

BEHAVIORAL NEUROSCIENCE

Choice strategies in *Drosophila* are based on competition between olfactory memories

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Abstract

The brain mechanisms by which animals deal with multiple experiences to predict outcomes are not yet fully understood. We explored the choice strategies that flies use to assess degrees of disadvantage, as well as how flies weigh past and recent experiences to guide decisions. *Drosophila* were exposed to two conditioning events in a T-maze: an odor paired with an electric shock followed by a second odor paired with an electric shock of a different intensity. Subsequently, flies were forced to choose between the two odors. We found that flies chose to avoid the more 'dangerous' odor by a linear subtraction mechanism that was based on two coexisting memories. We also found that flies weighed experiences of the same danger level (60 V electric shocks) according to the times when the experiences had occurred. More recent experiences had a greater impact and past experiences gradually became 'overlooked' during decisions as the time delay between the two events lengthened. However, the past memory was not so much disrupted as it was overshadowed by recent memories during decisions. Finally, when a past experience was more disadvantageous, wild-type flies were able to coordinate both the temporal factor and the degree of disadvantage into their decisions. By contrast, *amnesiac* mutant flies made choices completely according to the temporal factor, ignoring the degree of disadvantage. Taken together, wild-type flies are able to store multiple olfactory memories and can coherently evaluate learned experiences to guide their decisions according to the degree of disadvantage and/or the temporal factor.

Introduction

The survival of an organism, whether fly (Dickson, 2008) or human, depends heavily on its ability to evaluate previous experiences and thereby maximize utility and minimize danger. Some evidence has suggested that brains may employ two coexisting systems, intuition and reasoning, when making decisions (Kahneman, 2002; Sanfey & Chang, 2008). Several interesting studies on choice behaviors in *Drosophila* have provided an entry point for a systematic dissection of decision-making processes (Maimon *et al.*, 2008; Yang *et al.*, 2008). Thus, *Drosophila* is likely to be a good model system for exploring the processes behind simpler and more 'intuitive-like' decisions. The neural circuit underlying visual saliency-based decision-making in flies has also been studied (Tang & Guo, 2001; Zhang *et al.*, 2007). We are interested in the roles of previous experiences in choice behavior. It is well known that previous experiences usually have a strong influence on subsequent actions in *Drosophila*. For example, flies exhibit experience-dependent changes in fighting strategies and in visual feature extraction (Yurkovic *et al.*, 2006; Peng *et al.*, 2007). In *Drosophila*, classical olfactory conditioning in a T-maze is a well-established paradigm that leads to conditioned odor avoidance

behavior and produces robust aversive olfactory memory (Quinn *et al.*, 1974; Tully & Quinn, 1985). A memory formed by a single aversive training trial can last for several hours and can be dissected into three distinct phases: short-term memory (STM), middle-term memory (MTM) and anesthesia-resistant memory (ARM) (Quinn & Dudai, 1976; Quinn *et al.*, 1979; Dudai *et al.*, 1988; Folkers *et al.*, 1993; Dubnau & Tully, 1998; DeZazzo *et al.*, 1999; Waddell *et al.*, 2000; Heisenberg, 2003; McGuire *et al.*, 2005). Therefore, we developed new choice paradigms in the T-maze to reveal how previous experiences guide a fly's choice behavior in conflicting choice situations.

It has been reported that humans and monkeys may depend on a linear subtraction mechanism during simple perceptual decision-making (Romo & Salinas, 2003; Heekeren *et al.*, 2004). Using the new choice paradigms, we found that flies used different choice strategies for: (i) evaluating experiences according to the degree of disadvantage, (ii) weighing past and recent experiences according to temporal factors, and (iii) coordinating both the degree of disadvantage and temporal factor.

Materials and methods

Fly stocks and culture

Flies were cultured on standard food (Guo *et al.*, 1996) in a 12/12 h light/dark cycle at 25 °C and 60% relative humidity. The wild-type

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Drosophila strain was Canton-S. In addition, *amnesiac* (*amn*) mutant flies (i.e., *amn*¹ and *amn*^{X8}) (Quinn *et al.*, 1979; Moore *et al.*, 1998), which are defective for MTM (DeZazzo *et al.*, 1999), were also used. Canton-S flies were used as the wild-type control for the *amn* mutants (Keene *et al.*, 2004, 2006; Yu *et al.*, 2005). All of the flies were kindly provided by Dr Waddell (University of Massachusetts Medical School, USA). Male and female flies (3–5 days old) were used in the behavioral experiments. All behavioral experiments were performed under dim red light at 70% relative humidity. The animal studies were approved by the Institute of Biophysics, Chinese Academy of Sciences.

Apparatus

The T-maze (General Valve Corp., Fairfield, NJ, USA) is a previously described *Drosophila* olfactory learning and testing apparatus (Tully & Quinn, 1985; Connolly & Tully, 1998). An electric stimulator (Model S88K Square Pulse Stimulator; Grass Instrument Division of Astro-Med, Inc., West Warwick, RI, USA) was linked to an electrifiable copper grid in a T-maze training tube, which was controlled by a power switch. A vacuum pump (Resun Corp., Guangdong Province, China) was used to keep air flow at 750 mL/min in the training tube and collection tubes and to deliver odors to the training tube and collection tubes.

Odorants and conditioning parameters

The odors used here were 4-methylcyclohexanol (purity = 98%; Fluka), 3-octanol (purity 99%; Aldrich), benzaldehyde (purity 99%; Sigma-Aldrich Co.), ethyl acetate (purity > 99%; Sigma-Aldrich Co.) and isoamyl acetate (purity 98%; Aldrich), as wild-type flies can learn 4-methylcyclohexanol/3-octanol, 3-octanol/benzaldehyde and benzaldehyde/4-methylcyclohexanol combinations (Akmal *et al.*, 2006) as well as the ethyl acetate/isoamyl acetate combination (Schwaerzel *et al.*, 2003). Odors were dissolved in heavy mineral oil (Fisher Scientific Inc.) and delivered to the training tube or the two collection tubes with a 'bubbler' (see Supporting Information, Fig. S1). The concentration of 4-methylcyclohexanol, 3-octanol, benzaldehyde and isoamyl acetate was 1.0×10^{-3} [in mineral oil (v/v)]. The concentration of ethyl acetate was 1.1×10^{-3} . Flies learned each odor equally well at these concentrations (supporting Fig. S2). Multiple three-odor sets were used to preclude the possibility of odor set-specific results. The exposure time to each odor and/or electric shock (ES) in training sessions was 1 min, and exposure time to the two odors in testing sessions was 2 min, as previously described (Connolly & Tully, 1998). During choice protocols, the time interval between the two conditioning events was 1 min, unless otherwise stated. The ES mode was a series of 12×1.25 s 60 V pulses at 5 s intervals, yielding a total stimulus duration of 1 min, unless otherwise stated.

