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Aging Specifically Impairs *amnesiac*-Dependent Memory in *Drosophila*

Takuya Tamura,[1,2] Ann-Shyn Chiang,[3] Naomi Ito,[1,4] Hsin-Ping Liu,[3] Junjiro Horiuchi,[1,4] Tim Tully,[5] and Minoru Saitoe[1,4,*]
[1]Tokyo Metropolitan Institute for Neuroscience
Fuchu, Tokyo 183-8526
Japan
[2]Institute for Behavioral Sciences
Gunma University School of Medicine
Maebashi, Gunma 371-8511
Japan
[3]Department of Life Science
National Tsing Hua University
Hsinchu 30043
Taiwan
[4]Precursory Research for Embryonic Science and Technology (PRESTO)
Japan Science and Technology Agency
[5]Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

Summary

Age-related memory impairment (AMI) is observed in many species. However, it is uncertain whether AMI results from a specific or a nonspecific decay in memory processing. In *Drosophila*, memory acquired after a single olfactory conditioning paradigm has three distinct phases: short-term memory (STM), middle-term memory (MTM), and longer-lasting anesthesia-resistant memory (ARM). Here, we demonstrate that age-related defects in olfactory memory are identical to those of the MTM mutant *amnesiac* (*amn*). Furthermore, *amn* flies do not exhibit an age-dependent decrease in memory, in contrast to other memory mutants. The absence of AMI in *amn* flies is restored by expression of an *amn* transgene predominantly in DPM cells. Thus, we propose that AMI in flies results from a specific decrease in *amn*-dependent MTM.

Introduction

Although anatomical and physiological changes in age-related memory impairment (AMI) have been amply documented (Foster, 1999; Shimada, 1999), little is known about the molecular mechanisms underlying AMI. A major obstacle in performing genetic and behavioral analyses of AMI has been the long life span of animal models. For example, a number of genes involved in neuronal functions both in the hypothalamus and cortex of mice have altered expression upon aging (Jiang et al., 2001). It is difficult, however, to test whether mutations in these genes affect AMI, because the mouse lifespan exceeds 600 days. In contrast, the fruit fly, *Drosophila melanogaster*, has a short lifespan and is highly suited for genetic and behavioral analyses of aged individuals (Cook-Wiens and Grotewiel, 2002; Fresquet and Medioni, 1993; Guo et al., 1996; Savvateeva et al., 1999, 2000).

*Correspondence: saitoe@tmin.ac.jp

Genetic studies of memory formation in *Drosophila* have identified genes that function at distinct temporal phases of memory. These memory phases seem to form through at least partially distinct mechanisms since they can be separated by individual genetic mutations. Memory retention curves after olfactory conditioning of single-gene mutants show characteristic disruptions in specific memory phases. For instance, *linotte* (*lio*) (Dura et al., 1993), a mutant suspected to be defective for a putative receptor tyrosine kinase (Dura et al., 1995; Moreau-Fauvarque et al., 2002); *fasciclinII* (*fasII*), a mutant defective for cell adhesion molecule *Fas II* (Cheng et al., 2001); *leonardo* (*leo*), a mutant defective for a *Drosophila* 14-3-3 protein (Skoulakis and Davis, 1996); and *latheo* (*lat*), a mutant defective for a subunit of the origin recognition complex (Boynton and Tully, 1992; Pinto et al., 1999), show reductions in memory immediately after training (0 hr memory), but subsequent memory decay occurs roughly in parallel with that of wild-type control flies. Mutations in *rutabaga* (*rut*), which encodes Ca^{2+}/CaM-dependent adenylyl cyclase (Levin et al., 1992), *dunce* (*dnc*), which encodes cAMP-specific phosphodiesterase (Chen et al., 1986), and *volado* (*vol*), which encodes a subunit of cell adhesion molecule integrin (Grotewiel et al., 1998), result in larger reductions in short-term memory (STM) within the first hour after training, rather than in later memory, as compared to wild-type controls. In contrast, mutations in *amnesiac* (*amn*), which encodes a gene with homologies to vertebrate pituitary adenylyl cyclase-activating peptide (PACAP) and growth hormone-releasing hormone (GHRH) (DeZazzo et al., 1999; Feany and Quinn, 1995), show minimal defects in 0 hr and 7 hr memory, while memory retention between these time points, middle-term memory (MTM) (Li et al., 1996; Tully and Quinn, 1985), is significantly lower than wild-type controls. Finally, a mutation in *radish* (*rsh*), defective for phospholipase A2 (A.-S. Chiang et al., submitted), shows a greater disruption in 7 hr memory rather than at earlier time points. *rsh* has been shown to be defective in anesthesia-resistant memory (ARM), a consolidated form of memory that forms temporally after MTM. After single-cycle training (see Experimental Procedures), memory is not consolidated into protein synthesis-dependent long-term memory (LTM), which requires repetitive training with specific rest intervals between trainings and activation of the transcription factor CREB (Tully et al., 1994; Yin et al., 1994).

