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Effects of piracetam on behavior and memory in adult zebrafish

Leah Grossman[1], Adam Stewart[1], Siddharth Gaikwad, Eli Utterback, Nadine Wu, John DiLeo, Kevin Frank, Peter Hart, Harry Howard, Allan V. Kalueff*

Department of Pharmacology and Neuroscience Program, Zebrafish Neuroscience Research Consortium (ZNRC), Tulane Neurophenotyping Platform, Tulane University Medical School, 1430 Tulane Ave., New Orleans, LA 70112, USA

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ABSTRACT

Piracetam, a derivative of γ-aminobutyric acid, exerts memory-enhancing and mild anxiolytic effects in human and rodent studies. To examine the drug's behavioral profile further, we assessed its effects on behavioral and endocrine (cortisol) responses of adult zebrafish (*Danio rerio*) – a novel model species rapidly gaining popularity in neurobehavioral research. Overall, acute piracetam did not affect zebrafish novel tank and light–dark box behavior at mild doses (25–400 mg/L), but produced nonspecific behavioral inhibition at 700 mg/L. No effects on cortisol levels or inter-/intra-session habituation in the novel tank test were observed for acute or chronic mild non-sedative dose of 200 mg/L. In contrast, fish exposed to chronic piracetam at this dose performed significantly better in the cued learning plus-maze test. This observation parallels clinical and rodent literature on the behavioral profile of piracetam, supporting the utility of zebrafish paradigms for testing nootropic agents.

1. Introduction

Piracetam is a cyclic derivative of γ-aminobutyric acid (GABA). Since its discovery in the 1960s, it has been widely used in humans [38,39,43,47,55] and rodents [4,5,36] as a memory-enhancing (nootropic) agent. With low toxicity and few side effects, piracetam is effective in treating dementia and cognitive impairment [40,47], stroke [39] and ischemia [38]. Piracetam modulates neuroplasticity, neuroprotection and brain metabolism [53], has anticonvulsant effects [14], as well as reduces symptoms of clinical depression, anxiety and alcohol withdrawal [11,27].

Piracetam has also been extensively tested in various rodent models. In addition to nootropic activity [21,32], animal anxiety-like behavior has been found to be sensitive to this drug. For example, acute piracetam reduces anxiety in rat social interaction [16] and in rabbit conflict tests [46]. Similarly, mild chronic doses of piracetam reduce rat anxiety in the open field, elevated plus-maze, foot shock-induced fighting [4] and Vogel's conflict tests [29].

Despite numerous clinical and experimental studies, the mechanisms of piracetam's action remain poorly understood [32,52]. The drug is known to modulate membrane fluidity, which may affect receptor binding, and neurotransmitter release [10]. Another proposed mechanism of piracetam's action is at the benzodiazepine site of the GABAA receptor, since flumazenil inhibits its effects [30]. In addition to targeting GABA receptors, piracetam can also interact with glutamate receptors, suggesting another potential mechanism for its nootropic action [27].

A more comprehensive understanding of piracetam psychopharmacology requires further studies, utilizing novel approaches and new model species, in addition to humans and rodents. Although piracetam has been studied in several fish species (modulating their vestibular and feeding behavior [7,20,26]), its effects on fish anxiety and memory are currently unclear. As adult zebrafish (*Danio rerio*) are becoming popular screens for various psychotropic drugs [12,54], the behavioral effects of piracetam have not been tested in this model.

Given the sensitivity of zebrafish anxiety and cognition to various pharmacological manipulations [8,54], these fish may represent a promising novel model to study the effects of piracetam and similar psychotropic compounds. Additionally, zebrafish possess all major nuclei, neurotransmitters and receptors, allowing for translation of their behavioral and physiological modulation by nootropic compounds [1,34,35]. Our study focused on testing this possibility in a battery of zebrafish tests, also expanding the range of model species to examine the behavioral effects of piracetam. Furthermore, since zebrafish display robust endocrine (cortisol) responses to various stressors [8,12], we also examined their endocrine responses to acute and chronic piracetam treatment.

