FLUOXETINE MODULATES SEX STEROID LEVELS IN VITRO

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Abstract

Background and aims. Selective serotonin reuptake inhibitors (SSRIs) are antidepressants increasingly prescribed against depression during and after pregnancy. However, these compounds cross the placenta and are found in breast milk, thus reaching, and possibly affecting, the fetus and infant during critical developmental stages. Fluoxetine (FLX), a widely used SSRI, can interfere with estrogen signaling, which is important for the development of female sex organs and certain brain areas, among others. Interference with estrogen signaling can take place on different levels, e.g., by affecting receptor activity or hormone levels. FLX has previously been shown to induce estrogen receptor-dependent transcription in vitro at high concentrations. In this study we set out to assess effects of FLX on estradiol levels in vitro.

Methods. FLX was tested using the OECD recommended H295R model, a human adrenocortical carcinoma cell line that is able to produce all steroid hormones found in the gonads and adrenal glands, including estradiol and testosterone. H295R cells were incubated with different doses of FLX for 48h. Subsequently, concentrations of these two steroids were measured in cell culture medium after FLX exposure, using liquid chromatography coupled with tandem mass spectrometry. Aromatase mRNA expression was assessed using qPCR.

Results. Fluoxetine significantly increased estradiol secretion in H295R cells after a 48h exposure at low, submicromolar concentrations, but showed no effects on testosterone levels or aromatase mRNA expression.

Conclusion. Fluoxetine has the potential to interfere with estrogenic signaling by increasing estradiol secretion at low concentrations, which are relevant for fetal and adult human exposure.

Keywords: fluoxetine, steroidogenesis, estradiol, H295R, in vitro
about the potential effects of FLX on developmental outcomes in children exposed during these sensitive periods [5].

17β-estradiol (E2), an active sex steroid hormone, plays important roles during fetal growth and development, including control of sexual differentiation and the establishment of sexually dimorphic brain areas [6]. A number of studies in fish suggest that FLX interferes with E2 signaling by affecting levels of E2 and/or nuclear estrogen receptors (ERs) through which E2 exerts its main effects [Reviewed in 7]. Circulating E2 and gonadal aromatase expression significantly increased, and testosterone (T) significantly decreased in male goldfish exposed for two weeks to 1 or 175 nM FLX, concomitant with increased liver expression of ERα [8]. On the other hand, female goldfish exposed to 5 µg/g body weight FLX for 2 weeks showed decreased ER expression in the hypothalamus, in addition to decreased circulating E2 [9]. Female zebrafish exposed to 0.11 µM FLX for 7 days showed significantly decreased ovarian E2 and decreased ovarian aromatase mRNA [10], whereas Japanese medaka female fish exposed to 0.3 nM FLX for 28 days displayed a significant increase in circulating E2 [11]. Moreover, male fathead minnows exposed to low nanomolar concentrations of FLX had a significant increase in plasma vitellogenin, a known marker of estrogenic stimulation [12], shown to be under the control of ERα [13]. These in vivo findings have been complemented with in vitro data showing that the transcriptional response of nuclear ERs is significantly increased by FLX, albeit at concentrations above 10 µM, individually and in binary mixtures with E2 [14].

The present study aimed to assess whether FLX affects E2 levels by interference with the expression of CYP19A1 (aromatase), the enzyme responsible for one pathway of E2 biosynthesis. Sex steroid synthesis takes place in steroidogenic endocrine glands (e.g. adrenals, gonads) and uses cholesterol as the initial substrate and multiple enzymatic steps involving enzymes of the cytochrome P450 (CYP) family and β-hydroxysteroid dehydrogenases (β-HSD) [15]. E2 is one of the final products of the steroidogenic pathway and is produced by conversion of estrone, a reaction catalyzed by 17β-HSD, and through aromatization of testosterone, a reaction catalyzed by CYP19A1 [15]. We employed an OECD recommended in vitro model, the H295R steroidogenesis assay, which has been validated for identifying xenobiotics that affect E2 and testosterone (T) levels [16]. As one pathway to E2 synthesis is the conversion of T by CYP19A1, and both hormones can be reliably quantified using this assay, we further assessed if changes in CYP19A1 mRNA could account for potential effects of FLX exposure on E2 levels.

Materials and methods

Cell culture

H295R adrenal carcinoma cells were obtained from ATCC. Cells were grown in phenol red-free DMEM/F-12 medium (without glutamine, Gibco) supplemented with 2.5% Nu-Serum (Corning), 1% ITS+ Premix (Corning) and 2.5 mM L-Glutamine (Gibco). Cells were kept at 37°C with 5% CO₂ and passaged once per week.

