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Mice learn from the predator-attack experience to accelerate flight behavior via optimizing the strategy of environment exploration

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1. Introduction

Wild animals must risk exposure to predators to obtain access to food and mates. This requires maintaining vigilance so that imminent predators can be discovered in time to perform appropriate defensive responses. Though some animals may be sacrificed during their first encounter with predators, others may survive multiple predator strikes. Field studies in mice suggest that experience of predator attacks increase the chances of surviving future attacks [1,2]. Other work has shown that animals change their behavior to avoid risk after exposure to predator-related cues. For example, experiences of visual and/or olfactory predator cues enhances alertness and shortened foraging routes in damselfish [3] and experience of predator-related chemical signals reduce the rate of being caught by predators in tadpoles [4]. A single experience of capture results in Tetramorium Ants learning to avoid dangerous antlion pits [5]. However, little is known of how and why the experience of a predator attack would affect innate defensive behaviors. One hypothesis is that a predator attack causes sustained anxiety, and therefore leads to raised vigilance and improved defensive behaviors in response to danger cues [6]. Another hypothesis is that animals learn from predator attacks and optimize subsequent behavioral patterns for defense against potential predators.

Here, we evaluated the effects of simulated-predator-attack experience on responses to subsequent predator cues, anxiety levels, and behavioral pattern changes during environmental exploration in mice. Looming stimuli, a series of expanding dark disks which simulate an approaching object, were used as a predator cue. Previous studies have shown that looming stimuli can induce robust flight responses [7,8]. However, since looming stimuli cannot make contact with mice, we manually caught mice to simulate an actual predator attack. Anxiety levels were evaluated using both the elevated plus maze (EPM) and an open-field test.
that expanded from 246 cm above the arena (refresh rate 60 Hz, AOC) displaying a grey background, positioned with Psychtoolbox-3 and presented on a 42-inch LCD monitor with two open arms (23.5 cm2.3.2. Elevated plus maze (EPM) test

than 3 min. Maximum of 5 stimuli trials within 30 min with interval of no less 50 ms before disappearing and was repeated 15 times with in-

2. Methods

2.1. Animals

Male C57BL6/J mice (7 weeks of age, Beijing Vital River Laboratory Animal Technology, China) were group housed 5 mice per cage on a 12-hr light/12-hr dark cycle. Food and water were available ad libitum. All experiments were performed during day-
time (8:00–20:00). All animal experiments were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) and were conducted following protocols approved by the Institutional Animal Care and Use Committee at Shenzhen Institutes of Advanced Technology, CAS. Each mouse was tested in only one behavioral paradigm.

2.2. Predator-attack handling

Mice were randomly grouped. During one week before the experiment, the cages with experimental mice were not allowed to move; the food, water, and bedding were not changed. Mice in the experienced group were caught tail by hand and lifted up [12], whereas mice in the inexperienced group remained untouched on the day before the behavior test. Behavioral test was performed the next day. In the drug injection experiment, the catching and intraperitoneal injection were used as the simulation of predator attack.

2.3. Behavioral tests

2.3.1. Looming test

The looming test was performed in the AIBM system [13]. The behavior arena was made of acrylic and consisted of a circular open area (45 cm diameter, 30 cm height), adjacent to a refuge alley (10 cm × 5 cm × 30 cm). The floor was frosted white, the refugee walls were black, and the open area wall was transparent. Visual stimuli were the real time trajectory and speed of the testing mouse was detected by an infrared touchscreen frame. Looming stimuli was automatically triggered when the mouse walked in the trigger area (25 cm diameter at the center of the open area) with low speed (<0.15 m/s). The looming stimuli were generated using MATLAB with Psychtoolbox-3 and presented on a 42-inch LCD monitor (refresh rate 60 Hz, AOC) displaying a grey background, positioned 46 cm above the arena floor. The looming stimulus was a black disk that expanded from 2° to 40° in 300 ms and maintained this size for 50 ms before disappearing and was repeated 15 times with intervals of 30 ms between each repetition. Each mouse was allowed to freely explore the behavior arena for at least 5 min before the first looming stimuli trial was triggered. Each mouse could trigger a maximum of 5 stimuli trials within 30 min with interval of no less than 3 min.

