Neuron, Vol. 44, 521–533, October 28, 2004, Copyright ©2004 by Cell Press

Diverse Odor-Conditioned Memories Require Uniquely Timed Dorsal Paired Medial Neuron Output

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Summary

***amnesiac* mutant flies have an olfactory memory defect. The *amn* gene encodes a homolog of vertebrate pituitary adenylate cyclase-activating peptide (PACAP), and it is strongly expressed in dorsal paired medial (DPM) neurons. DPM neurons ramify throughout the mushroom bodies in the adult fly brain, and they are required for stable memory. Here, we show that DPM neuron output is only required during the consolidation phase for middle-term odor memory and is dispensable during acquisition and recall. However, we found that DPM neuron output is required during acquisition of a benzaldehyde odor memory. We show that flies sense benzaldehyde by the classical olfactory and a noncanonical route. These results suggest that DPM neurons are required to consolidate memory and are differently involved in memory of a volatile that requires multisensory integration.**

Introduction

Smelling influences the behavior of many animals. Olfactory cues are used for communication between animals, to find mates, and to avoid predation. Some odors, such as pheromones, have innate meaning. Others can be learned to become predictors of pleasant or unpleasant circumstance. Understanding how olfactory cues are perceived, integrated with other sensory cues, and stored as memories in the brain is a focus of considerable attention.

Drosophila melanogaster is an excellent model system to study the molecular and neural basis of olfactory perception and olfactory learning (Keller and Vosshall, 2003; Heisenberg, 2003). Olfactory memory-defective *Drosophila* mutants have been isolated using a paradigm in which flies associate an odor with electric shock punishment (Quinn et al., 1974; Tully and Quinn, 1985). The molecular characterization and anatomical localization of the affected gene products has highlighted the cyclic AMP cascade and the mushroom bodies (MBs) as key factors of olfactory memory (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993). The MBs are third order neurons of the olfactory system. Odors are sensed by olfactory sensory neurons in the antennae and maxillary palps. Sensory neurons that express the same odorant receptor project axons to bilaterally symmetrical structures called glomeruli in the antennal lobe of the fly brain (Vosshall et al., 2000; Gao et al., 2000; Scott et al., 2001). From there, projection neurons in two tracts relay information to the mushroom bodies (MBs) and the lateral horn (Heimbeck et al., 2001; Wong et al., 2002; Komiyama et al., 2003). The MBs are required for olfactory learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994), and a functional cAMP cascade in the adult fly MBs is required and sufficient for olfactory memory (Zars et al., 2000; McGuire et al., 2003). Strikingly, MB output is required during retrieval of olfactory memory but is dispensable during acquisition and storage (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). These data are consistent with the odor memories being represented in the presynaptic terminals of MB neurons.

Analysis of the *amnesiac* (*amn*) mutant uncovered another critical part of the memory circuit (Quinn et al., 1979; Waddell et al., 2000). The *amn* gene encodes a predicted preproneuropeptide with homology to mammalian pituitary adenylate cyclase-activating peptide (PACAP) (Feany and Quinn, 1995; Moore et al., 1998), and it is highly expressed in dorsal paired medial (DPM) neurons—large putative neuromodulatory neurons that ramify throughout the MB lobes (Waddell et al., 2000). *amn* mutant memory can be rescued with *amn* expression in DPM neurons (Waddell et al., 2000; Tamura et al., 2003), and blocking DPM output causes *amn*-like memory loss (Waddell et al., 2000). It is therefore plausible that DPM release of AMN peptide onto the MBs contributes to memory persistence. Here, we have determined the precise temporal requirement for DPM output in olfactory memory.

The vast majority of the memory mutants were isolated using a single odor pair—3-octanol (OCT) and 4-methylcyclohexanol (MCH) (Dudai et al., 1976; Quinn et al., 1979; Livingstone et al., 1984; Choi et al., 1991; Boynton and Tully, 1992; Dura et al., 1993; Folkers et al., 1993; DeZazzo et al., 2000; Dubnau et al., 2003). 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. Benzaldehyde (BA) is used by some groups in a BA-OCT combination (Skoulakis and Davis, 1996; Grotewiel et al., 1998; Cheng et al., 2001; Schwaerzel et al., 2002) or BA-MCH (Guo et al., 2000; Zars et al., 2000), 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 mem-

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ory 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 (Quinn et al., 1979; Tully and Gergen, 1986; DeZazzo et al., 1999; Waddell et al., 2000; Tamura et al., 2003). We previously demonstrated that blocking DPM neurons did not affect immediate memory 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 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 BA memory.

Results

amn Is Not Required for DPM Neuron Specification and MB Targeting

Our demonstrated acute role for DPM neurons in memory (Waddell et al., 2000) led us to investigate whether the memory defect of *amn* mutant flies results from the absence of DPM neurons.

We used confocal microscopy to analyze DPM morphology in *amn* mutant fly brains by driving a uas-mCD8:GFP transgene with the DPM driver c316{GAL4} (Figure 1A). For these experiments, we used *amn*^{1}, a strong behavioral allele that has not been molecularly characterized in detail, as well as two new *amn* alleles, *amn*^{ex1} and *amn*^{ex39}, generated here by imprecise excision of the single P element in the *amn*^{c651} mutant (Waddell et al., 2000). The *amn*^{ex1} and *amn*^{ex39} are not predicted to produce any functional AMN peptide (Figure 1F).

