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In vitro nuclear receptor activity and *in vivo* gene expression analysis in Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) after short-term exposure to fluoxetine

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ABSTRACT

Fluoxetine (FLX) is one of numerous pharmaceuticals found in treated municipal wastewater discharged to the environment. In the present study, we investigated the effects of short-term (96 h) waterborne FLX exposure (1 μg/L or 100 μg/L) on the expression of selected genes in brain, liver, and gonads of female Murray-Darling rainbowfish (*Melanotaenia fluviatilis*), a small-bodied teleost of ecotoxicological relevance in the Australasia region. Plasma 17β-estradiol (E2) levels were also determined. In the brain, no significant changes in mRNA levels were observed for the selected genes. In ovaries, 100 μg/L FLX caused a 10-fold downregulation of aromatase A (*cyp19a1a*) mRNA and a 4-fold upregulation of estrogen receptor α (*esr1*) mRNA levels. In liver, mRNA levels for vitellogenin A (*vtga*) and choriogenin L (*chgl*) were downregulated by 50-fold and 18-fold compared with controls, respectively, in response to 100 μg/L FLX. Concentrations of E2 in plasma were significantly lower than controls in response to 100 μg/L FLX. This could be attributable to a decrease in estrogen biosynthesis as a result of the observed downregulation of *cyp19a1a* mRNA. To establish whether the observed changes in gene expression could be explained by the modulation of selected nuclear receptors by FLX, we employed panel of reporter gene assays in agonistic and antagonistic modes. Apart from minor activation of ERα after exposure to high concentrations (5 μM), FLX did not activate or inhibit the nuclear receptors tested. Further study is required to determine whether the observed downregulation of ovarian aromatase expression and liver estrogen-regulated genes also occurs at environmentally relevant FLX concentrations over longer exposure periods.

1. Introduction

Active pharmaceutical ingredients (APIs) and their metabolites have been found in treated wastewater and effluent-receiving surface waters worldwide (for reviews, refer to Boxall and Ericson, 2012; Fent et al., 2006; Monteiro and Boxall, 2010). APIs used for the treatment of depressive disorders, anxiety and obsessive-compulsive disorders, collectively known as antidepressants, are widely prescribed in developed countries. Antidepressants that function as selective serotonin reuptake inhibitors (SSRI), such as fluoxetine (FLX), paroxetine and citalopram, have been found in surface waters receiving treated municipal wastewater at low ng/L concentrations (Kolpin et al., 2002; Schultz et al., 2010). At tens to hundreds of ng/L, SSRIs have been shown to alter the behaviour of aquatic vertebrates and invertebrates (Gaworecki and Klaine, 2008; Painter et al., 2009; Valenti et al., 2012; Weinberger and Klaper, 2014), and at tens to hundreds of μg/L are acutely toxic to aquatic organisms (reviewed by Brooks et al., 2003a; Silva et al., 2015; Sumpter et al., 2014). In particular, FLX has received much attention not only due to its widespread use, but also because of its toxicity to aquatic organisms from a range of trophic groups, including vertebrates, invertebrates and algae (Brooks et al., 2003b; Johnson et al., 2007; Nakamura et al., 2008).

FLX and its primary metabolite, norfluoxetine, have been detected at up to approximately 40 ng/L in effluent-impacted streams in the USA (Conley et al., 2008; Kolpin et al., 2002; Schultz et al., 2010; Vanderford and Snyder, 2006), Canada (Lajeunesse et al., 2008; Metcalfe et al., 2010), and Europe (Giebułtowicz and Nałęcz-Jawecki, 2014; González Alonso et al., 2010). In a study of organic micropollutants in Australian rivers, FLX was found in only 2% of samples, however the maximum detected concentration of 22 ng/L led the authors to conclude that FLX represented a potential risk to aquatic organisms (Scott et al., 2014). FLX and norfluoxetine have been shown to bioaccumulate in various species of fish in effluent-dominated surface waters (Metcalfe et al., 2010; Ramirez et al., 2009) and in Japanese