2.3.2. Elevated plus maze (EPM) test

The EPM apparatus was made of frosted white acrylic board, with two open arms (23.5 × 5 × 17 cm) and two closed arms of the same length extending from a central section (5 × 5 cm) to form a right-angled plus. The distance between the end of the open arms and the infrared frame was no less than 20 cm, and that between the sides of the open arms and the infrared frames was no less than 23 cm. The plus maze was elevated 85 cm above the floor. Individual mice were placed in the elevated plus maze at the beginning of the experiment and their movements were recorded for 5 min in the AIBM system.

2.3.3. Open field test (OFT)

The open field arena was circular (49 cm diameter, 30 cm height). The center area was defined as a circular area (10 cm diameter) at the center of the arena, and the edge area was defined as a ring-shaped area (width = 5 cm) at the edge of the arena. Individual mice were put in the center of the open field arena at the beginning of the experiment and their movements were recorded for 5 min in the AIBM system. We quantified the time spent in the center and the edge and the entry number to the center and the edge.

2.4. Histology, immunohistochemistry, and microscopy

To quantify the expression of c-Fos positive neurons, the experienced mice were perfused 90 min after being caught by tail, whereas the control mice were taken directly from the cage to perfuse. Mice were euthanized with 1% pentobarbital sodium (0.3 ml/10g body weight) and then perfused transcardially with phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde (PFA, Sigma, Germany). Brains were removed and submerged in 4% PFA at 4 °C overnight to postfix, and then transferred to 30% sucrose to equilibrate. Brains were mounted in tissue-tek® optimal cutting temperature (OCT) compound (4583, Sakura, United States) and coronal sections (thickness 40 μm) were cut using a cryostat microtome (CM1950; Leica, Germany). Brain sections were washed in PBS, blocked in blocking solution containing 0.3% Triton X-100 and 5% normal goat serum (NGS) for 1 h at room temperature, incubated in rabbit monoclonal anti-c-Fos (1:500, #2250; Cell Signaling Technology, United States), diluted in PBS with 1% NGS and 0.1% TritonX-100 at 4 °C overnight, washed in PBS, and stained with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:500; Jackson Laboratory, United States) for 2.5 h at room temperature. Then, brain sections were stained with DAPI (1:50,000, #62248; Thermo Fisher Scientific, United States) and cover slipped with aqueous mounting medium (Fluoromount™ F4680, Sigma, Germany). Brain slices were photographed using an Olympus VS120 virtual microscopy slide scanning system (Olympus; Japan). Images were analyzed using Imagej and Adobe Photoshop software. ROIs were traced with reference to the “The mouse brain in stereotaxic coordinates”, by Paxinos and Franklin, and c-Fos immunoreactivity was quantified by manual counts.

2.5. Drug injection

The protein synthesis inhibitor, anisomycin (150 mg/kg, MCE, HY-18982), was used to inhibit learning and memory and PBS was used as control. Anisomycin or PBS was intraperitoneal injected. The catching and drug injection were used as the simulation of predator attack.

2.6. Behavior analysis

All behavioral data from infrared frame were analyzed as previous studies [13], including the latency to refuge, latency to maximum speed, mean escaping speed, trajectory of the mice, distance ratio, and hiding time in the refuge. Videos were recorded in a side view. Behavior on each frame of the training video was manually labeled as one of the 7 behaviors, including being in
refuge, edge exploration, floor exploration, walking head down, walking head straight, searching head up, wall climbing and other unclassified behaviors. A ResNet50 artificial neural network was trained to label all the videos. The behavior labels were refined by the mouse location and speed recorded by the infrared touchscreen frame detector.