We found that DPM neurons are present in *amn* mutants ($n > 10$ per genotype; Figure 1B shows a typical *amn*^{ex1} brain). In both wild-type and *amn* mutant fly brains, each DPM neuron sends a single large-diameter neurite toward the MB lobes. The neurite splits and projects to the vertical and horizontal MB lobes. These neurites further divide and extend toward the vertically arranged α and α' lobes and the horizontally arranged β, β', and γ lobes. 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 *amn* is not essential for DPM targeting to the MBs during development. Furthermore, these data imply that *amn* mutant memory 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.

Throughout this study, we have primarily analyzed DPM neurons in memory using the c316{GAL4} fly line (Figure 1A). c316 mostly expresses GAL4 in DPM neurons, and blocking transmission from these neurons produces an *amn*-like memory defect (Waddell et al., 2000). This is currently the most specific driver line available to investigate DPM neuron function. In some experiments, we have also used a less specific line, Mz717 {GAL4} (Ito et al., 1998; Figure 1E) that labels DPM neurons and has additional expression in the antennal lobe. The c316 and Mz717 lines express GAL4 in the same DPM neurons because only two DPM neurons are visible when the driver lines are combined (data not shown). Other than DPM neurons, there is no obvious overlap between the neurons labeled in c316 and Mz717.

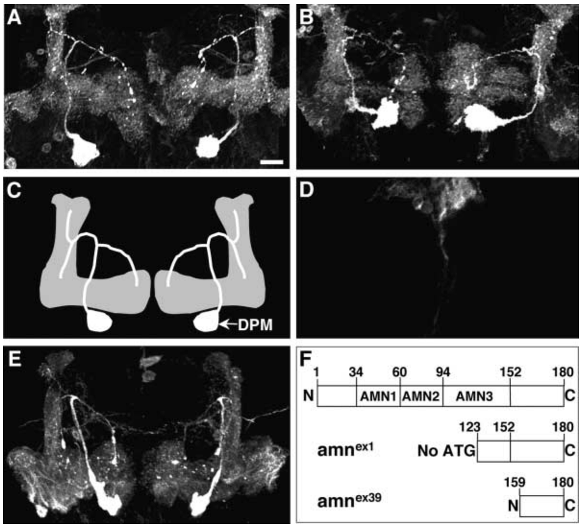

Figure 1. DPM Neurons Are Present in *amn* Mutants and Are Probably Cholinergic

(A) Morphology of DPM neurons in a wild-type fly brain revealed by driving mCD8:GFP with c316{GAL4}. The scale bar is 10 μm.

(B) Morphology of DPM neurons in an *amn*^{ex1} fly brain.

(C) Schematic representation of DPM neuron projections. Each neuron ramifies throughout the ipsilateral mushroom body lobe set, shown as gray.

(D) *Cha*$^{3.3kb}$-GAL80 represses c316{GAL4} activity in DPM neurons. Visible labeling is air sac material.

(E) Morphology of DPM neurons in a wild-type fly brain revealed by driving mCD8:GFP with Mz717{GAL4}.

(F) 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.

Although blocking DPM neurons produces an *amn*-like memory defect (Waddell et al., 2000), it is not known whether *shibire*^{ts1} affects dense core vesicle (and, by extension, AMN peptide) release. We therefore asked whether DPM neurons corelease a fast-acting transmitter. Because acetylcholine (ACh) is the predominant transmitter of the *Drosophila* central nervous system (CNS), we performed a genetic experiment to test if DPM neurons are cholinergic. The choline acetyltransferase gene (*Cha*) is expressed in large subsets of cholinergic neurons, and a *Cha* promoter construct (*Cha*$^{3.3kb}$) drives expression of transgenes in these cholinergic neurons (Kitamoto et al., 1992, 1995; Kitamoto, 2002). We generated flies carrying a *Cha*$^{3.3kb}$ promoter driving expression of GAL80, a GAL4 repressor (Kitamoto, 2002), and our c316{GAL4} driver. We reasoned that if DPM neurons are cholinergic, *Cha*$^{3.3kb}$-GAL80 would inhibit c316{GAL4}-

driven reporter expression. We analyzed brains of mCD8:GFP;*Cha*$^{3.3kb}$-GAL80/c316{GAL4} flies for GFP expression in DPM neurons. Figure 1D shows that *Cha*$^{3.3kb}$-GAL80 completely suppresses GAL4 activity in DPM neurons ($n = 14$ brains). These data suggest that DPM neurons express *Cha* and are therefore likely cholinergic.

DPM Neuron Output Is Required during Consolidation for 3 Hr OCT-MCH Memory and Is Dispensable during Acquisition and Retrieval

In this study, we use the 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 (Figures 3D–6), we present half scores to highlight odor-specific effects. Until then, all data presented for OCT and MCH memory are average scores from reciprocal 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 *shibire*^{ts1} transgene, uas-*shi*^{ts1} (Kitamoto, 2001), in DPM neurons using the c316{GAL4} or Mz717{GAL4} DPM drivers (Waddell et al., 2000; Ito et al., 1998). The *shi* gene encodes a dynamin that is essential for endocytosis and synaptic vesicle recycling (van der Bliek and Meyerowitz, 1991; Chen et al., 1991). The *shi*^{ts1} allele has a vesicle recycling defect above 29°C that results in a rapid cessation of synaptic transmission (Koenig and Ikeda, 1989). High-temperature inactivation of *shi*^{ts1} is reversible and allows 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 nonconditional *amn* mutant.