In this study, we present the first extensive behavioral-genetic characterization of AMI in *Drosophila*. We show that AMI results from memory defects very similar to those found in *amn* MTM mutants. Besides memory processing, the *amn* gene product has been shown to regulate ethanol sensitivity via the cAMP signaling pathway (Moore et al., 1998). In the fly brain, the *amn* gene product is preferentially expressed in the DPM cells that innervate the lobes of the mushroom body (MB) (Waddell et al., 2000), a neural center for olfactory learning and

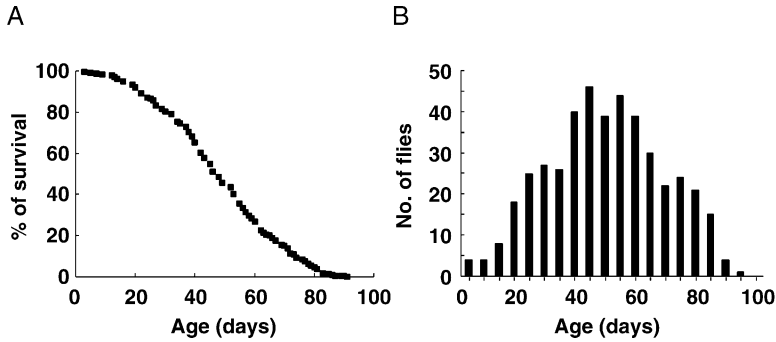

Figure 1. Lifespan of Wild-Type *Drosophila*

Survival curve (A) and frequency histogram of mortality (B) are derived from n = 483 flies. Average lifespan, premortality plateau phase (where less than 10% of mortality occurs), and maximum level of mortality (where more than 90% of mortality occurs) are 48 days, 22 days, and 74 days of age, respectively.

memory (Connolly et al., 1996; de Belle and Heisenberg, 1994). Presumably, the *amn* gene product modulates cAMP signaling in the MBs during olfactory memory formation. Significantly, AMI does not occur in *amn* flies, while it does in other memory mutants. Taken together, we propose that AMI results largely from a specific disruption in *amn*-dependent MTM.

Results

Memory Retention in Aged Flies

In our experimental conditions, the average lifespan of wild-type flies is 48 days after eclosion. The premortality plateau phase, in which less than 10% mortality occurs, encompasses the first 22 days, and the maximum lifespan, measured when 90% mortality has occurred, is 74 days (Figure 1A). To evaluate olfactory memory retention in aged flies, we first examined 0, 1, and 7 hr memory after a single-cycle training session in flies of 1, 10, 20 (near the end of the premortality plateau phase), 30, and 50 (around average lifespan) days of age. We found a statistically significant but slight impairment in 0 hr memory that first appeared in 10-day-old flies (Figure 2A). This impairment did not progress further during aging, however. We did not find any significant changes in 7 hr memory between flies of all ages (Figure 2C; $F[4,39] = 2.36$, $p > 0.05$ by one-way ANOVA). On the other hand, 1 hr memory was severely impaired in 20-day-old flies, and this impairment increased upon further aging (Figure 2B).

To analyze temporal memory retention more precisely, we generated memory retention curves for flies of 1, 10, 20, 30, and 50 days of age. As shown in Figure 2D, the memory retention curve of 20-day-old flies is similar to that of 1-day-old *amn* mutants, with a significant reduction in 1 hr memory but not much reduction in 0 and 7 hr memory. This similarity becomes much clearer in older flies such as 50-day-olds.

Apparent defects in learning and memory may be observed using this olfactory conditioning procedure if flies are defective for task-related skills, including olfactory acuity and shock reactivity. As shown in Table 1, 10-day-old flies showed normal olfactory avoidance at the odor concentrations used for training and testing (10^0) and also at 10-fold lower concentrations (10^{-1}). Likewise, they showed normal shock reactivity at the voltage used for olfactory conditioning (60 V) as well as at a lower voltage (20 V). These results reveal that the performance defect in 0 hr memory in 10-day-old flies cannot be explained by a reduction in the perception of, or re-

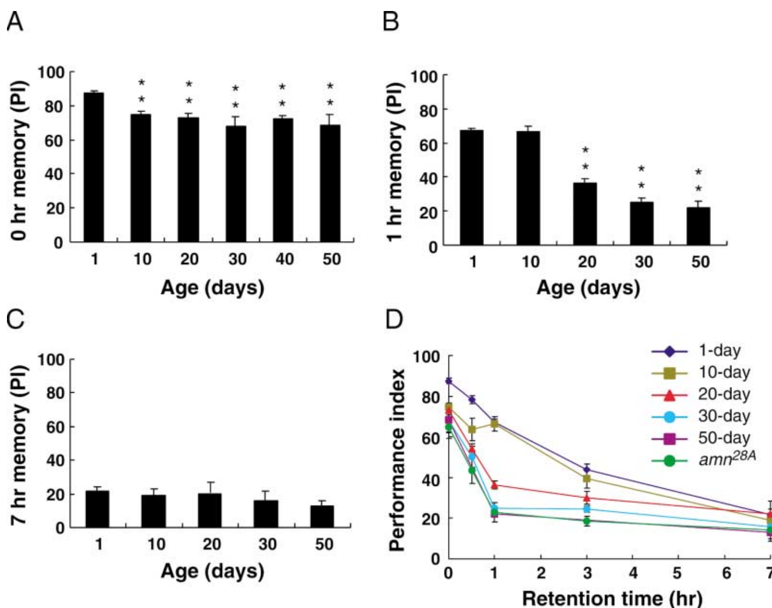

Figure 2. Age-Related Changes in Memory Retention

(A–C) Age-related changes in 0 hr (A), 1 hr (B), and 7 hr (C) memory. The slight impairment in 0 hr memory that appeared in 10-day-old flies did not progress further during aging (A). In contrast, the impairment of 1 hr memory, which appeared in 20-day-old flies, increased upon aging (B). PI scores of 50-day-old flies for 1 hr memory were significantly lower than those of 20-day-old flies ($p < 0.01$ by t test). Significant changes in 7 hr memory were not observed during aging (C).

