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Research Paper

Combinatory effects of low concentrations of 17α-etinylestradiol and citalopram on non-reproductive behavior in adult zebrafish (*Danio rerio*)

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ABSTRACT

Sewage effluents contain pharmaceuticals, personal care products and industrial chemicals, exposing aquatic organisms to complex mixtures. The consequences of exposure to combinations of different classes of drugs in fish are largely unknown. In this study, we exposed adult zebrafish (*Danio rerio*) males and females for two weeks to low, environmentally relevant concentrations of the endocrine disrupting chemical 17α-etinylestradiol (EE_2) and the selective serotonin re-uptake inhibitor (SSRI) citalopram, alone and in combination, and analyzed behaviors of importance for population fitness, scototaxis (light/dark preference), the novel tank test and shoal cohesion. Control water contained 0.4 ng/L EE_2 and the measured exposure concentrations were 0.9 ng/L EE_2 (nominal 0.1) and 1 ng/L EE_2 (nominal 0.5). The measured concentrations of citalopram were 0.1 (nominal 0.1) and 0.4 μg/L (nominal 0.5). Both EE_2 exposures increased anxiety in males in the scototaxis test, with significantly longer latency periods before entering and fewer visits to the white zone of the tank. The combined exposures (0.9 ng/L EE_2 + 0.1 μg/L citalopram and 1 ng/L EE_2 + 0.4 μg/L citalopram) resulted in abolishment of effects of EE_2, with shorter latency period and more transitions to white than for fish exposed to EE_2 alone. In the novel tank test, the results surprisingly indicated lower anxiety after both EE_2 and citalopram exposure. Significantly more transitions to the upper half of the tank observed in males exposed to 0.1 μg/L citalopram alone compared to control males. Males exposed to EE_2 (0.9 ng/L) had shorter latency period to the upper half. Combination exposure resulted in a longer latency and fewer transitions to the upper half compared to both control, EE_2- and citalopram-exposed males. Males exposed to the combination spent significantly less time in the upper half than males EE_2 or citalopram-exposed males. Females exposed to 1 ng/L EE_2 had fewer transitions to the upper half than the control group and females exposed to 0.4 μg/L citalopram. In the shoaling test, males exposed to 0.1 μg/L citalopram + 0.9 ng/L EE_2 showed more transitions away from peers than males exposed to 0.1 μg/L citalopram alone. In conclusion, low concentrations of EE_2, closely above the predicted no effect concentration (NOEC) of 0.1 ng/L, created anxiety-like behavior in zebrafish males. Citalopram showed marginal effects at these low concentrations but in the combination exposure the behavioral effects of EE_2 were abolished. This is an initial effort to understand the effects of cocktails of anthropogenic substances contaminating aquatic environments.

1. Introduction

Aquatic organisms are exposed to a wide range of chemicals from agriculture, industry and municipal sewage, often in complex mixtures. High concentrations of pharmaceuticals are present in effluents from sewage treatment plants (STPs) (Fick et al., 2011; Nikolaou et al., 2007; Weigel et al., 2004). The bioavailability and intended biological activity of pharmaceuticals give them high potential to cause sublethal effects on non-target organisms. Fish, as vertebrates, share many common physiological features with humans and are therefore very likely to be affected by waterborne pharmaceuticals designed to affect human physiology (Gunnarsson et al., 2008). Earlier studies on the effects of within-class mixtures of EDCs on fish exist (Brian et al., 2007; Kortenkamp, 2007; Lin and Janz, 2006; Santos et al., 2006; Thorpe et al., 2003). However we lack knowledge about the effects of mixtures of pharmaceuticals with different modes of action. In the present study,

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we analyzed the effects of low levels of the endocrine disrupting chemical (EDC) EE_2, close to the NOEC of 0.1 ng/L, and the SSRI citalopram, as well as a combination of the two, on non-reproductive behavior in zebrafish (*Danio rerio*).

