Research report

Analyzing habituation responses to novelty in zebrafish (*Danio rerio*)

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ABSTRACT

Analysis of habituation is widely used to characterize animal cognitive phenotypes and their modulation. Although zebrafish (*Danio rerio*) are increasingly utilized in neurobehavioral research, their habituation responses have not been extensively investigated. Utilizing the novel tank test, we examine intra- and inter-session habituation and demonstrate robust habituation responses in adult zebrafish. Analyzing the intra-session habituation to novelty further, we also show that selected anxiogenic drugs (caffeine, pentylenetetrazole), as well as stress-inducing alarm pheromone, attenuated zebrafish habituation. Some acute anxiolytic agents, such as morphine and ethanol, while predictably reducing zebrafish anxiety, had no effects on habituation. Chronic ethanol and fluoxetine treatments improved intra-session habituation in zebrafish. In general, our study parallels literature on rodent habituation responses to novelty, and reconfirms zebrafish as a promising model for cognitive neurobehavioral research.

1. Introduction

As the simplest form of learning, habituation has long been used in biological psychiatry research to examine animal cognitive phenotypes and their alterations produced by different experimental manipulations [1,2]. Habituation to novelty represents attenuation of innate behaviors, as subjects become accustomed to the environment [3,4]. Intra-session habituation reflects spatial working memory, whereas inter-session habituation is commonly used to assess middle- and long-term spatial memory [6]. In addition, habituation may represent deeper neurobiological constructs, such as adaptive processing of sensory information [7] and development of a cognitive map [5]. Impaired habituation can also be associated with increased anxiety [2], thereby providing valuable insight into anxiety-memory interplay [8–10].

Habituation has been extensively studied in various rodent experimental models, showing that animal habituation phenotypes are highly sensitive to various pharmacological manipulations [11–22]. For example, rats chronically exposed to fluoxetine, display increased habituation to novel environments [21,23], whereas

another agent with anxiolytic properties, morphine, appears to impair mouse habituation [11,18,24,25]. Although ethanol stimulates locomotion in mice and rats [24,26,27], its repeated administration impairs animal habituation [20,26], as do the psychostimulant/anxiogenic drug caffeine [12,24] or kindling with low doses of the anxiogenic agent pentylenetetrazole (PTZ) [16,17,22].

With immense genetic homology to humans and rodents, zebrafish (*Danio rerio*) present a highly efficacious model for human disorders [28,29]. In addition, zebrafish have proven to be beneficial in the study of behavior and its modulation by various endo- and exogenous factors [29–34]. Despite being widely studied in various rodents [1,3,4,26,35,36], habituation has not been extensively evaluated in zebrafish–a model species that is becoming increasingly popular in neurobehavioral research [29,33,37–41].

Until recently, fish behavior was generally assumed to be instinctively driven, with little cognitive ability (rev. in Ref. [42]). However, it is currently known that fish are capable of forming spatial memories and cognitive maps [42,43], providing an opportunity to explore their habituation behaviors in depth. Several recent studies suggest that zebrafish can habituate to various stimuli, including conditioned place preference [44,45], light/dark locomotion [46] and the startle reflex [47,48] testing. Novelty-based paradigms are commonly used in behavioral neuroscience to study both affective (e.g., fear, anxiety) [35,49] and cognitive (e.g., habituation) [1,3,4,36] phenomena. Since relatively little is known about adult zebrafish habituation, this study aimed to characterize their habit-

uation to novelty in detail, and to assess its sensitivity to different experimental manipulations.

2. Methods and materials

2.1. Animals and housing

A total of 210 adult (3–5-month-old; ≈50:50 male:female ratio) wild type short-fin zebrafish were used in this study. The animals were obtained from local commercial distributors (Petco, Rockville, MD and 50 Fathoms, Metairie, LA) and given at least 20 days to acclimate to the animal facility. The fish were housed in groups of approximately 20–30 fish per 40-L tank. All tanks were filled with deionized water, with room and water temperatures maintained at ≈25 °C and water pH at 7.0–8.0. Illumination (1170 ± 67 lux) was provided by ceiling-mounted fluorescent light tubes on a 12-h cycle (on 6:00 h; off 18:00 h), consistent with the zebrafish standard of care [50]. All animals used in this study were experimentally naïve and fed Tetramin Tropical Flakes (Tetra USA, VA) twice a day.

