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Aging accelerates memory extinction and impairs memory restoration in *Drosophila*

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ABSTRACT

Age-related memory impairment (AMI) is a phenomenon observed from invertebrates to human. Memory extinction is proposed to be an active inhibitory modification of memory, however, whether extinction is affected in aging animals remains to be elucidated. Employing a modified paradigm for studying memory extinction in fruit flies, we found that only the stable, but not the labile memory component was suppressed by extinction, thus effectively resulting in higher memory loss in aging flies. Strikingly, young flies were able to fully restore the stable memory component 3 h post extinction, while aging flies failed to do so. In conclusion, our findings reveal that both accelerated extinction and impaired restoration contribute to memory impairment in aging animals.

1. Introduction

In the process of aging, brain functions of learning and memory, gradually deteriorate [1–3], presenting substantial challenges to an ever-growing number of people, especially in aging societies. Studies in mammalian species, including human [4], monkeys [5] as well as mice [6], show that, upon aging, spatial memory is impaired. Intriguingly, a previous study reported that aging rats showed faster contextual-dependent memory extinction than young rats [7]. However, how memory processing is affected upon aging remains to be elucidated.

Drosophila is a well-established model organism to study olfactory learning and memory, and the short lifespan makes it an ideal system to study AMI. In aversive olfactory learning, simultaneous presentation of odor and electric shock leads to learned avoidance behavior [8]. The middle term memory (MTM), which is measured 3 h after conditioning, consists of a stable component named anesthesia-resistant memory (ARM) and a labile component named anesthesia-sensitive memory (ASM), according to their different sensitivities to cold shock [9]. Two mutant flies, *radish*^{1} (*rsh*^{1}) and *amnesiac* (*amn*^{X8}), were previously reported to be deficient in either ARM or ASM [10,11]. Upon aging, flies showed great impairment of ASM, whilst ARM remained intact, an observation similar to that in *amn*^{X8} mutant flies [12]. Furthermore, aging severely impaired long-term memory [13,14], which was proposed to rely on ASM [15]. Therefore, aging appears to specifically impair ASM, whilst leaving ARM intact.

Extinction, a procedure performed as repeated presentation of conditioned stimulus without the unconditioned stimulus, is recognized as an active inhibition of memory [16]. In *Drosophila*, the extinction paradigm was established earlier using a T-maze approach [8]. Using this experimental tool, it was found that cycles of repeated presentation of conditioned odor without electric shock (ES) after conditioning attenuated aversive olfactory memory [8]. Moreover, extinction was shown to require the output of projection neurons which mediated odor transmission [17], and long term memory was suppressed by cycles of extinction procedures [18]. However, little is known about the effects of aging on memory extinction. Here we report that, upon aging, flies exhibit higher ratios of memory extinction due to the specific suppression of ARM, and recovery from this memory loss is absent.

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2. Materials and methods

2.1. Fly strains

Flies were cultured on standard food [19] at 25 °C and 60% relative humidity with a 12 h light/dark cycle. Flies were collected at eclosion and aged for different days as required prior to start of experiments.

The wild-type strain used was *Canton-S*. The mutant lines rsh^1 and amn^{X8} were generously gifted from Gregg Roman (University of Houston) and Scott Waddell (University of Oxford), respectively. The *c739-Gal4* was provided by Ronald L. Davis (The Scripps Research Institute Florida), *UAS-Brp-RNAi* by Hiromu Tanimoto (Max Planck Institut für Neurobiologie). The *OK107-Gal4* and *c305a-Gal4* were obtained from the Bloomington Stock Center.

To generate *UAS-rsh* transgenic flies, we extracted total RNA from wild-type *Canton-S* fly heads and obtained total cDNA by reverse transcription PCR (RT-PCR). The *rsh* cDNA was amplified by PCR using the primers 5′-ATGTCAAAACTGCGGCATTAC-3′ and 5′-AAAATCAAAGTGCTCACCTGTCT-3′, and cloned into pUAST vector. The transgenic flies were raised and screened according to standard procedures.

2.2. Behavioral assays

Olfactory aversive learning and memory experiments were performed according to the standard T-maze apparatus [8]. Briefly, a group of approximately 100 flies with mixed sexes were used in all experiments except only male flies in *rsh* rescue experiments.

