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In utero and lactational exposure to fluoxetine delays puberty onset in female rats offspring

Alice Hartmann dos Santos[a], Milene Leivas Vieira[a], Nathália de Azevedo Camin[a], Janete Aparecida Anselmo-Franci[b], Graziela Scalianti Ceravolo[a], Gislaine Garcia Pelosi[a], Estefânia Gastaldello Moreira[a], Ana Carolina Inhasz Kiss[c], Suzana de Fátima Paccola Mesquita[d], Daniela Cristina Ceccatto Gerardin[a,*]

[a] Department of Physiological Sciences, State University of Londrina, 86051-980 Londrina, Paraná, Brazil
[b] Department of Morphology, Estomatology and Physiology, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
[c] Department of Physiology, São Paulo State University, Botucatu, São Paulo, Brazil
[d] Department of Biology, State University of Londrina, 86051-980 Londrina, Paraná, Brazil

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ABSTRACT

Depression is one of the most prevalent disorders in the world and may occur during pregnancy and postpartum periods. Fluoxetine (FLX) has been widely prescribed for use during depression in pregnancy and lactation. This study aimed to investigate if *in utero* and lactational exposure to FLX could compromise reproductive parameters in female offspring. Wistar rats received, by daily gavage, FLX 5 mg/kg or 0.3 ml of water (control group) from the first gestational day until weaning (21 days). Assessments in the female offspring included: body weight, anogenital distance, vaginal opening, first estrus, estrous cycle, reproductive organs weight, uterine morphometric analyses, ovarian follicle and corpora lutea counting, estradiol plasmatic concentration, sexual behavior, maternal behavior and fertility test. Exposure to FLX delayed the puberty onset in female pups. The present study demonstrated that developmental exposure to FLX can deregulate the neuroendocrine hormonal control of female offspring during prepubertal and pubertal periods.

1. Introduction

Pregnancy and postpartum period are risk factors for the development or exacerbation of mental disorders, such as depression [1], due to the emergence of biological, physiological and social alterations. Approximately 40% of women that suffer from postpartum depression develop the symptoms during gestation [2]. The prevalence of perinatal depression is 10–20% [1,3].

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor (SSRI) antidepressant and is the drug of choice during pregnancy due to its relative selectivity of action, efficacy, reduced side effects [4] and absence of morphological teratogenic activity [5]. FLX and its active metabolite, norfluoxetine, readily crosses the placenta in humans [6] and in experimental animals [7] and are also excreted in human milk [6]; therefore fetuses and neonates are exposed to these substances during important stages of development.

Fetal exposure to SSRI has been associated to low uterine fetal growth and symptoms related to changes in motor and somatosensory systems development [8]. Serotonin (5-HT) is known to be a trophic agent for the migration and synaptogenesis of monoaminergic neurons during brain development [9], whereas in adult life it is an important factor for neurogenesis and neuronal maintenance in the central nervous system [10]. Thus, it can be suggested that exposure to FLX during neurodevelopment may influence not only the 5-HT system, but all the monoaminergic systems. As a matter of fact, it has been reported that perinatal exposure to FLX can impair the dopaminergic (DA) system [11].

Abbreviations: 5-HT, serotonin; DA, dopamine; NA, noradrenaline; GABA, gamma-aminobutyric acid; CTR, control; FLX, fluoxetine; SSRIs, selective serotonin reuptake inhibitors; GD, gestational day; LD, lactational day; PND, postnatal day; AGD, anogenital distance; VO, vaginal opening; HPG axis, hypothalamic-pituitary-gonadal axis; GnRH, gonadotropin release hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; LM, lordosis magnitude; LQ, lordosis quotient; MB, maternal behavior; ANCOVA, analysis of covariance; ANOVA, analysis of variance; RMANOVA, repeated measures analysis of variance.

* Corresponding author at: Department of Physiological Sciences, State University of Londrina-UEL, 86051-980 Londrina, Paraná, Brazil.
E-mail addresses: dcgerardin@uel.br, danigerardin@yahoo.com.br (D.C.C. Gerardin).

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Additionally, 5-HT system may influence the reproductive function of vertebrate due to its communication with sex steroid system [12]. This interaction between the systems occurs through estrogen and progesterone receptors located in serotoninergic central neurons, demonstrating an important signaling pathway. These 5-HT central neurons can modulate several neural processes, including pituitary secretion and sexual behavior. In this way, estrogen and progesterone can interfere with these functions since these neurons are sensitive to ovarian hormones. Thus, the serotoninergic system may integrate signals from the hormonal system with the central nervous system.

Similarly to 5-HT, DA also regulates reproductive behaviors interacting with steroid hormones. Interactions between estradiol, progesterone and DA in the ventromedial nucleus of the hypothalamus regulate the lordosis reflex in rats [13]. Regarding maternal behavior (MB), the main components of the neural circuitry include the pre-optic area, the nucleus accumbens and other limbic and hypothalamic structures [14]. Despite the predominant role played by DA on MB [15], there are evidences that 5-HT is also involved in this behavior [16]. However, there are still few works evaluating the impact that developmental exposure to SSRI could have on the reproductive and central nervous systems functions. Based on these considerations, this study was carried out in order to evaluate if *in utero* and lactational exposure to FLX could disrupt the reproductive development of female offspring.

