Zebrafish as a behavioral model: Acute fluoxetine effects on behavior and influence of sex
Innehållsförteckning
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Abstract
The zebrafish as an animal model has recently become more noticed and used for behavioral studies and pharmacological manipulation in neuroscience. An increasing number of studies have shown that zebrafish can be used to better understand disease states in humans and assist in the development of new pharmacological drugs. However, this animal model is relatively new and needs to be further verified to increase its generalizability. In this study, we investigated the effect of an antidepressant (fluoxetine) on anxiety and aggression in zebrafish. We also investigated whether gender differences influence behavior. We treated zebrafish with fluoxetine dissolved in water for two hours and recorded the pattern of movement in an open field test and assessed aggression as number of attacks performed towards a conspecific in a dyadic interaction. We also measured monoamine activity in the brain after treatment with fluoxetine. Acute fluoxetine only had very modest effects on the behavior of the fish in the open field test and no effects on aggression, despite significant effects on serotonergic activity in the brain. Females were found to be less active in the open field test, but were just as aggressive as males. The results indicate that the effect of acute treatment with fluoxetine is still uncertain and that more studies are needed to possibly find an effective dose of this drug on the behavior of zebrafish. Furthermore, the results emphasize the importance of studying the sexes separately as difference in behavior was evident.

Populärvetenskaplig sammanfattning på svenska
1. Introduction

Zebrafish (Danio rerio) has become an increasingly used model organism in neuropharmacological and neurobehavioral research (Champagne et al. 2010; Sackerman et al. 2010). Due to its homology in physiology to humans, researchers can gain new insights to human pathological disorders while keeping maintenance and costs at a minimum (Egan et al. 2009; Lillesaar 2011; Shin and Fishman 2002). As a behavioral model, mounting data on pharmacological manipulations in zebrafish argue in favor of its sensitivity and translatability to rodents and humans (Stewart et al. 2011).

A wide variety of anxiogenic and anxiolytic compounds have been tested on zebrafish, including PTZ (Pentylenetetrazole), caffeine, pCPA (para-chlorophenylalanine), ethanol, morphine and selective serotonin reuptake inhibitors (SSRI) (Maximino et al. 2013; Wong et al. 2010).

SSRI having its effect on the serotonergic system (5-HT, 5-hydroxytryptamine) act to block the 5-HT reuptake in the synaptic cleft allowing 5-HT to act for a longer duration (Wong et al. 2013). The serotonergic system is involved in many aspects of zebrafish and mammal physiology and behavior including; integration with the endocrine system, stress and anxiety related behavior as well as aggressive responses (Maximino et al., 2013; Winberg et al., 1997). Although there are some differences in the serotonergic system between zebrafish and mammals (e.g., duplication of genes involved in synthesis and transport of serotonin) both species exhibit similar effects of drugs that target this system (Egan et al. 2009; Maximino et al. 2013; Stewart et al. 2012). SSRI being anxiolytic in humans, is frequently used in psychiatric disorders including depression and anxiety (Celada, Bortolozzi, and Artigas 2013).

Anxiolytic effects of chronic treatment with the SSRI Fluoxetine in zebrafish is paralleling that of human and rodent studies (Egan et al. 2009; Wong et al. 2013). On the other hand acute treatment with fluoxetine have not been as extensively studied and has yielded an unclear picture of effects on zebrafish behavior (Maximino et al. 2013; Stewart et al. 2011). Maximino et al. (2013) studied acute fluoxetine treatment with intraperitoneal injections in the scototaxis test (rectangular box divided into two equal compartments, one in black and one in white coloration where preference for the dark environment is shown for untreated zebrafish) and novel tank test (a narrow tank with demarcations on the outside dividing it horizontally into two or three equal sections where zebrafish initially show increased preference in the bottom of the tank). Fluoxetine was anxiogenic at the dose of 2.5 mg/kg in the scototaxis test but anxiolytic in the novel tank test (Maximino et al. 2013). In another study zebrafish were treated through fluoxetine-immersion (100-1000μg/L) for 20 min with no effect on behavior in the novel tank test (Stewart et al. 2011).

