Adding preferred color to a conventional reward method improves the memory of zebrafish in the T-maze behavior model

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ABSTRACT
Zebrafish have become a useful model for studying behavior and cognitive functions. Recent studies have shown that zebrafish have natural color preference and the ability to form associative memories with visual perception. It is well known that visual perception enhances memory recall in humans, and we suggest that a similar phenomenon occurs in zebrafish. This study proposes that adding a visual perception component to a conventional reward method would enhance memory recall in zebrafish. We found that zebrafish showed greater preference for red cellophane over yellow in the training session but could not remember the preferred place in the memory test. However, the test memory recall was greater when the zebrafish were exposed to the red cellophane with a food reward during the training session, when compared with the use of food reward only. Furthermore, the red cellophane with food reward group showed more predictable memory recall than the food reward only group. These results propose that visual perception can increase memory recall by enhancing the consolidation processes. We suggest that color-cued learning with food reward is a more discriminative method than food reward alone for examining the cognitive changes in the zebrafish.

Abbreviations: WM: working memory; LTM: long-term memory

Introduction
Recently, utilitarian models have been developed using zebrafish for studying cognitive functions and behavior (Levin and Chen 2004; Liu et al. 2012; Collier et al. 2014). Zebrafish exhibit various natural behavioral responses such as anxiety-like behavior, avoidance, scototaxis, geotaxis, stress-related freezing, habituation, food-seeking, place preference, startle response, and shoaling. Recently, Avdesh et al. (2012) demonstrated that zebrafish naturally preferred red and green colors over yellow or blue. Zebrafish are particularly suitable for forward genetics (Amsterdam and Hopkins 2006) because of their small size (4 cm when adult), ease of maintenance, transparency, and prolific nature (2–300 eggs per day). Considering the low cost, easy access to forward genetics, and the various behavioral responses of zebrafish, the zebrafish behavior model can be a beneficial tool for evaluating the mechanisms of cognitive disorders and for screening cognitive effects of drugs.

Memory is the process by which perceptions are encoded, stored, and retrieved. Memory is divided into two major components: working memory (WM) and long-term memory (LTM). WM generally lasts for durations in the order of seconds up to several minutes. Memories that have been retained in a more permanent manner become LTM. To promote memories of past events, LTM requires consolidation, the process by which memories become more stable and robust. Different perceptions can synergistically affect the consolidation of LTM. This effect is referred to as synesthesia or chromesthesia (Presti 2015). It is thought that visual perception enhances consolidation of memory by a structural change in the nervous system via the hippocampus. Synesthesia with visual perceptions is well-known in humans (Suzuki and Takahashi 1997; Brady et al. 2008). However, associative memory related to synesthesia has not been studied in the zebrafish model because of the belief that zebrafish lack complex brain processes due to the absence of the hippocampus. This idea was challenged by Sison and Gerlai (2010) who found that zebrafish have the ability to form associative memory with visual perception, even though synesthesia was not demonstrated.
In this study, we hypothesized that adding a preferred color to a conventional method that consolidates memory through food reward would enhance LTM in zebrafish and provide a more discriminative behavioral model for evaluating memory function.

**Methods**

**Animals**

Adult zebrafish (short-finned wild-type) of a heterogeneous genetic background were purchased from a local aquarium store (Jincheon, Chungcheongbuk-do, Korea). In all experiments, zebrafish were 6–8 months of age and were 2.5–3.5 cm long. Zebrafish were kept in water at 28.5°C on a 14-h-light–10-h-dark cycle in an aquarium container and were fed twice a day with brine shrimp. The container was equipped with a multistage filtration system that had a sediment filter, post-carbon filter, fluorescent UV light, and sterilizing filter (Zebrafish AutoSystem, Genomic Design, Daejeon, Korea).

**T-maze apparatus**

A three-armed T-maze was used. The stem of the maze (length 50 cm × width 10 cm × height 10 cm) included the start box (10 cm × 10 cm × 10 cm) separated from the edge of the stem by a transparent sliding door. Each arm of the maze (20 cm × 10 cm × 10 cm) included a target compartment (10 cm × 10 cm × 10 cm) at the end of the arm (Figure 1(A)). A transparent door was used to block off the arms of the maze from the stem. Green, yellow, or red cellophane sleeves were constructed to fit around target compartments at the end of the arms. The T-maze was filled with 3 L of aquarium water. The water temperature was maintained at 27 ± 1°C. All experiments were conducted between 10:00 and 16:00 h.