2. Material and methods

2.1. Animals and treatment

This study is part of a main study conducted in the Department of Physiological Sciences, State University of Londrina, which aimed to investigate reproductive, cardiovascular and neurological endpoints after developmental exposure to FLX. The experimental design adopted followed principles described in the guideline 426 (Developmental Neurotoxicity Studies) published by the Organization for Economic Co-Operation and Development [19] which, except for the lack of pre-mating treatment, complies with the guideline 443 (Extended One-Generation Reproductive Toxicity Study) from the same agency. All animal procedures were approved by the UEL Ethics Committee for Animal Research (16166.2012.12).

The experimental design is depicted in Fig. 1. A total of 17 male and 35 female Wistar rats (85-90 days) from the colony of the State University of Londrina (UEL) were used as parental generation. Animals were group housed five per cage in a polypropylene cage kept in a controlled environment with temperature at 21 ± 2 °C; 12 h light/dark cycle (lights on at 6:00 a.m.) and had free access to regular lab chow (Nuvital™, Paraná, Brazil) and tap water bottles. Autoclaved wood shaver bedding was used to prevent contamination from mycoestrogens in corncob bedding. Female and male rats were maintained separated by sex during 15 days prior to the beginning of mating. Rats were mated (2 females and 1 male per cage) and gestational day (GD) 0 was determined if there were sperm and estrus phase cells in vaginal smears. Dams were randomly divided into 2 groups:

- Control (CTR): 17 dams received tap water daily, by gavage, from GD 0 to postnatal day (PND) 21;
- FLX: 18 dams received FLX (5 mg/kg, Daforin™ oral solution, EMS Laboratory, Brazil), by gavage, from GD 0 to PND 21.

Dams were daily treated at 11:00-13:00 p.m. and doses were adjusted each 3 days according to weight.

In humans, FLX prescription ranges from 20 to 80 mg/day, which would correspond to approximately 0.29–1.14 mg/kg. Considering that the precautionary principle considers animals more resistant than humans, higher doses are tested in animals. Preliminary studies of our group showed that the dose of 10 mg/kg decreased the number of pups per litter (data not shown). Vorhees et al. reported that prenatal exposure to 12 mg/kg FLX resulted in a significant increase in offspring mortality from birth to PND 7 with no alterations observed in pups exposed to 5 mg/kg of FLX [17]. Bairy et al. demonstrated that prenatal exposure to 12 mg/kg FLX resulted in a significant reduction in birth weight and that this alteration was not observed at 8 mg/kg dose [18]. In this way, the dose of 5 mg/kg was chosen to ensure no influence on litter size and weight.

At birth (PND 0), pups were counted, the sex determined and the litters were weighed. On PND 4, litters were culled to 10 pups keeping 5 males and 5 females whenever possible. From each litter, 1–2 female pups were used for this study. The anogenital distance, body weight and vaginal opening were observed in the 2 female pups per litter, whenever possible. After that, one female was allocated to the behavioral evaluation and the other to the assessment of non-behavioral reproductive parameters.

2.2. Parameters analyzed in female offspring during development (PND 0–60)

2.2.1. Body weight

Female pups body weight was measured on PND 0, 7, 14 and 21 (CTR: 17 litters and FLX: 18 litters). This data is expressed as litter mean.

2.2.2. Physical sexual development

The anogenital distance (AGD, distance from the anus to the genital tubercle) was measured on PND 0 and 21 (CTR: 17 litters and FLX: 18 litters). AGD was normalized through its division by the cube root of body weight. From PND 30 on, females from both groups were daily evaluated for vaginal opening (VO, CTR: 27 litters and FLX: 28 litters) in order to determine the day in which complete VO occurred. In this day, females were weighed. Starting from the day of VO, daily vaginal smears were collected to detect the day of the first estrus (CTR: 11 litters and FLX: 11 litters), characterized by the predominance of cornified epithelial cells [20]. These parameters were evaluated in 2 littermates and data are expressed as litter means.

2.3. Parameters analyzed in female offspring during adulthood

2.3.1. Estrous cycle evaluation

On PND 75, the estrous cyclicity of female rats (CTR: 12 rats and FLX: 11 rats) was assessed through vaginal smears collected over a period of 15 days as previously described in Ref. [21]. The cycle phases were cytologically determined by the following characteristics: predominance of nucleated epithelial cells (proestrus); predominance of cornified epithelial cells (estrus); presence of cornified and nucleated epithelial cells and leukocytes (metaestrus); predominance of leukocytes (diestrus). The total frequency of each phase was used to calculate the total length (in days) of the proestrus, estrus, metaestrus and diestrus and the estrous cycle length.