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medaka (*Oryzias latipes*) following laboratory exposure (Nakamura et al., 2008). FLX was detected at up to 23 ng/g in liver tissue and norfluoxetine at up to 130 ng/g in muscle tissue of wild fish captured from effluent-dominated streams in the USA (Ramirez et al., 2009). FLX exhibited whole-body bioconcentration factors (BCF) of between 8.8 and 260 in Japanese medaka depending on the pH of the water, while the pseudo-BCF values for norfluoxetine were considerably higher (80 to 650) (Nakamura et al., 2008). The observed bioaccumulation of FLX in fish indicates that uptake rates exceed metabolic clearance rates, suggesting that over time FLX and its active metabolite could reach therapeutic levels in non-target organisms.

Drug targets such as receptors, enzymes and solute carriers often display a high degree of similarity even in distantly related organisms, meaning that the modes of action underlying the effects of APIs in aquatic vertebrates often mirror the intended mechanism of the drug in humans or livestock. This concept is the basis for the "read-across" hypothesis, which states that APIs only exhibit effects in non-target organisms if the drug target is conserved (reviewed in Rand-Weaver et al., 2013). The sequence similarity of serotonin transporters (SERTs) from fish compared to those from mammals, birds and reptiles is high according to first reports of cloning (Severinsen et al., 2008; Wang et al., 2006) and subsequent thorough phylogenetic analysis (Mennigen et al., 2011). However, while the modes of action of SSRIs may be similar across a wide range of vertebrates, the effects of any given drug may be manifested in unpredictable ways in non-target organisms due to differences in the regulation of fundamental biological processes such as reproductive endocrine signalling or neuroendocrine function.

SSRIs prevent the uptake of serotonin from the synapse into neurons by SERT-family transmembrane transporters, resulting in its accumulation and a concomitant increase in signalling through serotonin receptors (Stahl, 1998). In fish, experimental exposure to SSRIs via water has been shown to result in elevated whole-body serotonin levels (Winder et al., 2009). Since serotonin is involved in the regulation of reproductive function in vertebrates including fish (reviewed recently by Prasad et al., 2015), the increase in circulating serotonin caused by exposure to SSRIs could be expected to have some effect on reproductive processes. Western mosquitofish (*Gambusia affinis*) exposed to 71 μg/L FLX during juvenile-to-adult transition exhibited delayed onset of secondary sexual characteristics in both females and males (Henry and Black, 2008). Sexually mature female zebrafish (*Danio rerio*) exposed for 7 days to 32 μg/L FLX via water produced 4.5-fold fewer eggs and exhibited significantly reduced ovarian 17β-estradiol (E2) levels in compared to controls (Lister et al., 2009). At higher dose rates, female goldfish (*Carassius auratus*) exhibited a 75% reduction in circulating E2 concentration in response to repeated injections of 5 μg/g body weight over a 2-week period, and ERβ1 expression in the hypothalamus was downregulated significantly (Mennigen et al., 2008). Some subacute effects of FLX appear to be sex-specific, such as the stimulatory effect on estrogen-regulated pathways observed in FLX-exposed males. Dose-dependent increases in plasma E2 and hepatic ERα expression were observed in male goldfish exposed to up to 54 μg/L FLX, and plasma testosterone levels were reduced (Mennigen et al., 2010). In another study, environmentally relevant concentrations of FLX (28 ng/L) resulted in significantly elevated plasma vitellogenin levels in male fathead minnow (Schultz et al., 2011).