(D) Age-related changes in memory retention curves. Memory retention curves of 20-day-old and older wild-type flies show characteristics similar to those observed in *amn* mutants; minimal effects for 0 and 7 hr memory, but memory between these time points is significantly impaired. n = 6–14 for all groups. Error bars in all figures in this paper indicate SEM. **$p < 0.001$ when comparing to 1-day-old flies using the t test.

Table 1. Aversive Olfactory Avoidance and Shock Reactivity in Aged Flies

	Olfactory Avoidance					
	OCT		MCH		Shock Reactivity	
Age	10^{0} [a]	10^{-1}	10^{0}	10^{-1}	60 V	20 V
1 day	53 ± 5	21 ± 5	57 ± 4	21 ± 7	87 ± 3	68 ± 9
10 day	54 ± 9	25 ± 5	51 ± 7	17 ± 5	85 ± 5	69 ± 9
20 day	34 ± 6[b]	9 ± 4[b]	35 ± 8[b]	9 ± 5[b]	84 ± 9	68 ± 7

n = 8 to 12 per group. All scores are expressed as mean PIs ± SEM.
[a] 10^{0} is the concentration used in olfactory conditioning.
[b] Statistically different from 1-day-old flies ($p < 0.01$ by t test).

sponses to, the stimuli presented. On the other hand, 20-day-old flies showed decreased odor avoidance compared to 1-day-old flies, although they showed normal shock reactivity. This observation is consistent with a previous study that has reported the effect of age on odor avoidance (Cook-Wiens and Grotewiel, 2002). However, since 20-day-old flies have identical 0 hr memory scores to 10-day-old flies (performance index [PI] ± SEM = 75 ± 5 for 10-day-old versus 73 ± 6 for 20-day-old; $p = 0.60$; $n = 6$ for each group), it is likely that 20-day-old flies sense and distinguish the odors normally but have a lower aversive response to them. Likewise, comparable 0 hr memory in 30-day-old and 50-day-old flies implies that these flies sense and distinguish the two different odors normally but change their odor preferences upon aging. Taken together, the results from 0 hr memory and these sensoro-motor assays suggest that 20-day and older flies possess sufficient odor perception and shock reactivity to learn odor and shock association as well as 10-day-old flies. Thus, the severe reduction in 1 hr memory in these flies results solely from an age-related impairment in memory processing.

Minor Defects in Acquisition in 10-Day-Old Flies

As shown in Figure 2D, 10-day-old flies show a slight but significant reduction in 0 and 0.5 hr memory, but subsequent memory decay seems normal. This seemed to indicate that 10-day-old flies are slightly defective in acquisition but not subsequent memory processing, as has been shown previously for *fasII* mutants (Cheng et al., 2001). To evaluate acquisition more closely in 10-day-old flies, we varied the number of CS-US trials within a training session (Beck et al., 2000; Cheng et al., 2001; Tully and Quinn, 1985). 0 hr memory increases progressively in both 1-day and 10-day-old flies as a function of the number of training trials, with 0 hr memory of 10-day-old flies lagging slightly behind that of 1-day-old flies (Figure 3A). Therefore, 0 hr memory of 10-day-old flies can be set equal to that of 1-day-old flies by increasing the number of training trials. By training 1-day and 10-day-old flies for 6 and 8 trials, respectively, we were able to obtain similar levels of 0 hr memory from both groups. Under these conditions, memory retention curves of 1-day and 10-day-old flies were indistinguishable (Figure 3B). Identical results were obtained when 0 hr memory was normalized by training 1-day and 10-day-old flies for 4 and 6 trials, respectively (data not shown). Therefore, as reported for *fasII* mutants, these results confirm that acquisition is slightly reduced in 10-day-old flies, but subsequent memory processing is normal.

Memory Consolidation in Aged Flies Is Similar to *amn* Mutants

The severe reduction in 1 hr memory, which first appears at 20 days of age, progresses upon further aging ($p < 0.01$ by t test for PI in 20-day-olds versus 50-day-olds). This finding led us to suspect that this impairment is crucial for AMI and we decided to characterize it further. In addition, because of the characteristic similarities in temporal memory retention between aged flies and *amn* mutants, we wanted to probe their similarities further.

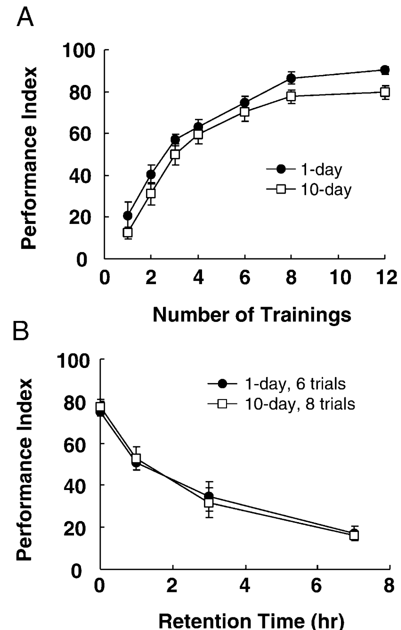

Figure 3. Memory Acquisition Is Impaired in 10-Day-Old Flies

(A) 1-day and 10-day-old flies were trained with various numbers (1 to 12) of CS-US trials within a training session and tested immediately after the final trail (0 hr memory). Although 0 hr memory increased progressively in both 1-day and 10-day-old flies, 10-day-old flies showed lower 0 hr memory than 1-day-old flies after multiple trials of "short program training" (n = 6–12 for all groups).