We previously showed that blocking DPM output throughout an entire operant olfactory conditioning experiment (Quinn et al., 1974) did not affect learning (3 min memory) but abolished 1 hr 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 hr memory experiments at 25°C (at which temperature we expected the neurons to function normally) and 31°C (under which conditions the *shi*^{ts1}-expressing neurons were expected to be synaptically silent). In each experiment, we compared the performance of c316;uas-*shi*^{ts1} double transgenic flies to wild-type and single transgenic c316 and uas-*shi*^{ts1} control flies. uas-*shi*^{ts1} 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 *amn*^{X8} flies to illustrate the effect of a null *amn* allele on 3 hr memory. At the permissive temperature of 25°C, both immediate (3 min) memory (wild-type $= 0.64 \pm 0.02$; c316;uas-*shi*^{ts1} $= 0.63 \pm 0.04$; uas-*shi*^{ts1}$= 0.63 \pm 0.03$) and 3 hr memory of c316;uas-*shi*^{ts1} flies were statistically indistinguishable ($p > 0.7$) from wild-type, c316, and uas-*shi*^{ts1} control flies, while all groups showed greater memory than *amn*^{X8} mutant flies ($p < 0.02$) (Figure 2A). At the restrictive temperature of 31°C, immediate (3 min) memory of c316;uas-*shi*^{ts1} flies (0.67 ± 0.04) was statistically indistinguishable ($p > 0.7$) from wild-type (0.69 ± 0.02), and uas-*shi*^{ts1} flies (0.66 ± 0.04). However, 3 hr memory was statistically lower ($p < 0.01$ for all groups) than wild-type, c316, and uas-*shi*^{ts1} flies and statistically indistinguishable ($p > 0.7$) from that of *amn*^{X8} mutant flies (Figure 2B). These results are consistent with our previous findings (Waddell et al., 2000) and demonstrate that DPM output is required for 3 hr but not for short-term OCT-MCH memory.

We next used the reversibility of uas-*shi*^{ts1} to test whether DPM output during training (Figure 2C) or testing (Figure 2D) was required for memory. To block DPM neuron output during training, we incubated c316;uas-*shi*^{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 hr memory was tested at 25°C. Blocking DPM output during training did not affect memory. The memory of c316;uas-*shi*^{ts1} flies was indistinguishable ($p > 0.9$) from uas-*shi*^{ts1} control flies that were trained at the restrictive temperature (Figure 2C). Therefore, memory acquisition does not require output from DPM neurons.

We similarly tested whether DPM output was required during memory recall (Figure 2D). We trained flies at 25°C, and 15 min before testing 3 hr memory we inactivated DPM neurons by shifting the flies to the restrictive temperature of 31°C. The 3 hr memory of c316;uas-*shi*^{ts1} flies was again indistinguishable ($p = 0.8$) from the uas-*shi*^{ts1} transgene control flies, suggesting that DPM output is not required for memory recall.

We also tested whether blocking DPM output during training and testing (Figure 2E) 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-*shi*^{ts1} control flies. Therefore, DPM output is not essential during training and testing for 3 hr OCT-MCH memory.

We finally tested whether DPM output was required in the period between training and testing (Figures 2F