Behavioral analyses

Single conditioning performance was assayed using a standard protocol (Tully & Quinn, 1985; Connolly & Tully, 1998). Two groups of flies were always tested in one complete run to produce one score. The first group of approximately 100 flies was conditioned by exposure to one odor (odor A) paired with an ES (conditioned stimulus, CS+) for 1 min, followed by exposure to a second odor (odor B) without the ES (CS-) for a further minute. Flies were then forced to choose between the two odors during a 2 min testing period. After the testing period, flies were collected from each T-maze collection tube, cold anesthetized at -20°C and counted. A second, reciprocal group of flies was trained with odor B as the CS+ and

odor A as the CS-. Memory performance index (PI) = $N_{CS-} - N_{CS+} / N_{CS-} + N_{CS+}$, where N_{CS-} represents the number of flies approaching the CS- odor and N_{CS+} denotes the number of flies approaching the CS+ odor. The average of the two PIs from the reciprocal experiments was taken as one complete PI. In order to avoid any possibility of odor bias, in all protocols the two odors used for testing were exchanged in the training sessions of the reciprocal experiments to generate one complete PI.

In the two-event choice paradigm, two groups of flies were always tested in one complete run to produce a Choice PI. The first group of about 100 flies was exposed to one odor (odor A) paired with a stimulus (ES1) for 1 min as the first conditioning event, and then exposed to a second odor (odor B) with a stimulus of different intensity (ES2) for 1 min as the second conditioning event. Immediately after training, choice performance was assayed by forcing the flies to choose between the two odors during a 2 min choice period (Fig. 2A). A second, reciprocal group of flies was trained with odor B paired with ES1 and odor A paired with ES2. The average of the two PIs from the reciprocal experiments yielded one complete Choice PI.

In the three-event choice paradigm, flies were conditioned with each of three odors paired with ESs of different intensities (in order, 60, 45 and 30 V), in three sequential events. Choice performance was then assayed between combinations of two odors (Fig. 3A). Again, two groups of flies were always tested in one complete run to produce a Choice PI. For example, for the 60 vs. 45 V Choice PI, the first group of about 100 flies was sequentially exposed to odor A paired with a 60 V ES, odor B paired with a 45 V ES and odor C paired with a 30 V ES during the training session. Flies were then made to choose between odors A and B in the testing session. A second reciprocal group of flies was trained with odor B paired with the 60 V ES, odor A paired with the 45 V ES and odor C again paired with the 30 V ES. The subsequent testing session was the same as that above. The average of the PIs from the two reciprocal experiments yielded one complete Choice PI. In brief, the two odors used for testing were exchanged in the training sessions of the two reciprocal experiments.

To retrieve the memories underlying choice, an unconditioned odor (CS-) was presented before two or three ES-associated conditioned odors (CS+) during the training session. In the testing session, flies were forced to choose between the CS- odor and one of the CS+ odors to retrieve the memory for the CS+ odor (Figs 2E and 3B). Again, the PIs from two reciprocal experiments were averaged to obtain one complete Choice PI. For example, to retrieve the first conditioning memory, the CS- odor and the first CS+ odor were exchanged in the two reciprocal experiments. As above, to retrieve the second conditioning memory, the CS- odor and the second CS+ odor were exchanged in the two reciprocal experiments.

Cold-shock anesthesia was delivered as described previously (Tully *et al.*, 1994; Isabel *et al.*, 2004). Flies were transferred to pre-chilled vials at 1 h after training and the vials were placed in an ice-water bath for 2 min. Flies were then transferred back to warm vials at 25°C and remained there until testing.

Performance index

Choice PI = $(N_f - N_l) / (N_f + N_l)$, where N_f represents the number of flies that approached the odor from the former conditioning event, whereas N_l denotes the number of flies that approached the odor from the latter conditioning event. This choice performance score was a 'half score' because normally a complete Choice PI data point represents the average score of two experiments. In the second experiment, the presentation sequence of the two odors was reversed in order to rule out non-associative effects. Accordingly, a positive or

negative Choice PI only meant that flies chose to approach the former or latter odor, whereas the absolute value of a Choice PI indicated decidability. If flies could not make a consistent choice between two options, then the Choice PI was zero.

Data analysis

Data were analysed using one-way ANOVA or Student's *t*-tests. Post-hoc analysis was performed using Tukey's HSD test for comparisons between groups with the same number of PIs, Scheffe's test for groups with different numbers of PIs and Dunnett's test for comparisons with the indicated control group. The statistical package SPSS 11.5 (SPSS Inc., Chicago) was used for these calculations. Error bars in figures are SEMs (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS indicates no significant difference). All *P*-values were two-tailed and $P < 0.05$ was considered to be statistically significant.

Results

Choice based on different degrees of disadvantage

Two-event-based choices

We designed a two-event choice paradigm in a T-maze to explore how flies weigh previous experiences. We exposed flies to one odor paired with an ES as one conditioning event. In the two-event choice paradigm, flies were conditioned in two separate conditioning events, in each of which a different odor was paired with an ES of a different voltage that was representative of a different degree of danger. Choice performance was assayed immediately afterwards by forcing flies to choose between the two conditioned odors. Previous studies have shown that immediate memory scores increase with shock intensity up to 60 V, suggesting that flies are able to sense differences in voltage (Tully & Quinn, 1985). Similarly to the above report, our data showed that both immediate memory PI and shock reactivity PI increased as a function of voltage in a linear manner up to 60 V (Fig. 1 and supporting Fig. S3, Pearson correlation, $r_{22} = 0.828$, $P < 0.001$; $r_{38} = 0.807$, $P < 0.001$, respectively), implying further that flies were able to sense voltage quantitatively. Thus, we used different voltages to represent different degrees of disadvantage in our choice paradigm.

First we investigated whether flies can choose between two conditioned odors (A and B) after exposure to two sequential

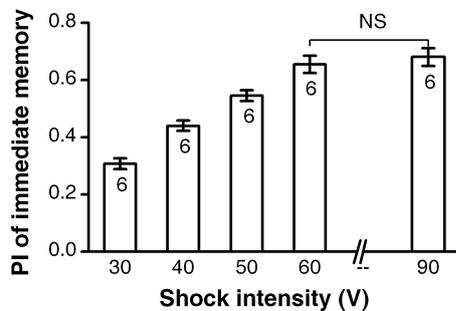


FIG. 1. Immediate memory PI increased with voltage up to 60 V, fitting a linear curve (Pearson correlation, $r_{22} = 0.828$, $P < 0.001$). Wild-type flies showed voltage-dependent immediate memory performance from 30 to 60 V. Different groups of flies received ES with 12 pulses of 1.25 s stimulus duration within 1 min at the indicated voltage during single conditioning training (see Materials and methods). There was no significant difference in immediate memory PIs between 60 and 90 V (Tukey's HSD test, $P = 0.943$). 4-methylcyclohexanol and 3-octanol were used here.

conditionings of 30 and 60 V (Fig. 2A, left). The 30–60 V choice was named according to the presentation order of the two voltages. The positive significant Choice PI (see Materials and methods) showed that flies chose to approach the former odor associated with the 30 V ES and to avoid the latter odor associated with the 60 V ES (30–60 V point in Fig. 2B, $F_{6,37} = 112.221$, $P < 0.001$, Dunnett's test against zero, $P < 0.001$). Together with the negative significant Choice PI of the 60–30 V choice (60–30 V point in Fig. 2B, Dunnett's test against zero, $P < 0.001$) showing that flies avoided the former odor associated with a stronger ES, our results demonstrate that flies can make consistent choices to avoid the odor associated with the greater voltage, regardless of the presentation order of the two ESs.

