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Long-term effects of pre-pubertal fluoxetine on behaviour and monoaminergic stress response in stress-sensitive rats

Badenhorst NJ, Brand L, Harvey BH, Ellis SM, Brink CB. Long-term effects of pre-pubertal fluoxetine on behaviour and monoaminergic stress response in stress-sensitive rats.

Objective: Although prescription rates of antidepressants for children and adolescents have increased, concerns have been raised regarding effects on neurodevelopment and long-term outcome. Using a genetic animal model of depression, this study investigated the long-term effects of pre-pubertal administration of fluoxetine (FLX) on depressive-like behaviour in early adulthood, as well as on central monoaminergic response to an acute stressor. We postulated that pre-pubertal FLX will have lasting effects on animal behaviour and monoaminergic stress responses in early adulthood.
Methods: Flinders sensitive line (FSL) rats received 10 mg/kg/day FLX subcutaneously from postnatal day 21 (PnD21) to PnD34 (pre-pubertal). Thereafter, following normal housing, rats were either subjected to locomotor testing and the forced swim test (FST) on PnD60 (early adulthood), or underwent surgery for microdialysis, followed on PnD60 by exposure to acute swim stress and measurement of stressor-induced changes in plasma corticosterone and pre-frontal cortical monoamine concentrations.
Results: Pre-pubertal FLX did not induce a late emergent effect on immobility in FSL rats on PnD60, whereas locomotor activity was significantly decreased. Acute swim stress on PnD60 significantly increased plasma corticosterone levels, and increased pre-frontal cortical norepinephrine (NE) and 5-hydroxyindole-3-acetic acid (5-HIAA) concentrations. Pre-pubertal FLX significantly blunted the pre-frontal cortical NE and 5-HIAA response following swim stress on PnD60. Baseline dopamine levels were significantly enhanced by pre-pubertal FLX, but no further changes were induced by swim stress.
Conclusion: Pre-pubertal FLX did not have lasting antidepressant-like behavioural effects in genetically susceptible, stress-sensitive FSL rats. However, such treatment reduced locomotor activity, abrogated noradrenergic and serotonergic stressor responses and elevated dopaminergic baseline levels in adulthood.

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Keywords: animal; antidepressive agents; child; fluoxetine; models

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Significant outcomes

- Pre-pubertal fluoxetine (FLX) does not have lasting antidepressant-like behavioural effects in Flinders sensitive line (FSL) rats.
- Pre-pubertal FLX administration has long-lasting effects on monoaminergic systems into early adulthood in FSL rats, allowing it to alter acute stress-related changes in pre-frontal cortical monoamine levels.
- FLX-induced elevated serotonin levels in pre-puberty likely underlie long-term neurodevelopmental effects, resulting in altered monoaminergic stressor response in early adulthood.

Limitations

- The study did not compare the results in genetically susceptible animals with normal control animals, so the role of genetic susceptibility in monoaminergic stress response is not determined.
- Although results in animal models of depression has well-described predictive validity and may generate working hypotheses for human counterparts, caution should be exercised in translation.

Introduction

Not only is major depressive disorder (MDD) the leading cause of disability world-wide, with an estimated 350 million individuals suffering from MDD globally (1), but epidemiological data also reflects a similar prevalence of MDD in adults *and* adolescents (2–5). The prevalence of MDD in pre-puberty is estimated to be 0–2% (6), increasing to 4–5% during mid to late adolescence (7). Concerns have been raised that MDD may be a growing problem in juveniles, in particular if one considers the rising trend in the number of antidepressant prescriptions for children and adolescents (8) already observed more than a decade ago. Meta-analysis of available epidemiological data from 1950 to 1990 has identified an increased suicide rate amongst adolescents in the United States (3). This study, as well as a more recent meta-analysis (9), failed to provide evidence of an impending epidemic of MDD in juveniles. Nevertheless, the apparent trend had been attributed to a growing awareness of this disorder where it had previously been underdiagnosed in children (3,9), as well as to the use of terminology associated with MDD and anxiety disorders in non-clinical settings and the rapid growth of the global population (9). These conservative projections, however, do not disregard that juvenile depression is currently a serious disorder of global significance.

Currently only FLX and escitalopram, both selective serotonin (5-HT) re-uptake inhibitors (SSRIs), have been approved by the Food and Drug Administration for the treatment of MDD in children and adolescents (10). Several studies have shown that SSRIs cause foetal developmental changes when used to treat MDD in pregnant woman (11–13). However, 5 year follow-up studies have not implicated *in utero* exposure with an SSRI to any lasting negative effects on cognition, temperament or internalising and externalising behaviours in offspring (14). Prenatal exposure of mice to inhibitors of 5-HT transporter protein (SERT), such as SSRIs, but not to inhibitors of norepinephrine (NE) transporter (NET), has produced undesirable behavioural outcomes later in life (15). Such undesirable developmental outcomes following late postnatal life administration of SSRIs (16) include alterations in reproductive behaviour (17), anxiety-like behaviour (17), social behaviour (18) and depressive-like behaviour (19). Some studies have even demonstrated neurochemical changes resembling those associated with autism in humans (18,20).