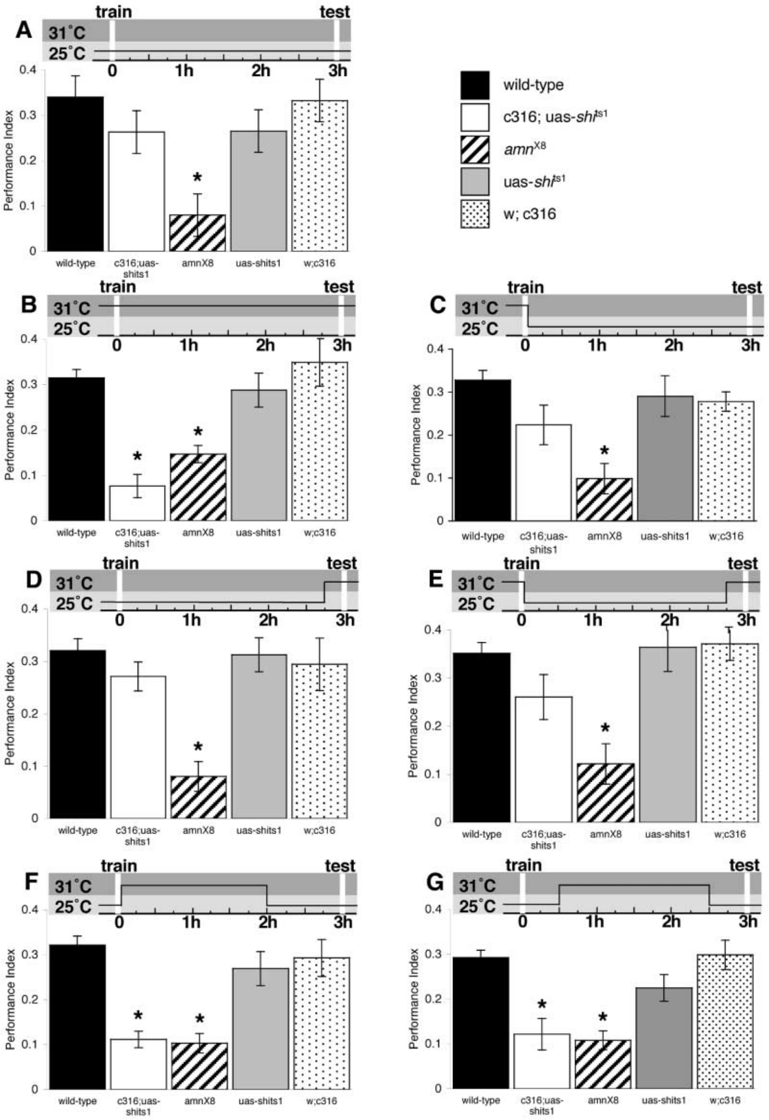

Figure 2. DPM Activity between Training and Testing Is Required for 3 Hr OCT-MCH Memory

All temperature shift protocols described below are shown pictographically above each graph. In each experiment, the genotypes shown were treated identically. (A) The permissive temperature of 25°C does not affect c316;uas-shi^{ts1} flies. All genotypes were trained and tested for 3 hr memory at 25°C. (B) Disrupting DPM output at the restrictive temperature of 31°C abolishes memory. All genotypes were trained and tested for 3 hr memory at 31°C. (C) Blocking DPM output during training does not affect 3 hr memory. Flies were incubated at 31°C for 15 min prior to and during training. Immediately after training, they were returned to 25°C and tested for 3 hr memory. (D) Blocking DPM output during testing does not affect 3 hr memory. Flies were trained at 25°C, and 165 min later they were shifted to 31°C. Fifteen minutes later, 3 hr memory was tested at 31°C. (E) Blocking DPM output during training and testing does not affect 3 hr memory. Flies were incubated at 31°C for 15 min prior to and during training. Immediately after training, they were returned to 25°C, and 165 min later they were shifted back to 31°C. Fifteen minutes later, 3 hr memory was tested at 31°C. (F and G) Blocking DPM output between training and testing impairs 3 hr memory. Flies were trained at 25°C, and immediately (F) or 30 min after training (G) they were shifted to 31°C for 2 hr. Flies were then returned to 25°C and tested for 3 hr memory at 25°C.

and 2G). This is the expected window of time in which memories become consolidated (Quinn and Dudai, 1976; Folkers et al., 1993; Tully et al., 1994). We trained flies at 25°C, and immediately following training we shifted them to 31°C for 2 hr. We then returned the flies to 25°C and tested them 1 hr later for 3 hr memory. Blocking DPM output between training and testing produced a dramatic loss of memory to levels statistically indistinguishable ($p = 1$) from that of *amn*^{X8} flies (Figure 2F). Therefore, DPM output is required between training and testing for 3 hr memory. We next tested whether blocking DPM output at later time points disrupted 3 hr memory. We delayed our 2 hr DPM blockade by 30 min into the middle of the experiment (Figure 2G). Blocking DPM output 30 min after training for 2 hr produced the same memory impairment as blocking output immediately after training.

To control for a nonspecific memory deficit produced by blocking DPM output for 2 hr at any point in the experiment, we incubated flies at 31°C for 2 hr, then returned them to 25°C and trained them 15 min later. This manipulation had no effect on 3 hr memory (wild-type flies = 0.33 ± 0.02; c316;uas-shi^{ts1} flies = 0.29 ± 0.06; $p = 0.4$). Therefore the intermediate 2 hr 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 at least 30 min after training is required for wild-type 3 hr OCT-MCH memory, consistent with the idea that DPM neurons are involved in memory consolidation.

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 nonclassical route (Figure 3).

We tested whether BA avoidance behavior was de-

Table 1. Sensory Acuity Controls

Genotype	Temperature	OCT Acuity	MCH Acuity	BA Acuity	Shock Avoidance
wild-type	25°C	PD	PD	71 ± 6	73 ± 2
c316;uas-*shi*^{ts1}	25°C	PD	PD	81 ± 2	72 ± 2
Mz717;uas-*shi*^{ts1}	25°C	83 ± 3	83 ± 4	81 ± 7	72 ± 6
uas-*shi*^{ts1}	25°C	PD	PD	70 ± 6	65 ± 3
amn^{X8}	25°C	PD	PD	75 ± 6	70 ± 2
c316	25°C	PD	PD	69 ± 9	63 ± 2
wild-type	31°C	86 ± 3	89 ± 2	91 ± 3	83 ± 2
c316;uas-*shi*^{ts1}	31°C	79 ± 4	89 ± 3	81 ± 4	76 ± 4
Mz717;uas-*shi*^{ts1}	31°C	85 ± 5	83 ± 5	88 ± 3	62 ± 2
uas-*shi*^{ts1}	31°C	90 ± 5	92 ± 3	85 ± 2	71 ± 5
amn^{X8}	31°C	69 ± 2	77 ± 7	84 ± 5	76 ± 7
c316	31°C	76 ± 11	86 ± 6	85 ± 6	79 ± 3

PD, previously determined. Our earlier studies (Waddell et al., 2000) showed that the olfactory acuity of these strains is not significantly different at 25°C.

