Coffee time: low caffeine dose promotes attention and focus in zebrafish

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Abstract

This study investigated the ability of zebrafish to discriminate visual signs and associate them with a reward in an associative learning protocol including distractors. Moreover, we studied the effects of caffeine on animal performance in the task. After being trained to associate a specific image pattern with a reward (food) in the presence of other images such as distractors, the fish were challenged to locate the exact cue associated with the reward. Distractors were same-colored patterns images similar to those of the target. Both the target and distractors were continually moved around the tank. Fish were exposed to 3 caffeine concentrations for 14 days: 0mg/L (control, n=12), 10mg/L caffeine (n=14) and 50mg/L caffeine (n=14). Zebrafish spent most of the time close to the target (where the reward was offered) under the effects of 0 and 10mg/L caffeine, and the shortest latency to reach the target was observed for the 10mg/L caffeine group. Both caffeine treatments (10 and 50mg/L) increased average speed and distance traveled when compared to the control group. This study confirms previous results showing that zebrafish demonstrate conditioned learning ability; however, low-dose caffeine exposure seems to favor visual cue discrimination and increase zebrafish performance in a multi-cue discrimination task, in which primarily focus and attention are required to obtain the reward.

Keywords: Adenosine antagonist; Vision, Conditioned learning; Associative learning

Introduction

Caffeine is one of the most consumed stimulants in the world (Ferré, 2008; Lieberman, 1992). It is present in a wide range of products including coffee, energy drinks, teas and chocolate. The popularity of this substance lies in its beneficial effects, such as heightened attention and alertness and decreased fatigue (Brunyé, Mahoney, Lieberman, & Taylor, 2010; Einöther & Giesbrecht, 2013; Smith, 2002). It is believed to affect reaction time and accuracy in a variety of tasks (Einöther & Giesbrecht, 2013), increasing consumer productivity (Dagan & Doljansky, 2006; Einöther & Giesbrecht, 2013; Franke et al., 2014; Souissi et al., 2014; Johnson et al., 2016).

Caffeine is almost completely absorbed by the body in the gastrointestinal system, rapidly reaching the brain, where it promotes its effects. The drug is a nonspecific antagonist of adenosine receptors, especially A1 and A2A, which are dispersed throughout the brain (Einöther & Giesbrecht, 2013). By blocking the inhibitory properties of adenosine, a number of neurotransmitters, such as dopamine, glutamate, acetylcholine and noradrenaline, increase postsynaptic potential in a large number of neural pathways, usually increasing brain activity (Brunyé et al., 2010; Einöther & Giesbrecht, 2013). However, caffeine exerts its effect in a dose-dependent manner: moderate amounts increase arousal, while large doses have anxiogenic effects (Lieberman, 1992). Furthermore, depending on caffeine dosage, locomotor behavior has exhibited a biphasic response: low to medium doses increase locomotor activity while high doses decrease it (Marin et al., 2011).

In the modern world we are constantly bombarded with information in a multi-tasking work environment, making it important to focus one’s attention even in the face of distractors, a valuable asset for enhanced learning. In this respect, studies have investigated

the effects of caffeine on cognition, primarily attention and learning (Angelucci, Cesario, Hiroi, Rosalen, & Cunha, 2002; Santos, Oliveira, Oliveira, Silva, & Luchiari, 2016).

In order to combine the effects of distractors and caffeine in a discriminating task, with translational relevance to humans, we used the zebrafish, an animal model at the vanguard of neuroethological research. Zebrafish (*Danio rerio*) are becoming more widely used for neuro-behavioral studies because they share psychopharmacologic, anatomic and genetic characteristics with mice and humans (Barbazuk et al., 2000; Caramillo, Khan, Collier, & Echevarria, 2015). Moreover, there are several recent studies using zebrafish for behavioral functions such as learning, memory and anxiety-like responses, in addition to a number of genetic, embryological and behavioral tools. Zebrafish are also considered a model for assessing drug effects because of easy substance dilution in water (Gerlai, Lahav, Guo, & Rosenthal, 2000) and similar genetic homology (more than 70%) with humans, resulting in a highly translational model. As such, the present study aimed to test the effect of a low and high dose of caffeine on zebrafish performance in locating a target in the middle of several distractors in order to obtain a reward.

