) reported an early forming cellular memory trace that is detectable in the axons of the $\alpha'/\beta'$ MBNs after olfactory conditioning. This memory trace is detected with G-CaMP expression in these neurons and therefore reflects increased calcium influx in response to the $CS^+$ odor due to prior conditioning (Figure 5). Animals that received explicitly unpaired conditioning with the $CS^+$ and US failed to exhibit this memory trace. The trace forms with either Oct or Ben as the $CS^+$ odors and is observed only in the lobes, not the calyx, of the MBNs. Thus, this trace is axon specific. This early-forming memory trace is not generated in the axons of the $\alpha/\beta$ or $\gamma$ MBNs. This trace is present up to 60 min after conditioning.

A peculiar aspect of this trace is that it is most easily extracted by calculating the ratio of the G-CaMP response in trained flies for the $CS^+$ and $CS^-$ (Wang et al., 2008), suggesting that calcium influx increases with the $CS^+$ and decreases with the $CS^-$. This aspect was confirmed by Tan et al. (2010). Indeed, if one examines the increased G-CaMP response to the $CS^+$ alone as compared to control flies (explicitly unpaired, naive, or backward conditioned animals), there is a trend toward an increased response but it often fails to reach significance. Conversely, the response to the $CS^-$ in conditioned flies compared to controls tends to be lower than the control. It is unclear at present what this means biologically. One possibility is that the decrease in response to the $CS^-$ may reflect a memory trace for inhibitory conditioning.

The $\alpha'/\beta'$ memory trace was also studied in a reduced preparation consisting only of a fly brain with AN and ventral nerve

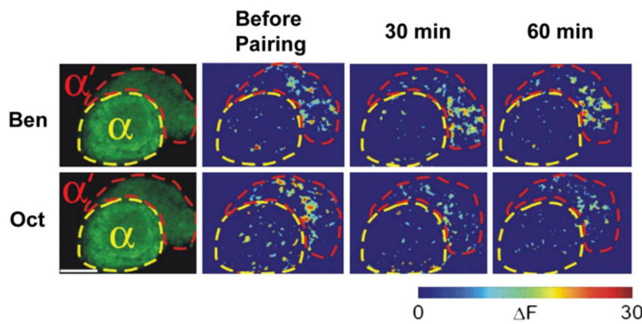


**Figure 5. Calcium-Based Memory Trace in the Axons of the α′ Neurons Detected with the Calcium Reporter G-CaMP**
Pairing the odor of benzaldehyde (Ben) as the $CS^+$ with mild electric shock increases the subsequent response to Ben and decreases the response to Oct (the $CS^-$) as shown by comparing the intensity of the responses in the α′ axons before and after pairing. Note the increased number of colored pixels within the area encircled by the red dotted line (region of α′ axons) at 30 and 60 min in the Ben row compared to the "before pairing" condition, and the decreased number of colored pixels within the same area in the Oct row. No changes were detected in the axons of the α/β neurons, also shown in this image, at these time points after conditioning. The magnitude of response is illustrated in pseudocolor on a pixel-by-pixel basis. The α′/β′ memory trace persists for at least 60 min after pairing. Adapted from Wang et al. (2008), with permission.

cords intact (Wang et al., 2008). Electrical stimulation of the AN, mimicking exposure of an intact fly to odors, along with stimulation of the ventral nerve cord, mimicking electric shock to the animal's body, produced an increased G-CaMP response to subsequent stimulation of the AN. Under these conditions, the memory trace forms by 5 min after conditioning and is similarly specific to the α′/β′ axons, with no changes occurring in the α/β axons, γ axons, or the calyx. Backward conditioning, or conditioning only with the "CS" (AN stimulation) or the "US" (VNC stimulation) fails to produce the increase. The time course for the memory trace in this reduced preparation is at least 60 min after paired stimulation. Later time points have not been assayed to ascertain its complete lifetime.

The in vivo and in vitro imaging results suggest that a memory trace forms in the α′/β′ neurons at the time of training or within minutes thereafter, and persists for at least 1 hr. The mechanistic basis for the memory trace is currently unknown. However, this memory trace requires signaling through G protein coupled receptors, since coexpression of a constitutively active $G\alpha_s$ ($G\alpha_s$*) subunit throughout the MBs eliminates the memory trace. Interestingly, expression of the $G\alpha_s$* subunit in the α′/β′ neurons reduces, but does not eliminate, behavioral performance when tested immediately after training (Wang et al., 2008), consistent with the hypothesis that this trace along with one or more other coexisting traces support behavior immediately after training. The α′/β′ memory trace also requires the activity of a casein kinase Iγ molecule since mutants of *gish*, the gene encoding this molecule, disrupt this memory trace (Tan et al., 2010).