2.7. Statistical analysis

Data are presented as boxplots. All behavior trials were treated independently for statistical analyses. Mann-Whitney U tests were calculated for comparisons between the experimental and control groups. Statistical analyses were performed using MATLAB. Asterisks indicate the level of statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001).

3. Results

3.1. The experience of predator-attack accelerated the looming-evoked flight behavior

To investigate whether the experience of predator-attack would affect the behavioral response to a cue of predator, we caught mice by tail to simulate a predatory attack and tested their responses to looming stimuli the next day (Fig. 1A). Looming stimuli evoked rapid flight-to-refuge behavior in both experienced and inexperienced (control) mice. However, the flight behavior in the experienced mice was enhanced compared to the control mice. The latency to refuge after looming stimuli was significantly decreased (Mann-Whitney U test: $U = 46$, $N_1 = 9$, $N_2 = 11$, $p < 0.8182$) or open arms (Mann-Whitney U test: $U = 36.5$, $N_1 = 9$, $N_2 = 11$, $p < 0.3366$) in the time spent in the closed (Mann-Whitney U test: $U = 47$, $N_1 = 9$, $N_2 = 11$, $p < 0.8792$) of the EMP between experienced and inexperienced mice (Fig. 2B–E). On the open field, there was no difference in either the number of entries to closed (Mann-Whitney U test: $U = 27$, $N_1 = 10$, $N_2 = 10$, $p > 0.0857$) or the edge (Mann-Whitney U test: $U = 31$, $N_1 = 10$, $N_2 = 10$, $p < 0.1615$), or in the time spent in either the center (Mann-Whitney U test: $U = 45$, $N_1 = 10$, $N_2 = 10$, $p > 0.0857$), indicating that the experience of being caught prolonged the fear emotion and made mice more cautious. These data suggest that the experience of predator-attack enhanced the vigilance of mice, making them respond more rapidly and intensively to the predator cue, and thus would increase the probability to survive.

3.2. The experience of predator-attack improve flight not by increasing the anxiety level

It has been reported that anxiety reduced the latency to flight in response to looming stimuli and increased the hiding time [6]. To investigate whether the experience of a predator attack enhances flight by enhancing anxiety level, we evaluated anxiety levels in tail-cought mice using both the elevated plus maze (EPM) and an open-field test (OFT) the following day (Fig. 2A, F). We found that there was no difference in either the number of entries to closed (Mann-Whitney U test: $U = 31$, $N_1 = 10$, $N_2 = 10$, $p < 0.1615$) or open arms (Mann-Whitney U test: $U = 47$, $N_1 = 9$, $N_2 = 11$, $p < 0.8792$) of the EMP between experienced and inexperienced mice (Fig. 2B–E).
Fig. 2. Evaluation of anxiety level in mice with or without predator-attack experience. A) Example trajectories from the control (top) and experienced (bottom) groups on the EPM. B-E) The number of entries to the closed arm B), the open arm C), time spent in the closed arm D), and the open arm E) of mice on the EPM. F) Example trajectories from control (top) and experienced (bottom) groups on the OF. G-J) The number of entries to the central area G) or the edge H) and time spent in the center I) or at the edge J) of the OF. Results are shown as box plots with individual data points. Each point represents one mouse; n = 9 mice in the control group for EPM; n = 11 mice in the experienced group for EPM; n = 10 mice in the control group for OF; n = 10 mice in the experienced group for OF; n.s., no significant difference, Mann-Whitney U test.
N1=N2=10, p < 0.7337) or edge (Mann-Whitney U test: U = 29, N1=N2=10, p < 0.1212) between the two groups of mice either (Fig. 2G–J). These results indicate that catching the tails of the mice in our experiments did not enhance anxiety levels. Therefore, the acceleration of flight in experienced mice was likely caused by learning. Following learning, mice may alter their behavior to optimize defensive strategies.

