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ORIGINAL ARTICLE

Effects of sub-chronic methylphenidate on risk-taking and sociability in zebrafish (*Danio rerio*)

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Abstract

Attention deficit hyperactive disorder (ADHD) is the most common psychiatric disorder in children affecting around 11% of children 4–17 years of age (CDC 2019). Children with ADHD are widely treated with stimulant medications such as methylphenidate (Ritalin®). However, there has been little research on the developmental effects of methylphenidate on risk-taking and sociability. We investigated in zebrafish the potential developmental neurobehavioral toxicity of methylphenidate on these behavioral functions. We chose zebrafish because they provide a model with extensive genetic tools for future mechanistic studies. We studied whether sub-chronic methylphenidate exposure during juvenile development causes neurobehavioral impairments in zebrafish. Methylphenidate diminished responses to environmental stimuli after both acute and sub-chronic dosing. In adult zebrafish, acute methylphenidate impaired avoidance of an approaching visual stimulus modeling a predator and decreased locomotor response to the social visual stimulus of conspecifics. Adult zebrafish dosed acutely with methylphenidate demonstrated behaviors of less retreat from threatening visual stimuli and less approach to conspecifics compared with controls. In a sub-chronic dosing paradigm during development, methylphenidate caused less robust exploration of a novel tank. In the predator avoidance paradigm, sub-chronic dosing that began at an older age (28 dpf) decreased activity levels more than sub-chronic dosing that began at earlier ages (14 dpf and 21 dpf). In the social shoaling task, sub-chronic methylphenidate attenuated reaction to the social stimulus. Acute and developmental methylphenidate exposure decreased response to environmental cues. Additional research is needed to determine critical mechanisms for these effects and to see how these results may be translatable to neurobehavioral toxicity of prescribing Ritalin® to children and adolescents.

Keywords Methylphenidate · Sub-chronic · Zebrafish · Behavior

Introduction

Attention deficit hyperactive disorder (ADHD) is a neurobehavioral syndrome characterized not only by inattentiveness and motor hyperactivity, but also by increased risk-taking and impaired social interactions (DSM-V-Task-Force 2013). The primary treatment for ADHD is stimulant medication with the percentage of children 4–17 years of age taking ADHD medication being 6.1% in 2011 (CDC 2019). The most widely used stimulant medication used for ADHD is methylphenidate (Greydanus et al. 2002), commonly known by the brand name Ritalin®. Methylphenidate medication is intended to increase concentration, lessen hyperactivity, and improve educational and personal outcomes for children with ADHD (Biederman and Spencer 2002). Methylphenidate is a re-uptake inhibitor of norepinephrine and dopamine in the presynaptic neuron. It has actions on serotonergic, glutamatergic, cholinergic, and opioid systems as well (Di Miceli et al. 2019; Faraone 2018). There is debate about whether methylphenidate is safe and effective for young children (Singh et al. 2015; Smith et al. 2000; Soileau Jr. 2008). Currently, methylphenidate is not approved by the Food and Drug Administration for use in children younger than six years of age. However, a significant number of three- to five-year-old children are being prescribed

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medication off-label by their physicians (Singh et al. 2015). Unfortunately, little research has been conducted on the effects of methylphenidate in this age group and there is concern of neurobehavioral toxicity (Garland 1998). Given the increasing number of young children who are exposed to methylphenidate, it is important to test the developmental effects of the drug. These effects are multifaceted. We chose to test risk-taking and sociability because these two aspects affect a child's personality and everyday interactions with parents, teachers, and peers. Children with ADHD are known to be increased risk-takers and have trouble acting in socially appropriate ways (CDC 2019). Alterations in these aspects of personality would be of particular interest. In addition, there is some evidence to suggest that methylphenidate like other simulants might act differently in healthy children than those with ADHD (Hughes and Hale 1998).

There are a variety of studies which have examined the neurobehavioral effects of methylphenidate exposure during juvenile and adolescent ages in rodents (Achat-Mendes et al. 2003; Adriani et al. 2006; Bolanos et al. 2003; Brandon et al. 2001, 2003; Gray et al. 2007; Moll et al. 2001; Vendruscolo et al. 2008; Zhu et al. 2010). They have found that juvenile and adolescent methphenidate exposure causes increased locomotor activity and risk-taking (Zhu et al. 2010) and persistently heightened responses to cocaine in terms of locomotor hyperactivity (Achat-Mendes et al. 2003; Adriani et al. 2006; Brandon et al. 2001) and reinforcing (Achat-Mendes et al. 2003; Brandon et al. 2001). Rats exposed to methylphenidate during early adolescence showed persisting changes in emotional responsiveness in adulthood with blunted responsivity to rewarding stimuli and increased sensitivity to stressful stimuli (Bolanos et al. 2003; Vendruscolo et al. 2008). These behavioral effects may be related to the finding of Brandon et al. (2003) that rats with adolescent methylphenidate exposure caused a short-term increase in dopamine D_2 sensitivity followed by longer-term decrease in D_2 responsivity. Also, rats given methylphenidate during juvenile and adolescent periods caused persisting neural alterations in the frontal cortex and striatum (Gray et al. 2007) and long-lasting decreased dopamine transporter density in the striatum that persisted into adulthood (Moll et al. 2001).