2.3.2. Collection of tissues and organs

After the estrous cycle evaluation and during an estrus phase (PND 90–95), females (n = 10/group) were weighed, deeply anesthetized with thiopental and laparatomized. Blood samples were collected from abdominal aorta for quantification of plasmatic estradiol. The ovaries and uteri (with fluid) were collected and weighed. After that, they were euthanized by decapitation. The right ovary and the medial portion of the right uterine horn were fixed in Bouin, dehydrated in ethanol and embedded in Paraplast®

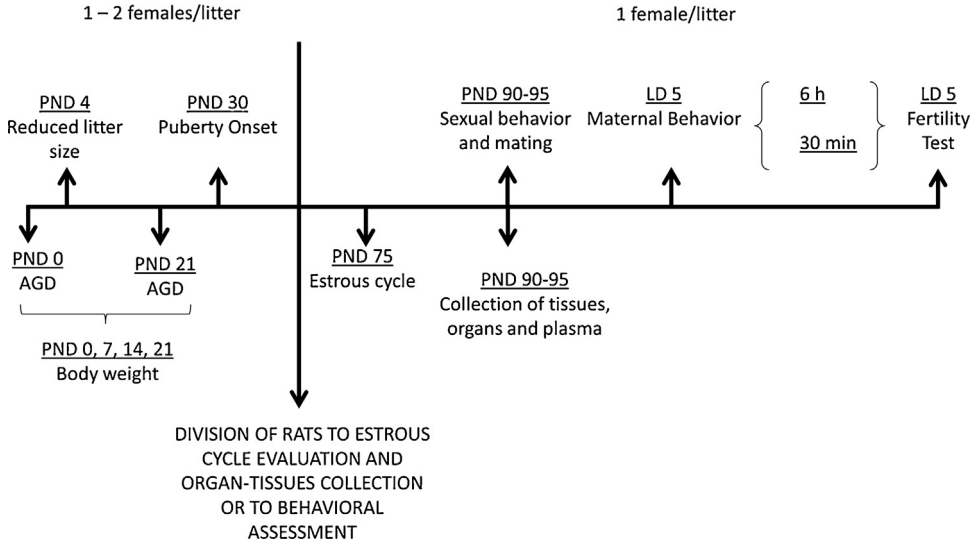

Fig. 1. Diagram of experimental design. PND: postnatal day; AGD: anogenital distance; LD: lactational day.

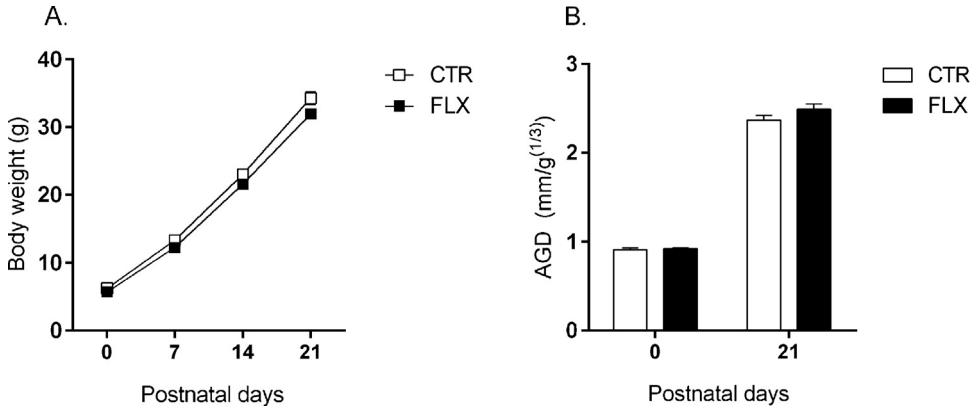

Fig. 2. Body weight (A) and anogenital distance (B) of female pups. Values are means ± S.E.M. RMANOVA ($p > 0.05$). CTR = saline, n = 17 litters; FLX = Fluoxetine 5 mg/kg, n = 18 litters.

(Sigma, St. Louis, MO, USA). Six sections (5 μm) per animal were obtained, mounted on glass slides and stained with hematoxylin and eosin (CTR n = 6/FLX n = 8). There was an interval of 100 μm between the first 3 and the last 3 sections.

In ovary, the corpora lutea and ovarian follicles (primordial, growing, antral and atretic) were counted under light microscopy (OSM-223287, Olympus; 100 × and 400 × magnifications) in accordance to Pedersen and Peters [22], as described in Ref. [23].

In uterine horn, it was measured the luminal epithelial height (400× magnification) and thickness of the endometrial stroma (100× magnification). For this, a light microscope (OSM-223287, Olympus) coupled to a digital camera and a computer with Bel view software was used.

2.3.3. Plasmatic estradiol quantification

Blood samples were collected from abdominal aorta into syringes containing heparin. Immediately after collection, blood samples were centrifuged (2500 rpm for 20 min at 2 °C) and the plasma was frozen until assayed for determination of serum estradiol levels. The estradiol plasmatic concentration (10 animals/group) was measured by radioimmunoassay, using the 17β-Estradiol (E2) Double Antibody RIA kit™ (MP Biomedicals, Santa Ana, CA, USA). The limit of detection was 1.2 pg/ml and intra-assay and inter-assay coefficients of variation were 2.5% and 7.0%, respectively.

2.3.4. Sexual behavior evaluation

Sexual behavior (CTR: 17 females and FLX: 16 females) was analyzed during the dark phase of a reversed light/dark cycle, under dim red light. The animals were allowed a 15-days period of adaptation to the reversed light/dark cycle before the evaluation. The sexual behavior was assessed in cycling rats 3–4 h after the diagnosis of proestrous through vaginal smear. The analysis always started 4 h after the onset of darkness and the behavior was recorded by a video camera linked to a monitor in an adjacent room. Sexually experienced males from the State University of Londrina colony (i.e., not the siblings obtained in our department) were used in these tests, which lasted until the occurrence of ten mounts. Results were expressed as the lordosis quotient (LQ, number of lordosis/ten mounts × 100) as well as the frequency of each lordosis magnitude (LM, on a scale of 0–3). The classification was as follows: 0 absence of lordosis; 1 the female showed little flex of spine, head and hips slightly elevated from floor; 2 the female showed spinal flex and head raise close of an angle of 30° with the floor; 3 maximum lordosis, with accented spinal flex and the head inclined at an angle of 45° or more relative to the floor.