An interesting observation currently at odds with the hypothesis that the α′/β′ neurons and the associated cellular memory trace mediate early memory formation comes from studies of the *ala* (*a*lpha *l*obes *a*bsent) mutant (Pascual and Préat, 2001). This mutant eliminates the lobes of the MBs with variable expressivity, with some flies missing only the α/α′ lobes and some missing only the β/β′ lobes. Surprisingly, flies missing the α/α′ lobes exhibit normal behavioral memory at 3 hr after conditioning, which is not predicted from the hypothesis that the α/α′ lobes are needed for memory formation at early times after conditioning. In the absence of the α′/β′ memory trace, it is possible that other coexisting traces support early behavioral memory.

Two other reports of plasticity observed early after conditioning have been published. A recent series of studies identified an inhibitory circuit that impinges upon and influences the responses of MBNs when sensory stimuli are presented to the animal. MBNs express a $GABA_A$ receptor named Rdl (*r*esistance to *d*ie*l*drin) at relatively high levels. Overexpression of the Rdl receptor in the MBs impairs acquisition during olfactory conditioning while reduction of Rdl expression (using RNAi) enhances acquisition (Liu et al., 2007). Reducing the GABA content of the APL neuron, which as described is thought to provide GABAergic input into the MBs (Figure 1B), by specific expression of an RNAi for glutamic acid decarboxylase (GAD) enhances acquisition during olfactory conditioning—much like reducing the expression of the Rdl receptor within the MBNs. Thus, the APL neuron via the Rdl receptor may function as an acquisition suppressor that constrains memory formation.

Functional optical imaging experiments suggest that learning overcomes this suppression by a learning-induced reduction in the activity of the APL neuron in response to the $CS^+$ odor. The APL neuron increases its activity measured optically with synapto-pHluorin to both odor and electric shock stimuli delivered to the animal (Liu and Davis, 2009), indicating that this neuron receives both CS and US information used for aversive conditioning. The most salient observation made in this study was that the calcium response of the APL neuron is reduced after conditioning specifically to the trained odor. This discovery indicates that the APL neuron displays training-induced plasticity that leads to a reduced release of GABA, presumably onto the MBNs expressing the Rdl receptor, after olfactory classical conditioning. The reduction in GABA release was observed to occur at time periods up to 5 min after conditioning (later time points were not assayed). Thus, learning releases an inhibitory constraint on the ability of MBNs to respond to the learned odor.

The changes in ability to learn about odors by altering the expression of Rdl in the MBs occurs for both aversive and appetitive conditioning, consistent with the possibility that the influence of inhibitory input is through the CS rather than the US pathway (Liu et al., 2009). Olfactory learning may therefore increase the response properties of the MBNs to the learned odor by reducing the inhibition. A similar strategy for learning may occur during auditory learning in vertebrates. The vertebrate auditory system, with cortical auditory neurons turned to respond to an optimal tone frequency, offers a unique system for exploring how tone learning alters the frequency receptive fields for primary auditory neurons (Weinberger, 2004). Froemke et al. (2007) reported that pairing the presentation of pure tones with electrical stimulation of the nucleus basalis, which provides cholinergic modulation to the cortex and acts as a substitute US, alters the receptive fields of cortical neurons toward the frequency of tone presented. The mechanism underlying this plasticity in frequency receptive fields is a rapid (within 20 s)

reduction in the inhibitory drive on these neurons with a subsequent increase in their excitability by the tone paired with cholinergic release. The net effect of pairing is to enhance the excitability of cortical neurons by the learned tone.

One report offers experimental support for learning-induced plasticity in the dopaminergic neurons (DA) that are thought to innervate the MBNs (Figure 1B). Riemensperger et al. (2005) expressed a calcium reporter in the DA neurons and imaged the DA fibers that innervate the MB lobes in flies before and after olfactory conditioning. Surprisingly, they observed calcium responses in these neurons when odors were presented to the flies, even though the DA neurons at the time were hypothesized to be part of the US pathway and not the CS pathway. Although there is no increase in the magnitude of the calcium responses of the DA neurons to the trained odor after conditioning, the data indicate that the duration of the calcium response may be prolonged. Multiple training trials were used to generate this plasticity, with the training-induced increase in calcium response forming by 15 min after the first pairing of odor and shock. This suggests that training alters the response properties of these neurons to the learned odor.

More recent studies indicate that the DA neurons are anatomically and functionally heterogeneous (Mao and Davis, 2009). The DA neurons reside in different clusters in the brain. One cluster with 12 DA neurons (PPL1) innervates distinct zones of the MB lobes (Figure 1B). Furthermore, functional imaging of the processes of DA neurons in the MB lobes at different focal planes, in attempt to isolate the responses of PPL1 neurons that innervate the tip of $\alpha$ lobe, the tip of $\alpha'$ lobe, the upper stalk, and the lower stalk/heel area, indicate significant heterogeneity in response properties (Mao and Davis, 2009). All four neuropil regions respond to odors presented to the fly, although the $\alpha'$ tip exhibits much stronger odor responses than other neuropil regions. All four regions similarly respond to electric shock stimuli presented to the fly, although the lower stalk/heel and the $\alpha$ tip displays strong responses compared to the very weak responses of the $\alpha'$ tip and the upper stalk. Notably, no plasticity in calcium responses within these four regions were observed due to conditioning.