We conducted the current study in a zebrafish model. There are advantages of including zebrafish in the spectrum of models with which to study methylphenidate neurobehavioral toxicity. With the rich array of molecular tools available in zebrafish, mechanistic studies are very approachable. Key to mechanistic studies is the determination of an important functional phenotype. Fortunately, an array of behavioral tests for zebrafish has been developed (Bailey et al. 2013). We chose zebrafish specifically because we can take advantage of their sociability and risk avoidance to test whether methylphenidate causes significant changes in these two parameters. Zebrafish are social animals and travel in shoals (fish that swim among one another). In addition, there is a strong necessity for zebrafish to avoid danger and stay away from predators. To assess the social affiliation to conspecifics and aversion to risk of zebrafish, validated measures have been developed (Bailey et al. 2013). An added benefit of zebrafish is that a large number of zebrafish can be tested efficiently and for a lower cost.

Zebrafish allow us to examine a longer dosing period without traumatizing the animals with injections because methylphenidate is dissolvable in water. This had not been done prior to this paper; however, there was some indication that developmental exposure to methylphenidate would cause behavioral changes in zebrafish in the literature. Previously, we (Levin et al. 2011) examined the persistent neurochemical and behavioral effect of embryonic exposure to methylphenidate because, as methylphenidate use becomes more widespread among adults, women on methylphenidate who get pregnant could be exposing fetuses to methylphenidate. We found that exposure to methylphenidate 0–5 days post fertilization caused a long-term behavioral effect in the novel tank diving test, predatory avoidance, and spatial learning.

In the current study, we examined the behavioral impacts of methylphenidate exposure during later development during the adolescent period and during adulthood. Persisting behavioral effects were evaluated. Using three behavioral paradigms: the novel tank diving task, the predator avoidance paradigm, and the social shoaling task, these tests have been shown to be sensitive for detecting neurobehavioral toxicological effects in zebrafish (Bailey et al. 2013). We aimed to examine whether methylphenidate in adulthood or during development had an effect on risk-taking and social behaviors in zebrafish.

Methods

Zebrafish husbandry

Zebrafish (AB strain *Danio rerio*) which were born and raised in our laboratory were kept at approximately 28.5 °C on a 10-h dark and a 14-h light cycle. Mature zebrafish (AB strain *Danio rerio*) were bred and eggs were collected the morning after fertilization. All viable eggs were selected and raised in an incubator set to 28 °C. At 6 days post fertilization (dpf), the larval zebrafish were moved to a water filtration system with the mature zebrafish. As larvae, they were fed brine shrimp and as they matured flake fish food (Tetra Fish Food, Blacksburg, VA, USA) was added. The mature zebrafish were housed in 3-L tanks in an Aquatic Habitats (Apopka, FL, USA) flow-through housing system. Each tank held fish from the same experimental or control group. Tanks were placed on a 6-tier dual filtration and constantly aerated rack unit. The tank water was made from deionized H_2O, sodium

bicarbonate, and sea salts (Instant Ocean, 1.2 g/20 L of water). All the experiments were performed during the light phase between 8:00 a.m. and 5:00 p.m. Fish from all of the treatment groups were tested throughout this period so that time of day effects would not be confounded with treatment. The procedures in these studies were approved by the Duke University Institutional Animal Care and Use Committee (Protocol number A006-13-01) in accordance with United States Federal regulations.

Dosing paradigm

For the acute dosing adult study, we separated mature zebrafish eighth months of age, into three experimental groups and a control group ($N = 11–12$ per group). Each experimental group was immersed in groups in a low, medium, or high dose of methylphenidate (2 mg/L, 4 mg/L, or 8 mg/L) for 30 min prior to behavioral testing. Methylphenidate was added to water taken from the tank housing system at the same pH and poured into a dosing beaker. A control group was immersed in water from the housing system for 30 min prior to behavioral testing.

For the sub-chronic developmental dosing study, we separated the zebrafish into three age groups ($N = 20$ per group). The first age group began dosing at 14 dpf, the second age group at 21 dpf, and the third age group at 28 dpf. We dosed the fish in groups housed in still water tanks by dissolving 0 mg/L, 2 mg/L, or 4 mg/L of methylphenidate in their water for two days, rinsed them, and then put the fish back in the flow-through housing system. They were dosed for two days twice a week. After dosing, the fish were kept in still water tanks without methylphenidate. There were control groups that underwent the same procedures without methylphenidate exposure for each age group. The developing zebrafish were dosed twice a week from their respective start points until they were twelve weeks of age. All behavioral testing took place shortly following the twelfth week post fertilization when the zebrafish were no longer given methylphenidate.