2.3.5. Maternal behavior

To investigate the effects of *in utero* and lactational exposure to FLX on mother-pup interaction, females were mated for analyzing the maternal behavior. Two types of MB analyses were conducted in

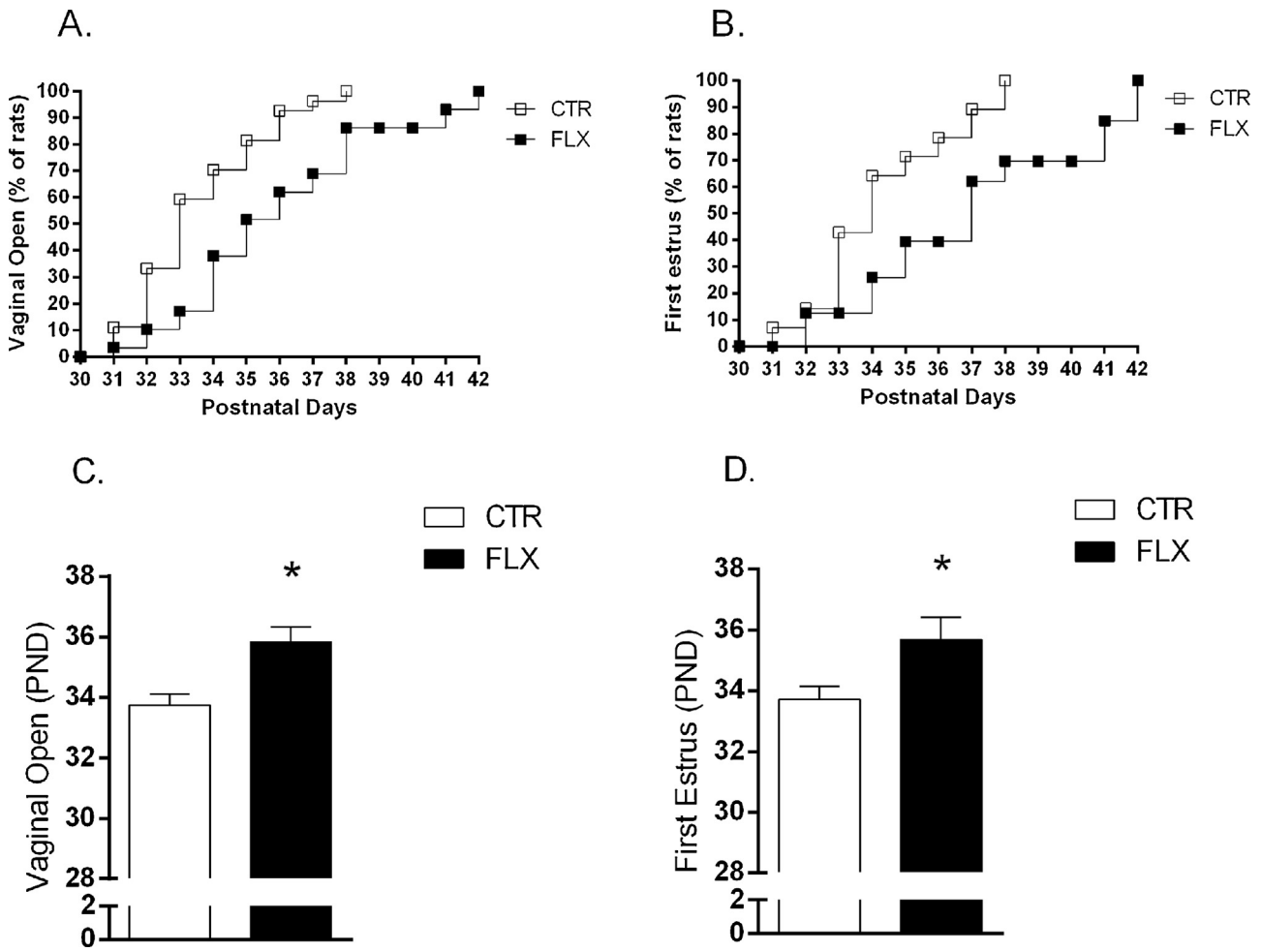

Fig. 3. Percentual representation of vaginal opening (A) and first estrus (B) of female pups. Average day of vaginal opening (C) and first estrus (D) of female pups. Values are means ± S.E.M. ANOVA (*p < 0.05). Vaginal Opening - CTR n = 27; FLX n = 28; First Estrus - CTR n = 11; FLX n = 11. PND = postnatal day; CTR = saline; FLX = Fluoxetine 5 mg/kg.

independent groups of dams and both were recorded at lactational day (LD) 5 between 7:30 a.m. and 1:30 p.m.