These discrepant results relative to memory trace formation in the DA neurons make it difficult to draw firm conclusions one way or the other. Differences in techniques and training protocols could underlie the discrepancy. However, the anatomical and functional heterogeneity of the DA neurons make clear that the *TH-GAL4* driver, which is broadly expressed in most of the DA neurons (Mao and Davis, 2009), is too blunt of a tool to obtain precise information for many types of experiments.

## Intermediate Term Cellular Memory Traces

Prior experiments suggest that the duration of behavioral memory is due to different phases of memory that are mechanistically distinct, at either a molecular, cellular, and/or systems level. An intermediate phase of memory forms in flies after olfactory conditioning that follows short-term memory. This memory phase forms within the first hour after conditioning and persists for a few hours. Studies of the *amnesiac* (*amn*) mutant have provided experimental support for this memory phase: flies carrying mutations at the *amn* gene acquire conditioned behavior at the same rate as control flies using short, repeated training trials, but forget faster than controls after reaching similar levels of acquisition (Figure 6). Similarly, the *amn* mutant flies, when tested using standard olfactory classical conditioning, perform immediately after conditioning at levels nearly equivalent to controls, but exhibit a rapidly decaying behavioral memory (Tully and Quinn, 1985). The mutants have therefore been considered to be impaired in an intermediate phase of memory, or alternatively in the process of consolidating STM into a form that is stable over the first few hours after conditioning.

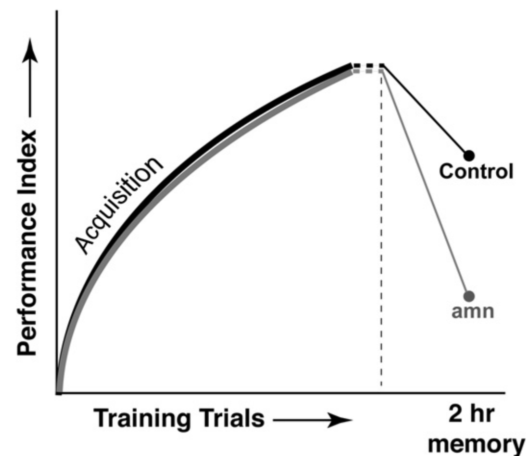


**Figure 6. Acquisition of Olfactory Memory in Control and *amn* Mutant Flies as a Function of the Number of Training Trials**
Memory increases as a function of trial number at the same rate in both control and *amn* mutant flies, indicating that memory formation in the mutant is essentially normal. However, memory measured at 2 hr after an equivalent amount of training is much reduced in the *amn* mutant flies compared to controls, indicating that the mutants are unable to consolidate their early forming memories into a stable form or that they are impaired in an intermediate and distinct phase of memory. Adapted from Yu et al. (2005).

Importantly, the *amn* gene product was found to be expressed and required in the DPM neurons for the formation of normal olfactory memory (Waddell et al., 2000). Additional experimental observations are consistent with a role for these neurons and the *amn* gene product in ITM. Synaptic transmission is required from the DPM neurons during the interval between conditioning and testing for normal performance at a few hours after learning. However, it is not required during acquisition or at testing, revealed by conditionally blocking synaptic transmission from these neurons with the expression of *Shibire*$^{ts}$. The time window over which synaptic blockade from these neurons impairs subsequent memory extends from 15–150 min post training (Keene et al., 2004; Yu et al., 2005).

The functional optical imaging experiments that revealed an intermediate-term memory trace in the DPM neurons were initially designed to challenge the now outdated hypothesis that the DPM neurons represent US input into the MBs, by testing the prediction that these neurons would respond with calcium influx and synaptic release to electric shock delivered to the body of the fly but not to odor stimuli delivered to the antennae (Yu et al., 2005). Although the neurons do respond to electric shock pulses as predicted, they also respond to odors, and they show little discrimination in their response between

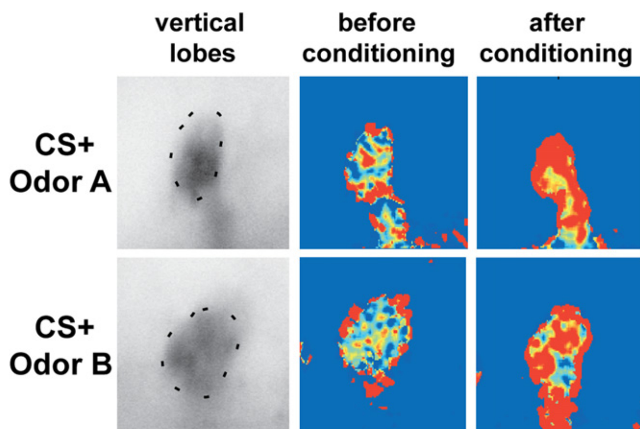


**Figure 7. Intermediate-Term Memory Trace Forms in the DPM Neuron Processes that Innervate the Vertical Lobes of the MBs**
Grayscale images of basal fluorescence of G-CaMP expression in the distal portion of the vertical lobes from a viewpoint above the fly (see Figure 3). Calcium influx detected in the DPM processes with odor stimulation before conditioning is illustrated in the middle column of images. The magnitude of the calcium response is illustrated in pseudocolor on a pixel-by-pixel basis. The enhanced calcium influx detected in these processes with odor stimulation after conditioning is illustrated in the right column of images. Adapted from Yu et al. (2005).