Novel tank dive test

The novel tank diving task is a paradigm that indexes the diving response of zebrafish when placed in a new environment. In this task, a single fish is placed in 5-L plastic tanks filled with 1350 mL of tank water for 5 min. The trapezoid-shaped tanks were 22.9 cm along the bottom and 27.9 cm along the top. The diagonal side of the tank was 15.9 cm and the opposite vertical side was 15.2 cm, the same design as used previously (Levin et al. 2007). During a 5-min trial in a novel tank, the fish's distance from the bottom of the tank and total distance traveled was tracked using EthoVision™ program (Noldus Information and Technology, Wageningen, The Netherlands). After the trial, the video—recorded by a Samsung camcorder—was analyzed. The mean distance from the bottom of the tank (cm) was calculated as well as the total distance traveled (cm). The total distance (cm) traveled each minute was converted to a swimming speed (cm traveled/min). Previous studies have shown that zebrafish tend to dwell near the bottom of a novel environment presumably as a predator avoidance strategy in a novel environment (Levin et al. 2007). As time passes without threat, the fish swim through the entire water column in an apparent foraging pattern (Levin et al. 2007).

Predator avoidance test

After dosing, the fish were placed individually into the predator avoidance paradigm, which measured the response of the zebrafish to a perceived predator. In the predator avoidance paradigm, each individual fish was placed into a tank with a monitor at one end for 5 min. The first minute allowed the fish to acclimate to the environment. In the second minute, the monitor displayed a blue dot that started small and grew larger within 5 s to mimic the sight of an oncoming predator getting closer. The screen was blank in the third minute and then the stimulus was presented again in minute 4. The two dependent measures were speed of swimming (cm/min) and average position in the tank relative to the stimulus (cm from screen) for each minute. The distance from the monitor, speed, and swimming pattern were tracked using EthoVision. For a detailed description of this test, please see Bailey et al. (2013). Our first dependent measure was the total distance traveled when the predator stimulus was on (min 2, 4) compared with when the predator stimulus was off (min 1, 3, 5). We also calculated a flee response defined as an increase in distance from the monitor when the perceived predator stimulus was presented (min 2, 4) compared with the control minute of the trial preceding the stimulus presentation when the screen was blank (min 1, 3 respectively). Both the first and the second flee responses are reported as the change in location (cm). The flee response indexes whether learning and memory played a role in the second trial. Published data indicate that control fish swim away from the perceived predator stimulus during the first presentation of the stimulus and then less so during the second presentation as the fish habituate to the stimulus that despite appearances presents no threat (Bailey et al. 2013). This measure also indicates that control fish are more likely to be closer to the screen when the predator stimulus is off compared with when the predator stimulus is on (Bailey et al. 2013).

Social shoaling test

The shoaling task measures the zebrafish's reaction to seeing a shoal following social isolation. An individual fish was

isolated for 30 min and then placed into a tank with a monitor at one end for 5 min. The first minute of the trial was a control minute that allowed the fish to acclimate to the environment. In the following 5 min, the monitor displayed a video of other zebrafish swimming in a shoal. The distance from the monitor, speed, and swimming pattern are tracked using EthoVision™. We measured the distance from the shoaling stimulus displayed on the screen by minutes. According to published data, normal behavior for control fish is to approach the shoal by swimming near the monitor (Bailey et al. 2013). In other words, control fish are closer to the monitor on average when the video of the shoal is being shown in comparison with the control image of moving dots. For a more detailed description of this test, please see Bailey et al. (2013).

Data analysis

All the statistics were calculated using IBM SPSS Statistics Software Version 22 (Armonk, NY, USA) and SuperANOVA/Statview (SAS Inc., Cary, NC, USA). The alpha level for statistical tests was set at 0.05. The behavioral tests were analyzed using a repeated measure model where the time course of the trial was the repeated measure. In the predator avoidance paradigm, periods when the predator stimulus was on or off was a repeated measure. Tests for simple main effects, between subjects contrasts and within-subjects contrasts were run. Analysis of the linear and quadratic trends of movement and position over time was evaluated (Keppel 1982). These trends assess the linear increase or decrease of movement or position over time of the test (linear trend) and the change in linear progression over time (quadratic trend). For example, a constant increase in position or speed over time would be detected as a linear trend and an accelerating increase in position or speed over time would be detected as a quadratic trend.

In the acute dosing paradigm, all the groups were included in the analysis. In the sub-chronic dosing paradigm, the highest dose of methylphenidate was fatal to the youngest group of zebrafish and all of them died. The methylphenidate did not cause significantly greater mortality in any of the other age groups than the casualties that occurred in the matched control groups (N dead = 1–2 fish/group). Thus, the highest dose 4 mg/L is excluded from all further analysis across age groups. Outliers with averages that were more than two standard deviations from the mean were re-tracked to ensure that there were no errors in their video quality. If the video was faulty, the trials were eliminated from further analysis. However, if their videos were accurate, their values were included in subsequent analysis. The number of zebrafish per age and treatment group was N = 11–16 per group for the acute study and N = 20 per group in the sub-chronic study.