2.3.5.1. Maternal behavior evaluation after pup removal. On the test day, all pups were removed from their home cage and the nest was destroyed (CTR n = 10/FLX n = 9). After 30 min, the pups were returned to the cage and mother-pup interaction was recorded for 30 min. Latency for retrieval behavior and total time grouping, pup grooming, self-grooming, crouching, off pups (defined as the amount of time that the rat spent without any kind of interaction with pups regardless of her position in the cage), and nest building were quantified. Full maternal behavior was scored if dams retrieved all pups to the nest and nursed them for 3 consecutive minutes. All behavioral analyses were performed using Etholog software [24].

2.3.5.2. Undisturbed mother-pup interaction evaluation. Mother and litter interaction was recorded on their home cage for 6 h (CTR n = 11/FLX n = 10). Videotaping began at 7:30 a.m. in the light phase. Behavior was scored every minute during this period according to the following categories: nursing, off pups, retrieval behavior, pup grooming and self-grooming. Total number of observations was used to calculate the percentage of observations in self-grooming, pup-grooming, nursing, building nest, retrieval and off pups.

2.3.6. Fertility test

On LD 5, after the MB analyses, female rats were deeply anesthetized with thiopental (CTR n = 17/FLX n = 16). After that the uterus and ovaries were collected, they were euthanized by decapitation and the numbers of corpora lutea (by gross morphology) and implantation sites were counted. From these results, the following parameters were calculated:

- implantation rate: number of implantation sites/number of corpora lutea × 100;
- pre-implantation loss rate: number of corpora lutea—number of implantation sites/number of corpora lutea × 100;
- post-implantation loss rate: number of implantation sites—number of live fetuses/number of implantation sites × 100;
- fetal viability: number of live fetuses/number of implantation sites × 100.

2.4. Statistical analysis

Initially, an exploratory analysis was conducted to evaluate normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test) of each variable. Variables that presented normal distribution and homogeneity of variance were analyzed by ANOVA. In the absence of normal distribution and/or homogeneity of variance, variables were transformed in order to achieve the criteria for parametric analysis. Conversely, for the other variables Mann-Whitney test was performed. For pups' body weight

Table 1
Estrous cyclicity evaluation in the female offspring exposed to fluoxetine during gestational and lactational periods.

Estrous cycling (days)	CTR [n = 12]	FLX [n = 11]
Estrous cycle length	3.75 (3.75–5.00)	3.75 (3.56–5.00)
Frequency of proestrus	4.00 (4.00–5.00)	4.00 (3.00–4.00)
Frequency of estrus	4.00 (3.00–4.00)	4.00 (3.00–4.25)
Frequency of metestrus	3.00 (2.00–3.00)	3.00 (3.00–3.00)
Frequency of diestrus	4.00 (4.00–5.00)	4.00 (4.00–5.00)

Values are expressed as median (1st–3st quartile). Numbers in brackets represent the number of animals/group. CTR = saline, FLX = Fluoxetine 5 mg/kg. There were no significant differences between groups, Mann-Whitney ($p > 0.05$).

(PND 0, 7, 14, 21) and AGD (PND 0, 21), repeated measures ANOVA (RMANOVA) was applied with day as the within-subject factor and treatment as the between-subjects factor. For wet organs weight (PND 90–100), analysis of covariance (ANCOVA) was applied with body weight as covariate. The lordosis reflex magnitude was analyzed by Fisher's test. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Parameters analyzed in dams

3.1.1. Body weight

Maternal body weight during gestation and lactation was unaffected by FLX treatment (data not shown, RMANOVA, $p > 0.05$).

3.2. Parameters analyzed in female offspring during development (PND 0–60)

3.2.1. Body weight and physical development

Body weight of female pups during the first 3 weeks of age was not influenced by FLX exposure as indicated by RMANOVA [$F(2.01,66.32) = 2043$; $p = 0.138$]. There was only an effect of age [$F(2.01,66.32) = 1970.373$; $p = 0.0001$] reflecting the weight gain of pups (Fig. 2A). Age was also the only significant factor observed in the AGD [$F(1,33) = 1248.423$; $p = 0.0001$] (Fig. 2B).

3.2.2. Physical sexual development

The repeated exposure to FLX cause significant alteration in days of VO (CTR: 33.78 ± 0.36, $n = 27$; FLX: 35.83 ± 0.49, $n = 28$) [$F(1,53) = 11.374$; $p = 0.001$] and first estrus (CTR: 33.71 ± 0.44, $n = 11$; FLX: 35.68 ± 0.73, $n = 11$) [$F(1,20) = 5.361$; $p = 0.031$] are given in Fig. 3. The *in utero* and lactational exposure to FLX delayed puberty onset in female offspring.

3.3. Parameters analyzed in female offspring during adulthood (PND 75-LD 5)

3.3.1. Estrous cycle and estradiol plasmatic concentration

FLX exposure did not affect the regular pattern of estrous cyclicity (Table 1) neither the plasmatic concentration of estradiol (pg/ml; CTR: 180.08 ± 36.31, $n = 10$; FLX: 267.53 ± 50.33, $n = 10$) [$F(1,18) = 1.985$; $p = 0.176$], FSH (ng/ml; CTR: 1.10 ± 0.25, $n = 9$; FLX: 1.03 ± 0.13, $n = 9$) [$F(1,16) = 0.71$; $p = 0.793$] and LH (ng/ml; CTR: 1.44 ± 0.97, $n = 9$; FLX: 0.38 ± 0.05, $n = 9$) [$F(1,16) = 1.18$; $p = 0.293$] in adulthood on estrus phases.