However, in the 60–60 V choice flies were unable to make a consistent choice, as the Choice PI was indistinguishable from zero (60–60 V point in Fig. 2B, Dunnett's test against zero, $P = 0.984$). To preclude an interference effect of the ES or the odor presentation from the second conditioning event, we performed an 'unpaired control' experiment. In the control experiment, the second conditioning event was replaced by a presentation of the 60 V ES alone followed by the second odor presentation 1 min later and flies were then made to choose between the two odors. The Choice PI of 60–60 V choice was significantly lower than the PI from the 'unpaired control' (supporting Fig. S4, $t_{11} = 23.177$, $P < 0.001$), indicating that the 60–60 V choice performance did not result from the interference effect of the ES or the odor presentation from the second conditioning event *per se*.

To explore the fly's choice strategy, we varied the voltage difference between the two ESs (ΔV) from 0 to 30 V (Fig. 2B). $\Delta V = |V_{1st} - V_{2nd}|$, where V_{1st} is the voltage in the first conditioning event and V_{2nd} is the voltage in the second conditioning event. Our data show that the Choice PI increased as a function of ΔV in a linear manner (Fig. 2B, Pearson correlation, $r_{42} = 0.965$, $P < 0.001$), suggesting a voltage difference-dependent choice. To further verify this result, we kept ΔV constant and compared the choice performances of different voltage-pair groups. The results indicated that the Choice PI in the 60–40 V choice was roughly equal to that in the 50–30 V choice (Tukey's HSD test, $P = 0.854$) ($\Delta V = 20$ V) and the Choice PI in the 60–50 V choice was statistically indistinguishable from that in the 50–40 V choice (Tukey's HSD test, $P = 0.999$) ($\Delta V = 10$ V) (Fig. 2C). The Choice PI in either the 60–40 or 50–30 V choice ($\Delta V = 20$ V) was significantly higher than that in either the 60–50 or 50–40 V choice ($\Delta V = 10$ V) ($F_{4,25} = 11.712$, $P < 0.001$, Tukey's HSD test, $P < 0.01$).

However, in the 10 V difference groups ($\Delta V = 10$ V), we found an exception in that the Choice PI in the 40–30 V choice was significantly higher than that in the other voltage-pair choices (Tukey's HSD test, $P < 0.01$) (Fig. 2C and supporting Fig. S5). Similarly, the Choice PI in the 20–30 V choice was significantly higher than that in the 30–40, 40–50 or 50–60 V choice (supporting Fig. S5, $F_{3,20} = 11.586$, $P < 0.001$).

Next, we addressed whether the appropriate choice could also be made after 1 h retention time. In these experiments, flies always chose to avoid the odor associated with the stronger ES in both the 90–60 and 60–90 V choices (Fig. 2D), whether choice performance was assayed at 2 or 60 min after training. A similar result was also obtained in both the 60–30 V choice and 30–60 V choice (supporting Fig. S6). Together, the results suggest that the decision resulted from stable rule-governed information processing rather than only an immediate reaction. Moreover, the results in Fig. 2D also indicate that flies were able to discriminate a 90 V ES from a 60 V ES, whereas the immediate memory PIs of 90 and 60 V were indistinguishable (Fig. 1).

We used a different protocol to address whether flies' choices were based on two relatively independent memories. Flies were presented