Methods

Subjects

Zebrafish (four months old, wild type, both sexes) were acquired from a local breeding farm (Natal-RN) and kept in stock tanks (80 x 25 x 40 cm, 50L) in the vivarium of the Fish Laboratory (Physiology Department of UFRN). The tanks were kept in a closed system using water recirculation with mechanical, biological and chemical filtering. The water temperature was maintained at 28ºC on a 12L/12D light/dark cycle photoperiod. Fish were

fed commercial food (38% protein and 4% lipids, Nutricom Pet) and frozen *Artemia salina* twice a day.

All the experimental procedures were evaluated and approved by the Animal Ethics Committee of Universidade Federal do Rio Grande do Norte (CEUA: 045/2017).

Caffeine exposure

Five days before the beginning of substance exposure, the animals were transferred from the stock tanks into three experimental tanks (40 x 25 x 30cm) with constant aeration and daily water changes to maintain quality. The following groups were tested: control (0mg/L caffeine; n=12), chronic 10mg/L (n=14), and chronic 50 mg/L (n=14). The caffeine concentrations used were based on the behavioral characterization of caffeine effects by Santos *et al*. (2016).To obtain these concentrations, the specific amount of caffeine powder (Sigma – Aldrich #cat C0507) was diluted in system water. The doses were gradually increased to prevent animal deaths (Tran & Gerlai, 2014), starting with 5mg/L and increasing by 50% every two days until the desired dosage was reached (10mg/L or 50mg/L). Caffeine exposure occurred for 60 minutes before and during the training/test sessions. Fish were individually transferred to a 2L tank containing the substance and then to the training/test tank, where caffeine concentration was kept constant.

Discrimination task

The learning task took place in three phases: tank acclimation (1), training (2), and test (3). The three groups (control, caffeine 10mg/L and caffeine 50mg/L) were submitted to all the phases for a total of 20 days. The experimental phases occurred in a 70 x 70 x 15cm tank (40L), which walls were covered with white paper to avoid external interference (Fig. 1).

The acclimation phase (1) lasted 5 days. Fish were placed in the tank in groups to prevent isolation stress, and were allowed to explore the tank for 15 min per day. On the following days, the size of the group was gradually reduced until a single fish explored the tank for 15 min on the last day (5th day). This procedure allowed fish to become familiar with the experimental arena and avoid any novelty effect. After the 15-min period, each fish was returned to its home tank.

The training phase (2) started on the 6th day, following the acclimation phase, and lasted 14 days, with two training trials per day (total of 28 training trials). Fish were always alone in the experimental arena. During the training trials, a different figure was placed on each side of the tank (set of figures in Fig. 1), one of which was the target. The target was the figure that indicated the reward, and although it was moved every training trial, it was always paired with the reward (*Artemia salina*), while the others were distractors. All figures were randomized at each training trial. The reward was only available when the fish entered the target area. A silicon tube connected to a syringe was used to deliver 2 units of artemia to the fish as soon as it entered the target area. All the 4 areas had the silicon tube so that no other cue than the figures could be used to learn the task. Fish behavior was recorded from above using a handycam (Sony DCR-SX45 Digital Video Camera Recorder). Fish were allowed to explore the arena for 15 min, after which they were returned to their home tank.

The test phase (3) was applied after on the 20th day (after 14-days training). All procedures were the same as in the training phase, except that individuals received no reward, even when they entered the target area. Fish explored the arena for 15 min. The test was filmed and later analyzed using the Zebtrack tracking program (Pinheiro-da-Silva, Silva, Nogueira, & Luchiari, 2016). To determine whether the animal chose either the

target or the distractors, we marked an area around each figure and the tracking software calculated the latency to enter each area and time fish spent in each area. The tank (4900 cm^2) was divided into four equal areas located around each visual cue (500 cm^2 each) plus the central and corner areas (2900 cm^2). We also measured average and maximum swimming speed, and freezing behavior.

Statistical analysis

All data were analyzed using the R program (Team, 2015). Statistical significance of $p<0.05$ was considered for all tests.

First, we evaluated data normality and homoscedasticity using Kolmogorov-Smirnov and Levene tests, respectively. We used One-way ANOVA to compare parameters such as intergroup freezing behavior, average swimming speed and maximum speed. For post hoc, Tukey's honest significance test was used to explore all possible pair-wise comparisons of means.