* Corresponding author at: Department of Pharmacology, Room 3731, Tulane University Medical School, 1430 Tulane Ave., New Orleans, LA 70112, USA.
Tel.: +1 504 988 3354; fax: +1 504 988 3354.
E-mail address: avkalueff@gmail.com (A.V. Kalueff).
[1] These authors contributed equally.

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2. Methods

2.1. Animals and housing

A total of 336 adult (3–5 month-old) wild type short-fin zebrafish (1:1 male:female ratio) were obtained from a local commercial distributer (50 Fathoms, Metairie, LA). All fish were given at least 10 days to acclimate to the laboratory environment and were housed in groups of 15–20 fish per 40-L tank. The tanks were filled with filtered (facility) water maintained at 25–27 °C. Illumination was provided by ceiling-mounted fluorescent light tubes on a 12-h cycle. Fish were fed Tetramin Tropical Flakes (Tetra USA, Blacksburg, VA). All fish used in this study were experimentally naïve. Following behavioral testing, the animals were euthanized in 500 mg/L Tricaine (Sigma–Aldrich, USA), and immediately dissected on ice for further analysis. This study was performed in full compliance with Institutional and National guidelines on animal experimentation.

2.2. Pharmacological manipulations and cortisol assay

Acute treatment was performed by immersing zebrafish for 20 or 90 min in a 3-L plastic beaker containing piracetam (100, 400, or 700 mg/L) prior to testing. Piracetam was also administered chronically by adding 200 mg/L piracetam to the home tank water for 7 or 8 days. The drug concentration was maintained by daily changing water and re-administration of this dose. The doses and treatment times were based on our pilot experiments with a wide range (25–1000 mg/L and 5–90 min, respectively), as well as on previously published studies using piracetam in rodents [4,25] and fish [7,20,26]. Behavioral testing in the novel tank was performed in all acutely exposed zebrafish, as well as daily (for 7 days) during the chronic treatment. The light–dark test was performed on acutely exposed fish, as well as one day later (day 8) after the 7-day chronic piracetam exposure (see further). Chronic 7-day treatment was used to study the effects of piracetam in the plus-maze memory test. All subjects were experimentally naïve and exposed only to one of the following behavioral paradigms (see below), to minimize stress and avoid habituation to the apparatuses. Immediately following behavioral testing, fish from all three experiments were euthanized with 500 mg/L Tricaine, their whole-body cortisol was extracted, and assessed using a human salivary cortisol ELISA kit (Salimetrics, PA), as described elsewhere [12].

2.3. Behavioral testing

2.3.1. Novel tank and light–dark anxiety tests

Behavioral testing was performed between 12.00 and 16.00. In all experiments, testing was performed in a tank containing standard facility water, adjusted to the holding room temperature. Zebrafish were placed individually in a novel tank test, representing a 1.5-L trapezoidal tank (15 height × 28 top × 23 bottom × 7 width cm; Aquatic Habitats, Apopka, FL) maximally filled with water. Novel tanks rested on a level, stable surface and were divided into two equal virtual horizontal portions, marked by a dividing line on the outside walls [54]. Zebrafish behavior was manually recorded by two trained observers (inter-rater reliability >0.85) for 6-min (standard novel tank test) or 30 min (extended novel tank test), scoring the latency to reach the upper portion of the tank (s), time spent in the upper portion of the tank (s), number of transitions (entries) to the upper portion of the tank, number of erratic movements, number of freezing bouts and freezing duration (s). Erratic movements were defined as sharp spontaneous changes in direction or velocity and repeated rapid darting behaviors. Freezing was defined as a total absence of movement, except for the gills and eyes, for 2 s or longer. Reduced exploration (longer latency to reach the top, fewer entries to the top, longer freezing) or elevated erratic movements in this test typically represent anxiety in zebrafish [54].