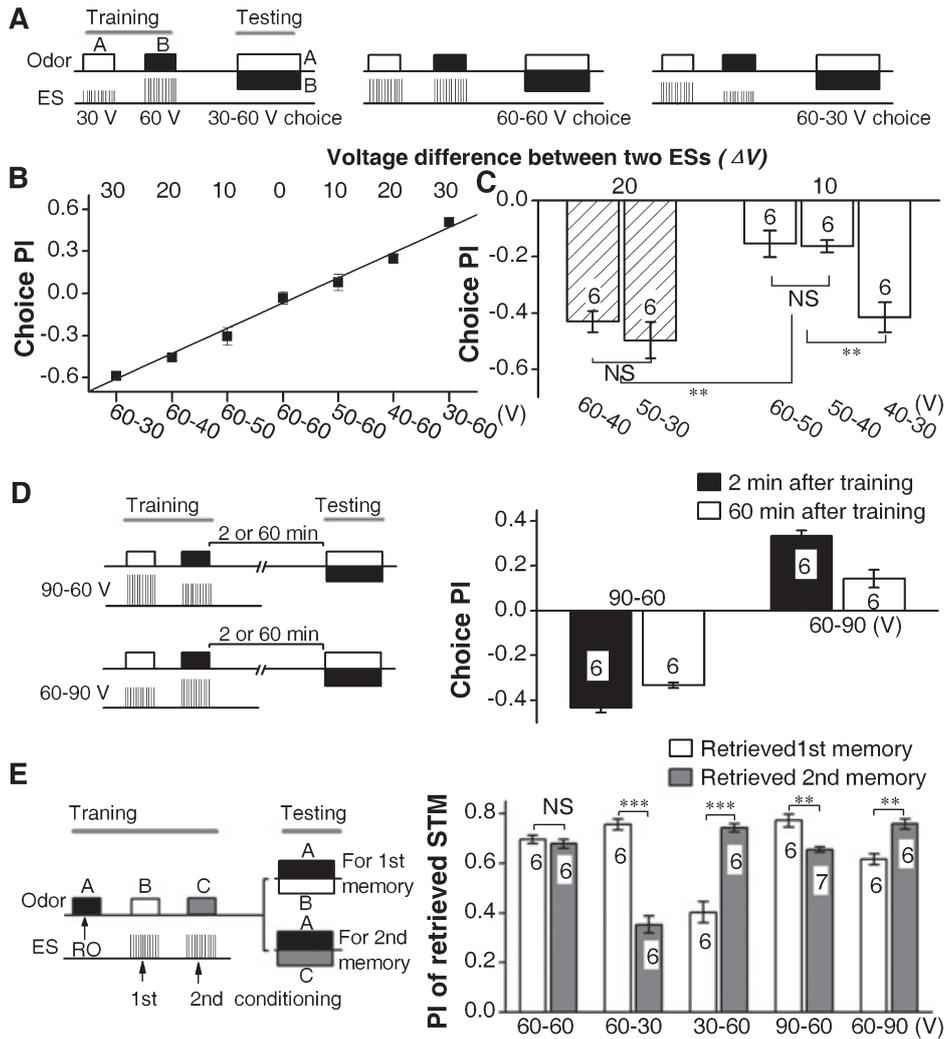


FIG. 2. Flies make voltage difference-dependent choices based on coexisting memories of two events. (A) Two-event choice protocols. Flies were conditioned by exposure to one odor (odor A) paired with an ES of a certain voltage and then exposed to a second odor (odor B) paired with an ES of a different voltage (or the same voltage). After that, odors A and B were presented to flies simultaneously to assay choice performance. For example, left: 30–60 V choice protocol; middle: 60–60 V choice protocol; right: 60–30 V choice protocol. (B) Choice PIs increased as a function of voltage difference (ΔV), fitting a linear curve (Pearson correlation, $r = 0.965$, $P < 0.001$; $n = 6$ for each point, except $n = 7$ for 60–30 and 30–60 V points). Here, one of the two ESs (whether the former or the latter) was fixed at 60 V and the other was changed from 30 to 60 V (lower abscissa), so that ΔV ranged from 0 to 30 V (upper abscissa). The upper abscissa indicates the voltage difference between the two ESs (ΔV), whereas the lower abscissa indicates the order of the two voltages used in the two-event choices. The time interval between the two events was 45 s. (C) Except for the 40–30 V choice, Choice PIs were significantly different between the 20 V difference (diagonal striped bars) and the 10 V difference (white bars) groups, and showed no significant difference within the 20 V difference groups or 10 V difference groups. However, in the 10 V difference groups, the Choice PI in the 40–30 V choice was significantly higher than that in either the 60–50 V choice or 50–40 V choice. (D) Flies chose to avoid the odor associated with the stronger ES, whether the choice performances were tested at 2 min (black bar) or 60 min (white bar) after training in both the 90–60 and 60–90 V choices. (E) Left: memory retrieval protocol. Flies were exposed to a reference odor (RO) before two CS+ odors in the training session. In the testing session, the RO and the first CS+ odor were presented to flies simultaneously for retrieving the first memory, or the RO and the second CS+ odor were presented for retrieving the second memory. Right: there was no significant difference between the two retrieved memories in the 60–60 V protocol. Significant differences were observed between two retrieved memories in the 60–30, 30–60, 90–60 and 60–90 V protocols. The three odors used here were benzaldehyde, 4-methylcyclohexanol and 3-octanol (for odor balances see supporting Fig. S2). Numbers in each bar indicate the number of PIs per group. ** $P < 0.01$, *** $P < 0.001$.

with a third unconditioned reference odor (as a CS-) before the two ES-associated odors (CS+) in the training session and were then made to choose between the CS- odor and either of the two CS+ odors in the testing session, in order to retrieve the memory for each CS+ odor (Fig. 2E, left). In this protocol, we first made sure that flies could learn each combination of two odors within the set of three odors equally well (supporting Fig. S2) so that we could use these balancing odors to retrieve each memory quantitatively. First, two relatively independent STMs were retrieved in the 60–60 V protocol, as shown by two equivalent significant PIs ($t_{11} = 0.753$, $P = 0.469$), indicating that flies

were able to remember two events simultaneously (Fig. 2E). Furthermore, the PI of the retrieved 60 V STM was higher than that of the retrieved 30 V STM in proportion to the voltages in both the 60–30 and 30–60 V protocols ($t_{11} = 8.930$, $P < 0.001$; $t_{11} = 7.530$, $P < 0.001$, respectively). Moreover, the retrieved 60 V memory was significantly weaker than the retrieved 90 V memory in both the 90–60 and 60–90 V protocols ($t_{12} = 4.514$, $P = 0.001$; $t_{11} = 4.236$, $P = 0.002$, respectively), despite the fact that there was no significant difference between the immediate memory PIs of either of the ES intensities (Fig. 1). To preclude the possibility of odor-specific effects,

we also used the same odor pair to retrieve the two memories in either the 60–60 or 60–30 V choice and obtained similar results (supporting Fig. S7). These results indicate that the STMs of two events coexisted and the strength of each retrieved STM was proportional to the voltage used for the conditioning that underlies the two-event-based choices.

Three-event-based choices

To further explore the flexibility of choice and memory capabilities of *Drosophila*, we conditioned flies with each of three odors paired with ESs of different intensities (in order, 60, 45 and 30 V) in three sequential conditioning events. We then assayed choice performance between each combination of two conditioned odors (Fig. 3A, left). Our results showed that flies always chose to avoid the odor associated with the relatively stronger ES, regardless of which two of the three conditioned odors were presented to the flies during the testing session. For example, after exposure to three sequential conditionings of 60, 45 and 30 V, flies chose to approach the odor associated with 45 V in the 60–45 V choice, whereas flies avoided the same 45 V-associated odor in the 45–30 V choice, demonstrating flexibility of choice (Fig. 3A). Next, we explored the memories underlying these choice behaviors. Three memories were retrieved and the strengths of these memories were proportional to the ES voltages used for conditioning (Fig. 3B), indicating that flies were able to form and store memories of three events simultaneously. These three coexisting memories underlie flexible choice behaviors.

We also determined that different time intervals between the CS– (i.e. reference odor) and each CS+ odor (i.e. 1, 3 and 5 min) in

the memory retrieval protocol had no significant influence on memory performance (supporting Fig. S8). Again, the same odor pair was used to retrieve all three memories in the 60–45–30 V protocol to preclude the possibility of odor-specific effects. Also, the three memories (60, 45 and 30 V) were retrieved in proportion to the voltages used for conditioning and these memory strengths were not significantly different from the results of Fig. 3B (supporting Fig. S9, $t_{11} = 0.407$, $P = 0.693$ for the first memory of 60 V; $t_{11} = 0.178$, $P = 0.863$ for the second memory of 45 V; $t_{11} = 0.074$, $P = 0.942$ for the third memory of 30 V). Moreover, the data from the 30–30–30 V and 60–60–60 V experiments showed that three memories conditioned at the same voltage were statistically indistinguishable from each other (supporting Figs S10 and S11). However, the third memory strength appears to be consistently slightly lower than the first memory strength, although there was no significance between them in both the 30–30–30 V and the two 60–60–60 V experiments (Tukey's HSD test, $P = 0.184$, 0.120, 0.347, respectively). However, the effect of order was small and could not account for the memory strength differences seen in Fig. 3B. Thus, the different memory strengths shown in Fig. 3B mainly resulted from the different voltages used for conditioning but not from the influence of the order of succession of three conditioning events.

Taken together, these results indicate that flies were able to flexibly extract and integrate relevant information from multiple memories, enabling them to make appropriate choices according to the degree of disadvantage.