Results

Acute methylphenidate exposure—novel tank diving test

No significant effects of methylphenidate treatment were seen on behavior in the novel tank diving test. The controls had an average position of 1.66 ± 0.48 cm from the bottom of the tank and swam at an average speed of 107.0 ± 14.2 cm/min. The fish exposed to 2, 4, and 8 mg/L of methylphenidate averaged 1.78 ± 0.51, 1.62 ± 0.50, and 1.92 ± 0.48 cm from the bottom respectively and had swimming speeds of 119.3 ± 19.9, 108.3 ± 20.1, and 93.0 ± 13.1 cm/min respectively.

Acute methylphenidate exposure - predator avoidance test

As shown in Fig. 1, there was a significant main effect of repetition of the stimulus where the first flee response was significantly greater than the second flee response ($F(1,44) = 4.24$, $p < 0.05$). There was also a significant interaction between methylphenidate and repeated stimulus presentation as displayed in Fig. 1 ($F(3,44) = 3.28$, $p < 0.05$). When the predator stimulus was first shown, the zebrafish in the control group fled 4.13 cm further from the screen on average than in the minute before the stimulus was shown. However, the control fish fled significantly less during the second encounter with the predator. They fled only 1.67 cm away from the screen compared with the minute before the stimulus was presented. The fish exposed to the lowest dose of methylphenidate reacted in a similar way as the control group. In contrast, the fish with the 4 and 8 mg/L methylphenidate doses had significantly ($p < 0.05$) fled less than controls. They fled an average of 1.51 ± 1.05 cm (4 mg/L) and 1.75 ± 0.67 cm (8 mg/L) compared with 4.13 ± 0.73 cm for controls. During the second exposure to the stimulus, the control fish showed less of a flee response and there were no significant methylphenidate effects.

Acute methylphenidate exposure—social shoaling test

As shown in Fig. 2, there were significant main effects of methylphenidate ($F(3,42) = 3.31$, $p < 0.05$) and minute ($F(1,42) = 15.50$, $p < 0.001$) on locomotor activity. Control fish increased their locomotor activity (cm/min) in response to viewing a video of the fish shoaling compared with their locomotor activity during the first minute of the test when there is a control image on the screen. On average, control fish traveled 118.8 cm in the first minute. In minutes 2–6, they swam an average of 144.2 cm/min, 214.4 cm/min, 194.3 cm/min, 209.3 cm/min, and 195.0 cm/min respectively. All three groups of zebrafish exposed to methylphenidate showed similar locomotor activity

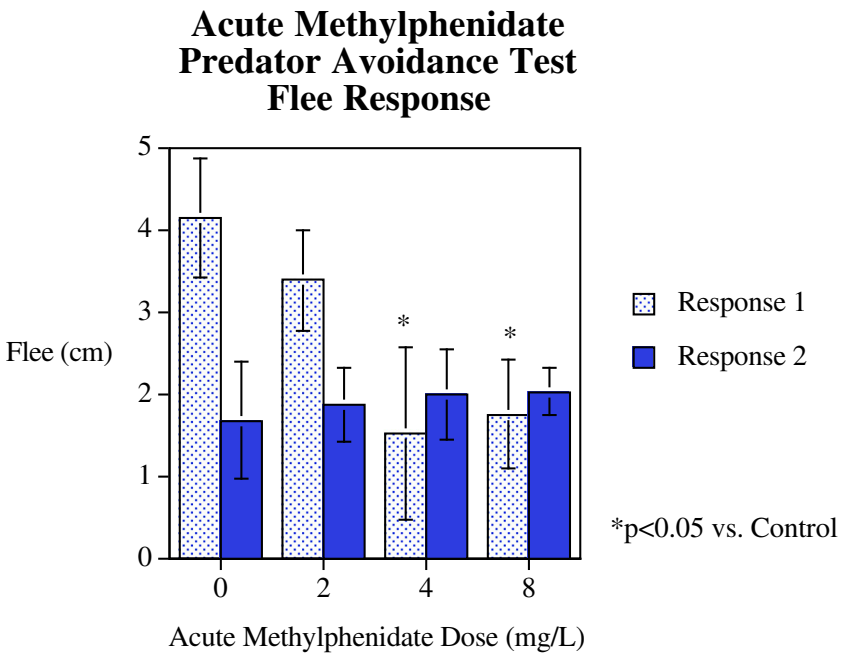

Fig. 1 Acute methylphenidate effects on the fleeing response in the predator avoidance paradigm (mean ± sem): There is a significant interaction between the dose of methylphenidate and flee response ($F(3,44) = 3.83$, $p < 0.05$). Flee response 1 is calculated by subtracting the average distance to the screen in minute 1 (predator stimulus off) from the average distance to the screen in minute 2 (predator stimulus on). Flee response 2 is calculated by subtracting the average distance to the screen in minute 3 (predator stimulus off) from the average distance from the screen in minute 4 (predator stimulus on) ($N = 11–12$ per group)

in the first minute compared with controls; however, they never increased their swimming speed in later minutes as the controls did such that the methylphenidate-induced differences became more pronounced. When the shoaling video was playing in minutes 2–6, all of the doses of methylphenidate caused significantly decreased locomotor activity compared with controls ($p < 0.05$ for 2 mg/L, 8 mg/L; $p < 0.005$ for 4 mg/L). These results are shown in Fig. 2. The locomotor activity when the control image played on the screen in minute 1 was not statistically different for the control fish and the fish exposed to methylphenidate. There were no statistically significant differences in how close the zebrafish were to the screen across time.