The light–dark test consisted of a rectangular tank, modified from the mouse light/dark box (15 height × 30 length × 16 width cm), and maximally filled with aquarium water [25]. The box rested on a level, stable surface and was divided into two equal vertical portions, demarcated by black and white coloration. Endpoints were recorded and scored over a 6-min period by two observers (inter-rater reliability >0.85) using USB LifeCam webcams (Microsoft, Redmond, WA) set 45 cm above the center of the light–dark box. Observers and laptops were located at least 2 ft away from the light–dark box to reduce possible confounding effects. Behavioral endpoints included latency to cross into the white half, time and number of transitions (entries) to the white. Reduced exploration (longer latency and fewer entries) of the white in this test reflects high anxiety states [54].

2.3.2. Analysis of novel tank habituation

The zebrafish novel tank test has previously been established as a sensitive model of intra/inter-trial habituation, reflecting their short-term or long-term spatial memory phenotypes, respectively [54]. To apply this approach here, zebrafish novel tank behaviors (recorded as described above in 6-min trials) were analyzed for their per-minute distribution, and then compared as the first vs. last minute for each behavioral endpoint (similar to traditional animal habituation assays [49]). To further assess habituation to novelty over a longer duration of time, 30-min novel tank trials were then conducted in another cohort of naïve zebrafish ($n = 23$ per group). Habituation responses were then assessed in a similar manner. Finally, in a separate cohort of experimentally naïve zebrafish ($n = 15$ per group), inter-trial habituation was analyzed using daily 6-min trials for 7 days, comparing the day 1 scores with those of subsequent days 2–7.

2.3.3. Plus-maze test

To assess zebrafish memory, we used a cued-learning plus-maze test, which consisted of a transparent, four-armed, plus-shaped maze with each individual arm (10 cm × 10 cm in height and width, 530 cm length) and a central square

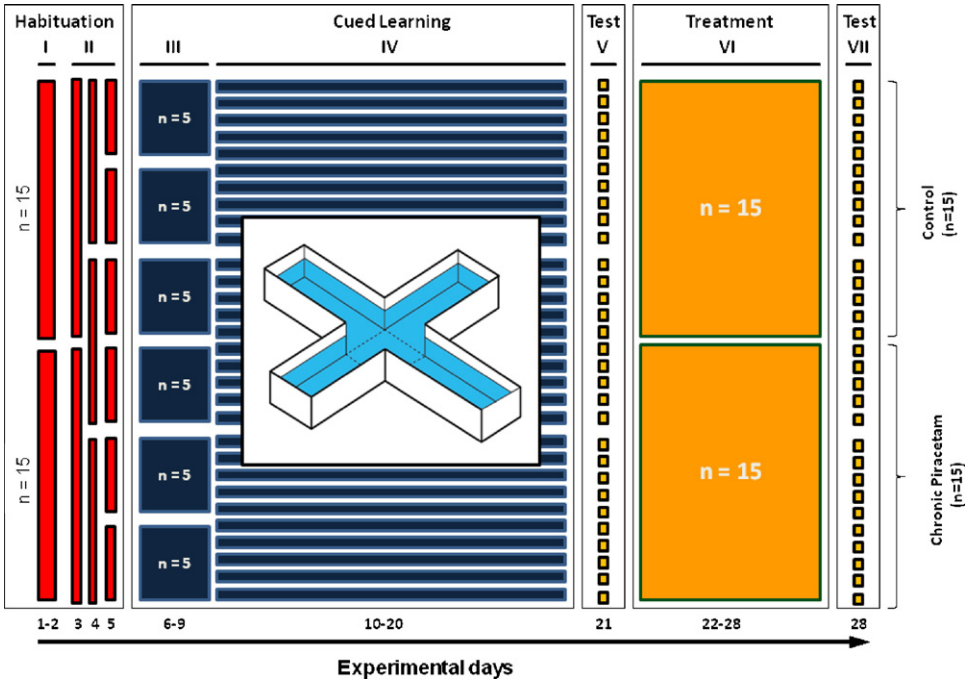

Fig. 1. Experimental design for the cued learning plus maze test. Schematic of the plus maze apparatus is shown in inset diagram. Experimental phases include: phase I – habituation to the plus maze apparatus (2-h trials, days 1-2); Phase II – habituation to the gelatin bait (15-min, days 3–5); phase III – group learning trials (6-min, 5-fish shoals, days 6-9); phase IV – individual learning trials (6-min, days 10–20); Phase V – pre-treatment testing trial (6-min, day 21); Phase VI – chronic piracetam (200 mg/L) treatment (days 21–28); Phase VII – final testing trial (6 min, day 28).