3.3.2. Reproductive organs weight and histology

The final body weight and reproductive organs weight of adult female are presented in Table 2. No significant difference as a result of *in utero* and lactational FLX exposure were revealed in body weight [$F(1,21) = 0.904$, $p = 0.353$]. ANCOVA indicated that the wet weight of uterus [$F(1,21) = 1.599$, $p = 0.221$] and right [$F(1,21) = 0.962$; $p = 0.338$] and left [$F(1,21) = 0.955$; $p = 0.356$] ovaries were similar between groups. Counting of ovarian follicle and corpora lutea as well as uterine morphometric analyses are given in Table 3. ANOVA indicated lack of treatment effect for all these evaluated parameters from FLX group when compared to CTR ($p < 0.05$).

Table 2
Body weight and wet organs weight from female rats at PND 90–95.

Weight (g)	CTR [n = 12]	FLX [n = 11]
Body	226.89 ± 7.92	217.35 ± 5.92
Uterus	0.48 ± 0.06	0.56 ± 0.07
Right ovary	0.05 ± 0.003	0.06 ± 0.003
Left ovary	0.05 ± 0.003	0.05 ± 0.003

Values are expressed as means ± S.E.M. Numbers in brackets represent the number of animals/group. CTR = saline, FLX = Fluoxetine 5 mg/kg. Body weight was used as a co-variate in the analysis of organs weight (ANCOVA, $p > 0.05$).

Table 3
Ovarian follicle and corpora lutea counting and uterine morphometric analyses of female rats in PND 90–95.

Parameters (count)	CTR [n = 6]	FLX [n = 8]
Primordial follicles	5.19 ± 0.41	4.35 ± 0.54
Growing follicles	4.36 ± 0.40	4.06 ± 0.54
Antral follicles	4.61 ± 0.38	3.63 ± 0.60
Atretic follicles	3.53 ± 0.42	3.56 ± 0.42
Corpora lutea	11.39 ± 0.45	11.85 ± 0.35
Parameters (μm)	**CTR [n = 6]**	**FLX [n = 8]**
Thickness of uterine epithelium	24.03 ± 2.49	24.86 ± 2.24
Thickness of endometrial stroma	913.39 ± 52.10	926.94 ± 83.13

Values are expressed as means ± S.E.M. Numbers in brackets represent the number of animals/group. CTR = saline, FLX = Fluoxetine 5 mg/kg. There were no significant differences between groups, ANOVA ($p > 0.05$).

3.3.3. Sexual behavior

The sexual behavior results are shown in Table 4. *In utero* and lactational exposure to FLX did not influence the parameters from this evaluation.

3.3.4. Maternal behavior

3.3.4.1. Maternal behavior evaluation after pup removal. ANOVA indicated that FLX exposure did not affect the parameters evaluated, i.e. retrieval behavior (total pups: [$F(1,14) = 1.671$; $p = 0.217$]), total time grouping [$F(1,15) = 1.786$; $p = 0.201$], pup grooming [$F(1,15) = 0.333$; $p = 0.573$], self-grooming [$F(1,10) = 0.595$; $p = 0.458$], crouching [$F(1,15) = 0.075$, $p = 0.986$], off pups [$F(1,15) = 0.098$; $p = 0.949$] and nest building [$F(1,14) = 0.167$; $p = 0.689$] (Table 5).

3.3.4.2. Undisturbed mother-pup interaction evaluation. ANOVA indicated lack of FLX exposure effect for all the parameters evaluated, i.e. nursing [$F(1,16) = 0.061$; $p = 0.940$], off pups [$F(1,16) = 0.082$; $p = 0.930$], retrieval [$F(1,16) = 3.452$; $p = 0.083$], pup grooming [$F(1,16) = 0.974$; $p = 0.338$], self-grooming [$F(1,16) = 0.153$; $p = 0.700$] and nest building [$F(1,16) = 1.308$; $p = 0.270$] (CTR group: $n = 10$; FLX group: $n = 9$, $p > 0.05$; Table 5).

3.3.5. Fertility test

The fertility test parameters are given in Table 6. They were not influenced by FLX exposure as indicated by Mann-Whitney test $p > 0.05$.

4. Discussion

The present study evaluated the effects of *in utero* and lactational exposure to FLX on physical and reproductive parameters in

Table 4
Sexual behavior of female rats at PND 90–95.

Parameters	CTR [n = 17]	FLX [n = 16]
Lordosis quocient	100.00 ± 0.00	99.38 ± 0.63
Lordosis reflex magnitude (LRM) (% of observations)		
0	0.00% (0/170)	0.63% (1/160)
1	1.76% (3/170)	4.38% (7/160)
2	34.71% (59/170)	26.88% (43/160)
3	63.53% (108/170)	68.13% (108/160)

Lordosis quocient values are expressed as means ± S.E.M. and were analyzed by ANOVA. LRM values are expressed as percentage of observations and were analyzed by Fisher's test. Numbers in brackets represent the number of animals/group. Numbers in parenthesis represent the number of observations presented in each lordosis grade/total of observations. CTR = saline, FLX = Fluoxetine 5 mg/kg. There were no significant differences between groups $p > 0.05$.

Table 5
Maternal behavior observations of rats from CTR and FLX groups.