Choice based on past and recent experiences

To investigate how flies weigh past and recent experiences, we assessed choice performance in a 60–60 V choice protocol with variably extended time delays between the two conditioning events (Fig. 4A). In this scenario, the two experiences represented the same danger (i.e. 60 V ES) and the time between the experiences was all that varied. Our data show that the Choice PIs were statistically indistinguishable from zero within a 2 min time delay (Fig. 4A inset magnified chart, Dunnett's test against zero, $P > 0.5$), indicating that flies could not make a consistent choice when the time delay between the two conditionings was < 2 min. Taken together with the result in Fig. 2E, that two indistinguishable STMs (underlying the 60–60 V 1 min delay choice) coexist, this suggests that these two experiences had equivalent weights within a 2 min delay. After 2 min, Choice PI increased rapidly as the time delay was extended. Once the time delay reached 30 min, the positive Choice PI was indistinguishable from the immediate memory PI of a single 60 V conditioning event (Fig. 4A, Dunnett's test, $P > 0.999$), indicating that flies made decisions that were solely based on the most recent experience from 30 min onwards.

The 'recent-takes-all' strategy is ascribed to 'overlooking' past experience

However, one more fundamental issue regarding memory of past events should be addressed. This issue is whether the second conditioning event (recent event) erased the memory of the first conditioning event (past event). Our data show that exposure to the second conditioning event resulted in a significant impairment of the 1 h memory of the first conditioning event (Fig. 4B, $t_{13} = 4.317$, $P = 0.001$). Therefore, memory of the first conditioning event was partially disrupted by the second conditioning event. However, most memory from the first conditioning event still existed during choice and the memory PI is 0.5 (Fig. 4B). Thus, this past memory was ignored by flies during the 1 h delay choice. Therefore, this suggests that flies used a 'recent-takes-all'

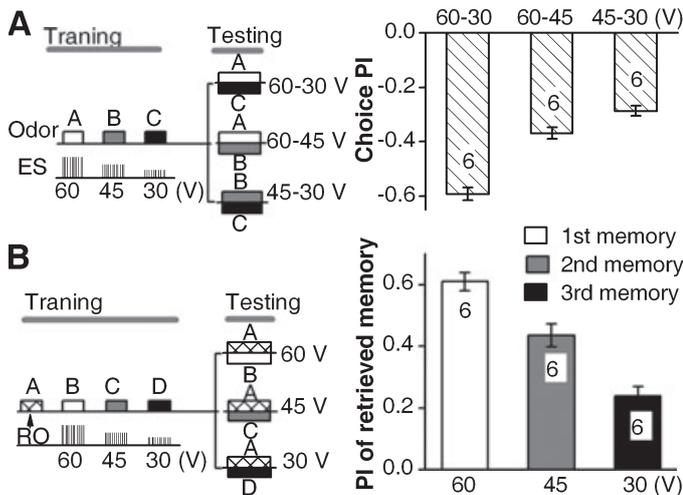


FIG. 3. Flexible choices are based on coexisting memories of three successive events. (A) Left: the three-event choice protocol. In the training session, flies were successively exposed to odor A paired with a 60 V ES, odor B paired with a 45 V ES and odor C paired with a 30 V ES. In the testing session, odors A and C were simultaneously presented to flies for the 60–30 V choice performance. Odors A and B or odors B and C were simultaneously presented to flies for the 60–45 or 45–30 V choice performance, respectively. Right: the negative Choice PI indicates that flies always chose to avoid the former odor associated with the relatively stronger ES irrespective of what the odor was. The odors used were 4-methylcyclohexanol (MCH), 3-octanol (OCT) and isoamyl acetate (IA). (B) Left: flies were exposed to a reference odor (RO) before three CS+ odors in the training session. The RO and first CS+ odor associated with the 60 V ES were presented to flies in the testing session for retrieving the 60 V memory. The RO and second CS+ odor associated with the 45 V ES as well as the RO and third CS+ odor associated with the 30 V ES were presented to flies for retrieving the 45 and 30 V memories, respectively. MCH, OCT, ethyl acetate and IA were used here (for odor balances see supporting Fig. S2). Right: the strengths of these retrieved memories were proportional to the ES voltages for conditioning.

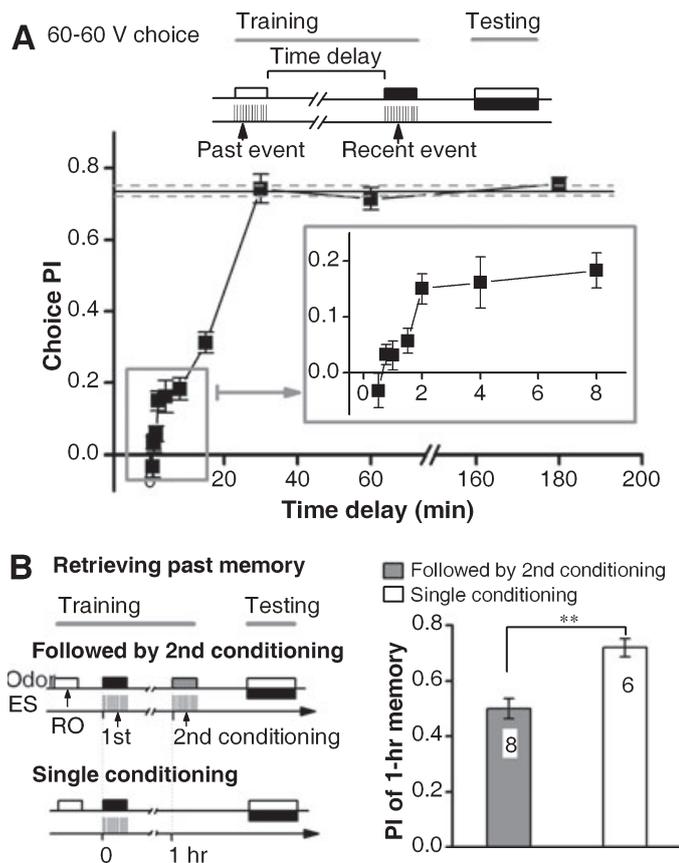


FIG. 4. The 'recent-takes-all' strategy is ascribed to 'overlooking' past experience. (A) Full weight was given to the recent experience when the time delay was 30 min or more. Upper: the time of occurrence of the recent event was fixed at 2 min before decision and the time delay between the past and recent events ranged from 30 s to 3 h. Flies were first exposed to an odor paired with a 60 V ES (past event); after a given time delay, they were exposed to a second odor also paired with a 60 V ES (the recent event); 2 min later, flies were forced to choose between these two conditioned odors. Lower: Choice PIs were statistically indistinguishable from zero within a 2 min time delay (inset magnified chart, Dunnett's test against zero, $P > 0.5$). Avoidance of the recent conditioned odor only became significant when the time delay was 2 min or more (Dunnett's test against zero, $P < 0.01$). The positive Choice PIs were statistically indistinguishable from the immediate memory PI for a 60 V single conditioning when time delays were 30 min or more (Dunnett's test, all $P > 0.99$). The upper three lines on the curve represent mean PI \pm SEMs of immediate memory in 60 V single conditioning ($n = 6$ for each point). We used the standard single conditioning protocol (see Materials and methods) to assay the 60 V immediate memory performance. (B) The 1 h memory of the first conditioning event still existed but was partially disrupted by a second conditioning event. Left: for retrieving the 1 h memory of the first conditioning event followed by a second conditioning event, flies were exposed to a reference odor (RO) at 1 min before the first conditioning and the second conditioning was performed 1 h later. Immediately after that, the RO and the first CS+ odor were simultaneously presented to flies in the testing session (upper). For 1 h memory of a single conditioning event, the protocol was the same as the above protocol except that the second conditioning event was omitted (lower). 4-methylcyclohexanol, 3-octanol and ethyl acetate were used here. Right: 1 h memory from a single conditioning event was significantly better than the retrieved 1 h memory followed by a second conditioning event. ** $P < 0.01$.

strategy, rather than a linear subtraction strategy, to evaluate past and recent experiences in the 60–60 V 1 h delay choice.