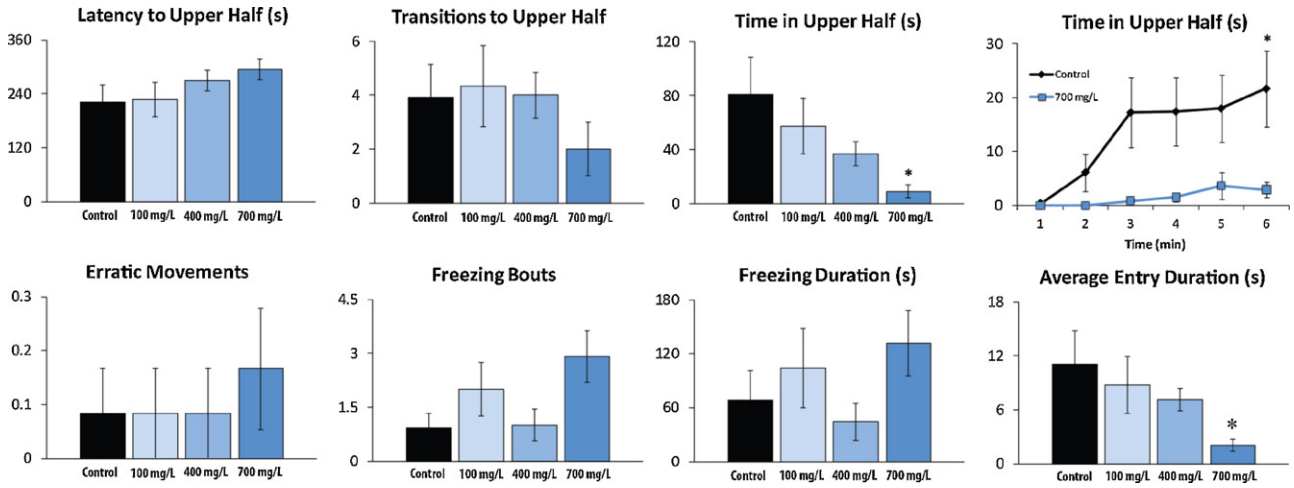

Fig. 2. Experiment 1: behavioral effects of acute piracetam (100–700 mg/L for 20-min) on adult zebrafish tested in a 6-min novel tank test ($n = 12$ per group; $^*P < 0.05$ vs. controls, Tukey's test for significant ANOVA data).

(10 cm × 10 cm; see Fig. 1). One arm was randomly designated as the target arm while the other arms were placed in the incorrect arms category. Using a custom-made (1 mm × 1 mm × 1 mm) gel bait as a reinforcement stimulus, the animals were successfully trained for cued learning for 20 trials (1 trial per day), reaching 80% correct response rate associating a visible cue (a red card placed at different arms) with a food reward (bait). To minimize procedural novelty stress, the fish first underwent a series of habituation trials, which also served to reduce handling stress. To acclimate fish to the plus-maze apparatus, 2-h initial habituation trials were administered on the first two days of the experiment (according to [41]). During these trials, the fish (in groups of 15) were allowed to freely explore the plus-maze. To minimize acute social isolation stress, zebrafish groups were only gradually reduced in size during the experiment (according to [17]), starting with 15 fish per group on days 1–3 to 10 fish per group on day 4, 5 fish per group on days 6–9, and individual testing starting from day 10. On days 3–4, the trials lasted 15 min, on days 5–9, the fish were tested in groups of 5 for 6 min. On days 10–20 of Experiment 3, there were individual-fish 6-min learning trials. To avoid chronic social isolation stress, the animals were returned to their tanks after each plus-maze trial, and housed in their home tanks in groups, as described earlier.