Sub-chronic methylphenidate exposure—novel tank diving test

As shown in Fig. 3, there was a main effect of minute where dive response of the zebrafish measured by the distance to the bottom of the tank (cm) varied as a function of time in the trial ($F(3,284) = 34.05$, $p < 0.0005$). The main effect of minute shows that the zebrafish engaged in a diving response and provided an internal validation check for the task. There was a significant interaction between time x methylphenidate dose ($F(4,284) = 3.79$, $p < 0.01$). Figure 3 shows the difference in trends for the distance to the bottom of the tank over time for

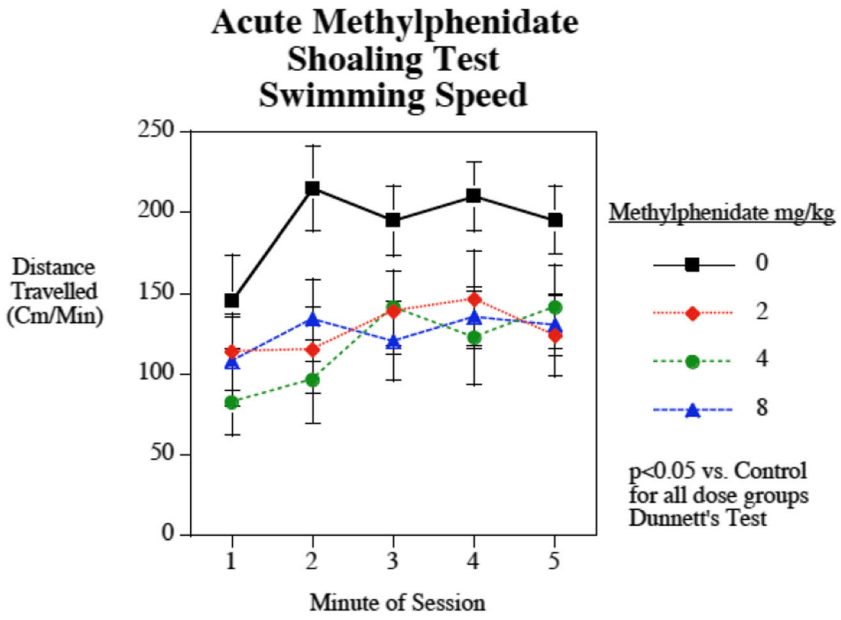

Fig. 2 Acute methylphenidate effects swimming speed (cm/min) in the shoaling test (mean ± sem): There is a main effect of time ($F(4,168) = 2.93$, $p < 0.025$) and dose ($F(3,42) = 3.75$, $p < 0.025$). When the shoaling video was playing, all of the doses of methylphenidate had significantly decreased locomotor activity compared with controls ($p < 0.05$, Dunnett's test, two-tailed) for 2 mg/L, 4 mg/L, and 8 mg/L ($N = 11–12$ per group)