Serotonin is believed to act inhibitory on aggressive behavior (Summers & Winberg, 2006; Summers et al., 2013; Winberg et al., 1992). However, various results on aggressive behavior have been reported using serotonin enhancing or inhibiting drugs respectively. In rats, chronic treatment with SSRI have been reported to result in increased aggressive behavior (Mitchell and Redfern 2005). However, in fish there is also reports suggesting that chronic SSRI inhibits aggressive behavior (Perreault et al., 2003).

Many methods to examine zebrafish behavior have been adapted from experiments to novelty with rodents, which display similar phenotypic behavior in these tests (Stewart et al. 2011). A model for assessment of stress/anxiety-like
responses in mammals and fish is the open field test (OFT), where a subject is introduced to an empty novel arena/tank. Anxiety-like behavior can then be measured as different movement patterns (Maximino et al. 2010), including: thigmotaxis (swimming close to the walls, avoiding the center of the test arena), freezing bouts and decreased locomotion (Maximino et al. 2010).

Apart from looking at the behavioral response of individuals to stress or pharmaceuticals, one can measure concentrations of brain monoamines and their respective metabolites (Clotfelter et al, 2007; Øverli et al, 2000). In the serotonergic system, 5-hydroxyindoleacetic acid (5-HIAA, 5-HT’s major metabolite) and the ratio 5-HIAA/5-HT are often measured, where a higher ratio corresponds to an increase in 5-HT-turnover and activity (Winberg et al., 2001).

Due to the zebrafish relatively new entry into the field of neuropharmacological research, the efficacy of different neuroactive drugs’ doses are still unknown (Stewart et al. 2011) Our main purpose of this study was to analyze the acute effects of two different doses of fluoxetine on zebrafish behavior and brain serotonergic activity. A disadvantage that injection treatment has compared to immersion treatment is that fish are subjected to pain and severe stress which naturally effects the results of subsequent behavioral testing (Stewart et al. 2011). We therefore chose immersion as our route of administration. Previous studies have shown discrepancies in behavior and brain monoamine activity between sexes in zebrafish (Dahlbom et al., 2012; Philpott et al., 2012). The secondary purpose of this study was therefore to investigate the impact of gender on behavior and brain monoamine turnover.

2. Materials and methods

AB-strain adult Zebrafish, bred in captivity was used in this experiment. The use of animals in this study was approved by Uppsala Ethical Committee (permit number C55/13)

The fish were held in the lab at Uppsala University Biomedical Center. Fish were housed in tanks with municipal tap water (pH≈7.3) of which 15% were exchanged daily and held at a temperature of 27°C. Fluorescent ceiling light tubes illuminating at 14h light and 10h dark cycles was provided. Fish were fed once a day with Tropical flake food (Sera San) after testing. A total of 79 fish were used (40 female and 39 males). Due to a limited number of equally sized tanks two different kinds were used to isolate and house the fish; one with the capacity of 2.8L (15cm height X 27cm top X 22cm bottom X 7cm width) and one with the capacity of 1.8L(14cm height X 26cm top X 23cm bottom X 5cm width)

Fish were left a minimum of one week to acclimatize to their own tank prior to tagging.

The drug used in this experiment was the SSRI Fluoxetine hydrochloride (sigma Aldricht, Sweden). Fish of each sex were then randomized into three different groups; high dose (1.5 mg/L fluoxetine, n=20), low dose (0.5mg/L fluoxetine, n=19), control group (no fluoxetine, n=40).

Tagging

Prior to tagging, fish were anaesthetized in Benzocaine (Ethyl p-aminobenzonate, 0.34 mg/ml).