odors. Indeed, they responded to all 17 odors that were tested (Yu et al., 2005), making them "odor generalists." These observations offered the possibility that the DPM neurons might form a memory trace, given their response to both CS and US stimuli. To probe this possibility, flies were trained with odors and electric shock and then the responses of DPM neurons to the trained odors were assayed at different times after training. Remarkably, the coincidence of electric shock with odor caused a significant increase in the subsequent response of the DPM neurons to the trained odor (Figure 7), but not to an odor unpaired with shock (Yu et al., 2005). Furthermore, this training-induced plasticity forms only after a delay of ~30 min. In other words, no increased calcium influx or synaptic release in response to the $CS^+$ is detectable immediately after conditioning; rather, this increase is detectable only 30 min later, indicating that this memory trace is "delayed" in its formation. The time course for the DPM memory trace coincides with intermediate-term behavioral memory. Initial experiments (Yu et al., 2005) indicated that the memory trace persists for at least 60 min after training with detectability becoming unreliable by 2 hr. More recent data show that the aversive memory trace persists to 70 min after conditioning and is undetectable at 90 min after conditioning (I. Cevantes-Sandoval and R.L.D., unpublished data). The DPM memory trace is dependent on the expression of a wild-type copy of the *amn* gene in the DPM neurons: *amn* mutants fail to exhibit the memory trace while expressing a wild-type version specifically in the DPM neurons rescues the formation of the memory trace (Yu et al., 2005). Most remarkably, the DPM memory trace is observed only in the DPM processes that innervate the vertical lobe of the MBs; the memory trace does not form in the processes that innervate the horizontal lobes. The role that this branch specificity plays in aversive olfactory memory remains unknown.

Thus, DPM neurons respond after training to a $CS^+$ odor presented to the fly with increased synaptic release and increased calcium influx into the processes that innervate the vertical lobes of the MBs between 30 and 90 min after training, and this training-induced plasticity requires the expression of the *amn* gene product within these neurons. But what does this memory trace represent to memory processes and subsequent conditioned behavior? Does it embody training-induced plasticity that forms independently of other memory traces and helps to determine the subsequent responses of the fly to the learned odor across the time window of its existence? Alternatively, might it embody training-induced plasticity that is required for the consolidation or stabilization of memories that form earlier, perhaps taking memories that form in the MBs, processing them, and reimplanting them back into the MBs in a consolidated form? In other words, is the DPM trace an independently forming, ITM trace that guides behavior or is it a consolidation trace? The time course for the existence of the DPM trace (30–70 min), the time window over which DPM synaptic transmission is required for behavioral memory (30–150 min), the requirement for the *amn* gene product, and the memory phenotype of *amn* mutants, are consistent with both models. So at present, the issue of whether the DPM trace represents a ITM trace or whether it is a fingerprint of consolidation is unresolved.

## Long-Term Memory Traces

As previously stated, LTM in *Drosophila* is produced by spaced conditioning and is dependent on normal protein synthesis at the time of training and on the activity of the transcription factor, CREB. An additional molecular requirement for this form of memory is on the *amn* gene product, since *amn* mutants fail to display normal LTM after spaced conditioning (Yu et al., 2006). Neuroanatomically, this memory is dependent on the vertical lobes of the MBs (Pascual and Préat, 2001), since the previously mentioned *ala* mutants without the vertical lobes of the MBs fail in LTM tests.

LTM traces have been studied using a "between group" experimental design, in which the neuronal response properties of animals receiving forward conditioning are compared to control animals, such as those that have received backward conditioning. An initial study searching for LTM traces by functional cellular imaging utilized expression of the G-CaMP reporter in the $\alpha/\beta$ neurons of the MBs (Yu et al., 2006). These neurons respond with calcium influx to odors presented to the living animal, as expected since the neurons are third order in the olfactory nervous system and receive input directly from the AL. In addition, this subset of MBNs responds to electric shock pulses delivered to the abdomen of the fly, indicating that they also are activated when US information is presented. Interestingly, this set of MBNs fails to form a detectable, calcium-based memory trace early after training (Wang et al., 2008), in contrast to the $\alpha'/\beta'$ neurons discussed previously. However, they do form a calcium-based LTM trace detected only after experimental animals receive spaced conditioning (Yu et al., 2006). This LTM trace exhibits several important and remarkable features:

(1) The LTM trace forms only after spaced conditioning. It does not form from a single training trial, from multiple

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training trials delivered in a massed configuration, or from backward-spaced training, in which the US precedes the CS. The conditions that generate the memory trace therefore match perfectly those that generate protein-synthesis dependent LTM. Indeed, six different training schedules were attempted and only spaced-forward conditioning produces the memory trace and long-term behavioral memory (Yu et al., 2006).