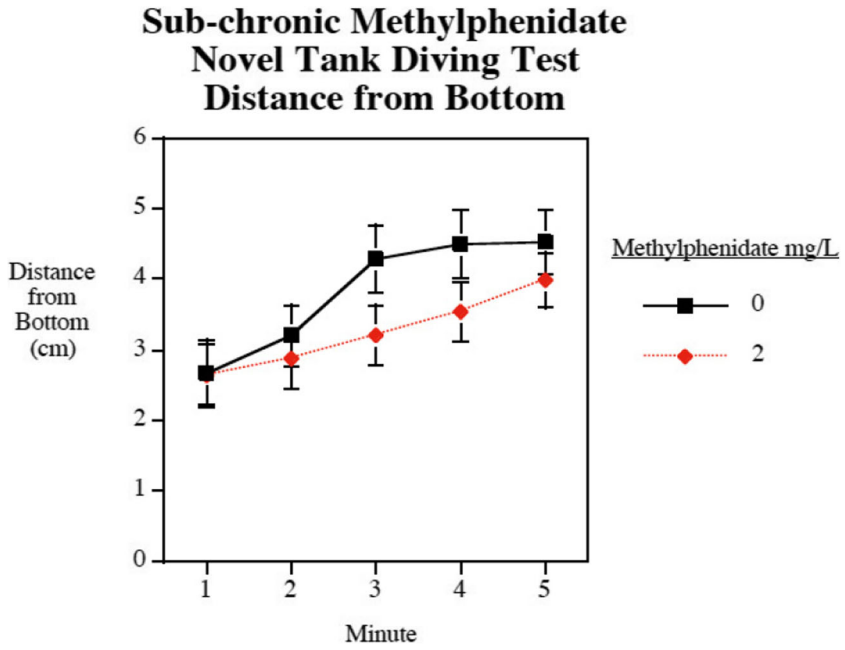

Fig. 3 Sub-chronic methylphenidate effects on the diving response in the novel tank diving test (mean ± sem): The quadratic interaction between time x methylphenidate dose is significant ($F(4,68) = 4.52$, $p < 0.005$) and a follow-up analysis revealed a quadratic trend for this affect ($F(1,71) = 12.44$, $p < 0.025$). In other words, the rate of increase of distance from the bottom of the tank over time of testing is significantly different between methylphenidate treatment groups. The control and experimental fish both dove to the bottom of the tank (mean 0 mg/L = 2.57 cm; mean 2 mg/L = 2.49 cm). As more time past, the control group explored the tank more rapidly in a quadratic fashion reaching a mean of 4.44 cm in minute 5 whereas the zebrafish dosed in 2 mg/L methylphenidate explored the tank more slowly in a linear fashion reaching a mean of 3.88 cm in minute 5 ($N = 20$ per group)

the methylphenidate-exposed and control groups. The control and experimental fish both dove to the bottom of the tank (mean 0 mg/L = 2.67 ± 0.47 cm; mean 2 mg/L = 2.62 ± 0.45). As more time past, the control group explored the tank more rapidly over the time of the test reaching a mean of 4.52 ± 0.56 cm in minute 5, whereas the zebrafish dosed in 2 mg/L methylphenidate explored the tank more cautiously reaching a mean of 3.98 ± 0.39 cm in minute 5. Figure 4 shows that across age groups, the fish dosed with methylphenidate were less excitable and did not explore as much following a dive response. There is an age effect where the younger fish (14 dpf and 21 dpf) show a more robust dive response and are slightly more willing to explore the tank than their 28 dpf counterparts. There were no statistically significant differences between swimming speeds between any of the groups.

Sub-chronic methylphenidate exposure—predator avoidance test

There was a main effect of whether the predator stimulus was on or predator stimulus was off on the total distance that the zebrafish traveled across all ages and doses ($F(1,54) = 6.50$, $p < 0.025$). When predator stimulus was on, the fish exhibited more freezing behaviors and moved significantly less compared with the amount they moved when the predator stimulus was off. Zebrafish in all the groups moved less when the threat stimulus was on. There were no significant differences between groups or main effects when measuring flee responses.

Sub-chronic methylphenidate exposure—social shoaling test

There was an interaction between age and methylphenidate dose ($F(1,59) = 2.81$, $p < 0.10$). The younger (14 day) zebrafish showed a significant ($p < 0.05$) reduction in activity with 2 mg/L of methylphenidate compared with controls (control = 309.3 ± 53.0, 2 mg/L methylphenidate = 210.0 ± 23.4), while the older zebrafish (21 days) dosed with 2 mg/L of methylphenidate did not exhibit much change in their locomotor activity (control = 263.5 ± 20.0, 2 mg/L methylphenidate = 261.5 ± 27.2). There were no significant differences between groups or main effects when measuring the distance to the screen that displayed the shoal.

Discussion

We found that methylphenidate exposure, with either acute adult dosing or as a residual effect after sub-chronic juvenile dosing, significantly diminished behavioral responses to environmental stimuli. The two studies demonstrated the