2.2. Experimental manipulations

The first experiment analyzed the normal habituation responses of zebrafish using 6- and 30-min trials in the novel tank test (see further). Our second study explored the effects on zebrafish ($n = 15$–16 in each group) habituation produced by ethanol (acute: 0.3% (vol/vol) × 5 min, chronic: 0.2% (vol/vol) × 2 weeks), acute morphine (2.0 mg/L × 15 min), acute caffeine (100 mg/L × 15 min), chronic fluoxetine (100 µg/L × 2 weeks), and acute PTZ (900 mg/L × 10 min). Ethanol and morphine were used here because both drugs have known anxiolytic effects in human, rodent and zebrafish models. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), was chosen for its known anxiolytic action in human, rodent and zebrafish (e.g., [38]) subjects following chronic administration. Caffeine and PTZ were used here as traditional anxiogenic drugs, known to provoke anxiety in various clinical and experimental models (see above).

Acutely exposed fish were pretreated in a 0.5 L plastic beaker for specified times. Chronic agents were dissolved in home tank water for 2 weeks. The doses were chosen based on our own pilot data and previously published literature (e.g., [38]) confirming the lack of non-specific toxic/sedative effects of these drugs (also see further). Acute exposure to the anxiogenic alarm "pheromone" substance was also used in this study ($n = 15$ in each group). The substance was extracted from epidermal cells of several euthanized zebrafish, using a razor blade to make 10–15 shallow incisions on one side of the body [51]. Incisions were carefully controlled to prevent contamination with blood. The cut body side was then placed down into a Petri dish filled with 10 mL of distilled water and shaken gently for 5 min. The Petri dish was then placed on ice to preserve the extracted substance, while the entire procedure was repeated on the opposite side of the zebrafish [51]. Extracted alarm substance was administered acutely by adding 7 mL of the collected solution to fresh water in the novel tank. Control fish tanks received 7 mL of distilled water.

2.3. Apparatus and behavioral testing

Behavioral testing was performed using the novel tank test, representing a 1.5-L trapezoidal tank (15.2 cm height × 7.1 cm width × 27.9 cm top × 22.5 cm bottom length; Aquatic Habitats, Apopka, FL) maximally filled with aquarium water [38]. Novel tanks rested on a level, stable surface and were divided into two equal horizontal portions, premarked by a line on the outside walls. Behavioral testing took place between 11:00 and 16:00 h. Following pre-treatment, the behavior of each fish was recorded for 6 or 30 min by two trained observers (inter-rater reliability >0.85), scoring the following endpoints: time spent in the top of the tank (s), number of transitions (entries) to the top, number of erratic movements, and number and duration (s) of freezing bouts. Erratic movements were defined as sharp changes in direction and/or velocity, representing rapid anxiety-like darting behaviors [38]. Freezing was defined as a total absence of movement, except for the gills and eyes, for 2 s or longer. In general, reduced exploration (fewer entries to the top, more freezing) or increased erratic movements correlate with high stress and anxiety [38,51–53].

2.4. Habituation analysis

To establish zebrafish as a model of habituation, and obtain a general profile of their habituation phenotypes, we first analyzed 6-min habituation responses in a relatively large cohort of 38 naïve zebrafish. Animal behaviors were recorded as described above and analyzed for their per-min distribution. Zebrafish habituation responses were then analyzed more specifically, by comparing the first vs. last 3 min for each behavioral endpoint measured. To further assess zebrafish habituation to novelty and visualize the change in behavior over a longer period of time, 30-min trials were next conducted in another cohort of naïve zebrafish ($n = 23$). Habituation responses were then assessed in a similar manner, by comparing the first vs. last 6-min measures for each endpoint. In a separate cohort of experimentally naïve zebrafish ($n = 15$), inter-session habituation was analyzed using daily 6-min trials for 7 days, comparing Day 1 scores with those of subsequent days. Finally, for experiments with different pharmacological manipulations, zebrafish habituation

responses (recorded in 6-min novel tank trials) were analyzed more specifically by two methods adapted from traditional protocols widely used to study habituation in rodents [1,3,4,36,54]. First, the ratio of behaviors for each individual animal during the first and last minute of the trial (single-minute habituation ratio, SHR) was calculated. Second, the ratio of behaviors during the first 3-min vs. the last 3-min (cumulative habituation ratio, CHR) was computed.