(B) When 0 hr memory of 10-day-old flies was set equal to that of 1-day-old flies by training 10-day and 1-day-old flies for 8 and 6 trials, respectively, memory retention curves of 10-day and 1-day-old flies were indistinguishable (n = 6–12 for all groups).

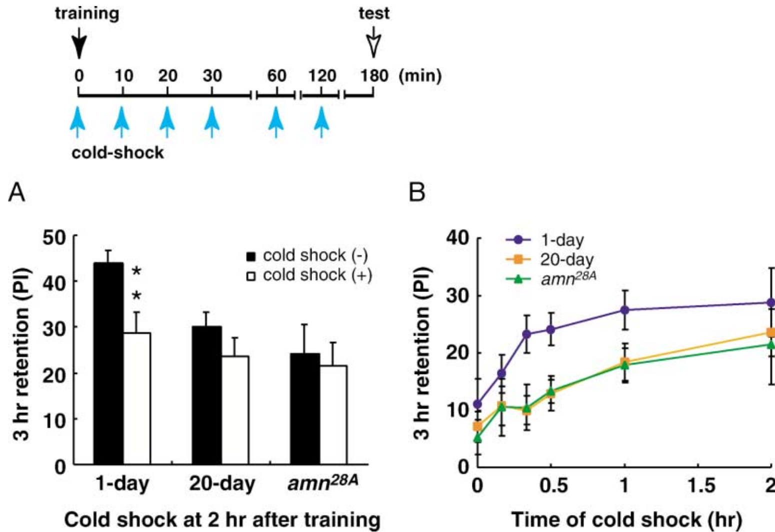

Figure 4. Memory Consolidation in Aged Flies Is Similar to Those of *amn* Mutants

After single-cycle training (black arrow), flies were subjected to cold shock anesthesia at 0, 10, 20, 30, 60, or 120 min (time of cold shock, light blue arrows) and then assayed for 3 hr memory (white arrow). 3 hr memory increases when cold shock is given at later time points and reaches an asymptotic maximum level within 2 hr after training (see B).

(A) Maximum levels of ARM (cold shock at 2 hr after training) did not differ between young (1-day-old) and aged (20-day-old) wild-type flies and *amn* (1-day-old) mutants ($F[2,15] = 3.18$, $p > 0.05$ by one-way ANOVA). In young flies, 3 hr memory in the absence of cold shock was significantly higher than in the presence of cold shock, indicating the presence of anesthesia-sensitive MTM, which is absent in aged and *amn* flies ($p < 0.01$ for young flies and $p > 0.05$ for aged and *amn* flies by t test).

(B) Progressive formation of ARM in young (1-day-old), aged (20-day-old), and *amn* (1-day-old) flies ($n = 6–8$ for all groups).

Memory at 1 hr can be separated into two components, an anesthesia-sensitive memory (ASM) component, consisting of STM and MTM, and an anesthesia-resistant memory (ARM) component. In flies as well as other organisms, newly formed memories are initially unstable and can be disrupted by administration of amnesia-inducing treatment, such as cold shock anesthesia. This initially labile ASM is eventually consolidated into a more stable, cold shock-resistant, ARM. In wild-type flies, ARM forms gradually over the first 2 hr after training, after which it has reached maximal levels. During this time, there is a concomitant decrease in ASM. Previously, it has been reported that *amn* mutants are defective for MTM, the primary form of ASM present between 1 and 2 hr after training (Tully et al., 1990). Thus, we wanted to determine whether defects in 1 hr memory present in old flies consisted of defects in anesthesia-sensitive MTM or in consolidation to ARM.

ARM can be measured directly in "retrograde amnesia experiments," in which 3 hr memory after one training session is quantified in flies subjected to a 10 min cold shock at various times after training (Folkers et al., 1993; Quinn and Dudai, 1976; Tully et al., 1990, 1994). When the cold shock is given at time points soon after training, memory is severely disrupted, because most of the memory at this time is ASM. As a consequence, 3 hr memory is low. However, as the time interval between training and cold shock increases, memory becomes more resistant to cold shock, reflecting the time-dependent consolidation of ASM to ARM. Therefore, 3 hr memory increases as the cold shock is administered at later times and reaches an asymptotic maximum level within 2 hr after training (see Figure 4B). In retrograde amnesia experiments, we compared the formation of ARM in young (1-day-old) and aged (20-day-old) wild-type flies and young (1-day-old) *amn* mutants. We used 20-day-old flies since they showed significant AMI, measured as reduction in 1 hr memory, and their memory retention curve was qualitatively similar to that of *amn* mutants.

As seen in Figure 4A, in young wild-type flies, 3 hr memory in the absence of cold shock can be quantified at about 45 PI units. When a cold shock is given at 2 hr after training, ARM has reached an asymptotic level of 27 PI units, which is 60% of normal 3 hr memory. In *amn*^{28A} mutants, maximal ARM levels are the same as maximal ARM levels in young wild-type flies. Memory in the absence of cold shock, however, remains the same as memory in the presence of a cold shock at 2 hr. This indicates that the main component of ASM present at 2 hr posttraining consists of *amn*-dependent MTM. 20-day-old wild-type flies behave identically to *amn*^{28A} mutants in that maximal ARM levels remain the same as young wild-type flies while anesthesia-sensitive *amn*-dependent MTM is absent (no differences between 3 hr memory with and without cold shock).