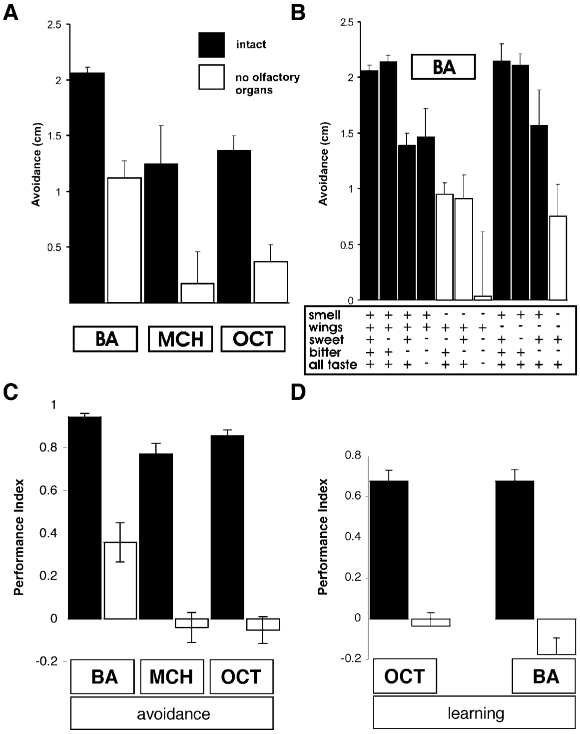

Figure 3. BA Is a Multimodal Stimulus

(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 *poxn*$^{70\text{-}23}$*/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 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.

pendent 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 3A and 3B) and the T maze used for olfactory learning (Figure 3C). Strikingly, significant BA responses were measured in both behavioral paradigms in the absence of olfactory organs, but OCT and MCH avoidance was abolished (Figures 3A, 3B, and 3C). This result 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 3B). 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 (Wang et al., 2004). Ablating ~30 sweet-sensitive gustatory neurons (Gr5a-driven ablation) did not affect the response to BA, whereas ablating ~25 bitter-sensitive gustatory neurons (Gr66a-driven ablation) significantly reduced BA avoidance. 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., 2004), 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 3A, 3B, and C), 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 3C). Unlike naive 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 4A). The half score data revealed that, whereas wild-type flies learned well with OCT and BA, OCT learning of *amn*^{X8} flies was indistinguishable ($p = 1$) from wild-type flies but BA learning was greatly reduced ($p < 0.01$) (Figure 4A).

It has previously been reported that *amn*^{1} 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*^{X8} mutant flies both before and after electric shock.

Prior to conducting a learning experiment, the odors are balanced so that naive flies distribute evenly between the odors. Wild-type flies and *amn*^{X8} mutant flies distributed evenly between BA and OCT prior to shock (Figure 4B). 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 s, 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*^{X8} mutant flies. Therefore, the BA learning defect of *amn*^{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*^{X8} mutant flies and tested avoidance of BA, MCH, and OCT (Figure 4C). *amn*^{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.

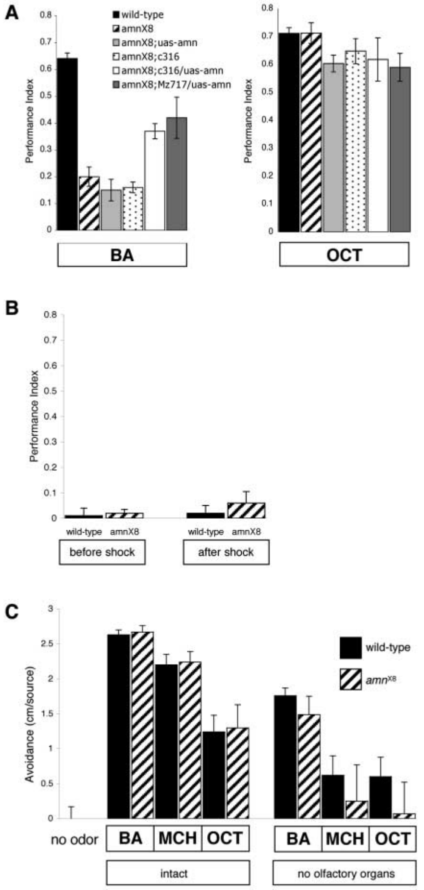

Figure 4. *amn* Mutant Flies Have a BA Learning Defect that Is Partially DPM Neuron Dependent

(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 *amn* gene in DPM neurons (*amn*^{X8};c316/uas-*amn* and *amn*^{X8};Mz717/uas-*amn* flies) partially rescues the BA memory defect of *amn* mutant flies.

(B) Olfactory acuity of wild-type and *amn*^{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) *amn*^{X8} mutant flies without olfactory organs retain BA avoidance. Wild-type flies and *amn*^{X8} mutant flies with or without olfactory organs were tested for OCT, MCH, and BA avoidance in the arena apparatus.