Food reward was chosen here as a known efficient reinforcement in zebrafish learning tasks. The need to localize the food position in the maze required a custom insoluble bait (rather than standard "Tropical flakes"). For this, a special jelly-like bait was developed and used in the present study. Briefly, 2 g of "Tropical flakes" fish food was dissolved in 10 ml of deionized water and vortexed for 2 min, 3 g of gelatin was then added to this solution and heated to 80 °C for 3 min. The mixture was again vortexed for 2 min, cooled overnight at −20 °C, and used as bait on subsequent days. Fresh bait was prepared on every second day of this study.

In addition to the apparatus and handling stress, novel food exposure may also confound animal behavioral performance. Therefore, to avoid food neophobia, habituation to the bait [41] was also performed on days 3–5 of Experiment 3, in parallel with fish acclimation to the maze apparatus. Since shoaling behavior is innate in zebrafish [13] and facilitates learning by social transmission [18], we started with a 15-fish shoal on day 3, after which the shoal size and trial duration were gradually decreased. Accordingly, the number of baited arms was also gradually reduced, as part of fish habituation to novel food (bait). On day 3, all four arms were baited, on day 4 – only three arms, on day 5 – two arms, and starting from day 6 – only one arm per trial. During individual learning trials (days 10–20), the fish were food-deprived to evoke hunger (as in [42]), and feeding was permitted only during trials, in order to facilitate procedural reinforcement (e.g., [48]). The learning task trials started after 5 days of habituation to the apparatus (days 1–2) and bait (days 3–5). Following 4 days of group learning (days 6–9), fish were food-deprived for 24 h before beginning individual learning trials, and were only fed in the plus-maze apparatus [24,42]. Cumulatively, there were ten 6-min one-arm baited trials over a period of 10 days (days 10–20), followed by an unbaited *testing* trial on day 21.

To evoke cued learning, a red plastic 10 cm × 10 cm cue card (chosen because zebrafish can see and react to the red color [6]) was placed adjacent to the reward arm. During the trials, the baited arm location (denoted by the red card) was randomly changed, to prevent bias. Overall, there were four 5-fish (days 6–9) and ten single-fish baited trials (days 10–20), followed by a final unbaited *testing* trial on day 21 (needed to assess the efficacy of zebrafish learning). During this trial, one of the four arms was denoted as the target (correct) arm, and the other three were grouped as incorrect arms. Behavioral quantification was performed for the following endpoints: latency to the target arm (s), the number of target arms, incorrect arm and total arm entries, as well as the duration in the target or incorrect arms (s). After the first testing trial on day 21, the fish were returned to their home tanks (containing 200 mg/L piracetam or drug-free water) for 7 days, food-deprived for 24 h, and retested in the plus-maze test on Day 28 of the experiment (as described previously; see Fig. 1 for details).

2.3.4. Statistical analysis

The experimental data was analyzed using Mann–Whitney U-test (for 2 groups) or a one-way ANOVA (factor: dose) with or without repeated measures (minutes of test or days of chronic test), followed by the Tukey test for significant ANOVA data. Data were expressed as mean ± SEM. Significance was set at $P < 0.05$.

3. Results

Experiment 1 aimed to determine the active dose range of acute piracetam by testing mild doses (100–700 mg/L) of this drug. In the novel tank test, there was significant dose effect for time spent in top ($F_{(3,47)} = 2.9$, $P < 0.05$), average entry duration ($F_{(3,47)} = 3.6$, $P < 0.05$), and a trend for freezing bouts ($F_{(3,47)} = 2.5$, $P = 0.08$), but not for the latency to enter the top, number of top entries, or erratic movements ($F_{(3,47)} = 0.2–1.2$, NS). While acute administration of 100 and 400 mg/L did not affect zebrafish activity, the highest dose of piracetam (700 mg/kg) significantly inhibited their novel tank swimming (Fig. 2).