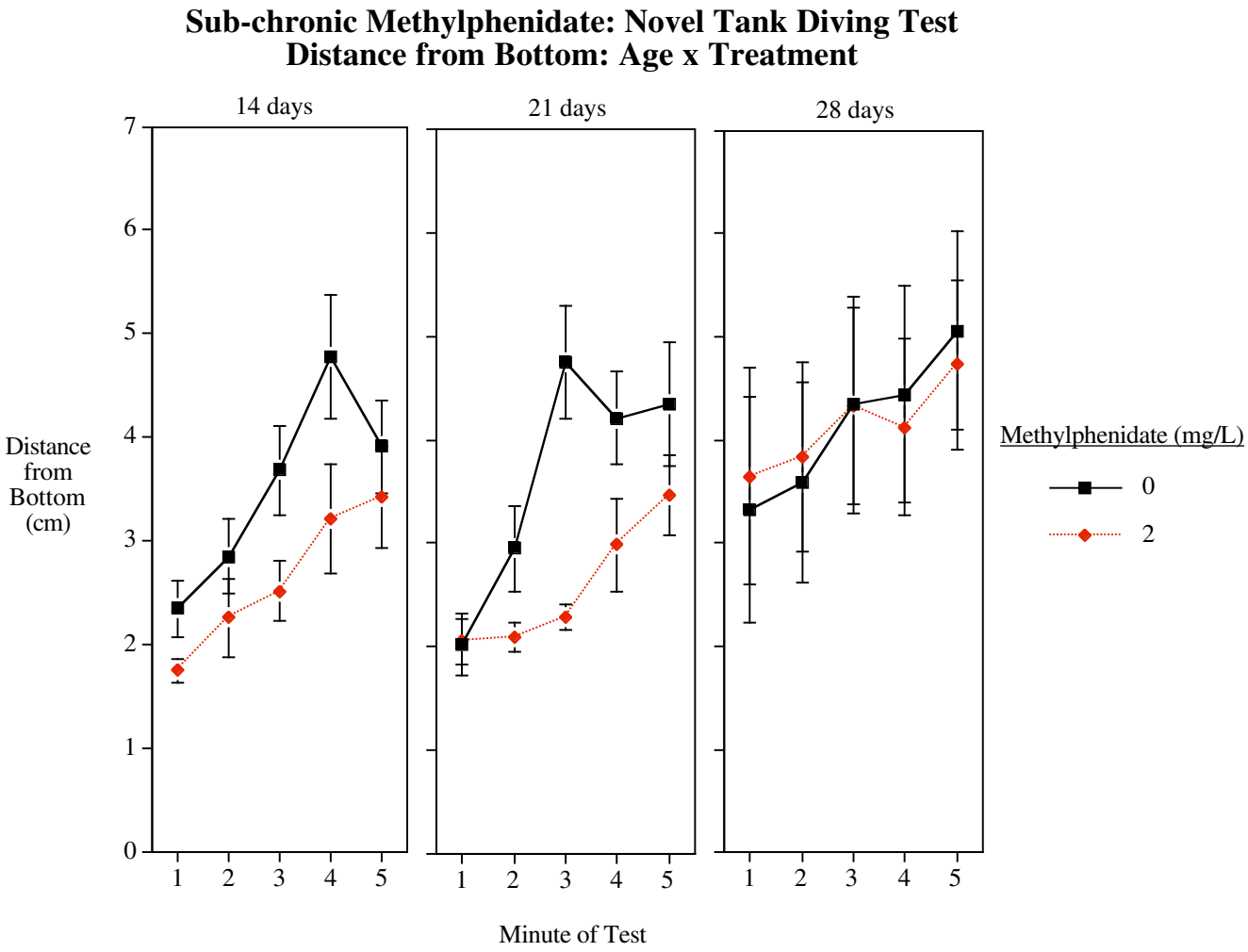

Fig. 4 Sub-chronic methylphenidate and age effects on the diving response in the novel tank diving test (mean ± sem): There is a significant age x time x methylphenidate interaction ($F(2,71) = 4.44$, $p < 0.025$). This figure shows that across age groups, the fish dosed with methylphenidate were less active and did not explore as much following a dive response. There is an age effect where the younger fish (14 dpf and 21 dpf) show a more robust dive response and are slightly more active in the tank than their 28 dpf counterparts ($N = 20$ per group)

pervasiveness of the behavioral actions of methylphenidate as a result of two quite different exposure regimens.

In summary, methylphenidate consistently lowered responsivity to environmental stimuli. With adult fish, acute methylphenidate significantly lowered fleeing response in the predator test and significantly lowered locomotor activity in response to a social stimulus in the shoaling test. With juvenile zebrafish, sub-chronic methylphenidate exposure produced significant persisting behavioral effects with decreased exploration of novel tank and attenuated response to a social stimulus.

The acute methylphenidate-induced lowering of escape and social behavior did not seem to be merely a result of general debilitation. Methylphenidate-treated fish did swim in response to the environmental changes; it is just that the response was not seen to change over the time course of the test as it did with controls. Endres et al. (2017) also found that acute methylphenidate blunted behavioral aspects of stress response in zebrafish. They found that acute methylphenidate exposure caused a lessened diving response in the novel tank test run after a stressful experience. Parker found in zebrafish that methylphenidate increased anticipatory responses on a five-choice task (Parker et al. 2014). Zebrafish are not the only fish species that show adverse behavioral effects of methylphenidate. Adverse behavioral effects of methylphenidate in terms of aggressive behavior are seen in tilapia (Batalhão et al. 2019) and methylphenidate-induced increased investigatory behavior is seen in guppies (De Serrano et al. 2016).

In the sub-chronic study with juvenile zebrafish, the age at which the methylphenidate dosing began in the zebrafish significantly affected their behavioral responses in the novel tank diving task and the predator avoidance paradigm. In the novel tank diving test, the age at which the zebrafish was first dosed

with methylphenidate changed the time course of response. In the predator avoidance paradigm, sub-chronic dosing that began at an older time point (28 dpf) decreased the cohort's activity levels more than sub-chronic dosing that began at earlier time points (14 dpf and 21 dpf). Overall, sub-chronic methylphenidate exposure during development and acute methylphenidate exposure in adulthood lead to a decreased response to environmental cues.