(2) The increased calcium influx observed in response to the $CS^+$ odor is also delayed, similar to the DPM neuron memory trace. The $\alpha/\beta$ neuron trace is undetectable at 3 hr after spaced conditioning but is observed by 9 hr and persists through 24 hr after conditioning. It decays by 48 hr after conditioning (Yu et al., 2006; Akalal et al., 2010).

(3) The increased calcium influx occurs only in response to the $CS^+$ odor and not to the $CS^-$ odor that is also presented during training but unpaired with the US.

(4) The $\alpha/\beta$ neuron LTM trace requires the normal function of the *amn* gene; the trace does not form in *amn* mutants. Interestingly, long-term behavioral memory is also strongly reduced in *amn* mutants, indicating that this disruption alters the memory trace and long-term behavioral memory in parallel and that long-term behavioral memory is dependent upon the *amn* gene product, either from the participation of *amn* in ITM and the potential serial organization between ITM and LTM or from a role for *amn* specifically to LTM processes, independent of any role in ITM (Yu et al., 2006).

(5) The $\alpha/\beta$ neuron LTM trace requires normal protein synthesis at the time of training. Feeding animals the drug cycloheximide blocks LTM formed from spaced conditioning (Tully et al., 1994; Yu et al., 2006) in parallel to the memory trace (Yu et al., 2006), yet has no effect on STM.

(6) The $\alpha/\beta$ neuron LTM trace requires the normal function of the transcription factor, CREB (Yu et al., 2006). Expression of a dominant negative form of CREB specifically in the $\alpha/\beta$ neurons blocks LTM formed from spaced conditioning in parallel to the memory trace and again, with no effect on STM.

(7) The $\alpha/\beta$ neuron LTM trace requires the normal function of the signaling enzyme calcium:calmodulin-dependent protein kinase II (CaMKII). Expression of a RNAi to CaMKII specifically in the $\alpha/\beta$ neurons blocks LTM formed from spaced conditioning in parallel to the memory trace, but again has no effect on STM (Akalal et al., 2010).

(8) Most interestingly, the increased calcium influx in response to the $CS^+$ odor after spaced conditioning occurs only in the vertical branch of the $\alpha/\beta$ neurons (Yu et al., 2006), similar to spatial organization of the DPM neuron memory trace. No changes in calcium influx occur after conditioning in the horizontal branch of these neurons, despite the fact that this memory trace is dependent upon transcription (CREB) and translation (protein synthesis inhibitors). An additional striking feature is that this anatomical specificity is aligned with the anatomical requirement of the vertical lobes for long-term behavioral memory (Pascual and Préat, 2001).

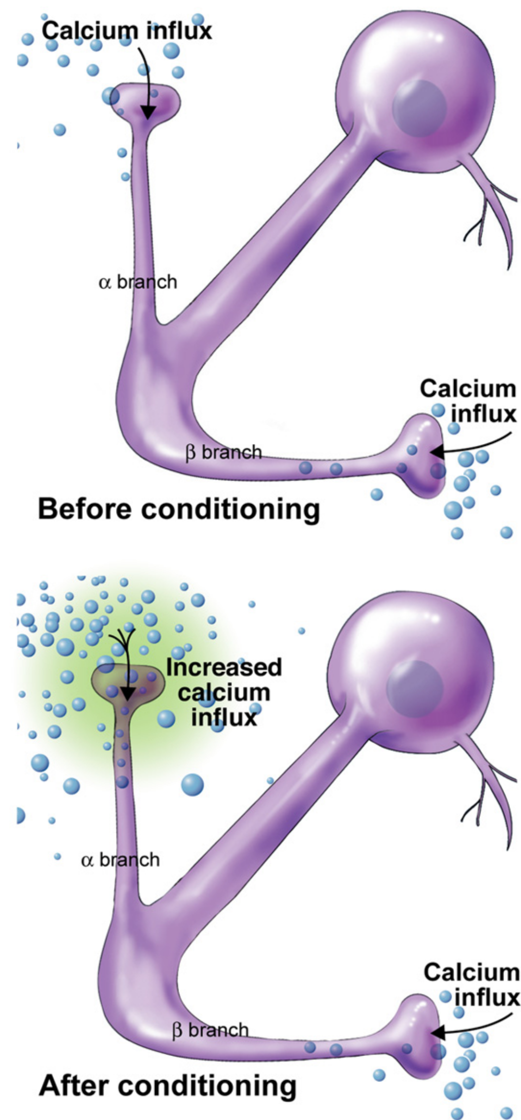


**Figure 8. A LTM trace, Detected as Increased Calcium Influx in Response to the Learned Odor, Forms in the $\alpha/\beta$ MBNs after Spaced Conditioning**
The increased calcium influx occurs only in the vertical ($\alpha$) branch of these neurons and not the horizontal ($\beta$) branch. A similar LTM trace forms in the unbranched axon of the $\gamma$ MBNs after spaced conditioning but becomes detectable only by 18 hr after spaced conditioning. The $\alpha/\beta$ LTM trace is detectable by 9 hr after spaced conditioning.