2.5. Statistical analysis

All data are expressed as mean ± SEM, and analyzed with a two-sample unpaired or paired Wilcoxon U-test for significance either between the groups or vs. the initial observation time (e.g., min 1), respectively. One-way ANOVA (factor: time) with repeated measures (minutes of test, or testing days) was used to analyze the significance of intra- and inter-session habituation, followed by post hoc U-test with Bonferroni correction. Significance was set at $P < 0.05$ for ANOVA and U-test, but was adjusted accordingly for Bonferroni-corrected post-hoc tests.

3. Results

3.1. Habituation trials

In 6-min trials, we found significant increases in exploratory behavior and decreases in freezing behavior over time (Fig. 1A). One-way ANOVA revealed significant time effects for transitions to top ($F(1,37) = 45.263$, $P < 0.0005$), time in top ($F(1,37) = 44.801$, $P < 0.0005$), erratic movements ($F(1,37) = 21.355$, $P < 0.0005$), freezing bouts ($F(1,37) = 7.246$, $P < 0.05$), and freezing duration ($F(1,37) = 8.798$, $P < 0.005$). In addition, significant differences were found between first and last 3-min intervals for freezing bouts and duration ($P < 0.05$, U-test).
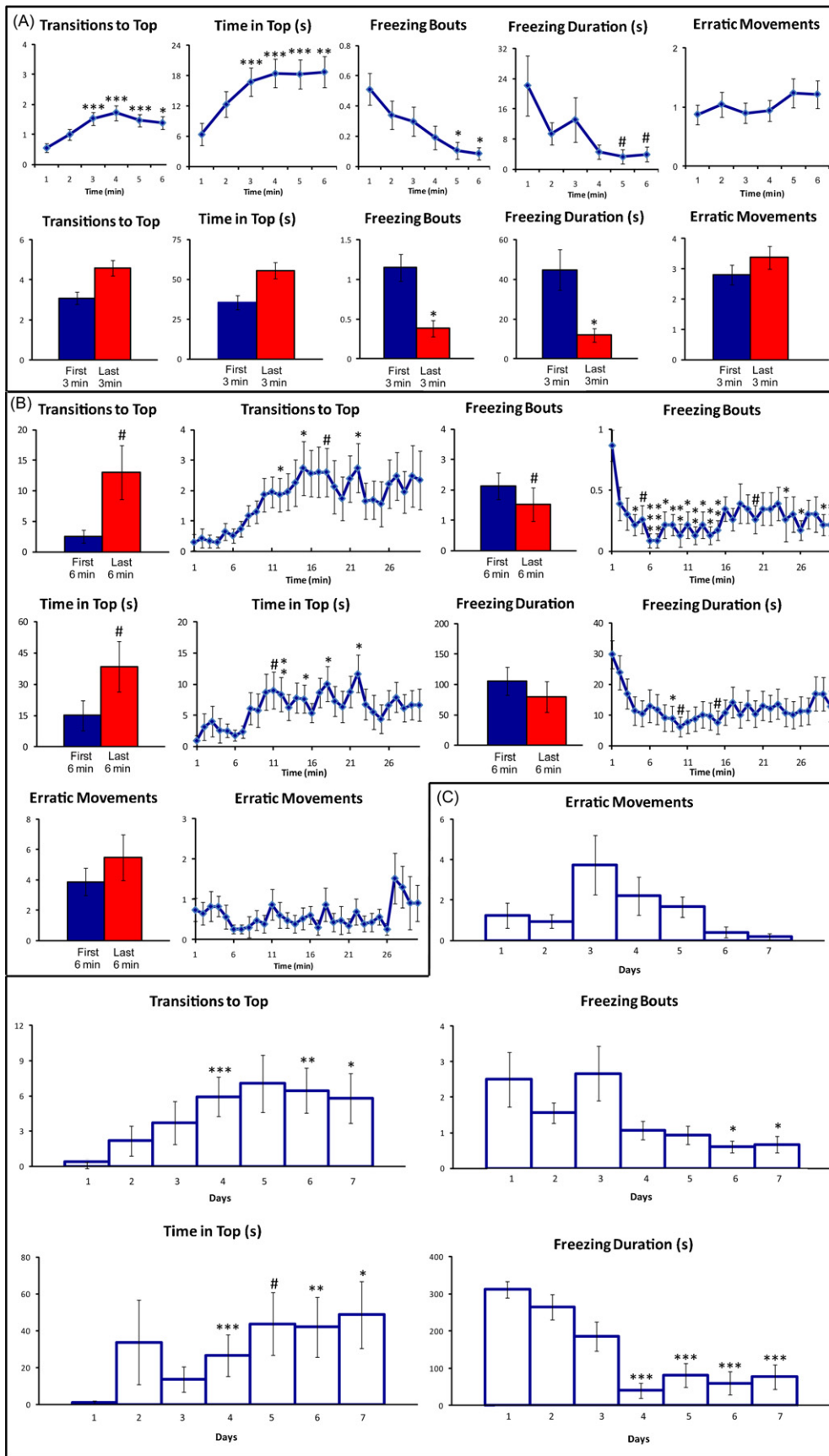
During the 30-min intra-session habituation trials, the zebrafish exhibited a steady increase over time in transitions to the top of the novel tank, time spent in the top, as well as a marked decrease in freezing scores, but not in erratic movements (Fig. 1B). One-way ANOVA revealed significant time effects for transitions to top ($F(1,22) = 17.856$, $P < 0.0005$), time in top ($F(1,22) = 22.023$, $P < 0.0005$), erratic movements ($F(1,22) = 15.575$, $P < 0.005$), freezing bouts ($F(1,22) = 13.583$, $P < 0.005$), and freezing duration ($F(1,22) = 16.465$, $P < 0.005$). Notable trends between the first and last 6-min intervals were also observed in the number of top entries and time in top ($P = 0.05$–0.1, U-test). In all endpoints, the greatest behavioral change was observed within the first 5–10 min of the trials (Fig. 1B).