H295R steroidogenesis assay

The H295R steroidogenesis assay was used to evaluate the ability of FLX to interfere with E2 and T synthesis in vitro. To this end, H295R cells were seeded in 24 well plates at a density of 2x10⁵ cells/mL in culture medium. Cells were left to attach for 24h and then the medium was replaced with exposure medium containing flutamide 1, 0.1 and 0.01 µM, forskolin 1 µM (inducer of hormone synthesis), prochloraz 1 µM (inhibitor of hormone synthesis) and DMSO 0.1% (solvent control). Cells were incubated with test chemicals for 48h and then medium was removed and used to quantify E2 and T secretion.

LC-MS/MS measurements of steroid hormones

E2 and T were quantified in H295R culture medium using ultra performance liquid chromatography coupled to electrospray tandem mass spectrometry (UPLC-MS/MS). Initially, 0.5 mL culture medium was mixed with 50 µL of internal standards d5-E2 and 3C-T (Sigma-Aldrich, Stockholm, Sweden) dissolved in methanol. Subsequently, 20 µL of 25% NH₄OH and 1000 µL of methyl-tert-buthyl ether were added to each sample, followed by vigorous vortexing for 4 min. Samples were then centrifuged for 2 min at 5000 g and the supernatants were transferred to glass inserts and vacuum centrifuged to dryness. The samples were reconstituted in 100 µL of methanol:H₂O (50:50, v/v) and 40 µL of each sample were injected on the LC column. The steroids were chromatographically separated on an Acquity™ (Waters, Milford, MA, USA) using gradient elution (solvent A: H₂O with 30 µM NH₄F; solvent B: methanol with 30 µM NH₄F) on a 100x2.1 mm HSS T3 column (Waters). The gradient was as follows (% solvent B/min): 65/0 65/1 95/3 95/4 65/4.01 65/5 at a flow rate of 0.5 mL/min. The mass spectrometer was a Xevo™ TQ-S (Waters) operating in multiple reaction monitoring, and it monitored the [M-H]- or [M+H]+ by polarity switching during the analysis. The capillary voltage was set to 2.7 kV and 2.2 kV for positive and negative mode respectively. Desolvation gas flow and desolvation temperature were set to 1000 L/h and 550 °C respectively. Collision gas flow was set to 0.15 mL/min. Two specific fragments from each native steroid and one fragment from each labeled steroid were monitored. The transition that generated the highest signal/noise ratio was used as quantifier transition and for constructing calibration curves whereas the second qualifier transition was included only for qualitative purpose. Quantification was achieved by external calibrators (n=9) made in human serum spiked with known amounts (~1 pM to 50 nM) of each steroid. A more detailed description of the UPLC-MS/MS settings for each of the steroids may be found in Table 1.
Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from H295R cells using the AllPrep DNA/RNA Mini Kit (Qiagen) and reverse transcription was accomplished with Superscript VlO cDNA Synthesis Kit (Thermo Fisher Scientific). PCR was performed using 5 ng of cDNA, self-designed (Primer 3.0 web) exon-exon spanning primers (see Table II) and KAPA SYBR Fast ABI Prism (Kapa Biosystems) mastermix, in a 7500 RealTime PCR System (Thermo Fisher Scientific). Threshold cycle (Ct) values for CYP19A1 were determined in triplicates and presented as average normalized to the average Ct values of the mRNA coding for P0 large ribosomal protein RPLP0 (36B4). Relative fold changes compared to controls were calculated using Pfaffl method.

Statistical analysis

Three independent experiments were performed with three biological replicates in each plate. Statistical differences between groups were analyzed by one-way ANOVA, using GraphPad Prism 6. P < 0.05 was considered statistically significant. Data are expressed as the mean ± SD.

Results

To study effects of FLX on steroid levels in vitro we used the OECD recommended H295R steroidogenesis assay. H295R cells were incubated with test chemicals for 48h hours. Subsequently, culture medium was collected for hormone quantification using UPLC-MS/MS. We tested FLX 1, 0.1 and 0.01 µM and included three controls: solvent control DMSO 0.1%, forskolin 1 µM (FOR) as an inducer of steroid biosynthesis and prochloraz 1 µM (PRO) as an inhibitor of steroid biosynthesis. The tested FLX concentrations were chosen based on reported plasma concentrations for this SSRI in humans and newborns, which are below 1 µM [17].

FLX increased E2 secretion at all concentrations tested, but only 0.01 µM FLX significantly increased E2 secretion in H295R cells after a 48h exposure, compared to solvent control (2.66 fold change, p=0.0290). No significant changes in T levels were observed at any of the FLX concentrations compared to solvent control (Figure 1).