Thus, a LTM trace—reflected by increased calcium influx in response to the learned odor (Figure 8)—forms in the $\alpha/\beta$ neurons after spaced-forward conditioning and exists during the 9–24 hr window of time after conditioning. It forms only in the vertical branch ($\alpha$ branch) of these neurons, is dependent on protein synthesis at the time of training, and on the activity of CREB and CaMKII in these neurons. The parallel between long-term behavioral memory and the $\alpha/\beta$ neuron trace is most striking, given the specificity of training protocols required to generate them along with the parallel effects of four disruptive treatments.

A recent study sought to probe the mechanism underlying the α/β neuron LTM trace by assaying the trace in 26 different mutant lines that impair LTM but preserve STM (Akalal et al., 2011). The lines included mutations in genes that encode a wide variety of cellular components, including transcription factors, cell adhesion molecules, translational regulators, signaling enzymes, and several novel proteins. Unexpectedly, all 26 mutants exhibit a diminished LTM trace! It was anticipated that at least some would exhibit a normal memory trace with impaired long-term behavioral memory and therefore represent cellular functions downstream of those involved in trace formation, i.e., they would be involved in reading the trace to potentially drive behavior. Although no new insight into the mechanism of memory trace formation emerged from this experiment, these and prior results firmly indicate that the LTM trace formed in the α/β neurons is truly fundamental to long-term behavioral memory. When the effects of the 26 LTM mutants are added to the four disruptive treatments described above, the amazing conclusion is that there exist 30 disruptions that simultaneously impair both long-term behavioral memory and the LTM trace.

A second LTM trace was recently discovered to form in the γ MBNs (Akalal et al., 2010). This memory trace exhibits many of the same properties exhibited by the α/β neuron LTM trace: (1) it forms only after spaced conditioning, (2) it is detected only with the learned odor and not to odors unpaired with the US, (3) it requires the activity of CREB, and (4) it requires the activity of CaMKII. It occurs only in the one major axon of the γ MBNs since these neurons are unbranched. The major difference between the two LTM traces is their time of onset and offset. The α/β neuron LTM trace is detectable between 9 and 24 hr after spaced conditioning. The γ MB neuron LTM trace is detectable between 18 and 48 hr after spaced conditioning. Thus, the γ MB neuron LTM trace covers a later window of time after conditioning and is thus referred to as a late-phase, LTM trace.

One additional form of molecular plasticity has been reported that may be associated with long-term behavioral memory. Ashraf et al. (2006) constructed a reporter transgene encoding YFP but carrying the sequences from the CaMKII gene in the 3′UTR that confer dendritic localization on the mRNA. Animals carrying this transgene were subjected to spaced conditioning and 1 day later the amount of reporter gene product in glomeruli of the AL was quantified relative to untrained animals. An odorant-specific, training-dependent increase in synaptic protein synthesis was observed. When Oct was used as the $CS^+$, an increase in synaptic protein synthesis was observed in glomeruli D and DL3. When Mch was used as the $CS^+$, an increase in synaptic protein synthesis was observed in DA1 and VA1. Remarkably, the increased synaptic protein synthesis occurred in essentially the same glomeruli that are recruited into odorant representation immediately after training (Yu et al., 2004; see above). Thus, the early events within PNs that cause their recruitment into the representation of the learned odor may lead to later molecular processes that increase synaptic localization of specific mRNAs and synaptic protein synthesis.

## Discussion

A unique and important feature of olfactory classical conditioning using *Drosophila* is that the ongoing learning is relatively simple compared to other popular learning models, such as spatial learning or contextual learning in rodents and insects, or novel object recognition in rodents or humans. In these and many other popular learning models, the information learned is complex and relational or occurs through many sensory systems that are difficult to separate. This complexity creates significant difficulty in mapping the functions that underlie memory formation—such as acquisition, consolidation, retrieval, or the various temporal forms of memory—to discrete regions of the brain. Mapping these and other learning functions to the neuroanatomy is necessary for understanding the logic behind the organization of the learning network and for effectively probing and understanding the meaning of the many molecular and cellular changes that occur within nodes of the network. Olfactory classical conditioning in flies provides learning about a single association, the smell of an odor and a mild electric shock, and affords the possibility of mapping memory traces with functional optical imaging to specific nodes of the olfactory nervous system.