**Procedure**

To minimize stress due to procedural novelty, all zebrafish underwent a habituation trial of 2 h before testing. After the habituation trial, each zebrafish was placed into an individual chamber for 1 h. During each trial, a zebrafish was placed in the start box for 1 min with the door closed. Then, a transparent door was raised and closed after the zebrafish had exited. As soon as the zebrafish swam into the left or right target compartment, another door was closed to prevent the zebrafish from re-entering the start box. If the zebrafish did not leave the start box for 5 min, it was excluded from the results. After 5 min, the zebrafish was netted and returned to its individual chamber. A stopwatch was used to measure the time spent in each target compartment. Each zebrafish underwent one trial per day during training. Training was conducted for four consecutive days. After the last training session, all experimental zebrafish underwent memory testing (Figure 1(B)).

**Color preference and food reward**

**Direction test (right or left compartment)**

We investigated whether zebrafish preferred to swim in a specific direction in the T-maze. During the direction test training session, there was no colored cellophane or food reward in the T-maze. The memory testing was not conducted in the direction test group.

**Color preference (red vs. green, red vs. yellow)**

First, to identify color preference (red vs. green, red vs. yellow), green or yellow cellophane was used in the left compartment and red cellophane in the right compartment of the T-maze. Second, to test food location memory, the right arm contained a food reward in the target compartment, while there was no reward in the left compartment. Third, to test whether color cues could
improve memory recall of an existing food reward, red or yellow cellophane was used with the food reward in the right compartment, and a food reward alone was placed in the left compartment. Finally, in one compartment we placed red cellophane with a food reward, and in the other compartment, yellow cellophane with a food reward.

In each trial of all experiments, the time taken to reach the chosen compartment was recorded. A certain amount of food (brine shrimp, 20 µL) measured with a micropipette was used in all food reward experiments before each zebrafish was put in the start box. After 5 min, the zebrafish was netted and returned to an individual chamber. In the memory test, there was no colored cellophane or food reward in the T-maze.

Statistical analysis
All data were expressed as the mean (line) and interquartile range (box); whiskers indicate 5% and 95% percentiles. The experimental data were analyzed using the t-test or Mann–Whitney test. The predictability of the color-cued food reward paradigm was analyzed using univariate logistic regression and expressed as odds ratios. All data were analyzed using SPSS 20.0 software. We calculated the power needed for an effect size at the recommended 0.95 level using G*Power 3.1 software. We chose to use sample sizes of at least 12 in all experiments as a result of this analysis. P values < 0.05 were accepted as statistically significant.

Results

Right vs. left
First, we confirmed whether zebrafish preferentially moved toward the right or left compartment of the T-maze. The total number of entries and time spent in each compartment showed no difference between the right and left compartments (Figure 2(A,B)). Zebrafish similarly visited the right and left compartments of the T-maze. This result showed that direction did not affect the learning of zebrafish using the T-maze.

Red vs. yellow
We evaluated whether zebrafish recognized colors using colored cellophane. A previous study indicated that zebrafish preferred the color red over yellow or green (Avdesh et al. 2012). First, we assessed whether zebrafish preferred red more than yellow. The total number of entries and time spent in each compartment were measured. During the training session, zebrafish spent significantly more time in the red than in the yellow compartment (Figure 3(A), P < 0.001), suggesting that zebrafish preferred red over yellow. However, in memory testing, no significant differences were observed between the red and yellow compartments (Figure 4(A)). The total number of entries showed no significant differences between the red and yellow compartments during the training session or memory test (Figures 3B and 4B).

Red vs. green
Next, we assessed whether zebrafish preferred the color red over green. During the training session, there was no difference in the time spent in the red and green compartments (Figures 3(C,D)). This result showed that zebrafish did not prefer red over green. Also, no significant differences were observed in the length of time spent or total number of entries in the red and green compartments in memory testing (Figures 4(C,D)).

Reward vs. no reward
We tested whether zebrafish were motivated to learn by a food reward. The food was present only in the right compartment. During the training sessions, zebrafish spent significantly more time in the right compartment.