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 *amn*^{X8};c316/uas-*amn* and *amn*^{X8};Mz717/uas-*amn* flies and tested BA and OCT immediate memory (Figure 4A). The *amn*^{X8};c316/uas-*amn* and *amn*^{X8};Mz717/uas-*amn* flies learned to avoid BA significantly better than *amn*^{X8} 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 con-

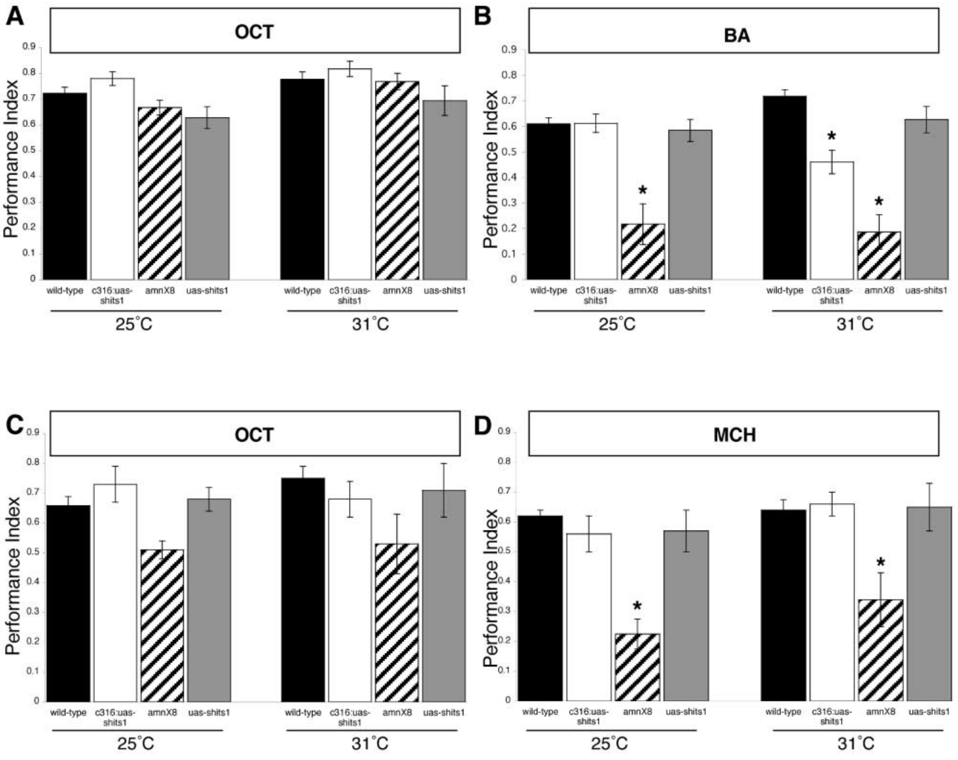

Figure 5. Blocking DPM Output Impairs BA Learning

(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.

trast, OCT immediate memory of *amn*^{X8} flies was indistinguishable from wild-type flies and *amn*^{X8};c316/uas-*amn* or *amn*^{X8};Mz717/uas-*amn* flies. This result implies that DPM neurons are involved in BA learning.

Blocking DPM Output Impairs BA Learning

We tested if directly blocking DPM output impaired BA learning (Figure 5). We expressed uas-*shi*^{ts1} 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 31°C. At 25°C, the BA learning scores of all genotypes, except *amn*^{X8}, were not statistically different ($p > 0.1$) (Figures 5A and 5B). However, blocking DPM output with c316;uas-*shi*^{ts1} specifically reduced BA immediate memory ($p < 0.01$) (Figure 5B) and left OCT immediate memory intact ($p > 0.2$) (Figure 5A). Crucially, the uas-*shi*^{ts1} control flies do not have a defect with BA or OCT at 31°C (Figures 5A and 5B). For comparison, we also tested whether blocking DPM output impaired OCT and MCH immediate memory (Figures 5C and 5D). The OCT and MCH performance of c316;uas-*shi*^{ts1} 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 *amn*^{X8} 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

DPM output is required to stabilize OCT-MCH memory but is not required during acquisition of these odor memories (Figures 2F and 2G). Having observed a significant BA learning defect when we blocked DPM output (Figure 5B), we tested whether DPM output was required during acquisition of BA memory (Figure 6). We blocked DPM neuron output 15 min before training by incubating c316;uas-*shi*^{ts1} and Mz717;uas-*shi*^{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 function. We tested olfactory memory 1 hr later and again analyzed individual odor half scores separately. Blocking DPM output during acquisition did not affect 1 hr OCT memory: c316;uas-*shi*^{ts1} and Mz717;uas-*shi*^{ts1} fly memory was indistinguishable ($p > 0.8$ for both genotypes) from wild-type (Figure 6A). However, DPM blockade in c316;uas-