Within this broad rationale, an attractive model on one extreme is that the various memory traces formed in different nodes of the olfactory nervous system that occupy discrete windows of time after conditioning are responsible, in part, for the different temporal phases of memory (Figure 9). The earliest memories after conditioning may be represented by coexisting traces within three nodes of the network—the PNs of the ALs, the α′/β′ MBNs, and the GABAergic APL neurons. It seems possible that because the APL neurons may provide GABAergic innervation of the α′/β′ MBNs, that these two memory traces are interrelated with one another. Because the earliest detectable changes after conditioning were discovered in these three nodes, it is also possible that the process of acquisition occurs within one or all of these nodes, although it is not yet possible to exclude acquisition occurring in an alternative node with rapid transfer of the acquired information to these nodes. An intermediate temporal phase of memory may be represented by the memory trace observed in the DPM neurons, given its emergence and disappearance across the time window that generally defines ITM at the behavioral level. Long-term and protein synthesis-dependent memory may be represented by two (or three, if one counts the increased dendritic protein synthesis in the AL), temporally overlapping memory traces in two other nodes of the olfactory nervous system—the α/β MBNs and the γ MBNs. Provisionally, we have named the memory traces occurring in these nodes as a long-term and a late-phase, long-term memory trace, respectively. Thus, an emerging model to explain temporal phases of memory is that these forms of memory are underlain by multiple memory traces that form at discrete times after conditioning in discrete nodes of the olfactory nervous system.

The evidence that these memory traces are truly related to behavioral memory ranges from fair to exceptionally strong. The evidence tying the APL and PN traces to STM resides in their coincidence in time after conditioning and in the requirement for a temporal association of the CS and US. In other words, training protocols that decouple the presentation of the CS and US like backward conditioning, CS-only, US-only, etc., fail to generate short-term behavioral memory and fail to generate the memory traces. Therefore, the memory traces cannot be assigned as

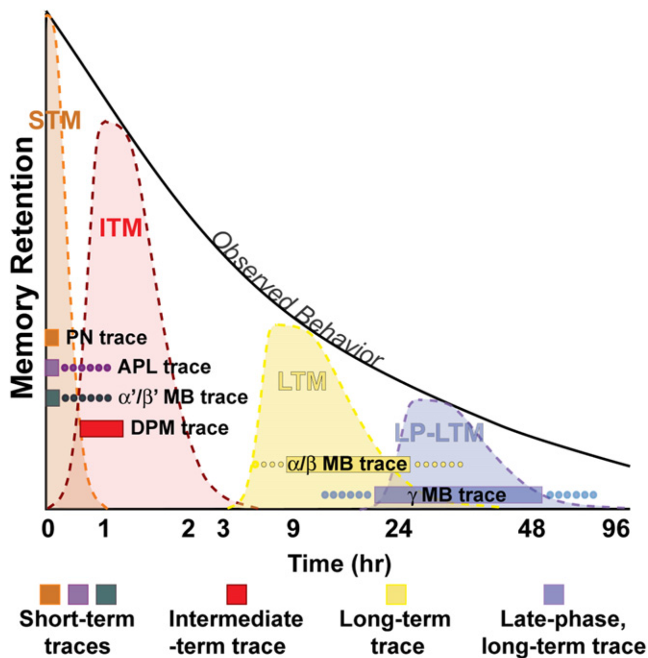


**Figure 9. Model for How Memory Traces May Underlie Temporal Phases of Memory after Olfactory Classical Conditioning**
*Drosophila* olfactory memory persists for at least 4 days after spaced conditioning and is thought to be comprised of short-term memory (STM), intermediate-term memory (ITM), long-term memory (LTM), and late-phase long-term memory (LP-LTM). These phases of behavioral memory exist across different windows of time after conditioning, as depicted in the figure. The coexisting traces that may underlie short-term memory after conditioning include a trace that forms in the AL PNs (0–5 min), a trace in the GABAergic neuron APL (0–>5 min), and a trace that forms in the α′/β′ neurons (0–>1 hr). Intermediate-term behavioral memory from 30–70 min after conditioning is associated with a memory trace that forms in the DPM neurons. Long-term memory generated by spaced conditioning may be underlain by two memory cellular memory traces. A LTM trace forms in the α/β MBNs by 9 hr after spaced conditioning and persists to at least 24 hr. This memory trace is dependent on normal protein synthesis, CREB, CaMKII, the *amnesiac* gene product, and the gene products of 26 different genes involved in long-term behavioral memory. The most persistent memory trace discovered to date forms in the γ MBNs. This trace, which is associated with late-phase, LTM, forms by 18 hr after spaced conditioning and persists to at least 48 hr. Thus, temporal forms of behavioral memory are associated with different cellular memory traces that form in the olfactory nervous system and occupy different windows of time after conditioning.

emerging from nonassociative, experience-dependent plasticity. In addition to time window coincidence and training protocol-dependence, the DPM memory trace is tied to ITM with results from experiments that block synaptic transmission from these neurons and ascertain the effects of the blockage on later memory. As described above, blocking synaptic transmission over the time window of 30- < 150 min (the endpoint has not yet been accurately established) after conditioning impairs 3 hr memory. This time window is very similar to the time window over which the DPM trace is detectable (30-70 min). Furthermore, the DPM memory trace is tied to ITM through the *amn* mutant: *amn* mutants do not form the DPM memory trace, they are impaired in ITM, and the introduction of a normal copy of the *amn* gene into the DPM neurons of an otherwise *amn* mutant animal rescues both ITM and the memory trace. With the time window coincidence, training-protocol dependence, and the disruption with the *amn* mutant and subsequent rescue of both behavioral memory and cellular memory trace, the strength of evidence tying the DPM memory trace to ITM is very strong. The evidence is also very strong for the argument that the γ MB neuron trace is relevant to late-phase LTM. Time window coincidence, training-protocol dependence, and bumping the system in two different ways —reducing CREB and CaMKII activity—alters both memory trace and long-term behavioral memory in parallel. The conclusion that the LTM trace of the α/β MBNs is fundamental to long-term behavioral memory is inescapable. Time window coincidence, training-protocol dependence, and 30 disruptions that alter the memory trace and long-term behavioral memory in parallel tie these together and elevate this trace to arguably the most convincing memory trace relevant to behavior discovered in any organism to date.