Table I. UPLC-MS/MS compound specific settings. The transitions are listed as the parent ion followed by the quantifier/qualifier ion.

| Steroid       | Polarity | Tr (min) | Transition (m/z) | Cone voltage (V) | Collision energy (V) | Calibration range (pM) |
|---------------|----------|----------|------------------|------------------|----------------------|------------------------|
| Estradiol     | ESI-     | 2.0      | 271.2à145.1/183.1| 60               | 40/42                | 2.5-50 500             |
| D5-Estradiol (IS) | ESI- | 2.0      | 276.2à147.1     | 60               | 40                   | -                      |
| Testosterone  | ESI+     | 2.2      | 289.1à97.1/109.1| 50               | 21/27                | 3.4-48 200             |
| 13C3-Testosterone (IS) | ESI+ | 2.2      | 292.1à100.1     | 50               | 21                   | -                      |

Table II. Primer sequences.

| Primers used for human cDNA | Forward | Reverse |
|-----------------------------|---------|---------|
| 36B4                        | GACACCCTCCAGGAAGCGA | GTGTTCGACAATGGCAGCAT |
| CYP19A1                     | TGGAATGCTGAAACCGGATAC | AATTCCCATGCAGTAGCCAGG |

Figure 1. Effects of FLX, FOR (positive control) and PRO (negative control) on E2 (A) and T (B) levels in H295R culture medium compared to solvent control (DMSO 0.1%). Results are represented as means ± SD of three independent experiments (3 replicates/plate, each measured in duplicate by UPLC-MS/MS). Significant differences are marked with asterisks (* for p<0.05 and **** for p<0.001, one-way ANOVA).
As only E2 levels were affected, we hypothesized that FLX could affect CYP19A1 (aromatase), the enzyme converting T to E2. To analyze the effects on CYP19A1 mRNA expression, lysates from the treated cells were collected and mRNA expression was measured by qPCR. However, the expression of aromatase was not significantly changed after FLX exposure at any of the concentrations tested (Figure 2).

**Figure 2.** Effect of FLX on CYP19A1 gene expression in H295R cells. Results are represented as means ± SD of three independent experiments (3 replicates/plate).

**Discussion**

Recent findings from in vivo studies suggest that at low submicromolar concentrations FLX interferes with estrogen signaling by affecting E2 levels [8-11]. The results of the current study also show that FLX has the ability to increase E2 levels at biologically relevant submicromolar concentrations in vitro. Therapeutic plasma concentrations of FLX are below 1 µM and vary depending on FLX dose and the polymorphism of metabolizing enzymes [18]. In the fetus and newborns, FLX concentrations reach approximately 90% of the maternal serum levels [17]. The observed increase in E2 levels at submicromolar concentrations corroborates in vivo studies on female Japanese medaka fish and male goldfish, both displaying endocrine disruption [19].

As only E2 but not T levels were affected in our study, we hypothesized that FLX affects CYP19A1 action, the enzyme converting T to E2. However, the fact that we did not observe an increase in CYP19A1 mRNA expression does not exclude that FLX influences the protein levels or its activity via other mechanisms such as post-transcriptional modifications or alternate pathways.

If this is the case, then it is also plausible that the E2 synthesis pathway involving estrone conversion could also be affected by FLX. Moreover, the fact that T levels were unaffected in vitro suggests that FLX does not affect 17β-HSD, but testosterone metabolism may have increased as a compensatory mechanism to reduce the substrate available for E2 synthesis. Thus, we cannot exclude an effect of FLX on 17β-HSD or other enzymes upstream of T synthesis in our system. H295R cells also metabolize E2 by use of two endogenous enzymes: E2-sulfotransferase and E2-glucuronidase and estrogen agonists such as bisphenol A have been shown to inhibit E2 metabolism in H295R cells [20], thus it is possible that FLX caused an increase in E2 levels by affecting its metabolizing enzymes. Therefore, the increase in E2 observed in H295R cell culture medium could also be mediated by FLX decreasing E2 metabolism. Further experiments with this in vitro model including quantification of intermediary steroids and expression of steroidogenic and metabolizing enzymes will reveal the target(s) and modality of FLX action on E2 synthesis and/or metabolism.

The limits of the H295R assay used in this study are that it does not provide mechanistic information beyond steroid levels and that it does not have the capacity to detect interferences with steroidogenesis via the hypothalamic-pituitary-gonadal (HPG) axis [16]. Thus, while the results from this study could indicate some mechanisms underlying the observed estrogenic effects in vivo, they do not exclude other explanations such as serotonergic stimulation of gonadotrophin releasing hormone (GnRh) release from the hypothalamus [21], which leads to an activation of the HPG axis, and, in turn, to increased steroidogenesis in the gonads.

**Conclusion**

In conclusion, this is the first in vitro study to report that FLX has the potential to interfere with estrogenic signaling by increasing E2 levels at low submicromolar concentrations relevant for human exposure.

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