The fish-tagging procedure consisted of inserting a 0.4mm cannula with a 0.11mm diameter nylon filament (fishing line) through the dorsal musculature just beneath the dorsal fin. The cannula was then removed but leaving the
monofilament in place. The ends of the monofilament were melted to prevent the nylon monofilament from falling off. These also served as color marking points at which different color combinations was painted using nail polish. After tagging the fish, they were directly put back in their housing tanks. They were allowed to recover for a minimum of 14 days before testing was commenced.

**Open field test (OFT)**

The open field test arena used in this experiment consisted of a rectangular (34cm length x 29cm width x 14cm height) Plexiglas box filled with system water (6cm in depth). Prior to introducing the fish to the test arena, fish were immersed for two hours in treatment beakers (20cm length x 9cm width x 7cm height), with lids from their own housing tank to lessen the stress. Open field boxes were made opaque using masking tape and subsequently placed on a white infrared table with a camera detector mounted in the ceiling above. Using Ethovision XT (Noldus, the Netherlands) different types of movement pattern were quantified (Distance moved, duration in the center zone, immobility duration and zone transitions). The center zone was defined as half of the area of the arena. Between tests, the open field arena was cleaned with 90% ethanol and rinsed twice with housing water.

Total time of recording was 20min per fish and immediately after testing fish was returned to respective housing tank to rest for a minimum of 12 days before the next test.

**Aggression test**

To test the effects of fluoxetine on aggression we set up fighting tanks (9cm length x 14cm width x 19cm height) with a vertical non-see-through plastic wall dividing it into two equally sized compartments. To be able to pair fish up with similar weight and size, they were weighed prior to the aggression test.

In this test 74 fish were size-matched in respective sex and paired (Control vs treated) and placed in the fighting tanks to acclimatize for three days. Each fish remained in the same treatment group as before. As in the previous test, prior to testing fish placed in treatment beakers for two hours and thereafter returned to the fighting aquarium to rest for one hour. As the wall was carefully removed recording of the tanks was commenced for one hour. Aggression was manually scored as attacks/chases in three different time periods; the first 5 minutes, 30th to 35th minute and 55th to the 60th minute (a total of 15minutes).

All tests were performed between 10am to 5pm to avoid circadian disturbances.

**Sampling**

Two months after the aggression test 48 (Males: n=24, females: n=24) of the fish from the start were randomly selected and randomized to a test group: High dose (1.5 mg/L fluoxetine, n=16), Low dose (0.5mg/L fluoxetine, n=16), Control group (n=16).

As in the previous testing, fish were placed in treatment beakers for two hours. Fish were then transferred to a beaker containing ice-cold housing water to quickly anaesthetize upon decapitation. Whole-brains were removed, wrapped in tin foil and immediately put on dry ice and stored at -80⁰C.

**Analysis of brain serotonin and serotonin metabolite concentrations**

Frozen brains were weighed and homogenized in a Potter-Elvehjelm homogenizer (Rudolph Grave AB, Stockholm) with 400µl of 4% ice-cold
perchloric acid containing 100ng/ml 3,4-dihydroxybenzylamine (DHBA, internal standard). The homogenized samples were then immediately centrifuged for 10 minutes at 15000 rpm and subsequently put on dry ice. The samples were thawed and centrifuged for a second time for 10 minutes at 15000 rpm before using the supernatant for high performance liquid chromatography with electrochemical detection (HPLC-EC). Analysis of 5-HT and 5-HIAA was made as described by Øverli et al, 2000, Dahlbom et al, 2012 (briefly explained below).

The HPLC-EC system consisted of; a solvent delivery system (Model 592, ESA, USA), a reverse phase column (Reprosil-Pur C18-AQ 3µm, 100mm x 4mm column, HPLC, GmbH, Germany) held at 40°C, an autoinjector Midas type 830 (Spark, Holland Emmen, Netherlands), and an ESA 5200 Coulochem II EC detector (ESA, USA) containing two electrodes at reducing and oxidizing potentials of -40mV and +320 mV, respectively. A guarding electrode was set up to oxidize any potential contaminants before the analytical electrodes. The mobile phase consisted of 1.4mM sodium octyl sulphate, 75mM sodium phosphate and 10µM EDTA in deionized water with 7% acetonitrile.