In extinction paradigm, flies were simultaneously exposed to odor A and electric shock for 1 min for conditioning. After 45s, one to nine cycles of odor A with 1 min-duration and 45s-interval were performed as post-conditioning odor presentation (PCOP). After 45s interval, flies were exposed to odor B for 1 min, and allowed to choose between the two odors (Fig. 1A). In comparison, control flies were exposed to air instead of odor A in the PCOP steps (Fig. 1A). The electric shock mode consisted of a series of 12 × 1.25s 60v pulses with 3.75s intervals session. Immediate memory and MTM were examined immediately after training as well as 3 h after training. A 2-mins cold shock at 2 h after training was used to examine ARM [20].

Odors used were 3-octanol (OCT, 1:1000, Aldrich) and 4-methylcyclohexanol (MCH, 2.6:1000, Fluka) diluted in mineral oil (Thermo Fisher Scientific). The Performance Index (PI) was measured by averaging the preference index of two reciprocally trained groups. Memory reduction was calculated as the PI in the PCOP group (PI_{PCOP}) minus the PI in the control group (PI_{Ctrl}). The ratio of memory reduction was calculated as memory reduction divided by PI_{Ctrl}.

2.3. Statistical analysis

Data were analyzed with SPSS (SPSS Inc.). For comparisons among multiple groups, one-way ANOVA was performed, followed by comparisons between the relevant groups with a Bonferroni *post hoc* test. For comparisons between two groups, independent-sample *t* tests were used.

3. Results and discussion

3.1. Higher proportion of memory loss in aging flies

Simultaneously exposing the flies with one odor (conditioned odor) and electric shock, then another odor (unconditioned odor)

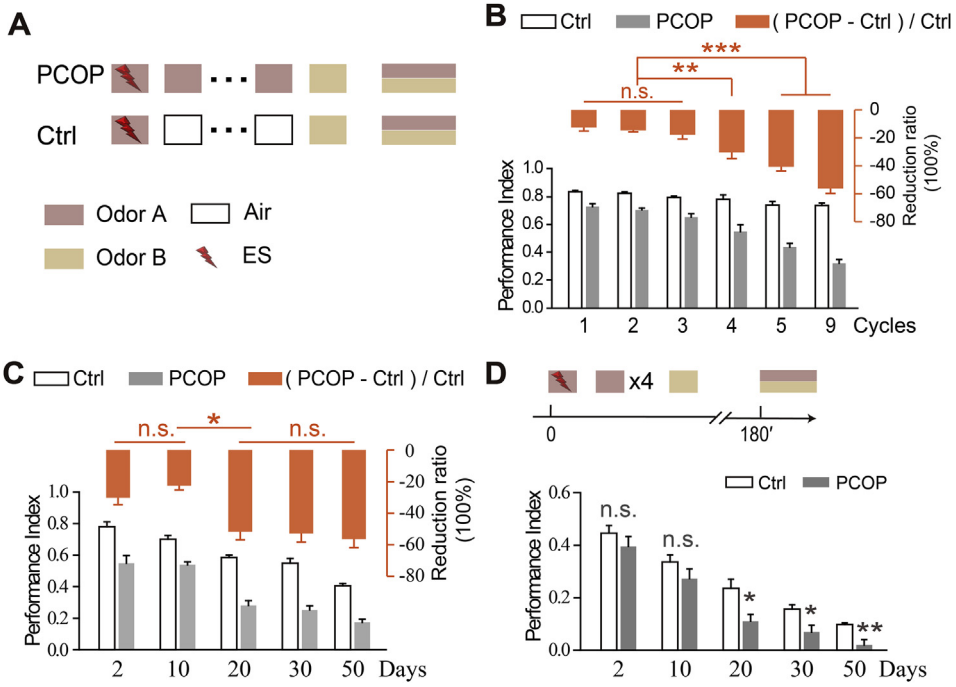

Fig. 1. Aging induced higher ratios of memory reduction and impairment of memory restoration in *Drosophila*. (A) Behavior paradigm for memory extinction in flies. In the PCOP group, following simultaneous presentation of odor A and electric shock, several cycles of odor A were presented to the flies (see Materials and methods). In the control group, odor A presentation was replaced with air. (B) With the increase of PCOP cycle number, memory decreased gradually. When the cycle number was equal or more than four cycles, the reduction ratio of memory was significantly higher. (C) The ratio of memory reduction was higher in flies at 20, 30 or 50 days of age. (D) Memory reduction was restored after 3 h in young flies at both 2 days and 10 days of age, while it remained un-restored in flies at 20, 30 or 50 days of age. Data were quantified using independent sample *t*-test between two groups, and one-way ANOVA followed by Bonferroni *post hoc* test among multiple groups. Data are shown as means ± standard error of the mean (s.e.m.). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