Intra-trial habituation in this 6-min test was not improved by lower doses (data not shown) but significantly inhibited with the highest dose tested (Fig. 2). To allow the drug more time to exert its behavioral effects, we extended the pretreatment time to 90 min in a separate experiment, but again failed to detect behavioral effects the two mild doses elicited (data not shown). To further explore the possibility of behavioral effects of piracetam in mild non-sedative doses, the drug (100 and 400 mg/L, administered acutely for 20 min) was examined using the 6-min light–dark box test. Again, no overt behavioral differences were observed for acute piracetam in this test (data not shown).

In Experiment 2, chronic piracetam administration (200 mg/L for 7 days) did not evoke sedation or anxiolysis in the novel tank (Fig. 3A and B), albeit showing a trend towards mild anxiolysis in the light–dark test (Fig. 3 C). Inter- or intra-trial habituation in the 6-min novel-tank test was not improved by chronic piracetam treatment used here (data not shown).

In contrast, the cued learning plus maze test (Experiment 3) showed that chronic piracetam (200 mg/L) exerts a robust nootropic effect on zebrafish, significantly increasing the number of target arm entries and time spent in the target arm. There was also a trend towards shorter latencies to enter the target arm of the maze for drug-treated animals (Fig. 4). Interestingly, overall locomotion