3.3. Simulated predator attack activates brain nuclei related to the processing of innate fear and learning

To test the hypothesis that mice can learn from the experience of a predator attack, we analyzed tail-caught-induced c-Fos expression in brain. We found that in innate-fear-related nuclei such as the superior colliculus (SC) (Mann-Whitney U test: U = 1, N1 = 5,

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Fig. 3. C-Fos expression in the brains of mice with or without predator-attack experience. A) C-Fos expression in the SC and the PAG, two nuclei related to innate fear. B) the LA, the basolateral amygdala (BLA) and the central nucleus of the amygdala (CeA). C) the dorsal hippocampus (D) and the ventral hippocampus, nuclei related to learning. E) C-Fos expression in the anxiety related nucleus, the LC. F) Statistical analysis of c-Fos expression in the SC (n = 5 in the control group, n = 6 in the experienced group), the PAG (n = 5 in the control group, n = 6 in the experienced group), the CeA (n = 6 in the control group, n = 6 in the experienced group), the BLA (n = 6 in the control group, n = 6 in the experienced group), the LA (n = 5 in the control group, n = 6 in the experienced group), the dorsal hippocampus (n = 6 in the control group, n = 6 in the experienced group) and the LC (n = 4 in the control group, n = 3 in the experienced group). Results are shown as box plots with individual data points. Scale bars = 200 μm, *p < 0.05, **p < 0.01, Mann-Whitney U test.
N2 = 6, p < 0.0087) [14–16] and the periaqueductal grey (PAG) (Mann-Whitney U test: U = 0, N1 = 5, N2 = 6, p < 0.0043) [17–19], c-Fos expression in experienced mice was significantly higher than in inexperienced mice (Fig. 3A, F). In brain nuclei involved in learning and memory, including the lateral amygdaloid nucleus (LA) (Mann-Whitney U test: U = 3, N1 = 6, N2 = 6, p < 0.0173, Fig. 3B, F) and the hippocampus (dorsal part, Mann-Whitney U test: U = 1, N1 = N2 = 6, p < 0.0043, Fig. 3C, F; ventral part, Mann-Whitney U test: U = 0, N1 = 5, N2 = 6, p < 0.0043, Fig. 3D, F) [20], experience of predator-attack also induced c-Fos expression. Meanwhile, in anxiety-related nuclei such as the locus coeruleus (LC) [6,21], there was no difference in c-Fos expression between experienced and inexperienced mice (Mann-Whitney U test: U = 3, N1 = 4, N2 = 3, p < 0.3429, Fig. 3E and F). These data are consistent with the EMP and OFT results which suggested that experience of a predator attack did not induce anxiety but may have initiated a learning process after which mice would increase caution and optimize their strategy to defend against potential predators.

3.4. Inhibition of protein synthesis weakened the effect of predator-attack on looming-evoked flight behavior

To test the hypothesis that the experience of predator-attack had formed fear memory to optimize the flight response to the predator cue, we inhibited the memory formation by intraperitoneal injection of the protein synthesis inhibitor anisomycin [22,23] and PBS was used as control (Fig. 3A). Injections were given before tails were caught to mimic a predator attack. Looming-induced flight behavior was tested the next day (Fig. 4A). We found that mice injected with anisomycin took longer to reach the refuge in response to looming stimuli compared to control mice (Mann-Whitney U test: U = 156, N1 = 20, N2 = 25, p < 0.0327, Fig. 4B). The latency to maximum speed was higher in anisomycin-injected mice (Mann-Whitney U test: U = 132, N1 = 20, N2 = 25, p < 0.0073, Fig. 4C) and mean speed during escape was lower (Mann-Whitney U test: U = 144, N1 = 20, N2 = 25, p < 0.016, Fig. 4D), compared to control mice. The time spent hiding in the refuge were unaffected by anisomycin (Mann-Whitney U test: U = 203.5, N1 = 20, N2 = 25, p < 0.2934, Fig. 4F). These results suggest that mice are able to learn from the experience of a predator attack to quicken response to a predator, and therefore, potentially increase the probability of survival.