Figure 2. Direction preference of the zebrafish during the training session. (A) Zebrafish spent similar amounts of time in both compartments (n = 17). (B) Zebrafish showed similar numbers of entries into both compartments.
than in the left (data not shown). In addition, zebrafish spent significantly more time in the right compartment than in the left in the memory test (Figure 5(A), \( P < 0.05 \)). This result showed that zebrafish could learn by a food reward during the training session. However, the total number of entries showed no significant differences between the reward and no-reward compartments during the memory test (Figure 5(B)).

Red cellophane + reward vs. reward alone

To enhance the learning effects, we tested a combination of color-cues and food reward to determine whether zebrafish preferred the color red combined with a food reward to the food reward alone. The red cellophane was only present in the right compartment, but the food reward was present in both right and left compartments. During the training session, zebrafish spent more time in the right compartment than in the left (data not shown). During memory testing, zebrafish spent significantly more time in the right compartment than in the left (Figure 5(C), \( P < 0.01 \)). This result showed that zebrafish preferred the color red over white. However, the total number of entries showed no significant differences between the right and left compartments during the memory test (Figure 5(D)).

Yellow cellophane + reward vs. reward alone

We tested whether zebrafish preferred the yellow color combined with a food reward to the food reward alone. The yellow cellophane was only present in the right compartment, but the food reward was present in both right and left compartments. During the training session, zebrafish spent more time in the right compartment than in the left (data not shown). However, during memory testing, the total number of entries and time spent in each compartment showed no difference between the right and left compartments (Figures 5(E,F)).

Univariate logistic regression

We analyzed whether the color-cued food reward method can improve memory compared with food
reward alone. We calculated odds ratios to evaluate discrimination capacity between color-cued food reward and food reward alone (Table 1). The red cellophane with food reward showed a higher odds ratio (mean time 1.05, frequency 1.31, \( P < 0.01 \)) than the food reward alone group.

**Discussion**

This study focused on a simple behavioral testing method examining associative memory of zebrafish. Zebrafish have a highly developed visual system and are an important model for studying vision using easily manipulated visual stimuli (Fadool and Dowling 2008). The present study showed that zebrafish have natural preferences among colors (i.e. red, yellow, green, and no color). In particular, red and green were similarly preferred over other colors (Avdesh et al. 2012).

To the best of our knowledge, this study was the first to confirm that zebrafish prefer red over yellow in a color preference test. In the training sessions, zebrafish spent more time in the red compartment than in the yellow compartment. In contrast, zebrafish did not prefer red over green. Although zebrafish did not remember the previously red compartment in the memory test, we reconfirmed that zebrafish preferred red over yellow.

We hypothesized that color preference would carry over to a learning paradigm, but there was a limit to zebrafish color-cue learning using our methods. It seems that the experiment duration and the number of iterations were insufficient to trigger recognition of the preferred color. However, in the food reward test, zebrafish swam to the target compartment where food was located, and most entered and stayed in the food reward compartment during the training sessions. Furthermore, during testing, many zebrafish swam directly to the compartment where there was previously a food reward, and despite the absence of food, stayed in the compartment. These results suggest that zebrafish acquire and remember spatial cues when a food reward is used. Food rewards have been successfully used in animal research in behavioral tasks as a positive motivator, and food reward preference is a simple behavioral method demonstrating the appetitive behavior of zebrafish (Fernandes et al. 2015). In this study, zebrafish were starved for 1 d prior to the training session and were given a limited amount of food during the training session. Limiting food prevents problems with satiation. Thus, this paradigm could be effective in judging the performance of learning and memory.

We also tested whether a color-cued stimulus can increase mnemonic ability. According to recent studies,
zebrafish have natural color preferences, which directly affect learning and decision-making in zebrafish (Colwill et al. 2005; Spence and Smith 2008). In one study, it was shown that zebrafish learned that red visual stimuli predicted the presence of food (Sison and Gerlai 2010). However, research in associative learning in zebrafish is lacking. Though zebrafish do not have a hippocampus, the region of the brain associated with spatial learning in mammals, the lateral pallium is considered to be structurally homologous and suggests the conservation of some cognitive function (Tropepe and Sive 2003). For instance, zebrafish acquired avoidance behaviors in a Y-maze in which they were presented with a visual cue on the floor paired with an electric shock (Aoki et al. 2015). In humans, reward association improves visual working memory performance (Gong and Li 2014),

Table 1. Univariate logistic regression.

| Dependent variables | Factors         | Odds ratio | C.I.     | P value |
|---------------------|-----------------|------------|----------|---------|
| No reward vs. Reward| Frequency       | 1.03       | 0.93–1.14| 0.570   |
|                     | Mean time       | 1.08       | 1.00–1.16| 0.056   |
| Reward vs. Red      | Frequency       | 1.31       | 1.01–1.70| 0.044*  |
|                     | Mean time       | 1.05       | 1.00–1.09| 0.031*  |
| Reward vs. Yellow   | Frequency       | 1.14       | 0.83–1.57| 0.419   |
|                     | Mean time       | 1.06       | 0.98–1.13| 0.133   |

Note: The data representing the effect of the red cellophane with food reward paradigm.