We next compared the rates of ARM formation between 1-day-old, 20-day-old, and *amn*^{28A} flies. Although maximal levels of ARM did not differ among all flies, ARM at 20 min, 30 min, and 1 hr after training was significantly lower in *amn* mutants than in 1-day-old wild-type flies ($p < 0.05$ by t test, Figure 4B). Importantly, these *lower* ARM scores at these time points were also observed in 20-day-old wild-type flies, and curves plotting ARM formation in 20-day-old wild-type flies and *amn* mutants are indistinguishable. Thus, although neither mutations in *amn* nor aging alters maximum level of ARM, they equally lower the rate of ARM formation, suggesting a linkage between AMI and a decrease in *amn*-dependent memory.

The observation that robust ARM is formed in *amn* mutants and aged wild-type flies, suggests that ARM can be produced, albeit with slower kinetics, in the absence of MTM. This further suggests that ARM may be produced from an earlier form of memory such as STM while MTM facilitates ARM formation.

Genetic Dissection of AMI with Memory Mutants

Because of the extreme similarity in memory performance between aged wild-type flies and *amn* mutants, we hypothesized that aging results in a specific reduc-

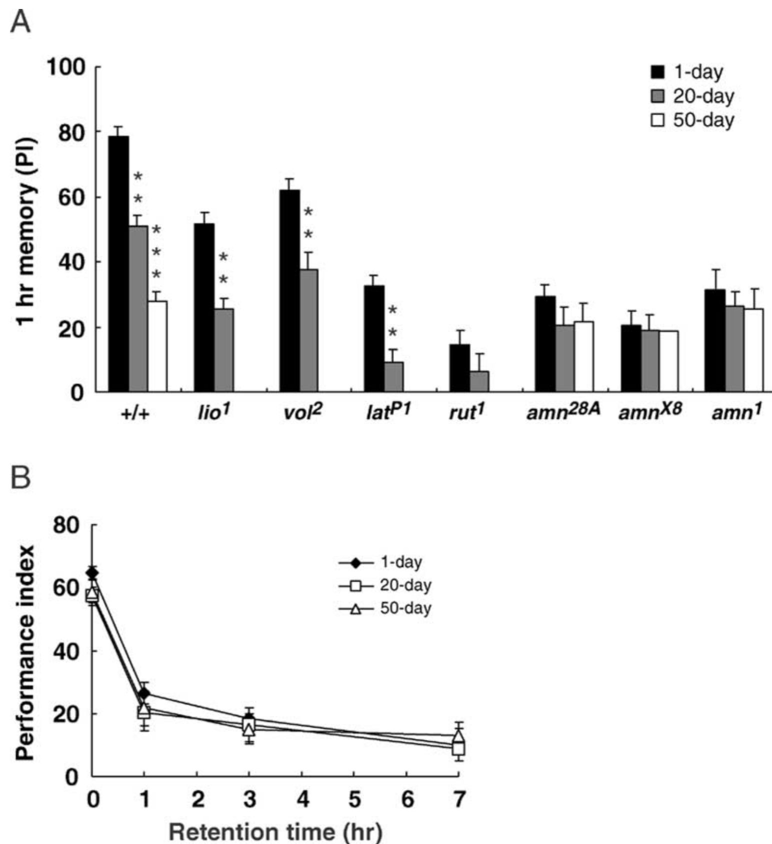

Figure 5. Absence of AMI in *amn* Mutants

(A) Age-related change in 1 hr memory in *lio*^{1}, *vol*^{2}, *lat*^{P1}, *rut*^{1}, *amn*^{28A}, *amn*^{x8}, and *amn*^{1} mutants. In contrast to other memory mutants, 1 hr memory does not change upon aging in *amn* mutants, even at 50 days of age.

(B) Memory retention curves of *amn*^{28A} mutants at 1 day, 20 days, and 50 days of age. There are no significant changes in memory retention among the groups (n = 6–12 for all groups, $^{**}p < 0.002$, $^{***}p < 0.001$).

tion in *amn*-dependent memory. If this is the case, *amn* flies should not show any further decreases in memory due to aging. In contrast, if other *amn*-independent memory components contribute to AMI, *amn* flies should show an AMI effect. Therefore, we compared 1 hr memory in young and aged flies of various memory mutants.

As shown in Figure 5A, *linotte* (*lio*^{1}) mutants show decreased 1 hr memory that is further reduced upon aging. Likewise, *volado* (*vol*^{2}) and *latheo* (*lat*^{1}) also showed reduced 1 hr memory and normal AMI. The AMI seen in these mutants resulted in a quantitatively similar reduction in memory to that seen in wild-type control flies (difference in PI scores between 1-day and 20-day-old flies is about 25). In contrast, 1 hr memory in aged *amn* flies (*amn*^{28A}, *amn*^{x8}, and *amn*^{1}) was not significantly different from that in young *amn* flies even at 50 days of age. Notably, while both 1-day-old *amn* and *lat* mutants had comparable 1 hr memory, 20-day-old *lat* but not *amn* mutants revealed significant AMI. In addition to *amn*, we did not observe significant differences in PI scores between 1 day and 20 day in *rutabaga* (*rut*^{1}) mutants. However, since 1 hr memory in 1-day-old *rut*^{1} is much lower than the amplitude of AMI, we cannot conclude whether AMI is absent in *rut* mutants.