3.5. The experience of predator-attack regulated the behavior pattern during environment exploration

Experienced mice had a more rapid and intense response to the predator cue compared to inexperienced mice (Fig. 1). We hypothesized that the experience of predator-attack could regulate the behavior pattern during the environment exploration. To test this hypothesis, we analyzed the behavioral repertoire in mice (Fig. 4A). We labeled the behavior on each frame of the training videos and trained a ResNet50 artificial neural network [24] to label behaviors in all the videos. The locations and velocity of mice which were recorded by the infrared touchscreen frame were used to check and refine the behavior labels (Fig. 4A). Seven behaviors were labeled, including being in the refuge, edge exploration, floor exploration, walking with head down or head straight, searching with head up, wall climbing and other unclassified behaviors (Fig. 5B). The ratio of time spent at each behavior to the total time

![Fig. 4. Analysis of flight behavior induced by looming stimuli following the injection of anisomycin or PBS.](image)

A) Schematic diagram showing the experimental design. Mice were caught by their tails and injected (i.p.) with anisomycin or PBS (control) on the first day. Looming-stimuli-induced flight behavior was tested on the second day. B) The latency for mice to enter the refuge after each looming stimuli trial. C) The latency for mice to reach their maximum speed during flight. D) Mean speed during flight. E) The ratio of the actual flight trajectory to the straight-line distance from the trigger position to the refuge. F) The time spent in the refuge following looming stimuli. Results are shown as box plots with individual data points. Each point represents one trial, n = 20 trials from 4 mice in the control group; n = 25 trials from 5 mice in the experienced group; *p < 0.05, **p < 0.01, Mann-Whitney U test.
during a 5-min chamber exploration period before looming stimuli was analyzed. We found that experience of a predator attack led to a tendency of higher refuge ratio compared to mice with no experience (Mann-Whitney U test: $U = 19$, $N_1 = 10$, $N_2 = 9$, $p < 0.0942$, Fig. 5C). Experience had no effect on the edge-exploration ratio (Mann-Whitney U test: $U = 41$, $N_1 = 10$, $N_2 = 9$, $p < 0.7751$, Fig. 5D). Floor-exploration ratio was significantly lower in experienced mice compared to inexperienced mice (Mann-Whitney U test: $U = 19$, $N_1 = 10$, $N_2 = 9$, $p < 0.0373$, Fig. 5E). The ratio of walking-related behaviors (head down, Mann-Whitney U test: $U = 27$, $N_1 = 10$, $N_2 = 9$, $p < 0.1530$; head straight, Mann-Whitney U test: $U = 37$, $N_1 = 10$, $N_2 = 9$, $p < 0.5403$; Fig. 5F and G), upward-searching-related behaviors (head up, Mann-Whitney U test: $U = 33$, $N_1 = 10$, $N_2 = 9$, $p < 0.3477$; climb, Mann-Whitney U test: $U = 35$, $N_1 = 10$, $N_2 = 9$, $p < 0.4379$; Fig. 5H and I) and other unclassified behaviors (Mann-Whitney U test: $U = 41$, $N_1 = 10$, $N_2 = 9$, $p < 0.7751$, Fig. 5J) were no different in experienced mice than in inexperienced mice. These results suggest that the experience of a predator attack did not affect general environmental exploration, except for a reduced focus on the ground. During floor exploration, mice touch the floor with whiskers, nose, and mouth, which is likely helpful for discovering resources such as food and mates. However, focusing on the floor can potentially lead to ignorance of cues present in the upper visual field, such as predators. Therefore, the experience of a predator attack significantly reduces focus on the floor, which can facilitate the discovery of predators.