MB after pup removal	CTR [n = 10]	FLX [n = 9]
Time to retrieve the first pup (s)	49.11 ± 13.07 (10/10)	78.33 ± 25.34 (8/9)
Time to retrieve half of the litter (s)	175.49 ± 30.00 (10/10)	139.06 ± 31.64 (8/9)
Time to retrieve all pups (s)	352.00 ± 66.98 (8/10)	246.84 ± 46.17 (8/9)
Total time grouping (s)	163.76 ± 19.26 (10/10)	213.56 ± 33.15 (8/9)
Total time self grooming (s)	19.59 ± 4.08 (8/10)	14.54 ± 4.19 (5/9)
Total time pup-grooming (s)	490.15 ± 70.62 (10/10)	546.35 ± 66.03 (9/9)
Total time crouching (s)	372.98 ± 125.61 (6/10)	369.06 ± 169.98 (3/9)
Total time off pups (s)	729.48 ± 86.17 (10/10)	737.55 ± 88.88 (9/9)
Total time nest building (s)	147.36 ± 34.80 (7/10)	168.67 ± 38.72 (7/9)
Undisturbed MB	CTR [n = 11]	FLX [n = 10]
Off pups (% of observations)	14.75 ± 1.02 (11/11)	14.97 ± 2.15 (10/10)
Grouping (% of observations)	1.91 ± 0.33 (11/11)	2.25 ± 0.27 (10/10)
Total nursing (% of observations)	69.75 ± 1.19 (11/11)	69.51 ± 3.51 (10/10)
Self-grooming (% of observations)	2.04 ± 0.25 (11/11)	2.19 ± 0.31(10/10)
Pup-grooming (% of observations)	11.51 ± 1.02 (11/11)	10.06 ± 1.06 (10/10)
Nest building (% of observations)	0.86 ± 0.14 (11/11)	1.17 ± 0.23 (9/10)

Values are expressed as means ± S.E.M. with the number of animals that displayed the behavior per total number of animals in the group given in parenthesis. Numbers in brackets represent the number of animals/group. The parameters were calculated using only animals that presented that behavior. CTR = saline, FLX = Fluoxetine 5 mg/kg. There were no significant differences between groups (ANOVA, $p > 0.05$).

Table 6
Fertility parameters evaluated in adult female rats from CTR and FLX groups on lactational day 5.

Parameters	CTR [n = 17]	FLX [n = 16]
Implantation rate	61.36 (52.59–66.30)	60.87 (54.55–76.19)
Pre-implantational loss (%)	38.64 (33.70–47.41)	39.13 (23.81–45.45)
Post-implantational loss (%)	4.17 (0.00–19.55)	0.00 (0.00–7.14)
Fetal viability rate	95.38 (80.45–100.00)	100.00 (92.86–100.00)

Values are expressed as median (1st–3st quartile). Numbers in brackets represent the number of animals/group. CTR = saline, FLX = Fluoxetine 5 mg/kg. There were no significant differences between groups (Mann-Whitney, $p > 0.05$).

the female offspring. Firstly, clinical signs and non-invasive indicators of maternal toxicity (such as changes in behavior—agitation, lethargy and hyperactivity; autonomic signs—lacrimation, piloerection, pupil-size, unusual respiratory patterns; body weight) were evaluated during the FLX treatment period and no signs of maternal general toxicity were observed.

The best indicator for the evaluation of pups' physical development has been suggested to be the body weight gain. In the present study, FLX exposure did not induce body weight changes in female offspring. Our results are in agreement to those observed by several studies with different FLX employed doses, such as 1 [17], 5 [17], 7.5 [25] and 16 mg/kg [26]. However, it was observed, at doses of 10 and 12 mg/kg, an increase [27] and a decrease [17,18] respectively in the body weight. These divergences can be related to differences in the exposure protocol (dose, route, exposure period) as well as animal strain used.

AGD is an important hormonal-sensible developmental measure which is influenced by endocrinal deregulators, and this parameter has being commonly used to verify sexual dimorphism changes in rodents. Although it has been demonstrated a possible estrogenic activity of FLX in immature rat uterotrophic assay [28], the present work did not found any statistical difference in AGD of female offspring from both groups at different ages.

The activation and coordination of hypothalamic-pituitary-gonadal (HPG) axis is required to the puberty establishment [29], which is the acquisition of reproductive capability. Some authors state that puberty onset occurs when the gonadotropin release hormone (GnRH) is secreted by hypothalamus [30], while others believe that the first step is the ovary estradiol production and release that will further stimulate the hypothalamic GnRH release [31]. Once released, GnRH will stimulate luteinizing (LH) and follicle stimulating (FSH) hormones release by adenohypophysis, which will induce germinative follicles growth and maturation, leading to ovulation and ovarian steroidogenesis. The enhanced estradiol serum levels leads to vaginal opening (VO) and estrous cyclicity, which are parameters used to assess puberty establishment. Herein, the *in utero* and lactational FLX exposure delayed VO and the first estrus of female offspring.