To characterize the lack of AMI in *amn* mutants further, we generated memory retention curves for 10-day, 20-day, and 50-day-old *amn* flies. As shown in Figure 5B, these curves are indistinguishable, implying that *amn* mutants are disrupted for a memory component that is impaired by aging. Importantly, AMI was restored in *amn* mutants when the *amn*$^{+}$ transgene is induced predominantly in DPM cells (*amn*^{x8}*;c316/uas-amn*$^{+}$, *amn*^{1}*;c316/uas-amn*$^{+}$) as well as under *amn*^{28A} promoter control (*amn28A;uas-amn*$^{+}$) (Figure 6). The *amn*^{28A} mutation results from the insertion of a P-GAL4 element near the *amn* gene transcription start site (DeZazzo et al., 1999;

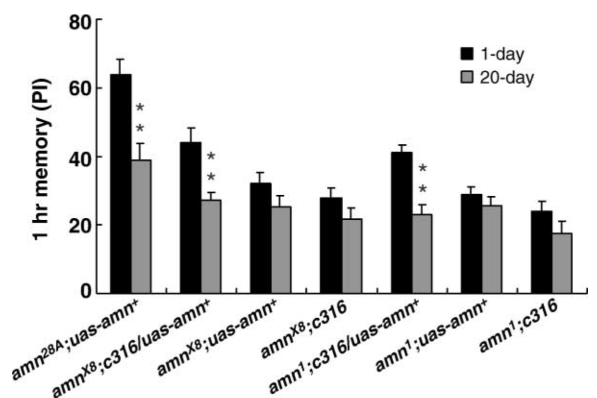

Figure 6. Restoration of AMI in *amn* Mutants

AMI was restored in *amn*^{28A} mutants when an *amn*$^{+}$ transgene is induced under *amn*^{28A} promoter control (*amn*^{28A}*;uas-amn*$^{+}$). The *amn*^{28A} mutation results from the insertion of a P{GAL4} element near the *amn* gene transcription start site (DeZazzo et al., 1999; Moore et al., 1998). Likewise, AMI was also restored when the *amn*$^{+}$ transgene is predominantly induced in DPM cells by a c316-GAL4 driver (*amn*^{X8}*;c316/uas-amn*$^{+}$ and *amn*^{1}*;c316/uas-amn*$^{+}$) while the *uas-amn*$^{+}$ transgene (*amn*^{X8}*;uas-amn*$^{+}$ and *amn*^{1}*;uas-amn*$^{+}$) or the c316-GAL4 driver alone (*amn*^{X8}*;c316 and amn*^{1}*;c316*) have no effects on AMI (n = 6–8 for all groups and $^{**}p < 0.01$).

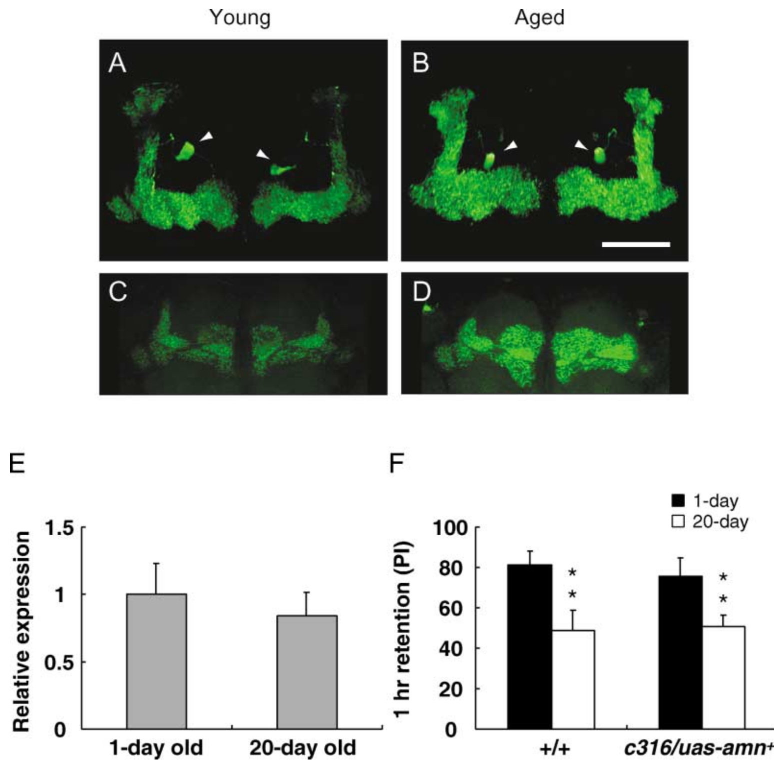

Figure 7. Morphology of DPM Cells and *amn* Gene Expression in Aged Flies

(A–D) Confocal images showing morphological changes in DPM cells during aging. (A and B) Overall morphology of DPM cells in young and aged wild-type flies. Volume rendering images from stacks of confocal images of mCD8-GFP reporter expression in DPM cells (driven by c316-GAL4). Arrowheads indicate DPM cell soma. Aged (20-day-old, B) flies show more extensive arborization into MB lobes than young (2 days of age, A) flies. (C and D) Single confocal sections of MB lobes showing neuronal-synaptobrevin-GFP reporter expression in DPM neurons. Scale bar equals 50 μm.

(E) Age-dependent change in *amn* gene expression. Semiquantitative RT-PCR analysis showed no significant change in *amn* gene expression upon aging.

(F) Overexpression of the amn^+ transgene in DPM neurons did not suppress AMI in a wild-type background. *c316-Gal4/uas-amn*$^+$ transgenic flies showed similar significant reductions in 1 hr retention when compared to a wild-type control ($p < 0.01$ by t test, $n = 6$–8).

Moore et al., 1998), resulting in GAL4 expression under *amn* promotor control. Therefore, the amn^+ transgene is expressed in regions normally expressing *amn*. Taken together, our present results provide strong evidence that AMI results from a specific reduction in the *amn*-dependent memory component.