Anesthesia-resistant memory of past events remains intact

The current model of memory phase dynamics suggests that in *Drosophila*, by 1 h after a single conditioning event, only two forms

of memory exist, i.e. MTM and ARM, as STM disappears gradually within 1 h (Dubnau & Tully, 1998). Therefore, we investigated which of the two types of past memory was left intact by the second conditioning event. We used *amn* mutants as well as cold-shock anesthesia to identify the ARM component of past memory. The Choice PI in a 60–60 V 1 h delay choice was not statistically different from the immediate memory of a 60 V single conditioning event in either *amn^{XS}* or *amn¹* mutant flies (Fig. 5A, $t_{15} = 1.976$, $P = 0.068$; $t_{15} = 0.360$, $P = 0.725$, respectively). Therefore, similarly to wild-type flies, *amn* mutants made choices according to their recent experience and completely ignored the past experience in the 60–60 V 1 h delay choice. In *amn* mutants, which are defective for MTM, ARM is the only type of memory that remains by 1 h after conditioning (DeZazzo *et al.*, 1999; Waddell *et al.*, 2000). No significant difference was observed between the 1 h ARM of a single conditioning event and that followed by a second conditioning event in either *amn^{XS}* or *amn¹* mutants (Fig. 5B, $t_{11} = 1.532$, $P = 0.157$; $t_{12} = 0.256$, $P = 0.802$, respectively), indicating that ARM was not disrupted by the second conditioning event. Moreover, we used cold-shock anesthesia in wild-type flies to further dissect the ARM component of past event memory. Similarly to previous reports (Folkers *et al.*, 1993; Isabel *et al.*, 2004), 2 h memory performance with cold shock was significantly lower than 2 h memory performance of a single conditioning event (without cold shock) (Fig. 5C, $F_{2,18} = 8.363$, $P = 0.003$, Scheffé's test, $P = 0.030$). As cold-shock anaesthesia disrupts MTM in wild-type flies, ARM was the only memory form observed at the 2 h retention time-point. We found that 2 h ARM (with cold shock) was not statistically different from 2 h ARM (with cold shock) followed by a second conditioning event (Fig. 5C, Scheffé's test, $P = 0.722$). Taken together with the result in Fig. 4B, this further demonstrated that ARM was not disrupted by the second conditioning event, suggesting indirectly that the disrupted form of past memory is MTM. Therefore, we concluded that ARM of the past event is intact but overshadowed completely by the recent memory during the choice process (Figs 4A and 5).

Choice based on both the degree of disadvantage and temporal factors

From a neuro-economic point of view, relying solely on the most recent experience is not sufficient for optimal decision-making. In some situations where historical information is very important, one should consider experiences that have occurred over extended periods of time. Therefore, we used a 60–30 V choice with a 1 h delay between the two events to test the influence of a past, more dangerous experience on current choice by comparing the 60–30 V Choice PI with the 30 V immediate memory PI from a single conditioning. The positive 60–30 V Choice PI indicated that wild-type flies still chose to avoid the latter odor associated with the 30 V ES (the recent experience) but the Choice PI was significantly lower than the immediate memory PI for 30 V single conditioning (Fig. 6A, $t_{11} = 5.254$, $P < 0.001$), demonstrating that past experience of a 60 V conditioning event exerted a significant influence on current choice performance. In this case, past information was evaluated by flies and it contributed to the strength of the decision that they reached.

The role of the *amn* gene in the multiply-weighted decision

The *amn* mutant flies were also forced to make choices in this type of situation. Our data show that the Choice PI in the 60–30 V 1 h delay

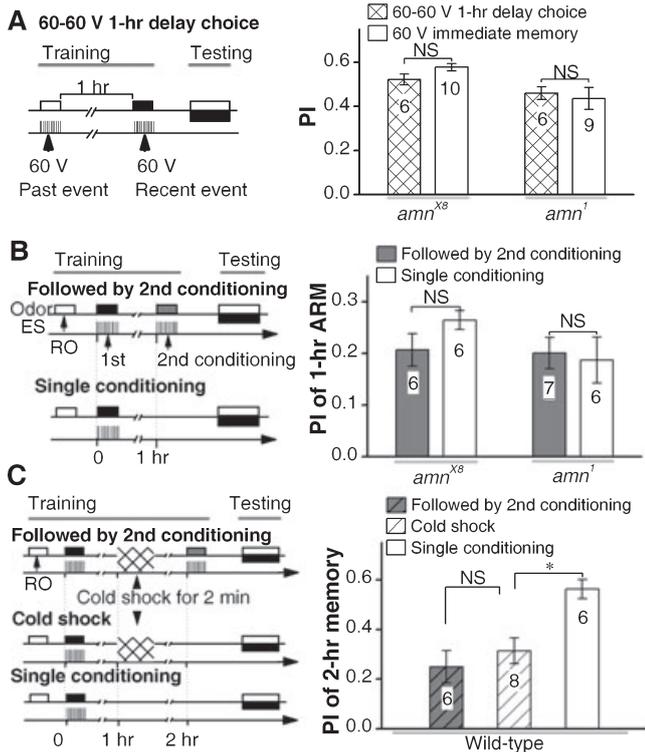


FIG. 5. The ARM of the first conditioning event is not disrupted by a second conditioning event in the 60–60 V choice. (A) Left: the protocol for the 60–60 V 1 h delay choice. There was a 1 h delay between the two 60 V conditioning events. After two conditioning events, flies were forced to choose between the two conditioned odors. Right: the choice performance (cross-hatched bar) was not statistically different from the immediate memory of a 60 V single conditioning event (white bar) in either *amn^{X8}* or *amn¹* mutant flies. For the 60 V immediate memory, we used the standard single conditioning protocol (see Materials and methods). (B) Left: experimental protocols are the same as those in Fig. 4B. 4-methylcyclohexanol, 3-octanol and isoamyl acetate were used here (for odor balances see supporting Fig. S2). Right: there was no significant difference between the 1 h memory from a single conditioning event (white bar) and the 1 h memory followed by a second conditioning event (grey bar) in either *amn^{X8}* or *amn¹* flies. For wild-type performance, please see Fig. 4B. (C) Left: wild-type flies were subjected to 2 min of cold-shock anesthesia at 1 h after the first conditioning event and received a second conditioning event at 2 h. Immediately after that, 2 h ARM was retrieved (upper). For 2 h ARM (with cold shock), the protocol was the same as the above except that the second conditioning event was omitted (middle). For 2 h memory of a single conditioning event (without cold shock), the protocol was the same as the above except that both the second conditioning event and the 2 min cold shock were omitted (lower). Right: the retrieved 2 h ARM followed by a second conditioning event (diagonal striped grey bar) was not significantly different from the 2 h ARM with cold shock (diagonal striped white bar) (Scheffe's test, $P = 0.722$), which was significantly lower than the 2 h memory of a single conditioning event (without cold shock, white bar) (Scheffe's test, $P = 0.030$). RO, reference odor. * $P < 0.05$.