Analyzing inter-session habituation (Fig. 1C), with each successive day, we found significantly increased transitions to the top, and time spent there, as well as significantly reduced freezing behaviors. One-way ANOVA revealed significant time effects for transitions to top ($F(1,14) = 41.222$, $P < 0.0005$), time in top ($F(1,14) = 27.544$, $P < 0.0005$), erratic movements ($F(1,14) = 34.977$, $P < 0.0005$), freezing bouts ($F(1,14) = 67.35$, $P < 0.0005$), and freezing duration ($F(1,14) = 170.18$, $P < 0.0005$).

3.2. Anxiogenic treatments

As shown in Fig. 2A, administration of alarm substance lowered overall transitions to and time spent in the top of the tank. Although we saw some reduction in erratic movements over time, freezing behavior remained unaltered (data not shown), and neither SHR nor CHR demonstrated significant trends in any of the observed endpoints.

While still exhibiting an increase in transitions and time spent in the top of the tank over time, the slopes of behavioral curves of the caffeine-treated zebrafish were reduced (Fig. 2B). Although freezing frequency and duration remained unaltered (data not shown), caffeine also exhibited a robust upward effect on the number of erratic movements, significantly lowering both SHR and CHR (Fig. 2B).

Fig. 1. Habituation behavioral responses in zebrafish tested in the novel tank test. (A) Six-minute intra-session habituation trials ($n = 38$). (B) Thirty-minute intra-session habituation trials ($n = 23$). (C) Inter-session habituation responses in zebrafish tested in daily 6-min trials for 7 days ($n = 15$). $*P < 0.05$, $**P < 0.005$, $***P < 0.001$, $\#P = 0.05–0.1$ (trend) vs. min 1, U-test with Bonferroni correction.
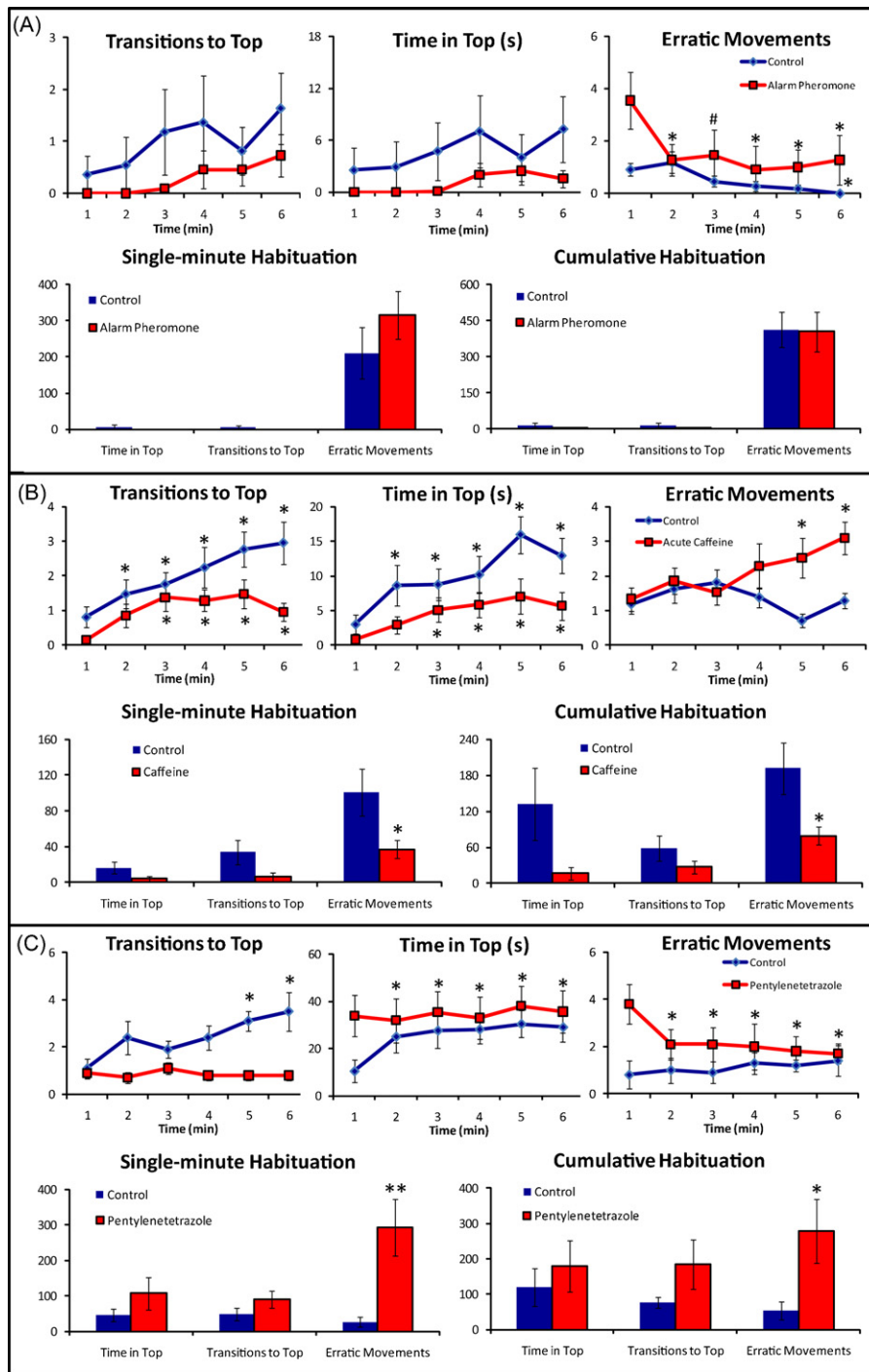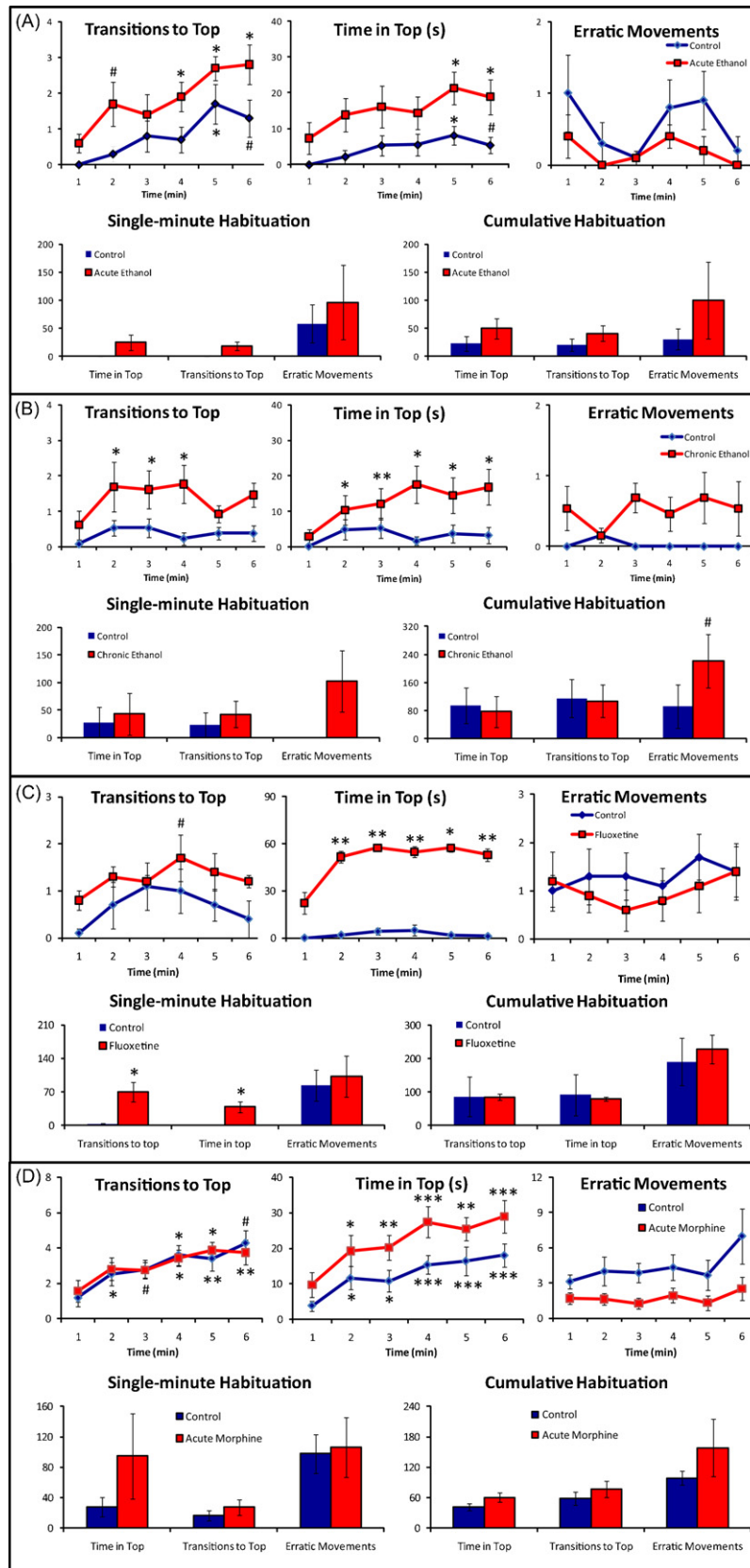
Fig. 2. Anxiogenic drugs effects on zebrafish intra-session habituation response in 6-min trials. (A) Alarm "pheromone" substance (7 mL × 15 min). (B) Caffeine (100 mg/L × 15 min). (C) Pentylenetetrazole (PTZ, 900 mg/L × 10 min). *$P < 0.05$, **$P < 0.005$, #$P = 0.05$–0.1 (trend) vs. min 1, U-test ($n = 15$–16 in each group). For behavioral scores, significant difference is shown vs. min 1. For habituation scores, significant difference is shown vs. control group. All anxiogenic drugs used here produced significant anxiety-enhancing effects compared to control zebrafish (for a better clarity, statistical significance for these behavioral effects is not shown in this diagram).