**Fig. 5.** Behavioral repertoire analysis in mice with or without predator-attack experience. A) Schematic flow chart showing the behavioral repertoire analysis. B) Example showing the refined behavioral labels of an experienced mouse. C) The ratio of time spent in the refuge, D) the ratio of exploration at the edge of the chamber, E) the ratio of exploration on the floor, F) the ratio of walking with head down, G) the ratio of walking with head straight, H) the ratio of head up searching, I) the ratio of wall climbing, and J) the ratio of other undefined behaviors. Results are shown as box plots with individual data points; $n = 10$ mice in the control group; $n = 9$ mice in the experienced group; *$p < 0.05$, Mann-Whitney U test.
4. Discussion

In this study, we show that a single predator-attack experience led mice to respond more rapidly to a subsequent predator cue. A simulated predator attack did not induce sustained anxiety but formed an experience which the mice used to optimize behavioral patterns during environmental exploration to facilitate the discovery of predator cues, and therefore, potentially increase their probability of survival.

Our data suggests a new mechanism other than chronic stress which quickens flight behavior in response to visual danger cues. Chronic stress caused by a four-day repeated treatment that consisted of restraint, forced swim and foot shock, led to significantly elevated anxiety levels in mice. Chronic stress accelerates the flight response via activation input of LC (Locus Coeruleus) neurons to the SC [6]. We show that a single predator attack did not change anxiety levels (Fig. 2). It is likely that a single predator attack led to quick strategy formation because nuclei related to both innate fear and learning were activated by the tail-catch manipulation (Fig. 3).

A single predator attack activates the hippocampus and the LA (Fig. 3), which have been reported to participate in fear memory acquisition and consolidation [20,23,25,26]. The effect of the predator experience on learning is inhibited by anisomycin, which inhibits more than 90% of protein synthesis in the brain in the first 2 h following injection, thereby reducing plasticity in synapses, and blocking learning [23,27,28]. Therefore, our data suggests that the experience of a predator attack elicited a rapid learning process. In contrast to well-studied associative learning in humans and animals in the laboratory, the experience of a predator attack did not associate a specific cue with punishment or reward, such as in Pavlovian conditioning [29]. Instead, the learning process led to a revised risk assessment which leaned towards caution during exploration and a quickened flight response to danger cues. Rodents pay attention to the overhead space via vibrant eye movements which allows for the immediate discovery of predator cues [30]. At the same time, rodents probe cues on the ground which can lead to resources such as food and potential mates. Danger defending and resource searching alternate all the time. In the floor exploration segments, mice focus on cues on the ground and touch the floor with their nose and mouth, which might reduce the chance to discover danger from above. By reducing floor exploration, mice optimized attention to the overhead space, which facilitated predator-cue discovery. Thus, the predator-attack experience led to a more efficient strategy for predator avoidance in mice, which may enhance the chance of survival when facing predators in the wild.

Learning from experience happens throughout the whole life of animals and human beings. Young mammals simulate hunting or escaping when playing with parents and siblings, which could shape their behavior patterns and increase the chance for survival [31,32]. We show that the learning following the predator attack seems to start an abstractive thinking process. Here, we demonstrated a simple paradigm in mice to evaluate the effect of learning from experience, and we discovered what may be the brain nucleus responsible for learning from experience. However, the detailed mechanism of how these brain nuclei encode the learning process for predator-attack experience remains unclear. Future work should record the electrophysiological responses of experience-related nuclei after the predator attack and other experiences. Thus, the neural basis of abstract thinking and experience formation may be decoded on a neuronal level.

Catching mice by tail, which is used in our experiments to mimic the predator attack, is a basic operation in handling the laboratory mice. Previous studies have shown that tail catching for several times can induce aversion [12], stress [33,34], and increased blood glucose levels [35]. Even a single instance of tail catching can induce acute stress in mice [36]. Our data suggest that tail catching can affect behavioral patterns during both environmental exploration and predator cue defense via advanced cognitive processes. Therefore, our results can be used for reference in animal welfare and laboratory experiments.

Author contributions

J. Z., X. Y., Q. L., and L. W. designed the study; J. Z., J. S. and X. L. executed the experiments; J. Z. and X. Y. analyzed the data; Q. L. Z., X. Y., and L. W. wrote the paper; and all authors read and commented on the manuscript.

Data availability

All data are available in the main text.

Declaration of interest statement

All authors declare that they have no competing interest.

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