protocol was not significantly different from the 30 V immediate memory PI in both *amn^{X8}* and *amn¹* mutant flies (Fig. 6A, $t_{12} = 0.374$, $P = 0.716$; $t_{12} = 0.302$, $P = 0.769$, respectively). Therefore, a past experience with more danger had little influence on current choice in *amn* mutant flies, indicating that their choices were based completely on recent experiences. We also tested the ability of *amn* mutant flies to discriminate between 60 and 30 V (Choice PI = -0.23 ± 0.04 and -0.15 ± 0.03 in the 60–30 V choice with 1 min delay in *amn^{X8}* and *amn¹* mutants, respectively). This result suggested that the *amn* gene may be indispensable for integrating past information into current decisions.

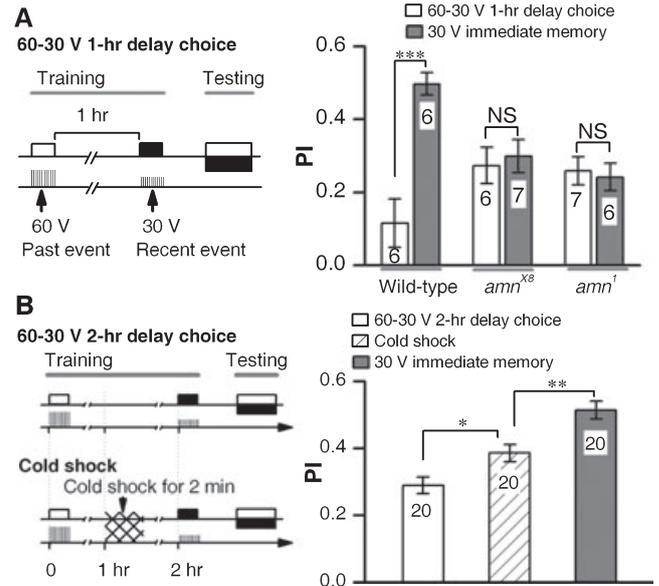


FIG. 6. Choice Performance in the 60–30 V choice with a 1 or 2 h delay. (A) Left: the 60–30 V 1 h delay choice protocol. Flies were first exposed to one odor paired with a 60 V ES as the past event; 1 h later the other odor was presented to flies along with a 30 V ES as the recent event in the training session. Immediately after that, flies were forced to choose between these two odors. Right: the Choice PI was significantly lower than the immediate memory PI of 30 V conditioning in wild-type flies. However, the Choice PI was statistically indistinguishable from the immediate memory PI of a 30 V single conditioning event in either *amn^{X8}* or *amn¹* mutant flies. (B) Left: the 60–30 V 2 h delay choice protocol is the same as that above, except for a 2 h delay between the two events (upper). The 60–30 V 2 h delay choice with cold-shock protocol. Flies were subjected to 2 min of cold-shock anesthesia at 1 h after a first conditioning of 60 V and received a second conditioning of 30 V at 2 h. Immediately after that, choice performance was tested (lower). Right: the Choice PI for the 60–30 V 2 h delay choice with cold shock (diagonal striped bar) was significantly higher than that without cold shock (white bar) (Tukey's HSD test, $P = 0.032$) and was significantly lower than the 30 V immediate memory PI (grey bar) ($P = 0.003$). The Choice PI for the 60–30 V 2 h delay choice (white bar) was significantly lower than the 30 V immediate memory PI (grey bar) ($P < 0.001$). For the 30 V immediate memory, we used the standard single conditioning protocol (see Materials and methods). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Anesthesia-resistant memory and middle-term memory of a past event may contribute to decisions

To identify which form of past memory contributes to the choice process, we used cold shock at 1 h after a first conditioning of 60 V to disrupt the MTM component of memory. Immediately after a second conditioning of 30 V at 2 h, wild-type flies were forced to choose using only ARM of the past 60 V conditioning event and immediate memory of the 30 V conditioning event. Our data showed that the Choice PI with cold shock was significantly lower than the immediate memory PI for a single 30 V conditioning event (Fig. 6B, $F_{2,57} = 18.147$, $P < 0.001$, Tukey's HSD, $P = 0.003$), suggesting that the ARM of the past 60 V conditioning event had a significant influence on the decision. Also, the Choice PI with cold shock was significantly higher than the Choice PI without cold shock (60–30 V 2 h delay choice) (Tukey's HSD, $P = 0.032$), implying that the MTM of the past 60 V conditioning event also had an influence on the decision. Therefore, the results suggest that both ARM and MTM of past disadvantageous events may contribute to current decisions when past experience represents a high degree of disadvantage.

Discussion

Our novel paradigms have given us an opportunity to conduct a series of quantifiable assessments of choice behavior and its underlying memories, and thereby clarify the choice strategies adopted by flies.

In this work we demonstrated that previous experiences play a crucial role in decisions in *Drosophila*. Based on their previous experiences, flies can interpret different odors as representations of different levels of danger and use these representations to choose the lesser of two egregious outcomes. Our experiment showed that the Choice PI increased as a function of ΔV in a linear manner and the Choice PIs were also roughly equal within the groups with the same ΔV (Fig. 2), demonstrating that flies are able to quantitatively evaluate two previous events and use a linear subtraction strategy to make a decision. This capability is similar to what monkeys do during the flutter discrimination task (Hernandez *et al.*, 2002), which may imply evolutionary conservation in choice strategy.

We found that flies weigh two equally dangerous experiences according to the relative times when the experiences occurred. Once the delay between the two experiences reached 30 min, full weight was given to the more recent experience (Fig. 4A). Although under these conditions the past memory does not contribute to the decision, our data indicate that most of the past memory still existed during the decision (Fig. 4B). This suggests that the 'recent-takes-all' character of this choice process mainly depends on an 'overshadowing' effect, rather than an 'erasing' effect, to magnify the influence of the recent experience. Similar types of heuristics and biases are characteristic of the neuro-economic activities of human beings, which systematically give more weight to familiar, recent or easy-to-conjure-up examples (Tversky & Kahneman, 1974).