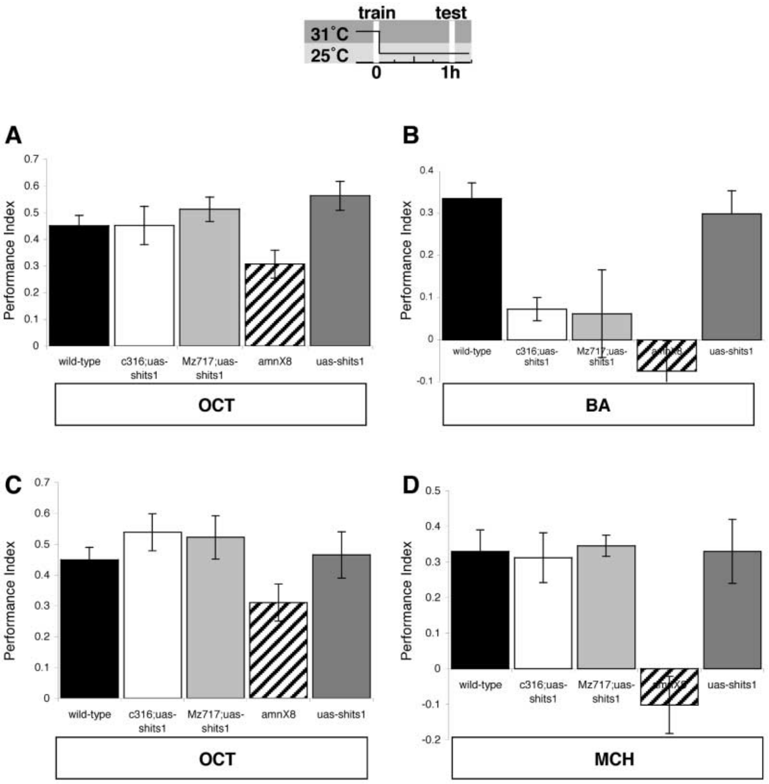

Figure 6. Blocking DPM Output during Acquisition Impairs BA Memory

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 hr 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.

shi^{ts1} and Mz717;uas-*shi*^{ts1} flies severely impaired 1 hr BA memory ($p < 0.05$ for both) (Figure 6B). In contrast, DPM blockade during acquisition did not significantly affect memory with OCT-MCH (Figures 1C, 6C, and 6D). Furthermore, olfactory acuity and the response to electric shock of c316;uas-*shi*^{ts1} and Mz717;uas-*shi*^{ts1} flies were unaffected by temperature (Table 1). Therefore, neurons expressing GAL4 in c316 or Mz717 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, we previously identified the DPM neurons—large putative modulatory neurons that innervate the mushroom bodies—as being the critical site of *amn* function in olfactory memory (Waddell et al., 2000). Using uas-*shibire*^{ts1} (Kitamoto, 2001) as a temperature-sensitive blocker of DPM neuron function, we showed that DPM output was not required for OCT-MCH learning but was required for extended (up to 1 hr) memory (Waddell et al., 2000). In this report, we demonstrate that DPM output is dispensable during training and recall for 3 hr OCT-MCH memory (Figures 2C, 2D, and 2E). Strikingly, DPM output is required at least 30 min into the period between training and testing (Figures 2F and 2G). 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 that of MB neuron output. MB output is not required for acquisition or during storage but is required for memory recall (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). DPM neuron output is required during storage but is dispensable during acquisition and recall (Figure 2). Therefore, DPM neuron action on the MBs does not occur at a time when output from the MBs is required. This suggests that DPM-dependent memory processes in the MBs do not rely on transmitter release from MB synapses.

How Is AMN Involved?

Blocking DPM output with the uas-*shi*^{ts1} transgene produces a similar effect to 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 (Quinn et al., 1979; Tully and Gergen, 1986; DeZazzo et al., 1999). These data are consistent with the possibility that AMN peptides contribute to the memory process.

In support of an acute role for AMN peptides in odor memory, we found that DPM neurons are present in *amn*^{1}, *amn*^{ex1}, and *amn*^{ex39} mutant flies (Figure 1). The main branches of the major neurite are present, and the lobes of the MBs are ensheathed in the characteristic putative DPM-MB synapses. Therefore, *amn* is not required for DPM neuron survival or for the gross targeting of DPM neurons to the MB lobes. In addition, our behavioral analysis shows that DPM neurons are acutely required for memory. Restricting *amn* gene expression spatially and temporally with TARGET (McGuire et al., 2003) or GeneSwitch (Osterwalder et al., 2001; Roman et al., 2001) should resolve whether *amn* has an acute function in adult flies and/or is involved in development.

Although blocking DPM neuron output with uas-*shi*^{ts1} produces an *amn* mutant-like memory defect (Waddell et al., 2000; and this study), it is not known if the *shi*^{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-*shi*^{ts1} blocks AMN release and/or blocks release of an essential cotransmitter.

It is plausible that AMN peptides are coreleased from DPM synapses with a classical fast-acting transmitter. Glutamate is used in the CNS of *Drosophila*, but it is not the predominant transmitter (Strausfeld et al., 2003). Instead, this role appears to be taken by acetylcholine (Buchner et al., 1986; Gorczyca and Hall, 1987). We showed that a DPM neuron marker is coexpressed with a cholinergic neuron-specific marker (Figure 1D), suggesting that the DPM cotransmitter is Ach. Assuming that DPM neurons corelease Ach and AMN transmitters, DPM neuron release would trigger a postsynaptic response in receptive MB neurons that involves Ach receptors and AMN receptors. Genetic and pharmacological experiments suggest that the *amn* gene affects cAMP synthesis (Feany and Quinn, 1995; Moore et al., 1998), and we previously 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 (Kawatani et al., 1985; Gurantz et al., 1994; Margiotta and Pardi, 1995; Liu et al., 2000). In a hippocampal slice preparation, PACAP38 enhances excitatory CA3-CA1 synaptic transmission, and the facilitation can be blocked by inhibiting muscarinic receptors (Roberto and Brunelli, 2000; Roberto et al., 2001). Perhaps AMN peptides 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 OCT or MCH (Figure 6). Therefore, the temporal requirements for DPM output show some odor specificity. This finding implies that DPM neurons may be differentially involved in odor memory.

Why Is BA Different?