One complication in this study is that duration of dosing was not the same for zebrafish who began dosing at different ages (14 dpf, 21 dpf, and 28 dpf). The reason they were not exposed for the same duration of time is to avoid testing them when they were different ages or had been withdrawn from the medication for different lengths of time. In our design, all the zebrafish were tested after being exposed until 12 weeks of age. This dosing regimen more closely models the human experience because children who get diagnosed with ADHD at a young age and begin medication usually take it for a longer period of time than their ADHD peers who are diagnosed later. However, the confound created is that the youngest dosed cohort was on methylphenidate for two weeks longer than the oldest dosed cohort. Thus, we cannot tease out whether the effect is due to the developmental time points at which dosing was started or if it is an effect of the duration of the dosing regimen. Another caveat in this study is that when we ran a novel tank diving task for acutely dosed zebrafish, the control fish did not exhibit a diving response, which indicates that the task did not work properly. We hypothesize this is a product of our dosing paradigm. Since zebrafish were placed in a new tank to be dosed for 30 min prior to the novel tank diving task, they had already become acclimated to a novel environment and were no longer reacting to the novelty of the second new tank. Finally, it is important to note that there is a wide gap between fish behavior and human behavior. These results may not extrapolate to humans directly and it is necessary to conduct multiple translational studies to clarify whether the results are conserved across species. However, the characterization of the behavioral effects of methylphenidate in zebrafish opens the way for further mechanistic studies to elucidate the molecular and cellular bases for those effects. Much of the neurotransmitter receptor and transporter systems are conserved across vertebrate species. Zebrafish also provide an economical model with which to screen the neurobehavioral impact of the ever-increasing number of methylphenidate analogues being synthesized for drug abuse (Davoli et al. 2018).

While the effect of long-term sub-chronic developmental exposure to methylphenidate in zebrafish was previously unknown, our study does not conflict with other studies in rodents. These studies also show that methylphenidate decreases response to environmental cues. Adriani et al. (2006) found that a 16-day exposure to methylphenidate during adolescence decreases the rewarding properties of cocaine in adulthood. Interestingly, they also noted that rodents had increased "economical efficacy" and enhanced "behavioral flexibility" in a choice behavior paradigm that may be the result of a decreased response to rewarding properties (Adriani et al. 2006). This indicates that decreased responses to certain properties of environmental stimuli, such as the rewarding properties of cocaine, could be beneficial as opposed to detrimental. Bolanos et al. (2003) also showed that preadolescent rodents exposed to methylphenidate for 15 days were less responsive to natural rewards of sucrose, novelty-induced activity, and sex. Additionally, they showed an increase in stress and anxiety-like behaviors in an elevated plus maze in these preadolescent rats, which parallels our finding that sub-chronically dosed zebrafish in the novel tank diving task appeared to be more anxious as evidenced by their increased diving response (Bolanos et al. 2003). However, a lack of anxiety-like behaviors in an elevated plus maze in adolescent rats exposed to a 28-day exposure to methylphenidate was shown by Gray et al. (2007). Vendruscolo et al. (2008) found that the increase in anxiety-like behaviors was dependent on task because rodents exposed to methylphenidate for 16 days during adolescence exhibited anxiety-like behaviors in an open field test, but not in the elevated plus maze. Taken together, these results indicate that the anxiogenic properties of methylphenidate depend on the experimental paradigm including both the task and the dosing conditions, specifically the age at which the rats were dosed. These results are supported by our findings that acute and sub-chronically dosed zebrafish reacted differently in response to a predatory stimulus. Unfortunately, it is difficult to know whether an increase in exposure period from 15–28 days to 56–70 days modified the effect since the model animals and behavioral paradigms are different in each of these studies. In addition, the observed differences in the behavioral effects of the acute and sub-chronic methylphenidate exposure might have resulted from differences of age between animals at the time of testing. The acute test was conducted with animals of 8 months of age, whereas the sub-chronic, 4-month-old animals. The fish in both studies were full adults when tested. It is possible that the adult age difference itself may have been critical in the differential expression of the behavioral effects, but there is currently no available dataset that would support differential methylphenidate effects in adult zebrafish over this timespan.

Future studies are needed to vary the duration of sub-chronic dosing with methylphenidate to explore how much exposure is needed before a decreased response to environmental cues can be observed. In addition, future studies are needed to test how these findings relate to changes in children's behavior after being exposed to sub-chronic methylphenidate. One possible mechanism by which methylphenidate could be decreasing the response of the zebrafish to environmental cues could be by decreasing the importance of other tasks or stimuli in the surrounding environment. If this is accurate, we would

hypothesize that depressing the environment cues would be one way that the drug allows people to focus on a specific task. Methylphenidate could make zebrafish, and perhaps humans, have trouble switching tasks. However, future studies would be necessary to elucidate this point. In addition, a decreased response to environmental cues could be related to a feeling of apathy in children, which is also an interesting target for future investigation. It is important to examine the effects of methylphenidate on children who are increasingly being prescribed methylphenidate as a treatment for ADHD. If methylphenidate were found to cause behavioral changes, such as decreasing responses to environmental stimuli or increasing apathy, we would need to reconsider the ethics of prescribing methylphenidate to young children.

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Authors' contribution RGB conducted the experiments and lead the writing. ANO supervised the drug exposures and data gathering and helped with the data analysis. WSA helped with the experimental design and writing. EDL designed the study, analyzed data, and helped with writing. All authors read and approved the manuscript.

Compliance with ethical standards This research project and article is compliant with the ethical standards of the journal. The procedures were approved by the Duke University Animal Care and Use Committee under United States of America regulations.

Conflict of interest The authors declare that they have no conflicts of interest.

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