without electric shock sequentially make them learn to avoid the conditioned odor [8]. Cycles of extinction procedures, which are performed as the presentation of conditioned odor without electric shock, impair aversive olfactory memory [8,17,18]. In a previous report, memory was reduced about 10% following 10 cycles of odor presentation [8]. To improve extinction efficiency, we modified the original paradigm by performing the extinction procedures between the presentation of the conditioned odor and unconditioned odor, and named this treatment PCOP (see Materials and methods). To avoid odor sensory adaptation, the PCOPs were performed in 60s-odor presentation/45s-interval, and the same cycles of air ventilation were used for the control group (Fig. 1A). We found that the performance index decreased gradually with the increase of extinction cycle numbers. Furthermore, when equal or more than four cycles of PCOP was performed, the ratio of memory reduction (see Materials and methods) was more than 30% (Fig. 1B), which was a more significant decrease than previous paradigm. These findings suggested that the presenting time of PCOP and the unconditioned odor affected extinction efficiency. We thus adjusted the sequence of 4 cycles of PCOP and the unconditioned odor, and found that the earlier presentation of PCOP, the more significant memory extinction induced (Supplementary Fig. 1). Therefore, we used four cycles of PCOP before the unconditioned odor in all subsequent experiments.

To investigate the effect of aging on extinction, we next measured the memory index upon extinction procedures in flies at 2, 10, 20, 30 and 50 days of age. We found that the aversive olfactory memory was reduced significantly by PCOP among these flies (Fig. 1C). Strikingly, the memory reduction ratio in flies at 20, 30 and 50 days of age was statistically higher than the younger flies (Fig. 1C). These results indicate that memory extinction in aging flies is more severe than in younger flies, in accordance with the faster extinction performance in aging rats [7].

3.2. Memory reduction was restored in young flies, while restoration was absent in aging flies

Several earlier reports showed that extinguished memory can be restored, in the presence of an unconditioned stimulus [21,22]. To test whether the extinction effect changed over time in flies, we evaluated the memory 3 h post conditioning. We found that PCOP-induced memory reduction was spontaneously recovered within 3 h in flies at 2 days or 10 days of age (Fig. 1D). Strikingly, this memory restoration was not observed in flies at 20, 30 and 50 days of age (Fig. 1D), suggesting more severe memory deficiencies in aging flies.

3.3. PCOP specifically suppressed ARM, but not ASM

It was reported that aging specifically impaired ASM while leaving ARM intact [12]. Given our findings that aging flies exhibited higher ratios of memory extinction (Fig. 1C), we then examined whether extinction affected ARM and ASM differently, using amn^{X8} and rsh^{1} mutant flies. We found that amn^{X8} mutant flies exhibited significant memory extinction, with a reduction comparable to that in wild-type flies (Fig. 2A). Unexpectedly, little memory extinction was observed in rsh^{1} mutant flies (Fig. 2A). These results suggested that PCOP specifically suppressed ARM, whereas ASM was unaffected.

Radish was reported to be strongly expressed in both the mushroom body (MB) and ellipsoid body in the adult fly brain [23]. We therefore tested if expression of Radish was able to re-establish memory extinction that was absent in the rsh^{1} mutant flies. Firstly, we generated *UAS-radish* transgenic flies (see Materials and methods), and expressed this protein in the rsh^{1} mutant background. Our results showed that expression of radish with *c739-Gal4* in the MB α/β lobes [24] rescued the ARM formation (Supplementary Fig. 2), and re-established PCOP-induced memory extinction (Fig. 2B). In contrast, expressing Radish in the MB α′/β′ lobes and ellipsoid body with *c305a-Gal4* [25] failed to do so (Fig. 2B). Together, these findings suggested that Radish expression in MB α/β lobes was required for memory extinction.