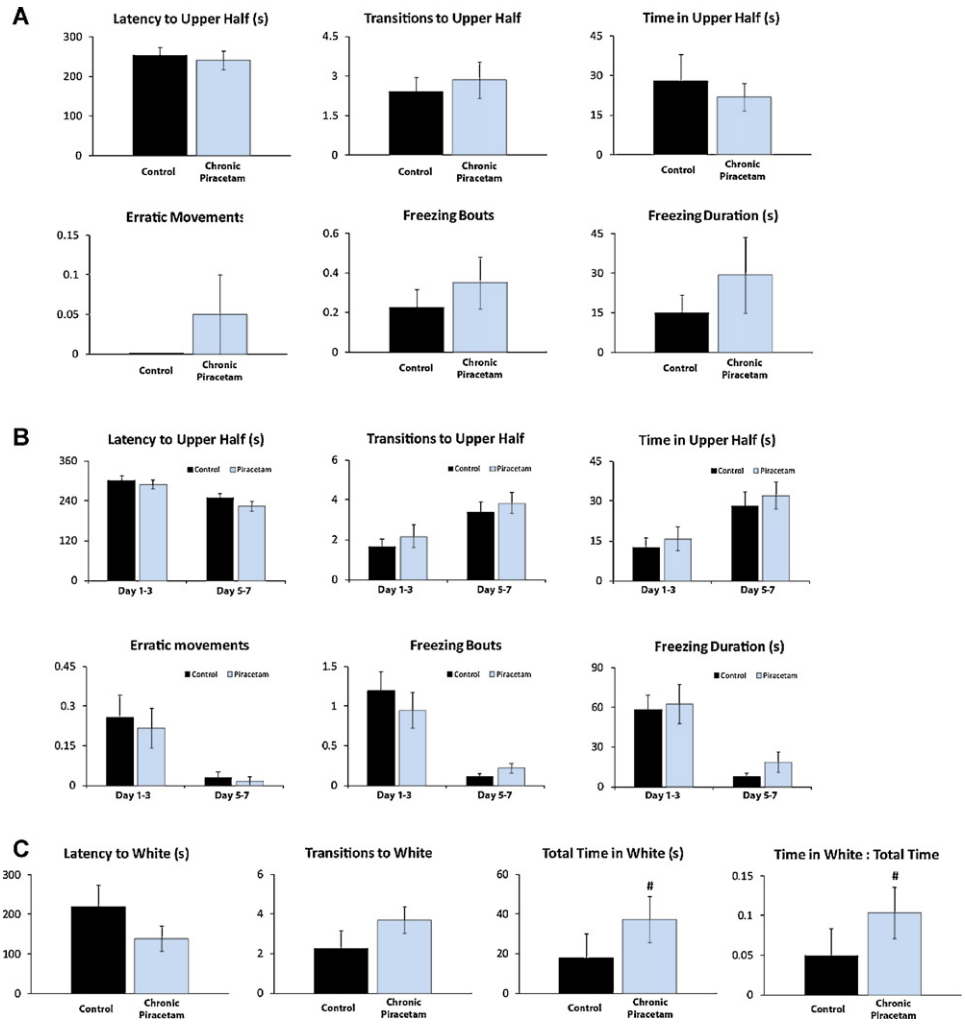

Fig. 3. Experiment 2: behavioral effects of chronic piracetam (200 mg/L for 7 days; $n = 20–23$ per group) on adult zebrafish tested in the 6-min novel tank test (A – day 7; B – days 1–3 vs. 5–7) and light–dark box (C; day 8). #$P = 0.05–0.1$ (trend) vs. control, U-test.

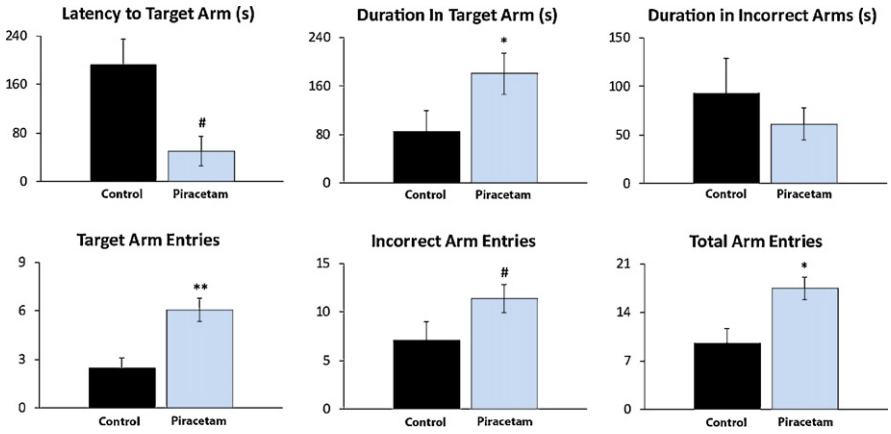

Fig. 4. Experiment 3: Memory-enhancing (nootropic) effects of chronic piracetam (200 mg/L for 7 days; $n = 15$ per group) on adult zebrafish tested in a 6-min cued learning plus-maze test (see Section 2 for details). *$P < 0.05$, **$P < 0.005$, #$P < 0.05–0.1$ (trend) vs. control, U-test.

was higher in the piracetam-treated cohort as entries to each arm were increased (e.g., target, empty, and total arm entries; see Fig. 4), consistent with elevated mobility observed in rodents for this drug [4]. Finally, no effects of acute or chronic piracetam were observed for whole-body cortisol levels in all three experiments (data not shown).

4. Discussion

While the precise mechanisms and sites of action of piracetam remain poorly understood, previous studies suggest that it indirectly modulates neurotransmission and neuroplasticity [9,28,37,44]. Piracetam also increases the number of postsynaptic

receptors [52], modulates the GABA-ergic [33] and glutamatergic [27] systems, and has been suggested to exert anxiolytic and nootropic effects via several different mechanisms [45].

Despite the growing popularity of fish paradigms in neuroscience research [12,54], piracetam has not been extensively tested in these models. Our study is the first report on the behavioral effects of piracetam in adult zebrafish, as the only other published study utilized larvae, reporting increased acoustic startle habituation [3].

Overall, testing acute doses of piracetam in our study failed to produce immediate anxiolytic or habituation-enhancing effects. However, the high dose of 700 mg/L produced clear sedation and non-specifically impaired habituation (Fig. 2) without affecting cortisol levels. This observation seems to contradict earlier reports in mice (e.g., [15,19,31]) on piracetam as a potential anxiolytic agent without sedative effects, but with corticoid-reducing activity.