EE_2 from human oral contraceptives is present in effluents from STPs in concentrations from less than 1 ng/L up to 300 ng/L (Hannah et al., 2009; Kolpin et al., 2002; Laurenson et al., 2014; Sun et al., 2013). The predicted no effect concentration (NOEC) of EE_2 for water-living organisms is as low as 0.1 ng/L (Caldwell et al., 2012). EE_2 is environmentally persistent, bio-magnification has been observed (Aris et al., 2014) and is regarded as the EDC contributing the highest ecological risk in waste water (Laurenson et al., 2014; Sun et al., 2013). EDCs have the potential to interfere with the function of the hormone systems of all vertebrates (Guillette and Gunderson, 2001; Waring and Harris, 2005; Vos et al., 2000). Human fetal exposure to estrogenic compounds has been associated with depression (Wolstenholme et al., 2012) and distractibility, affected verbal skills, learning and memory, reduced masculine play (Xu et al., 2010). Behavioral variables shown to be affected by estrogenic compounds in rodents include aggression, anxiety, play behavior, attention, learning and memory and sexual behavior (Dugard et al., 2001; Ryan and Vandenbergh, 2006; Wolstenholme et al., 2012, 2011; Xu et al., 2010). Environmental-like levels of EE_2 caused alterations in female sexual behavior in adult rats exposed during development (Della Seta et al., 2008) and juvenile rats showed an anxiety-like response in a novelty preference test after developmental exposure (Zaccaroni et al., 2016). Developmental exposure of EE_2 has also showed to cause anxiety-related behavior, alter spatial memory, disturbed maternal behavior and a lack of discrimination between gonad-intact and castrated males in female mice (Ryan and Vandenbergh, 2006). Male mice developmentally exposed to low doses of EE_2 showed an increase in sexual behavior and modifications of neuronal networks. The effects were also transgenerationally transmitted to the F4 generation (Derouiche et al., 2015). In fish EE_2 has shown to cause reduced fertility and fecundity, feminization in male fish, skewed sex ratios and decreased egg and sperm production as well as behavioral changes (Aris et al., 2014). EE_2 exposure has caused alterations in risky behavior in the threespine stickleback (Bell, 2004) and guppies (Heintz et al., 2015) as well as boldness in Siamese fighting fish (Dzieweczynski et al., 2014). We have previously found that EE_2 increases anxious behavior in guppies (*Poecilia reticulata*) and zebrafish (*Danio rerio*) exposed as adults (Hallgren et al., 2011; Reyhanian et al., 2011) or during development (Volkova et al., 2015b; Volkova et al., 2012). Developmental exposure resulted in irreversible effects (Volkova et al., 2015b), which were shown to be transgenerationally transferred (Volkova et al., 2015a).

Citalopram has been detected in STP effluents in concentrations ranging from 9.2 ng/L (Vasskog et al., 2006) to 720 ng/L (Wahlberg et al., 2008) and in surface waters between 4 ng/L (Giebułtowicz and Nałęcz-Jawecki, 2014) and 76 µg/L (Fick et al., 2009); more typical concentrations in polluted recipients are around 10–150 ng/L (González Alonso et al., 2010; Grabicova et al., 2015; Metcalfe et al., 2010; Nödler et al., 2011). Several SSRIs are present in STP effluents, and the combined load of 7 major SSRIs and their metabolites was up to 3.2 µg/L downstream a Canadian STP (Brooks et al., 2005). The predicted water concentration needed to obtain human therapeutic levels in fish is 141 ng/L (Fick et al., 2010). Brain SSRI bioaccumulation has been observed in fish caught downstream an STP (Brooks et al., 2005), and citalopram has been found in the liver of perch (*Perca fluviatilis*) caught in the inner parts of the Stockholm archipelago (Woldegiorgis et al., 2006). SSRIs are psychoactive drugs prescribed for treating depression and other psychiatric disorders. SSRIs reduce the re-uptake of the neurotransmitter serotonin (5-hydroxy-tryptamine; 5-HT) into the pre-synaptic nerve terminal by inactivating 5-HT transporters (5-HTT), resulting in an increased concentration of extracellular 5-HT in the synapse. 5-HT is ubiquitous to all vertebrate groups and influences a wide range of behaviors and endocrine functions (Anon., 2010). In fish, SSRI have been shown to affect behaviors like feeding (Kellner et al., 2015), aggression (Winberg and Thörnqvist, 2016) and anxiety (Barbosa et al., 2012; Kellner et al., 2016; Olsén et al., 2014; Sackerman et al., 2010). Citalopram, an abundantly prescribed SSRI, has given an anxiolytic response in the novel tank test in zebrafish (Sackerman et al., 2010), guppies (Olsén et al., 2014) and three-spine sticklebacks (Kellner et al., 2016).