Our learning experiments suggested that BA might be unique for flies. Importantly, our data (Figures 3B and 3C) 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 drastically effective insecticide (Dettner et al., 1992) and also a potent trigeminal stimulus in humans (Doty et al., 1978). Therefore, our 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; Dunipace et al., 2001; Scott et al., 2001; Thorne 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 will be initially processed by distinct brain regions. Antennal and palp input projects to the antennal lobe (Gao et al., 2000; Vosshall et al., 2000), but labelar gustatory neurons project to the subesophageal ganglion (Thorne et al., 2004; Wang et al., 2004). 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 *acj6* 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 *acj6*. *ptg*^{7} 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 Gqα 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 nonagitated naive ants (Blum et al., 1969). However, we have no evidence that BA is a constituent of a similar emission in *Drosophila*.

DPM Neurons and Odor Memories

In conclusion, the results presented here demonstrate that DPM neuron output is differentially involved in odor memory. DPM activity 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. In contrast, DPM output is required during acquisition of BA memory. Taken with our finding that BA is sensed by an olfactory and nonolfactory route, we speculate that DPM neurons are uniquely involved in the memory of odors that require multisensory integration.

Experimental Procedures

Fly Strains

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*^{c651}, and *amn*^{X8} alleles were described previously (Quinn et al., 1979; Waddell et al., 2000; Moore et al., 1998). *amn*^{X8} is a behavioral *amn* null allele generated by imprecise excision of the *amn*^{28A} P elements. DeZazzo et al. (1999) reported that *amn*^{X8} lacks the entire *amn* open reading frame (ORF). Imaging DPM neurons in *amn*^{X8} brains was not possible, because *amn*^{X8} flies have GAL4 activity in the mushroom bodies (data not shown). Therefore, *amn*^{X8} flies must retain P element sequence that was not described by DeZazzo et al. (1999). This residual GAL4 activity is not sufficient to rescue *amn* memory (Waddell et al., 2000; and this study; Figure 4A) but c316{GAL4};uas-CD8:GFP-labeled DPM projections into the MB lobes cannot be readily distinguished from intrinsic MB labeling. We therefore made new deletion alleles of the *amn* ORF by imprecise excision of the *amn*^{c651} P[w^{+}] element. Briefly, *amn*^{c651} females were crossed to transposase-bearing *Sb*(Δ2-3)/TM3*Ser* males. Dysgenic *amn*$^{c651;Sb}$(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, we isolated putative *amn*^{ex} males and prepared genomic DNA. We analyzed fifty of these putative excisions for the integrity of the *amn* locus by PCR and sequence analysis. Two of these lines—*amn*^{ex1} and *amn*^{ex39}—contained near complete deletion of the *amn* ORF. *amn*^{ex1} deletes a region of DNA extending from -661 nucleotides upstream of the ATG to position $+369$ within the *amn* ORF. *amn*^{ex39} deletes a region of DNA extending from -785 nucleotides upstream of the ATG to position $+477$ within the *amn* ORF. *amn*^{ex39} leaves only a small C-terminal fragment that is not expected to have function. The uas-mCD8:GFP flies are described in Lee and Luo (1999). The uas-*shi*^{ts1} flies were those previously used by us (Waddell et al., 2000) and described in Kitamoto (2001). We previously described the DPM neuron-restricted c316{GAL4} and the uas-*amn* flies (Waddell et al., 2000). The uas-*amn* flies are those previously denoted as "uas-*amn*#1." Mz717{GAL4} flies were described by Ito et al. (1998). Gr5a-Gal4;uas-DTI and Gr66a-Gal4;uas-DTI strains were tested as stable homozygous stocks generated as described in Wang et al. (2004). Transheterozygous *pox-neuro* mutant progeny from a cross between the hypomorphic allele and the deficiency were analyzed in behavioral assays.

Histochemistry

Adult brains expressing transgenic mCD8:GFP were removed from the head capsule and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl) for 15 min and rinsed in PBS-T (PBS containing 0.25% Triton X-100). Fixed brains were mounted in Vectashield. Confocal analysis was performed on a Leica TCS-SP laser scanning confocal microscope.

Preparation of Transgenic Flies for Behavioral Analysis

We generated flies expressing *shi*^{ts1} in DPM cells 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*;c316{GAL4} and *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 *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.

Behavioral Analysis

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 PI was calculated as described in the text. 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. Experiments involving uas-shi^{ts1} were performed while the behavior room temperature was shifted from 25°C to 31°C.

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

To test olfactory acuity, untrained flies were given 2 min 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 bubbled through mineral oil in the T maze. Percent avoidance was calculated according to Tully et al. (1994). Electroshock avoidance was performed and calculated similarly. Untrained flies chose between a tube containing an electrified grid and a tube containing a nonelectrified grid. To assess relative odor avoidance, we gave untrained (or previously electric-shocked) flies 2 min 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 fly's position 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 $p < 0.05$.

Acknowledgments

We are grateful to B. Leung, V. Budnik, and D. Guarnieri for comments on the manuscript. We thank T. Kitamoto, J. Hodge, K. Scott, and H. Amrein for fly stocks. This work was partly supported by grants to S.W. from the Mallindkrodt Foundation and the NIH (MH09883-01) and to L.B.V. from the John Merck Foundation and the NIH (5RO1 DC05036-03). A.K. is a M.S. Stoffel Fellow in Mind, Brain, and Behavior.

Received: March 23, 2004
Revised: July 21, 2004
Accepted: September 9, 2004
Published: October 27, 2004

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