One explanation for this discrepancy is that zebrafish and rodents may have different species-specific responses to piracetam. Notably, this is not the first report of psychotropic drugs exerting somewhat dissimilar profiles in zebrafish compared to rodents. For example, the benzodiazepine agents chlordiazepoxide [2] and diazepam (own unpublished observations) did not produce anxiolysis in zebrafish over a broad dose range, but do evoke sedation. Therefore, our present piracetam data (Figs. 2–4) seem to be in line with this notion. In contrast, chronic piracetam treatment exerted robust nootropic effects on zebrafish in the cued-learning plus-maze test, strikingly paralleling its nootropic profile in rodents [4] and humans [27]. Moreover, increased number of total arm entries in this experiment suggests that some mild activation of exploratory locomotion (together with a similar trend in the light–dark box) may be a part of behavioral action of chronic piracetam.

The lack of behavioral effects of acute or chronic piracetam on zebrafish novel tank test habituation was interesting, and merits further studies. Given the memory-enhancing effects of piracetam in humans and rodents [4,27,47], it was logical to expect improved habituation in piracetam-treated fish. However, it is important to consider the manner in which zebrafish habituate to novel environments. Unlike rodents, which gradually *reduce* exploratory locomotion over time, zebrafish *increase* their swimming during habituation [54]. While this zebrafish phenotype may reflect a shift from exploration to normal locomotion (as they habituate), it makes it difficult to dissect the two factors by observing zebrafish behavior in novelty-based tests. On one hand, zebrafish paradigms may be more sensitive to habituation-impairing experimental manipulations (e.g., [54]). At the same time, non-specific behavioral inhibition (such as observed here for 700 mg/L piracetam) may resemble habituation deficits, and a special attention should be paid to both habituation and overall activity levels, to avoid misinterpretation of data. For example, this situation is common in rodent studies [22,23], and researchers should be aware of this general problem with habituation assays. While these limitations must not preclude extensive testing of drug effects on zebrafish memory, knowing species-specific behavioral phenotypes may help to better dissect and interpret the observed responses in such studies. Given increasing activity during habituation in zebrafish, this also suggests that some novelty-based aquatic models may be less sensitive to pro-habituation effects due to ceiling effect, thereby requiring more specialized memory tests, such as the cued learning paradigm used here to detect nootropic effects of piracetam (Fig. 3).

The lack of overt effects of piracetam on cortisol levels in our study was also unexpected, given the drug's ability to reduce the levels of corticoids reported in rodent literature [19,31]. However, this phenotype may be related to species differences in central regulation of stress neuroendocrine axes. For example, the human and rodent hypothalamo-pituitary-adrenal (HPA) axis is regulated by multiple neurotransmitters, including the GABA-ergic system thought to be modulated by piracetam [30]. In contrast, the hypothalamo-pituitary-interrenal (HPI) axis, the zebrafish homolog of HPA, is most tightly controlled by the central serotonergic system [50,51], which may not represent the primary target of piracetam, and therefore remain unaffected here. The lack of robust anxiolytic effects of piracetam in our zebrafish study is also consistent with unaltered cortisol levels reported here.

Overall, our study shows that piracetam exerts specific behavioral effects on adult zebrafish, depending on the dose, paradigm and duration of treatment. This is generally in line with previously published reports on the effects of piracetam in various rodent models [4], especially its nootropic action [46]. Clearly, future studies are needed to dissect the effects of various doses of piracetam on zebrafish anxiety, memory and motor activity. This research, utilizing diverse and novel models such as the zebrafish, will foster a better understanding of the complex actions of this agent, eventually leading to more effective treatments for various cognitive and affective brain disorders. The sensitivity of some zebrafish memory-related behaviors to piracetam supports their utility in developing novel screens for compounds with potential nootropic properties. Finally, the use of other zebrafish strains (e.g., high-anxiety *leopard* zebrafish) as well as various mutant or transgenic zebrafish in this paradigm may enable further characterization of genetic and physiological mechanisms involved in learning and memory, as well as in fish sensitivity to piracetam and related compounds.

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