Statistical analysis
All statistical analyses were performed using the software IBM SPSS Statistics 21. Behavioral data was analyzed with non-parametric tests, Kruskal-Wallis or Mann-Whitney U-test, when appropriate. Data on monoamine and monoamine metabolite concentrations, as well as the ratio between monoamine metabolites and parent monoamine were log-transformed prior to two-way ANOVA analysis to meet the demand of normal distribution. Tukey’s post hoc analysis were performed when necessary. Results were considered statistically significant if p < 0.05.

3. Results
The color of the tag had no effect on the number of attacks performed in the aggression test (Mann-Whitney U-tests, lowest p-value: 0.344). There was no difference in weight within pairs, across both males and females, in the aggression test (Independent samples t-test, p = 0.642, N = 37, mean difference (g): 0.01351). Mean weight (g) ± SEM controls: 0.3189 ± 0.01852, treated: 0.3324 ± 0.02228.

In the HPLC-analysis, four samples of the 48 from the start were destroyed due to machine-malfunction. The samples destroyed and excluded from analysis were: two low dose females, one control female and one low dose male.

Open field test (OFT)
Fluoxetine had no significant effects on immobility duration, total distance moved, or zone transitions neither at 0.5 mg/l nor at the dose of 1.5 mg/l. However, there was an effect on time spent in the center zone (“in-center zone duration”, p = 0.021). Subsequent post hoc comparisons revealed that fish treated with the lower dose (0.5mg/L fluoxetine) tended to spend more time in the center zone compared to fish treated with the higher dose (1.5mg/L fluoxetine) (Mann Whitney U-test, adjusted significance p = 0.022, Fig 1). Further analysis also revealed that fish housed in larger tanks spent more time in the center zone than fish housed in smaller tanks (p = 0.017). No other effects of tank size were observed.

When comparing sexes regardless of treatment however, female fish showed significantly lower distance moved (p=0.002), longer immobility duration (p=0.021) and fewer zone transitions (p=0.003) than male fish (Fig 1).

Aggression test
Treatment with fluoxetine had no effects on the number of attacks initiated at the doses tested (p = 0.126). Neither were
there any difference in number of attacks initiated between males and females (p = 0.970; Fig 2).

**Brain levels of 5-HT and 5-HIAA, and brain 5-HIAA/5-HT ratios**

Fluoxetine treatment had a significant effect on 5-HIAA/5-HT ratios (p < 0.001) and 5-HIAA concentrations (p = 0.014, two-way ANOVA, Fig 3). Further, post hoc analysis revealed that fish treated with fluoxetine, at 0.5 as well as 1.5 mg/l, showed significantly lower brain 5-HIAA concentrations (0.5 mg/l, p=0.030; 1.5 mg/l, p=0.033) and 5-HIAA/5-HT ratios (0.5 mg/l, p=0.003; 1.5 mg/l, 0.001) than controls. However, there was no difference in either 5-HIAA concentrations or 5-HIAA/5-HT ratios between fish receiving different doses of fluoxetine (p=0.982 and p=0.915, respectively). Moreover, there was no significant effect of fluoxetine on Dopamine or 3,4-Dihydroxyphenylacetic
acid (DOPAC, a dopamine metabolite) levels, or DOPAC/dopamine ratios. No significant interactions between sex and treatment were evident on concentrations of brain monoamines, monoamine metabolites or monoamine-to-monoamine metabolite ratios.