In this study, adult zebrafish were exposed to low, environmentally relevant concentrations of two pharmaceuticals commonly found in the environment, EE_2 and citalopram, and analyzed for impact on non-reproductive behavior. The aim of the study was to investigate if the behavioral effects previously found of the two substances could still be seen at very low concentrations. We also further wanted to investigate if the effects of the two compounds, the anxiolytic effects of citalopram and anxiogenic effects of EE_2 would counteract and affect the behavioral outcome in the combinatory exposure. We utilized two tests assessing anxiety, the scototaxis test (Maximino et al., 2010) and the novel tank (NT) test (Egan et al., 2009), and one test analyzing social behavior by mean of shoal cohesion (Moretz et al., 2007). We studied both effects on single-substance exposures as well as a combination of the two.

2. Materials and methods

2.1. Animals and treatments

Adult 6-month-old zebrafish (*Danio rerio*) of the wild type strain AB were obtained from the Karolinska Institute Zebrafish Core Facility, Stockholm, Sweden. Fish of different sex were kept separate under standardized conditions (tap water, 25–27 °C, pH 7.8, conductivity 20.7 mSi) with 12/12 h light/dark cycles, and fed three times daily with Sera Dry Flakes (Vipan, Germany) and newly hatched Artemia nauplii (Artemia International LCC, USA). The fish were allowed to acclimatize to the new environment for 7 days before the experiment was started. All treatment and handling of the animals was performed according to the Swedish Animal Care legislation and approved by the Southern Stockholm Animal Research Ethics Committee (DNR S28-15).

Solutions of EE_2 (Sigma-Aldrich, USA) and citalopram (a racemic mixture of the citalopram bromide R- and S-enantiomers, kindly donated by H. Lundbeck A/S, Copenhagen, Denmark) were made by stepwise dilutions from stock solutions of EE_2 in acetone and citalopram dissolved in distilled water. All stock solutions were kept refrigerated in dark bottles before the dilution with aquarium water. The final working solutions, obtained by a 1:1000 dilution from the refrigerator stocks with temperate aquarium water, had nominal concentrations of 0.1 and 0.5 ng/L EE_2 and 0.1 and 0.5 µg/L citalopram, respectively, and the two combinations of the two: 0.1 ng/L EE_2 + 0.1 µg/L citalopram and 0.5 ng/L EE_2 + 0.5 µg/L citalopram. Since EE_2 solutions contained acetone, all other solutions including the water control were adjusted to contain equal concentrations of acetone, 10 ppm.

Fish were exposed in 3L aquaria with a semi-static model of 1/2 volume exchange per day. We exposed seven fish per aquarium. For each sex, one aquarium started per exposure per day for three consecutive days, rendering 21 fish of each sex per treatment in total (3 replicate aquaria with 7 fish in each). When water was changed, the aquaria were also cleaned from feces and food residues. After the 14 days exposure period, the fish were subjected to three behavior tests, and locomotor activity was analyzed within one of the test sessions. Water samples for chemical analyses were collected at three occasions during the exposure period and stored at −20 °C until analysis.

2.2. Chemical analyses

All reference standards including citalopram, EE_2 and EE_2-d6 were purchased from Cerilliant Co (via Sigma-Aldrich Sweden AB, Sweden). Stock and working solutions were prepared in methanol and stored at