The other extreme to the model presented above is that perhaps each node forms traces representing all temporal forms of behavioral memory, such that each node would have at least one trace representing STM, ITM, and LTM. This requires that all of the neurons have the capability of forming multiple temporal forms of memory, but there are precedents for this. *Aplysia* sensory neurons are capable of forming short-term, intermediate-term, and long-term facilitation in response to the application of serotonin, although the mode of induction determines which types of plasticity will emerge (Stough et al., 2006; Puthanveettil and Kandel, 2011). Similarly, different temporal forms of synaptic plasticity are evident in the hippocampus depending on the type of stimulation used to produce the plasticity (Roberson et al., 1996; Sacktor, 2008). Of course, the ability of individual neurons to form different temporal forms of synaptic plasticity does not necessarily mean that this expansive role will be adopted when in the context of the brain of a behaving animal.

Do the memory traces described above drive behavior over the window of time of their existence? This critical question, of course, is extremely difficult to answer with current technology. It would be necessary to implant the memory trace in some artificial way and then determine whether the organism exhibited behavioral memory. Although progress has been made in activating neural circuits used for memory formation using optogenetic approaches (Schroll et al., 2006; Claridge-Chang et al., 2009), the advances made to date have been limited to activating circuitry representing the reinforcer rather than the sensory information that is learned, which may be represented in a more complex way by the nervous system. It is likely that an understanding of the mechanisms by which the memory traces are generated will be needed before approaching the aforementioned question. For instance, one hypothesis for explaining the increased calcium influx into the α branch of the α/β MBNs after spaced conditioning is that such training causes an increase in local protein synthesis and the insertion of additional voltage-dependent calcium channels. If true, then an artificial way of producing this effect would be needed to show that the memory trace drives behavior. Little is currently known about the mechanisms by which these the various traces are generated. This is clearly a prime area of exploration for the future.

Another open question is whether the memory traces are generated in parallel and independently of one another or

whether they are generated in serial with later forming traces being dependent on the formation of early traces. Only one observation has been made relative to this issue: the DPM neuron memory trace fails to form in the *amn* mutant, and the LTM trace of the $\alpha/\beta$ MBNs fails to form in this mutant. This observation is consistent with the possibility that the formation of the $\alpha/\beta$ MBN LTM trace is dependent on the earlier formation of the DPM neuron memory. However, too little evidence is currently available to make a convincing argument for either serial or parallel modes of formation. Although the bias in the field is to emphasize serial formation, it should be noted that there exists significant evidence for parallel processing (McGaugh, 2000). Prior studies using invertebrate and vertebrate systems have revealed that late forms of synaptic plasticity and memory can form in the absence of earlier forms (Emptage and Carew, 1993; Mauelshagen et al., 1996; Grünbaum and Müller, 1998; Sherff and Carew, 2004; Sossin, 2008). For instance, serotonin application that is restricted only to the cell bodies of sensory neurons generates long-term facilitation in the absence of short- and intermediate-term facilitation. Ho et al. (2007) reported that LTM of olfactory learning forms in the absence of STM in flies expressing the GAP-related domain of neurofibromin, whereas the C-terminal domain of neurofibromin is required for STM.

It is important to note that the cellular memory traces described above must be a small subset of the changes that occur due to learning. At present, the most reliable and thoroughly characterized optical reporters for monitoring changes due to learning detect changes in calcium influx (e.g., G-CaMP) or synaptic release (synapto-pHluorin). It could be that calcium influx is well downstream in the series of physiological changes that occur due to learning and may even prove to be the optimal surrogate for evaluating where changes in activity occur, but it could also be that plasticity of calcium influx is not a currency valued highly within the memory trace market, making any model emphasizing calcium-based traces as much too simplistic. Indeed, molecular genetic evidence is consistent with the idea that there are cellular traces that have gone undetected to date: The *rutabaga*-encoded adenylyl cyclase is required for acquisition of memory (Yu et al., 2005; Buchanan and Davis, 2010); it can function as a biochemical coincidence detector of CS and US stimuli consistent with a role in acquisition (Tomchik and Davis, 2009), and its function in acquisition is required in the $\alpha/\beta$ and $\gamma$ neurons of the MBs (Akalal et al., 2006). Thus, it seems likely that there exist STM traces in these neurons that have not yet been detected.


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