4. Discussion

The results of this study showed small modest effects of acute fluoxetine on the behavior of zebrafish in the open field test but no effects on aggression. Zebrafish treated with 0.5mg/l fluoxetine showed anxiolytic behavior as indicative to spending more time in the center of the open field arena “In center zone duration” when compared to the 1.5mg/l treated fish. However, this significant difference should be considered weak as we also detected an effect of housing tank size on this variable as well. This does not mean there is no effect of fluoxetine on aggression and anxiety related behavior. It could be that we failed to detect an effect because of i) an insufficient dose of fluoxetine, ii) insufficient time of exposure to the drug, or both. Since the 5-HIAA/5-HT ratio was affected, 5-HT-turnover was decreased at least to some extent in the treatment groups. If the reuptake of 5-HT by the presynaptic neuron is blocked, less of the released 5-HT can be metabolized to 5-HIAA by the enzyme monoamineoxidase (MAO) in the presynaptic neuron. Thus,
the fact that fluoxetine treatment resulted in reduced 5-HIAA concentrations and 5-HIAA/5-HT ratios confirms that it had effects on the brain 5-HT system of the fish. However, the effect might not have been strong enough to detect an effect on behavior. Important to note however is that this lack of effect by fluoxetine is in line with a previous study in the fighting fish Betta splendens (Clotfelter et al. 2007). Using chronic treatment they did not detect any significant changes in aggressive behavior although brain 5-HIAA levels were decreased. However, elevated dietary levels of L-tryptophan, the precursor of 5-HT, inhibit aggressive behavior in rainbow trout (Oncorhynchus mykiss), but only after seven days of treatment. Three days of elevated dietary L-tryptophan had no effect on aggressive behavior, even though brain 5-HT activity was elevated. (Winberg et al. 2001). Lepage et al. (2005) showed that a seven day treatment with the SSRI citalopram also had an inhibitory effect on aggression in rainbow trout. Thus, effects of acute as well as chronic stimulation of brain 5-HT activity on aggressive behavior are still ambiguous.

In our study, female zebrafish were significantly less mobile in the open field test. Philpott et al. (2012) showed no significant difference in locomotion between sexes in zebrafish in the open field test. However when analyzing this parameter with age as a covariate, a discrepancy was found. Ten month old male zebrafish had a significantly higher locomotor activity than females of same age. In contrast the inverse outcome was shown when comparing 22 month old zebrafish (Philpott et al. 2012). The zebrafish used in this study were approximately 12 month old, which might support the work of Philpott et al. The findings of this current study are also consistent with those of Palanza who found individually housed female mice to be less exploratory of an open field arena than individually housed males (Palanza 2001). This is also supported by studies in rats where females had higher levels of corticosterone (the equivalent to cortisol in humans and zebrafish) when individually housed as compared to group housed. Interestingly the opposite were true in male rats, i.e. higher levels of corticosterone in crowded conditions (Road 1995). A possible explanation to this sex difference could be that male isolation mimic that of establishment of a territory, hence acquiring dominance and being less stressed (Palanza 2001). Subsequent behavioral outcome could then be affected by males being less anxious from the start than females thus increasing their exploratory behavior. Another aspect of this discrepancy is that male zebrafish are generally bolder than females when exposed to novelty (Dahlbom et al. 2011).

One unanticipated finding was that the duration of immobility was surprisingly high throughout the open field test in both sexes. Although as gently handled as possible, fish will always experience some amount of stress in these kind of behavioral testing when transferred from different confinements. The handling of the fish and the relatively small treatment beakers used in this study could explain this high immobility duration observed. Contradictory to this observation is a study by Champagne et al. who showed that zebrafish subjected to restraint stress prior to the open field test increased their activity in all zones of the arena, suggesting an anxiolytic effect of acute stress (Champagne et al. 2010).

Conclusion
We found acute fluoxetine to decrease 5-HT-turnover in zebrafish without affecting their behavior in regards to stress and aggression. However, further experimental investigations are needed to estimate effective doses of acute SSRI. In addition we found female zebrafish to be less mobile in the open field test. Since there are male and female behavioral differences
in zebrafish we suggest that more research in this area would yield increased understanding of this species. It would be interesting to assess the effects of housing conditions on cortisol levels in both sexes to see if this parallels that of rodent findings.

5. References