Our 60–60 V choice protocol (Fig. 4A) appears to be similar to the previously described reversal learning paradigm (Dubnau & Tully, 1998), as both involve two conditioning events. However, the maximal Choice PI in reversal learning is significantly lower than the immediate memory PI of single conditioning, a finding that differs from the results obtained using our protocol (Fig. 4A). Our explanation is that after an odor is presented as CS+ in the first conditioning event, the same odor presented as CS– in the second conditioning event in the reversal learning paradigm might retrieve the first memory before the choice test and activate the first memory. This active first memory might then exert some influence on the decision process. This could cause the Choice PI to be lower than the PI of single conditioning in reversal learning.

Moreover, we found that the Choice PI in the 40–30 V choice or 20–30 V choice was significantly higher than that of the other voltage-pair choices in the same 10 V difference groups ($\Delta V = 10$ V) (supporting Fig. S5). Why did flies overestimate the same difference at a lower voltage range? We hypothesized that there might be a threshold (or voltage-change-sensitive point) near 30 V, so that a small increase above the threshold was evaluated as a non-linear multiple of the voltage difference, whereas a small increase above some higher voltage was evaluated as a linear addition to the higher voltage. Thus, voltage differences near the threshold might be overestimated. Also, the comparison between two memories (first and second memories) might not be completely parallel. It might be possible that the first memory was slightly dominant during decision. Therefore, in $\Delta V = 10$ V groups, the 40–30 V choice performance is at the high point in the stronger/weaker voltage choice, whereas the high point was shifted to the 20–30 V choice in the weaker/stronger voltage choice. In addition, the PIs of the three memories retrieved in the 60–60–60 V choice were lower than expected (supporting Fig. S11). However, the three coexisting memories in the 30–30–30 V choice were normal (supporting

Fig. S10), indicating that flies can learn three events well at 30 V ES. Therefore, we suspect that three sequential 60 V conditionings (with 1 min intervals) might induce strong inhibition for each learning event. Moreover, *amn* mutant flies showed some STM defect (Figs 5A and 6A), although they have long been known as MTM-defective mutants. There is also some evidence of an STM defect in *amn* mutant flies in previous reports (DeZazzo *et al.*, 1999; Tamura *et al.*, 2003). However, it is not clear how the *amn* gene affects STM. We suppose that it may be due to a developmental effect, or it may be relative to the cAMP signaling pathway, as the *amn* gene encodes homologies to vertebrate pituitary adenylyl cyclase-activating peptide and growth hormone-releasing hormone (Feany & Quinn, 1995; DeZazzo *et al.*, 1999). However, our result showed that *amn* mutant flies were able to discriminate the 60 V ES from the 30 V ES and make an appropriate choice according to the degree of disadvantage in the 60–30 V 1 min delay choice, although they have a partial STM defect.

Our research focused not only on choice strategies but also on the memories underlying decisions. First, we found that two coexisting STMs contribute to a decision according to the degrees of disadvantage that they each represent. Previous studies have shown that two independent STMs can be stored in the fly's brain (Ejima *et al.*, 2005; Masek & Heisenberg, 2008). Our results indicate that these coexisting STMs not only represent the separate memory traces for different odors (or cues) but also provide the possibility for relative value computation. Moreover, we demonstrated that flies make choices using a linear subtraction mechanism that is based on competition between two coexisting STMs that represent different degrees of disadvantage. Second, coexisting memories of three events contribute to the flexibility of decisions (Fig. 3). To our knowledge, this is the first report that three memories can coexist in the fly's brain. More importantly, it demonstrates the fly's ability to store complex information and then extract it from multiple locations and integrate it into a single decision process. Third, we found that the role of ARM of past events in current decisions is varied depending on the degree of disadvantage and ARM is stable as a retentive memory. In addition to being resistant to cold shock and aging (Folkers *et al.*, 1993; Tamura *et al.*, 2003), we found that ARM is also resistant to interference from new conditioning events (Fig. 5). We demonstrate here that ARM of a past event and STM of a new conditioning event can coexist as memory traces for past and recent events. The ARM of a past experience does not contribute to decisions when the past experience and recent experience represent the same danger (Figs 4A and 5). However, the ARM of a past experience is activated and has significant influence on decisions when the past experience represents a high degree of disadvantage (Fig. 6). Thus, whether the ARM of a past event contributes to the current decision may depend on the degree of disadvantage of past and recent experiences.

Therefore, we demonstrate here that flies use different choice strategies to guide decisions, and which strategy they use depends on a competition between coexisting memories. The availability of powerful genetic tools, sophisticated techniques for imaging the working brain and electrophysiological techniques (Fiala *et al.*, 2002; Greenspan, 2004; Wilson *et al.*, 2004; Yu *et al.*, 2005; Keene & Waddell, 2007; Wang *et al.*, 2008) all make the fly an ideal model organism for understanding the fundamental brain mechanisms of decision-making. Additionally, many aspects of this process remain elusive. How are two memories activated simultaneously and compared to reach a quantitative decision? Is there working memory during decision-making in *Drosophila*? What region in the fly brain is in charge of the comparison? Our research provides insight into the nature of value computation in the fly's brain and may serve as a valuable starting point for studies that will eventually reveal the nervous mechanisms underlying the 'intuitive' level of decision-making.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. The apparatus for the application of odors.

Fig. S2. Odor balances within the odor sets.

Fig. S3. The shock reactivity PI increased as a function of voltage in a linear manner up to 60 V.

Fig. S4. Flies did not make a consistent choice in a 60–60 V protocol and this was not due to an interference effect of the 60 V ES or the odor presentation from the second conditioning event.

Fig. S5. In the 10 V difference groups, the Choice PI in the 20–30 V choice was significantly higher than that in the 30–40, 40–50 or 50–60 V choice, and the Choice PI in the 40–30 V choice was significantly higher than that in the 30–20, 50–40 or 60–50 V choice.

Fig. S6. Flies always chose to avoid the odor associated with 60 V in either the 60–30 or 30–60 V choices, whether choice performance was assayed at 2 or 60 min after training.

Fig. S7. Using the same odor pair, two memories were retrieved proportionally to their voltages in both the 60–60 and 60–30 V protocols.

Fig. S8. The time interval between CS– and CS+ did not affect memory retrieval.

Fig. S9. Using the same odor pair, three memories were retrieved proportionally to their voltages in the 60–45–30 V protocol.

Fig. S10. Three indistinguishable memories were retrieved in the 30–30–30 V protocol.

Fig. S11. Using either different odor pairs or the same odor pair, three indistinguishable memories were retrieved in the 60–60–60 V protocol.

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Abbreviations

amn, *amnesiac*; ARM, anesthesia-resistant memory; CS, conditioned stimulus; ES, electric shock; MTM, middle-term memory; PI, performance index; STM, short-term memory.

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