Data of latency to enter the target and distractor areas and residence time in the target and distractor areas needed to be transformed for normality, so that a LMM (Linear Mixed Model) could be applied. Thus, we used the maximum likelihood-like approach of Box and Cox (1964) to select a transformation index using powerTransform command (Team, 2015). For latency data we found the coefficient (λ) to be 0.192, and for time data the coefficient (λ) was 0.585. After transformation, data presented Gaussian distribution and we used the lmer command from the lme4 package (Bates, Maechler, Bolker, & Walker, 2015) to analyze it. In all cases, the post-hoc comparisons between treatments of each model were made using the Tukey post hoc test (lsmeans package) (Lenth & Hervé, 2014).

Results

Figure 2 shows the time fish spent in each area of the arena during the test trial and Figure 3 presents the latency to enter the target or any distractor area during the test. Mixed model comparison showed that time spent in each area showed statistical significance due to the area of the tanks (target or distractors 1, 2 and 3) (LMM, $\chi2 = 9.29$, df = 3, p=0.02) but was not significantly related to treatment (control, caffeine 10mg/L and caffeine 50mg/L) (LMM, $\chi2 = 4.58$, df = 2, p=0.10). The interaction terms treatment vs. areas of the tank was show to be statistically significant (LMM, $\chi2 = 21.88$, df = 6, p=0.001). The post-hoc comparison test (Tukey) indicated that time spent in the target area was higher for the control and caffeine 10mg/L than for caffeine 50mg/L. The fish treated with caffeine 50mg/L spent statistically similar time in the target and distractors 1 and 2 areas, but less time at the distractor 3 area ($p<0.05$) (Fig. 2).

The mixed model applied to latency to enter each area showed that statistical significance was found among treatment (control, caffeine 10mg/L and caffeine 50mg/L) (LMM, $\chi2 = 28.16$, df = 2, $p<0.001$) but there was not statistical significance related to the areas of the tanks (target or distractors 1, 2 and 3) (LMM, $\chi2 = 5.01$, df = 3, p=0.17). The interaction terms treatment vs. areas of the tank was show to be statistically significant (LMM, $\chi2 = 46.58$, df = 6, $p<0.001$). Tukey post-hoc comparison test indicated that the shorter latencies were shown by the control group to enter the distractor 1 area, the caffeine 10mg/L to enter the target area and the caffeine 50mg/L to enter the distractor 1 and 2 areas ($p<0.05$) (Fig. 3).

The values for average speed, maximum speed and freezing behavior are presented in figure 4. One-way ANOVA showed statistical significance for average swimming speed

($F_{40,2}$=6.70, p=0.003), and the post hoc Tukey HDS indicated that caffeine 10mg/L group presented higher average speed than the other groups ($p<0.05$; Fig. 4a). Maximum speed was not statistically significant between groups (One-way ANOVA: $F_{40,2}$=0.89, p=0.42; Fig. 4b). Freezing behavior, a trait related to anxiety response, was shown to present statistical significance between groups (One-way ANOVA: $F_{40,2}$=8.60, $p<0.001$), while Tukey HDS indicated that caffeine 10mg/L group presented the lowest freezing response compared to the other groups($p<0.05$; Fig. 4c).

Discussion

In this study, we evaluated the effect of caffeine on zebrafish performance in a task requiring focus and attention. Zebrafish display a natural tendency to explore and the ability to associate an unconditioned stimulus (food) with a previously neutral cue (the target) in order to process it as a conditioned stimulus. We added distractors, that is, objects resembling the target, which can confuse fish and impair conditioning. Our results show the associative learning ability of zebrafish, corroborating other literature studies (Al-Imari & Gerlai, 2008; Braubach, Wood, Gadbois, Fine, & Croll, 2009; Chacon & Luchiari, 2014; Gómez-Laplaza & Gerlai, 2010; Karnik & Gerlai, 2012; Luchiari & Chacon, 2013). In addition, we show that fish can discriminate the visual target in the presence of distractors and that their performance in terms of time to reach the correct choice improves at a low dose of caffeine (10 mg/L).

Although a number of studies have investigated distractors in fish decision-making and a few others in zebrafish under the effect of caffeine, none have studied these subjects in tandem. Apart from its effect of preventing fatigue, society also uses caffeine to maintain focus on certain activities, such as studying (Hameleers et al., 2000), driving (Liu, Yao, &

Spence, 2014) and similar attention and vigilance tasks (Foxe et al., 2012). In an environment filled with stimuli, attention allows individuals to process and respond only to what is relevant (Thiele & Bellgrove, 2018).