Bruchpilot (Brp), a ubiquitous presynaptic active zone protein, has been reported to be specifically required in the MB for ARM formation [26]. Similar to rsh^{1} mutant flies, MB-specific *brp*-knocking down flies exhibited no significant memory extinction in the PCOP assay (Fig. 2C). Taken together, these results suggest that prolonged odor presentation specifically impairs ARM, but not ASM.

3.4. Extinction-induced ARM impairment was not recovered in aging flies

Our findings showed that in young wild-type flies, extinction-induced memory impairment was recovered within 3 h (Fig. 1D), and extinction procedures specifically suppressed ARM (Fig. 2). To test if the impaired ARM was restored, or whether ASM was elevated after 3 h, we introduced a 2-min cold shock 2 h after the conditioning

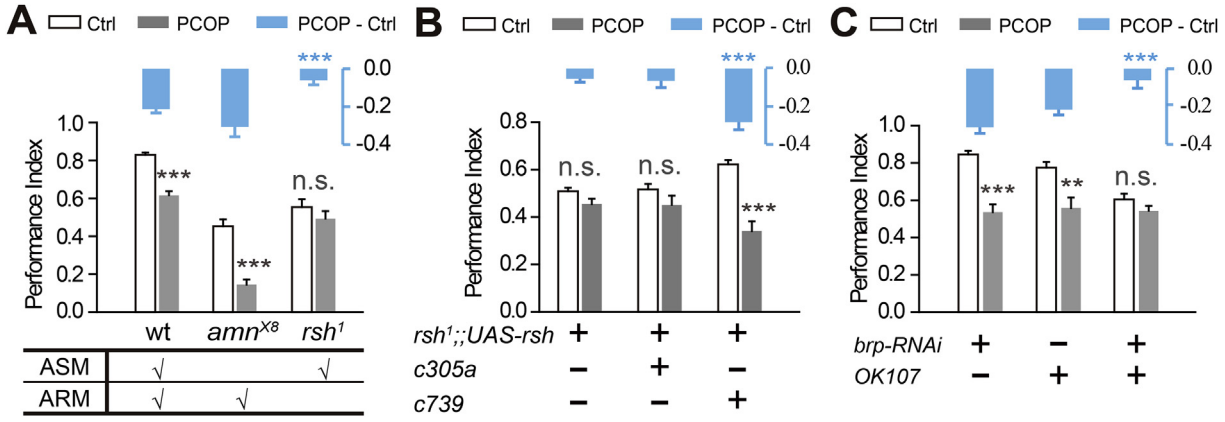

Fig. 2. PCOP specifically suppressed ARM, while ASM was unaffected. (A) Memory reduction in wild-type flies was comparable to that in ASM-deficient amn^{X8} mutant flies, with no reduction observed in ARM-deficient rsh^{1} mutant flies. (B) Expression of radish by *c739-Gal4* re-established memory extinction which is absent in rsh^{1} mutant flies. (C) No memory extinction was observed in ARM-deficient *brp-RNAi/+; OK107/+* flies. Data were quantified using independent sample *t*-test between two groups, and one-way ANOVA followed by Bonferroni *post hoc* test among multiple groups. Data are shown as means ± standard error of the mean (s.e.m.). $^{**}P < 0.01$, $^{***}P < 0.001$.