Morphology of DPM Cells and *amn* Expression in Aged Flies

The absence of AMI in *amn* mutants prompted us to examine the morphological changes of DPM cells during aging, since the *amn* gene product is predominantly expressed in these cells (Waddell et al., 2000). If DPM cells are unusually sensitive to age-associated cell death, they may be selectively lost during aging, resulting in AMI. However, mCD8-GFP expression in DPM cells (using the c316-Gal4 driver line) shows that DPM cells grew significantly upon aging (Figures 7A and 7B, $n = 9$ and 15, respectively). In agreement with previous reports (Waddell et al., 2000), volume rendering from stacks of confocal images of brains from 2-day-old (young) and 20-day-old (aged) flies shows that DPM cells innervate all lobes of the ipsilateral MB but not the calyx or peduncle. In aged flies, DPM cells had significantly more arborizations than in young flies, leading to an increase in MB lobe size. The presynaptic feature of DPM fibers in the MBs was also demonstrated by using *n-syb*-GFP, the presynaptic vesicle-specific neuronal synaptobrevin protein fused to GFP, as a reporter (Ito et al., 1998). We find that *n-syb*-GFP is expressed more in MB lobes of aged flies (Figure 7D, $n = 5$) rather than in young flies (Figure 7C, $n = 9$), suggesting that synaptic connections between DPM cells and MB cells increase as flies age. Since DPM cells are not lost, we next examined whether *amn* gene expression is reduced upon aging. To test this possibility, we compared the expression of *amn* transcripts between 1-day-old and 20-day-old flies and also examined whether overexpression of an *amn* transgene in DPM cells suppresses AMI. Semiquantitative RT-RCR, however, revealed no significant differences in the *amn* expression between young and old flies (Figure 7E). Moreover, c316-Gal4/uas-amn^+ transgenic flies, in which the *amn* gene product was specifically overexpressed in DPM cells, showed normal AMI (Figure 7F). These results suggest that although the memory pathway mutated in *amn* flies is important for AMI, it is probably not alteration in the expression of the *amn* gene itself that leads to AMI. Thus, *amn* is necessary for the memory pathways involved in AMI but is not by itself sufficient to prevent AMI.

Discussion

Although *Drosophila* is known to be an excellent model for genetic studies, it has not been well studied for AMI. In a previous study, a significant age-related decay in courtship learning was observed in mutants of the kynurenine pathway upon aging. However, this was not observed in wild-type flies (Savvateeva et al., 1999, 2000). In the current study, we observe significant AMI in wild-type flies for Pavlovian olfactory memory. Initially, a performance deficit appears immediately after training in 10-day-old flies. This effect is slight, however, and does not increase upon further aging, suggesting a minor contribution to AMI (Figure 2A). In contrast, the disruption of 1 hr memory in flies 20 days old and older is much more severe (Figure 2B). In temporal dynamics and magnitude, this type of disruption is similar to that

observed in *amn* mutants (Figure 2D), suggesting a linkage between AMI and *amn*-dependent memory.

Since 1-day-old *amn* flies already resemble aged flies, it is possible that *amn* flies age prematurely. However, we have not observed any shortening of average lifespan, but rather we have seen an extension of lifespan (M.S., J.H., and N.I., unpublished observations). Given the behavioral similarities between *amn* and aged flies and the absence of AMI in *amn* flies (Figure 5), it is likely that AMI is neither a general nor nonspecific disruption of memory processing upon aging, but rather a disruption of a specific phase of memory formation or its underlying neuroanatomy. This *amn*-dependent memory component has been characterized as MTM (DeZazzo and Tully, 1995; Dubnau and Tully, 1998; Li et al., 1996; Saitoe and Tully, 2001).

An alternative interpretation for AMI is that aging simply affects the ability to acquire information, due to a less-attentive state during training or difficulties with sensory perception. In fact, 20-day-old flies show a reduction in odor avoidance as previously reported (Cook-Wiens and Grotewiel, 2002). However, flies 20 days and older showed 0 hr memory comparable to that in 10-day-old flies, which have normal odor avoidance and shock reactivity (Figure 2A). Aging may also affect motor activity (Cook-Wiens and Grotewiel, 2002). However, shock avoidance was normal in flies up to 50 days of age (data not shown). These observations strongly suggest that flies 20 days and older retain sufficient attentive state, sensory perception, and motor activity to perform this Pavlovian task.

Since the *amn* gene product is preferentially expressed in DPM cells, it was possible that DPM cells degenerate upon aging, resulting in AMI. As shown in Figure 7, however, we found significant growth of DPM terminals rather than degeneration. Interestingly, despite the growth of DPM terminals during aging, we did not observe a concomitant increase in *amn* expression. If the amounts of *amn* gene products per synapse are reduced during aging due to the increase in numbers of release sites, one might expect that overexpression of the *amn* transgene would ameliorate AMI. However, we could not reverse AMI by overexpressing the *amn* transgene either in DPM cells (driven by c316-GAL4) or in panneuronal cells (driven by elav-GAL4) in a wild-type background (data not shown). Therefore, it is unlikely that changes in expression of *amn* per se are responsible for AMI, but rather an attenuation of cAMP signaling downstream of *amn*.