*P < 0.05, **P < 0.01.
and a study in primates demonstrated the memory consolidation process using a visual association task consisting of visual stimuli with fruit juice as a reward (Miyashita et al. 1998). Recently, associative learning based on color preference was used in a study of the behavioral and physiological mechanisms in a Parkinson’s-like model (Li et al. 2015), demonstrating the importance of associative learning and memory based on color preference in the study of neurodegenerative disorders.

In all experiments of the present study, the number of entries did not differ during memory testing. We analyzed the correlation between the time spent and the number of entries. The results showed that both color (red and yellow) and food reward affected the time spent (Data not shown). Also, food reward affected the number of entries. In all food reward experiments (reward vs. no reward, red + reward vs. reward, yellow + reward vs. reward), zebrafish remained in the location of the food reward for extended periods. We tested whether the number of entries was affected by the lengthy duration spent in the preferred arm of the T-maze. We found that the number of entries did not increase, therefore concluded that it was not an important metric in evaluating learning.

Our study indicates that combined reward and color association learning is an effective method for increasing learning ability. When zebrafish were exposed to the red cellophane with food reward (right compartment) and reward alone (left compartment), zebrafish preferred the red cellophane with food reward compartment over the reward alone compartment in training sessions, and recalled that location in memory testing (Figure 5(C)), indicating that color, especially red, can improve recognition in this task. The significant increase in the time spent in the target compartment reveals that the experimental zebrafish learned and remembered the association between the red cellophane and food reward. In contrast, when zebrafish were exposed to the yellow cellophane with food reward (right compartment) and reward alone (left compartment), zebrafish preferred the yellow cellophane with food reward more than the reward alone compartment only in the training session and not during memory testing (Figure 5(E)). This result demonstrates the utility of food reward and color-cuing in an appetitive learning task for zebrafish. In addition, we conducted the color-cued food reward test, in which the red (right) and yellow (left) cellophane compartments each had a food reward, and in the training session, zebrafish preferred the red cellophane with food reward to the yellow cellophane with food reward compartment. Furthermore, zebrafish recalled the location of the compartment (data not shown). Although both compartments contained food rewards, zebrafish preferred the red compartment over the yellow. These results demonstrate that the color red, the more preferred color, effectively improved the acquisition of a situation and the recall of the acquired memory.

For further analysis, we calculated the odds ratio between the color-cued food reward groups and the reward only group and found that the red cellophane with food reward group exhibited a higher odds ratio compared to that of the reward alone group (Table 1). Therefore, we conclude that the learning and memory ability of zebrafish was more enhanced by color-cued (especially red) food reward than food reward alone. In addition, we inferred that the color-cued food reward learning and memory paradigm is a more discriminative tool to assess cognitive function than the reward only method.

Another study showed that zebrafish required repeated trials (a total of 20 trials) to demonstrate learning (Sison and Gerlai 2010). In our study, four consecutive days of training was sufficient to reach an appropriate level of learning in the color-cued food reward T-maze apparatus. Due to time constraints (10:00 to 16:00 h), the number of zebrafish that can be tested per day is limited. Our methods showed that learning is possible without repetitive training within a day. In general, a single trial may elicit only a short-term (a few minutes) memory, whereas repeated training trials may result in a long-term memory that lasts for hours to days. Therefore, repeated food reward and color stimulations could elicit memories lasting for 24 h in zebrafish.

Conclusions

This study demonstrates that the zebrafish is an excellent vertebrate model to study the complex processes of learning and memory, and suggests that color-cued food reward learning is a more discriminative method than food reward alone for examining cognitive changes in zebrafish. We believe that the color-cued food reward learning paradigm in zebrafish will be a new screening tool for analyzing the cognitive functions of zebrafish in the future.

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