Following acute PTZ administration, zebrafish failed to habituate to the novel environment, displaying no change in frequency and duration of exploratory transitions over time (Fig. 2C). No significant effects were seen in freezing behaviors (data not shown) or in the SHR of CHR scores for these endpoints. In contrast, both habituation scores for erratic movements were significantly higher in PTZ-treated fish.

3.3. Anxiolytic treatments

Although acute ethanol-treated zebrafish displayed robust anxiolytic responses, they showed similar habituation curves for transitions and time spent in top, compared to control fish (Fig. 3A). There were also no alterations in the SHR or CHR scores in this experiment. When the animals were chronically treated with

Fig. 3. Anxiolytic drugs effects on zebrafish intra-session habituation response in 6-min trials. (A) Acute ethanol (0.3% (vol/vol) × 5 min). (B) Chronic ethanol (0.2% (vol/vol) × 2 weeks). (C) Selective serotonin reuptake inhibitor (SSRI) fluoxetine (100 μg/L × 2 weeks). (D) Morphine (2.0 mg/L × 15 min). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{\#}P = 0.05$–0.1 (trend) vs. min 1, U-test ($n = 15$–16 in each group). For behavioral scores, significant difference is shown vs. min 1. For habituation scores, significant difference is shown vs. control group. All anxiolytic drugs used here produced significant anti-anxiety effects compared to control zebrafish (for a better clarity, statistical significance for these behavioral effects is not shown in this diagram).

ethanol, the experimental cohort again had a greater number of transitions and longer time spent in top (Fig. 3B), consistent with reduced anxiety levels. In both groups, there was no noticeable change in the number of erratic movements or freezing bouts (data not shown). In both ethanol experiments, the drug treatment did not produce behavioral sedation, as assessed by increased (rather than non-specifically inhibited) exploratory locomotion (Fig. 3A and B). Interestingly, chronic ethanol tended to slightly increase the CHR, but not SHR, scores for the erratic movements (Fig. 3B).

Following chronic fluoxetine treatment, animals showed higher exploratory behavior with increased frequencies and durations of entries to top, in addition to a reduction in both freezing behaviors (data not shown). The fluoxetine-treated cohort also demonstrated significant alterations in exploratory endpoints (but not in erratic movements or freezing) in SHR (Fig. 3C).

Despite producing anxiolytic effects on time spent in top, acute morphine exposure did not affect habituation responses of zebrafish (as assessed by unaltered SHR and CHR scores). In line with this, the behavioral curves for transitions to top, time spent in top and the number of erratic movements in the experimental cohort generally paralleled the slopes of the control group (Fig. 3D).

4. Discussion

Although habituation is traditionally utilized to examine rodent exploration and cognition [36,54,55], it has not been comprehensively evaluated in adult zebrafish. This study was the first in-depth systematic analysis of habituation phenotypes, attempting to provide insights into zebrafish responses to spatial novelty.

To establish zebrafish model of habituation, we first analyzed intra-session habituation using both short (6-min) and long (30-min) trials, and showed steady increases in exploration of the novel tank within the first minutes of the test (Fig. 1). Likewise, freezing behavior steadily decreased until min 10, with no changes observed afterwards. Low habituation scores for the exploratory behaviors demonstrate that there were higher values in the last interval of the trial, therefore lowering habituation ratios. Similarly, the higher scores in the freezing behavior were due to the substantial amount of freezing during the first interval, and greatly reduced freezing during the last portion of the trial (Fig. 1A and B). Taken together, this shows that zebrafish display robust habituation to novelty, and that methods of habituation analysis adopted from rodent tests can be applied to analyze zebrafish behaviors. The observation that zebrafish habituation responses were most robustly affected during the first minutes of testing (Fig. 1A) justified the 6-min trials used in zebrafish research [38,52] as an accurate, high-throughput, and time-efficient assay of zebrafish habituation.

Establishing the utility of the short 6-min habituation trials then allowed us to assess long term habituation of zebrafish by repeating novel tank trials daily over the course of 7 days. Re-exposure of zebrafish to the tank demonstrated steadily increasing locomotion as well as reductions in freezing behaviors with each subsequent day (Fig. 1C), most notably during the last 4 days. Overall, this experiment not only demonstrated the ability of zebrafish to alter their behaviors (as they became familiar to the novelty), but also confirms their capacity to construct and retain a cognitive spatial map. Consistent with spatial habituation reported in some other fish species [42,43], these findings support zebrafish as a valid model for studying short- and long-term spatial memory and its modulation.

Interestingly, erratic movements showed relatively stable temporal distribution across intra- and inter-session habituation trials in naïve zebrafish (Fig. 1A–C). While this observation suggests that erratic behavior may represent a baseline *tonic* "vigilance" behavior in the novel environment, further studies are needed to examine whether this behavior (and its habituation) can be affected by stronger experimental and/or pharmacological challenges.