Although we could not conclude whether AMI is absent in mutants of *rut*-adenylyl cyclase (AC), our present results suggest the importance of cAMP signaling in AMI, since the *amn* gene encodes a putative peptide with sequence homology to PACAP, which exerts its effects via AC (Zhong, 1995). Supporting this possibility, AMI is ameliorated by the drugs that facilitate cAMP signaling in aged rodents (Bach et al., 1999; Barad et al., 1998). The expression of *rut*-AC is required exclusively in the MBs for normal olfactory memory (Zars et al., 2000), and synaptic output from MBs (MB lobes) is required for retrieval of olfactory memory for up to 3 hr (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). Therefore, one possible explanation is that a PACAP-like peptide released from DPM cell terminals may prolong rut-AC activation in the MB lobes to process olfactory memory. Notably, expression of the amn^+ transgene predominantly in DPM cells was sufficient to restore AMI to similar levels to wild-type. Taken together, we propose that *amn*-dependent processing of MTM, probably involving signaling between DPM cells and MB lobes via cAMP, is important in young flies and decays at 20 days of age, leading to AMI.

Similar to the situation in *Drosophila*, PACAP, the putative homolog to the *amn* gene product in mammals, has been shown to be critical for memory retention in rodents. Mice lacking the PACAP receptor show normal learning (0 hr memory) for one-trial contextual fear conditioning, but memory decay thereafter is abnormally rapid (Sauvage et al., 2000). In addition, administration of PACAP-38 immediately after acquisition of a passive avoidance task improves memory retention in rats (Sacchetti et al., 2001). In the rodent model, severe memory decay appears around 10 to 12 months after birth (Bach et al., 1999; Frick et al., 1995). Given the average lifespan in rodents, 10 to 12 months of age is roughly equivalent to 20 days in flies. Hence, our findings may be conserved in mammalian systems, and it will be of great interest to examine whether mouse mutants lacking the PACAP receptor show the absence of AMI.

Experimental Procedures

Fly Stocks and Rearing Condition

The Cantonized w^{1118} strain, *w*(*CS10*) (Dura et al., 1993), was used as the wild-type control since all mutants in this study except vol^1 were derived from the w^{1118} parental strain. vol^1 was derived from ry^{506}. All fly stocks were maintained at 22 ± 2°C and 60 ± 10% relative humidity under a 12:12 hr light-dark cycle. About 50 flies (for behavior analyses) or 20 flies (for measuring lifespan) were reared in food vials and transferred to fresh food vials every 2 or 3 days. All behavior analyses were carried out in conditioned environmental room in which flies performed olfactory conditioning at 25°C and 60% relative humidity under red light.

Behavioral Analysis

Pavilovian Olfactory Conditioning

Standard single-cycle training was performed as previously described (Tully and Quinn, 1985) with minor modifications. Briefly, about 100 flies were exposed sequentially to two aversive odors (3-octanol [OCT] or 4-methylcyclohexanol [MCH]) for 60 s with 45 s rest intervals after each odor presentation. During exposure to the first CS^+ odor (either OCT or MCH), flies also received the US, 1.5 s pulses of 60 V DC electric shocks, every 3 s. To test for memory retention, the trained flies were tapped into the choice point of a T maze in which they were exposed simultaneously to the CS^+ and CS^-. As previously described (Tully et al., 1994), a performance index (PI) was calculated so that a 50:50 distribution (no memory) yielded a PI of zero and a 0:100 distribution away from the CS^+ yielded a PI of 100.

Memory Acquisition Test

Short program training was performed according to previous studies (Beck et al., 2000; Cheng et al., 2001) with some modification. Briefly, for one training trial, flies were exposed to the CS^+ for 5 s paired with a single 1.5 s electrical shock delivered at a 3.5 s delay from the onset of the CS^+. After a 30 s rest interval, they were then exposed to the CS^- without electrical shock. For multiple training trials, the intertrial interval was 30 s.

Memory Consolidation Test

Cold shock retrograde amnesia experiments were performed as previously described (Tully et al., 1990, 1994). Different groups of flies were subjected to a 10 min cold shock anesthesia at various times after single-cycle training by transferring them to glass test tubes and submerging the tubes in ice water. After cold shock

treatment, flies were transferred back to food vials for the duration of the retention interval. 3 hr memory retention was assayed for each group.

DPM Neuron Morphology

Whole-mount preparation and confocal imaging of fly brains were performed according to Chiang et al. (2001) with minor modifications. Briefly, dissected brains were fixed with 4% paraformaldehyde in PBS for 2 hr and fixed again with 4% paraformaldehyde in PBS containing 0.25% Triton-X 100 in a vacuum chamber for another 2 hr. Fixed tissues were than cleared by direct incubation in a drop of FocusClear (Pacgen, Vancouver) and then mounted in the MountClear (Pacgen). Whole-mount brains were imaged with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena) using a 40× C-Apochromat water immersion objective lens (NA 1.2). The "votex" module in Amira 2.3 (TGS, San Diego) was used for volume rendering and rotation of volume images.

Semiquantitative RT-PCR

5 μg of total RNA was used for the RT reaction with 25 pmol of random hexamers. To obtain a linear response to template concentration, RT products used varied from 1/3000 to 1/125, and the cycle number varied from 25 to 33 cycles of PCR. For both *amn* (forward 5′-ATGCCGTGGCGAAAACTTTG-3′ and reverse 5′-TCTTTTTCGCT CATGCGGT-3′) and control GAPDH1 (forward 5′-GCGAACTGAA ACTGAACGAG-3′ and reverse 5′-CCCTTGCGGATTATGCAACA-3′) primer sets, linear response was obtained at a 1/750 volume of RT products and 30 cycles (data not shown). Therefore, 1/750 volume of RT products and 30 cycles were used for a subsequent PCR amplification, with *amn* or GAPDH1 primers.

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