Analysis of changes in gene expression in zebrafish ovaries in response to 32 μg/L FLX revealed a downregulation of luteinising hormone and follicle stimulating hormone levels after 7 day exposure (Lister et al., 2009). The expression of isotocin in the brain of female goldfish was also strongly downregulated after repeated injection of FLX over 2 weeks (Mennigen et al., 2008). Taken together, these observations have led researchers to conclude that FLX modulates neuroendocrine function in fish (Mennigen et al., 2011), as has been observed in mammals (reviewed by Raap and Van de Kar, 1999). The potential for FLX to adversely affect the neuroendocrine reproductive axis may have implications for wild fish populations in environments receiving regular inflows of treated sewage. Based on the relatively high concentrations used in laboratory studies, it is unlikely that FLX would be expected to be present in the environments at concentrations that could immediately impact reproductive parameters in fish. However, the effects of numerous SSRIs present at low levels requires further investigation to establish whether interactions are additive or synergistic, and the potential for these compounds to act through pathways other than those that involve known targets needs to be determined.

The Murray-Darling rainbowfish (*Melanotaenia fluviatilis*, [Castelnau, 1878]; Atheriniformes: Melanotaeniidae) is a small-bodied teleost widely distributed in rivers and wetlands throughout south-eastern Australia. We have previously validated a number of molecular biomarkers in *M. fluviatilis* related to reproductive health (Bhatia et al., 2014; Woods and Kumar, 2011; Woods et al., 2009), identified and characterised the function of androgen and progestogen receptors (Bain et al., 2015a, b), and generated a high-quality reference transcriptome to facilitate the rapid development of new potential biomarkers (Bain et al., 2015c).

To identify responses to high FLX concentrations that may aid in the selection of endpoints for subsequent environmentally relevant FLX exposures, we exposed sexually mature female *M. fluviatilis* to 1 μg/L or 100 μg/L FLX for 4 days and investigated changes in mRNA levels in brain, liver and ovaries for genes involved in the serotonin pathway, steroidogenesis, reproduction and growth. We also determined plasma E2 levels to establish a causative link between the observed changes in mRNA levels in ovaries and liver. A comprehensive *in vitro* screening study was also undertaken using a panel of reporter gene assays to evaluate the potential for FLX to activate or inhibit nuclear receptors. The results indicate that high FLX concentrations can impact estrogen biosynthesis and signalling in female *M. fluviatilis* after short-term exposures. These findings are discussed with reference to the reported effects of FLX on reproductive processes in other fish species.

2. Materials and methods

2.1. Animals

Adult female *M. fluviatilis* were obtained from a commercial supplier (Aquarium Industries Pty. Ltd., Victoria, Australia). Fish were maintained in aerated synthetic water (modified FETAX solution; Dawson et al., 1988) under a 16:8 h light:dark period on a diet of tropical fish flakes (TetraMin; Seaview Aquarium, South Australia) and frozen brine shrimp (AquaOne®; Seaview Aquarium, South Australia).

2.2. Fluoxetine exposures

Female *M. fluviatilis* with fork lengths between 40 and 65 mm were exposed to two concentrations of FLX, 1 μg/L and 100 μg/L, or solvent control, for 96 h in a semi-static experimental design. Solutions of FLX (Sigma-Aldrich Pty. Ltd., Australia) were prepared in methanol and added to well-aerated water in 5 L glass tanks such that the solvent concentration (0.005%) was identical in all treatment groups including controls. Nominal concentrations are presented herein, since previous studies in our laboratory have shown that measured FLX concentrations were close to nominal concentrations over similar timeframes using similar apparatuses; analytical methods described in Roberts et al. (2016) were used to measure FLX concentrations in aquarium water, which after 24 h were determined to be 93 and 440 μg/L for nominal concentrations of 100 and 500 μg/L, respectively (A. Kumar, unpublished data). Four fish were randomly allocated to each tank, with three replicate tanks prepared for each condition. A semi-static experimental design was implemented wherein 100% of the water in each tank was renewed every 24 h (*i.e.* the fish were transferred to a freshly prepared tank every 24 h). Fish were not fed for the duration of the experiment. Water quality parameters (pH, DO and EC) were monitored and fish observed daily for signs of stress.

All procedures were conducted in accordance with the recommendations and approval of, the CSIRO Food and Nutrition Animal Ethics