To further characterize zebrafish habituation, we studied the effects of various experimental manipulations on their behavior. Using standard 6-min habituation trials, we found that acute exposure to alarm substance inhibited transitions to the top of the tank and increased erratic movements (Fig. 2A). The low SHR and CHR values observed in this experiment imply somewhat attenuated zebrafish habituation generally consistent with higher anxiety/vigilance in animals exposed to anxiogenic stimuli. Similar to the effects of alarm substance, both caffeine and PTZ attenuated increases in exploratory behavior (Fig. 2B and C). Caffeine administration also resulted in an increase in zebrafish erratic behavior over time (Fig. 2B). Detected by both habituation scoring methods, this suggests poorer habituation in the caffeine group due to increased anxiety and hyperactivity. Similarly, PTZ markedly inhibited the habituation of exploratory behavior (Fig. 2C). Although we found a greater effect on CHR for erratic movements, a higher level of significance was seen in SHR scores, implying that suppression of habituation by PTZ may be due to higher anxiety levels and impaired spatial awareness. Importantly, all anxiogenic drugs tested here provoked robust effects on zebrafish anxiety (Fig. 2). Likewise, all anxiolytic drugs showed strong anti-anxiety action on zebrafish behavior (Fig. 3), confirming the effectiveness of drug treatments chosen for this study, regardless of their effect on habituation.

Although anxiogenic agents displayed consistent attenuation of habituation in zebrafish, the anxiolytic drugs used in this study demonstrated substantial variability in their effects (Fig. 3). For example, with no relative change in the habituation ratios, acute ethanol appears to have minimal effect on zebrafish habituation (Fig. 3A). However, when exposed to ethanol chronically, zebrafish exhibited significant increases in exploratory transitions and time spent in the top, consistent with improved habituation behavior (Fig. 3B). Although erratic behavior displayed no general alteration, the slight increase affected CHR, suggesting that chronic ethanol may improve habituation by activating exploratory behavior in the top, and stabilizing baseline erratic behavior. Following chronic administration of fluoxetine, significant changes were again observed in SHR and CHR of exploratory endpoints (Fig. 3C). These collective behavioral and habituation data suggest that habituation-enhancing effects of SSRIs may be due to significant reduction of zebrafish anxiety, including increased baseline exploration, and reduced erratic and freezing behaviors. Finally, in contrast to chronic ethanol and fluoxetine, but similar to the effects of acute ethanol treatment, acute morphine exposure had no effect on zebrafish habituation (Fig. 3D).

The differences in modulatory effects of fluoxetine, morphine, and ethanol on habituation generally support rodent data [21,23], suggesting that anxiolytic agents may affect anxiety and habituation through different mechanisms. Interestingly, while we found similar habituation-impairing effects produced by anxiogenic agents, marked variation in responses to anxiolytic drugs across animal models must also be noted. For example, our results contradict mouse data [19] that fluoxetine inhibits habituation, but support its pro-habituation effects observed in rats [21,23]. In addition, our ethanol data (Fig. 3A and B) in zebrafish contradict rodent findings that alcohol attenuates habituation [20,26], but mirror the hyperlocomotion found in mice after chronic ethanol exposure [27].

It is likely that several factors can explain these discrepancies, including species differences, as well as differences in testing apparatuses, and drug administration methods (e.g., acute vs. chronic, i.p. injection vs. adding to the water). Behavioral manifestation of habituation responses to novelty may also be different in rodents vs. zebrafish. For example, rodents usually reduce locomotion as they become familiar with the novel environment [3,17,23,37], whereas zebrafish appear to do the opposite (Fig. 1). Such behavioral inhibi-

tion in zebrafish immediate responses to novelty may represent a primitive, evolutionarily ancient form of "passive" adaptive behavior (compared to more complex and "proactive" exploration-driven rodent behaviors). Clearly, further comparative analyses of habituation phenotypes across animal species (e.g., [49,56]) may provide important insights on this topic.

Several other conclusions can be made based on the results of this study. For example, robust habituation within and between novel tank sessions strongly supports the utility of zebrafish and the novel tank test to study habituation phenotypes. Our findings show that zebrafish can be utilized to assess the effects of pharmacological agents on habituation as effectively as rodent models, and collectively support zebrafish as a new translational model in behavioral pharmacology. Future investigation may further characterize habituation response modulation by environmental factors such as novelty properties (e.g., tank size), other stressors (e.g., crowding stress, predator exposure or drug withdrawal) or genetic factors (e.g., different inbred strains and/or genetically modified zebrafish). Taken together, this study emphasizes the use of adult zebrafish as a useful model for neurobehavioral cognitive research.

Acknowledgements

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