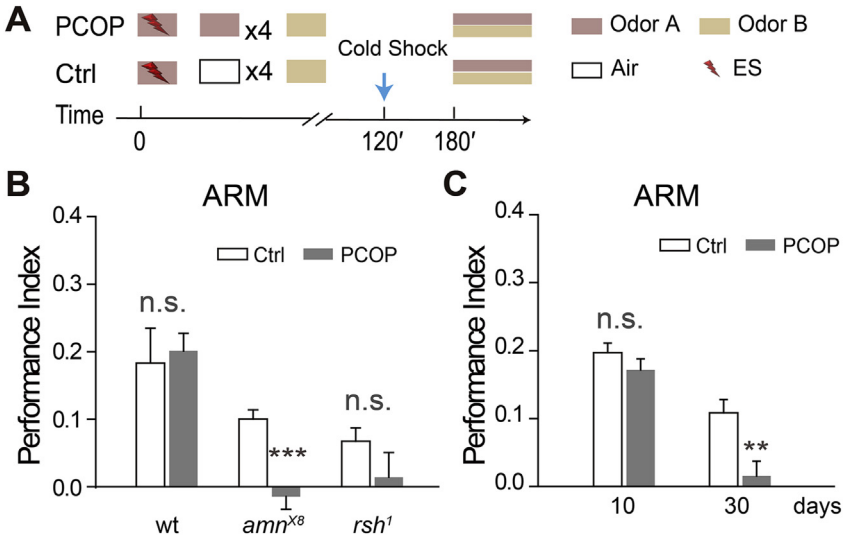

Fig. 3. Impaired ARM was recovered in young wild-type flies, but remained un-restored in aging flies and amn^{X8} mutant flies. (A) Paradigm for testing ARM upon extinction. Following four extinction procedures, a cold shock was added 2h post conditioning. (B) In wild-type flies, the ARM in PCOP group was comparable to that in the control group, but was still impaired in amn^{X8} mutant flies. In rsh^1 mutant flies, little ARM was observed in both the PCOP and air control group. (C) PCOP-induced ARM impairment was not restored in flies at 30 days of age. Data were quantified using independent sample *t*-test between two groups, and shown as means ± standard error of the mean (s.e.m.). $^{**}P < 0.01$, $^{***}P < 0.001$.

step [20] to examine ARM (Fig. 3A). In young wild-type flies, the PCOP group showed comparable ARM to that in the air control group (Fig. 3B), indicating that the extinction-induced impairment of ARM was restored. In contrast, amn^{X8} mutant flies still exhibited significant PCOP-induced reduction of ARM, and showed almost no detectable ARM in the PCOP group (Fig. 3B). Since amn^{X8} mutant flies are deficient in ASM [11], we propose that recovery of the suppressed ARM requires the presence of ASM. In comparison, the rsh^1 mutant flies showed little ARM in both the PCOP and air control group, which is in agreement with earlier reports [10].

Next, we tested ARM recovery in aged wild-type flies at 30 days of age. Similar to amn^{X8} mutant flies, no recovery from impairment of ARM upon PCOP was observed, as in their younger siblings (Fig. 3C). Taken together, these results suggest that in aging flies, ARM is suppressed by extinction, and once impaired, ARM cannot be restored.

Simultaneous presentation of odor and electric shock results in the acquisition of aversive olfactory memory in flies [8]. In aging flies, the formation of ASM is impaired whereas ARM is left intact. Here we find that the ARM is more sensitive to extinction, resulting in severe memory reduction in aging flies. Moreover, the impaired ARM is recovered after 3 h in young wild-type flies, while it remains impaired in amn^{X8} mutant flies and aging WT flies. On the basis of these findings, we propose that both the severity of memory extinction and inability of memory restoration contribute to memory decline in aging flies.

In a previous study, extinction exerts suppressive effects on 24 h memory in rsh^1 mutant flies [18]. In contrast, we showed that rsh^1 mutant flies were resistant to the extinction procedure in our experiments. We propose that this disparity is due to the fact that extinction was performed at different time points, and different memory components were examined in the two studies. In rsh^1 mutant flies, memory tested at 24 h post spaced training is long term memory, which is defined as a type of consolidated memory [20]. However, the memory we tested in rsh^1 mutant, either right after or 3 h after one cycle of conditioning, is a labile memory [10]. Further studies show that our extinction procedure selectively affects ARM, which is a type of consolidated memory [20]. Thus, the two studies both support the hypothesis that extinction inhibits consolidated memory components.

The formation of ASM and ARM was reported to be mediated by two separate molecular mechanisms [10,11,23,26,27], and based on distinguished neural circuits [28,29], suggesting that these two types of memory are processed individually. Our findings that a PCOP approach specifically interrupts ARM, without affecting ASM, provide additional evidence to support this model. Different studies showed that extinction involves either an active learning process [30], or a reversion of the previous conditioning process [16,31]. In our study, the specific interruption of ARM upon extinction suggests a modification of the previous conditioning process.

Furthermore, we observed a restoration of ARM after extinction, and more interestingly, this restoration required the presence of ASM. These results suggest a crosstalk between these two types of memory in a later stage of memory processing, which allows efficient memory formation under interferences. The underlying reason for this crosstalk might be the oscillation in the activity among the MB neurons.

Overall, our findings reveal that upon aging, memory extinction is becoming more and more severe, and once in place, this reduction cannot be restored. Our work should facilitate future investigation on the processes underlying memory decline in aging animals and human.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.131.

Transparency document

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