The increased attentional performance provoked by caffeine is related to its effects on adenosine receptors. In fact, during prolonged alertness and attention, firing neurons accumulate a byproduct called adenosine, which acts by binding adenosine receptors and signaling that brain activity should decrease, such as when the body needs rest (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). However, when caffeine is available, it binds the adenosine receptors (antagonist), and the brain's own stimulants, such as glutamate and dopamine, are more likely to function (Fredholm et al., 1999). Another neuromodulatory effect of caffeine is in the brain levels of acetylcholine (Carter, O'Connor, Carter, & Ungerstedt, 1995; Murray, Blaker, Cheney, & Costa, 1982). Methylxanthines such as caffeine increase acetylcholine metabolism and activity (Acquas, Tanda, & Di Chiara, 2002; Murray et al., 1982). Activation of the cholinergic system has been associated with different cognitive functions, including attention, memory and learning (Herlenius & Lagercrantz, 2004).

These positive caffeine effects occur only in controlled amounts, since high caffeine levels increase receptor binding in many parts of the brain and body, raise heart rate and blood pressure, and release hormones such as epinephrine and cortisol (Benowitz, 2008; Butt & Sultan, 2011; Franco, Oñatibia-Astibia, & Martínez-Pinilla, 2013; Rosa et al., 2018). In this respect, high amounts of caffeine are usually related to stress and anxiety (Wood, Sage, Shuman, & Anagnostaras, 2014).

In the present study, the low caffeine dose seems to have ameliorated the ability of fish to discriminate cues and reach the target, while the higher dose, instead of further

enhancing performance, impaired their ability to find the target and may demonstrate a side effect of the substance, namely, increased anxiety (Lieberman, 1992). This biphasic effect of caffeine on zebrafish behavior has been reported in other studies, showing that high doses negate its beneficial effects, giving rise to learning impairment and increased anxiety (Santos et al., 2016; Santos, Ruiz-Oliveira, Silva, & Luchiari, 2017).

It is important to underscore that in our study caffeine affected locomotor parameters, increasing average speed and decreasing freezing behavior in the groups treated with 10mg/L. The increase in zebrafish swimming could have led to the shortest time to reach the target (Fig. 3), however, this response would induce fish to continue exploring the tank regardless the presence of the visual cue, what was not observed (Fig. 2). In fact, after reaching the target area, fish stayed there longer (as the control group; Fig. 2). Also, the longer time in the same place could have been interpreted as higher freezing behavior, what as not observed for the 10mg/L caffeine group, suggesting that burst locomotion may be caused by a decrease in fatigue (Claghorn, Thompson, Wi, Van, & Garland Jr, 2017), rather than an anxiogenic response. The possible decrease in fatigue, together with improved focus to find the area of interest, confirms the positive effect of the low caffeine dose, suggesting that caffeine acts mainly in areas related to attention and alertness at this dose. On the other hand, the high dose (50 mg/L caffeine) may act on other areas of the brain domains, thereby augmenting stress. Rosa et al. (2018) found that 50 mg/L of caffeine increases whole-body cortisol levels in zebrafish. In this regard, we can expect a similar alteration in our experimental fish. However, we cannot confirm this hypothesis, since the levels of freezing and locomotors behavior were the same for 50mg/L caffeine and control groups. Therefore, new tests are required to thorough understand how 50mg/L caffeine impact on the fish cognitive ability.

Caffeine is a widely used psychostimulant (De Luca, Bassareo, Bauer, & Di Chiara, 2007), consumed daily by a large part of the population and drunk excessively by people seeking improved physical or cognitive performance. We demonstrate that a low concentration of caffeine helps fish select what is important in their environment in order to obtain a reward. On the other hand, high concentrations seem to create a stress response, preventing individuals from learning the task. However, these effects were not observed for locomotor behavior. In this respect, studies using techniques to show changes in the brain (neurotransmitters, proteins, neuroplasticity) and body (cortisol levels) caused by different doses of caffeine are crucial for a better understanding of the effect of caffeine on attention and learning shown here.

Finally, our study confirms the importance of zebrafish as a model for drug screening and cognition studies. We show that low caffeine consumption may help perform tasks demanding focus and attention, but chronic consumption of high amounts may have the opposite effect. For future studies, we suggest investigating the effects of different concentrations in order to determine the most appropriate dose and regime, in terms of focus and attention, and avoid its negative consequences.

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