The hypothalamic neurotransmission systems regulate the puberty onset during prepubertal period by stimulatory activity

on GnRH, LH and FSH release [31,32]. Cell bodies of serotoninergic neurons are found in raphe nuclei and emit projections to several brain regions [33], including hypothalamus [34]. It is known that hypothalamic GnRH-producing neurons receive serotoninergic innervation and this neurotransmitter is found in hypophysis and ovaries [29]. In this way, some studies have evidenced that 5-HT plays an important role in the regulation and control of HPG axis due to its influence on gonadotropin release [35]. Hence, Moran et al. observed that 5-HT subcutaneous administration (25 and 37.5 mg/kg) from PND 30 until the VO day in prepubertal rats, delayed VO, first estrus and reduced the estradiol plasmatic levels [29]. Furthermore, lesions on the dorsal raphe nucleus of female rats in prepubertal period induced a delay in the puberty onset [36]. Thus, 5-HT may have an inhibitory activity on gonadotropins release, since it was demonstrated that LH release should be inhibited by 5-HT neurons from medial raphe nucleus, via GABAergic system [37]. On the other hand, 5-HT neurons from dorsal raphe nucleus can stimulate the LH release by a noradrenergic (NA) mechanism [38] involving locus coeruleus [39].

In the present study, it is suggested that the delay in puberty onset may be due to a delay in estradiol release, which can occur by two mechanisms. The first one considers an inhibitory pattern of 5-HT on GnRH release, since FLX inhibits 5-HT reuptake, leading to an increase in extracellular concentrations of this neurotransmitter. This increase promotes stimulation of 5-HT1A somatodendritic inhibitory autoreceptors and 5-HT1D/B presynaptic terminals, culminating in reduced 5-HT synthesis and release. However, repeated treatment with FLX promotes downregulation and desensitization of these autoreceptors mechanisms, so 5-HT becomes no longer effective to inhibit its own release and then the serotoninergic neuron ceases to be inhibited [40,41]. Furthermore, it has been reported that repeated exposure to SSRIs could reduce the expression of the 5-HT transporter (SERT), resulting in enhanced serotoninergic transmission [42]. Thus, the neuronal impulse flow should be increased and the transporters diminished, leading to 5-HT release by axon terminals and this 5-HT can inhibit GnRH secretion. Secondarily, this inhibitory autoreceptors desensitization enhances the 5-HT release, leading to a postsynaptic receptors desensitization (5-HT2, 5-HT3, among others) [43]. This second hypothesis should explain how a serotoninergic stimulatory tone can delay puberty onset, since postsynaptic receptors desensitization would lead to a loss of this excitatory tone on gonadotropin secretion.

It is very important to clarify that these effects described above should not be related to FLX or norfluoxetine plasma concentrations, since the exposure was stopped approximately nine days before the parameters of puberty onset were checked (FLX and its metabolite have a half-life of around 7 and 14.4 h, respectively) [44]. However, delays in VO and in the first estrus may result from possible neuroadaptations coming from the repeated exposure, since the latency between the end of exposure and the puberty onset parameters assessment may not have been sufficient to normalize the number of receptors as well as its sensitivity [40]. Thus, further investigations are required for a better understanding of how the serotoninergic transmission controls the puberty onset and the estrogen release in this phase.

Thereby, it is evident the influence of serotoninergic pathways on the HPG axis control during brain development [29,35,45]. Although the estradiol dosage was not conducted in pubertal females in the present study, we suggest that 5-HT delays the estradiol release by ovaries due to its influence on the gonadotropins release and consequently delayed sexual maturity. This hypothesis could be strengthened with Matagne et al., which demonstrated that estradiol administration anticipated the VO date and first estrus in prepubertal rats [46].

Although there was a delay in puberty onset, all the parameters analyzed in these adult females have not changed. This result may be due to the fact that 5-HT system modulation on gonadotropin secretion is controversial (some studies showed an inhibitory [36,47] or stimulatory pattern [35,47] or even the absence of activity [35,48] and varies with the animal's age—prepubertal [35,39,47], peripubertal [35,48] and adult [35,47].

To evaluate these results it is important to consider that VO and first estrus were assessed in prepubertal/pubertal periods while the estrous cycle was evaluated in adult life and the 5-HT influence on gonadotropic hormones release is not the same in both phases. In this way, it should be emphasized that the delay in puberty onset can be due to the proximity between the end of treatment and the pubertal period in which the parameters were evaluated (the interval was 9 days). Thus, it can be assumed that even though FLX and its active metabolite are no longer present in circulating plasma levels (since they have a half-life of around 7 and 14.4 h, respectively) [44], the neuroadaptations resulting from repeated administration can still be present (e.g. receptor desensitization) [40], as well as 5-HT central levels may not yet returned to normal. In contrast, the estrous cycle was assessed in adult females (PND 75), 54 days after the end of FLX exposure.

Likewise, the estradiol plasmatic levels, ovarian follicular quantification and uterine morphometric analysis, body weight, reproductive organs weight, sexual and maternal behaviors and fertility test from adult female rats of FLX group were not changed when compared to CTR group. Therefore, it's suggested that although 5-HT has an effect on puberty onset, it did not have deleterious effects on neuroendocrine and reproductive systems of females in adulthood.

5. Conclusion

The present study revealed that *in utero* and lactational exposure to a human-relevant dose of FLX delays puberty onset in female rats' offspring. Since a single dose was evaluated, a no-observed adverse effect level could not be determined. The delay in sexual maturity could be related with the 5-HT influences on gonadotropin secretion and reinforces the relevance of further investigations into the mechanisms by which 5-HT acts on HPG axis, controls the GnRH release and delays puberty onset.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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