SSRIs also differentially inhibit the reuptake of NE and dopamine (DA), have variable interactions with 5-HT, muscarinic, histamine H_1, α_1 adrenergic and other receptors, promote the expression of brain-derived neurotrophic factor and its receptor TrkB, as well as differentially inhibit various cytochrome P450 enzymes (21). SSRIs prescribed to children and adolescents with MDD, and thereby modulation of serotonin levels in concert with other neuro-developmental factors, may therefore have profound impact on neurodevelopmental outcome (16), particularly stemming from altered neurogenesis, apoptosis, axon branching and dentritogenesis (22). A recent rat study demonstrated that prenatal exposure of rat pups to FLX in stressed dams prevented increased depressive-like behaviour in offspring later in life as compared with untreated controls (23). The latter study also suggested that prenatal FLX exposure may in fact enhance hippocampal neurogenesis and cell proliferation, as determined in adolescence (23), prompting a working hypothesis that appropriate use of FLX in early-life of at-risk individuals may be beneficial from a neuro-developmental perspective.

Ongoing psychosocial and other environmental stressors, and their associated effects on mono-aminergic neurotransmission, inflammation (due to the effects of the hypothalamic–pituitary–adrenal axis on immune system functioning), and neurogenesis and neuroplasticity (24–28), are an important prodromal events leading to the later development of MDD (29). The serotonergic system has especially been implicated in a number of important neurodevelopmental and neuroplastic processes, including neural cell proliferation, migration and differentiation, neurite outgrowth, axonal guidance, synaptogenesis and transsynaptic signalling, all eventually culminating in manifested neural control of perception, cognition, emotional regulation, autonomic response and motor activity (30). Furthermore, stress- and anxiety-induced changes in monoaminergic neurotransmission and corticosterone release have been well documented, and have also been shown to be affected by early-life events (31). Moreover, differences in sensitivity to acute stressors underlie differences in stress-susceptible

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and stress-resilient individuals and their subsequent proneness to developing a stress-related disorder (29).

The current clinical use of SSRIs early in life, as well as evidence suggesting neurodevelopmental effects of SSRIs and of the key role of the serotonergic system in early development, warrant studies on the long-lasting effects of early-life SSRIs on monoaminergic function later in life. The current study investigated the possible developmental effects of FLX in a genetic rat model of depression, the FSL rat, an animal with a known hyper-responsivity to stress (32). The FSL rat has been shown to exhibit reduced serotonin synthesis (32) and express lower levels of D_2 DA receptors than control Sprague–Dawley rats (33). Following sub-chronic pre-pubertal FLX administration, we explored long-lasting effects on depressive-like behaviour and locomotor activity in adult FSL rats. In an attempt to study early-life FLX use and its effects on late-life acute stress challenges, we first confirmed the corticosterone stress response in these animals following an acute forced swim stressor, and then studied long-lasting FLX-associated changes in monoamine release following said acute stressor. In this regard, we implemented intracerebral *in vivo* microdialysis to investigate extracellular monoaminergic levels in the pre-frontal cortex in awake, freely moving rats before, during and after exposure to the acute stressor. To our knowledge, this is the first study to examine long-lasting brain monoaminergic stressor response following pre-pubertal SSRI administration.

Materials and methods

Animals

Male FSL ($n = 56$) rats were bred, supplied and housed at the Vivarium of the Pre-Clinical Drug Development Platform of the North-West University. The original rat colonies were obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, NC, USA. All experiments were approved by the Ethics Committee of North-West University (ethical approval numbers: NWU-00045-10-5S and NWU-0028-08-A5). The authors assert that all procedures contributing to this work comply with the ethical standards of the South African national and North-West University institutional guides on the care and use of laboratory animals. Rats were pair-housed with the environmental temperature maintained at 22 ± 1°C and humidity at 50%. A 12 : 12-h light/dark cycle (lights on at 06:00 h and off at 18:00 h) was followed and food and water were available *ad libitum*. On postnatal day (PnD) 57 the rats used for the *in vivo* microdialysis and corticosterone assays were moved to the microdialysis laboratory maintaining the light/dark cycle and temperature, whereas rats for behavioural analyses were housed in the Vivarium until testing on PnD60. In addition, at the start of the study we confirmed the presence of an exaggerated 8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT)-induced hypothermic response in FSL rats relative to control Flinders resistant line (FRL) rats. Over and above their differences with respect to behaviour in the forced swim test (FST), the OH-DPAT procedure is also recommended to separate FSL from FRL rats on the basis of 5-HT_{1A} receptor supersensitivity in FSL rats (34), resulting in the aforementioned hypothermic response, measured rectally, within a matter of 15–30 min.

Study design

In the first part of the study, depicted in Fig 1a, we examined depressive-like behaviour and locomotor activity in FSL rats on PnD60, following treatment with saline (SAL) or FLX from PnD21 to PnD34, as described under 'Drug treatment' below.

In the second part of the study, depicted in Fig 1b, the acute corticosterone response to a forced swim stressor was assessed in untreated FSL rats between PnD59 and PnD62, following microdialysis guide cannula placement surgery and exposure to halothane (to facilitate microdialysis probe placement).

In the third part of the study, depicted in Fig 1c, the monoaminergic response to the forced swim stressor was assessed FSL rats between PnD59 and PnD62, following treatment with FLX from PnD21 to PnD34.

Drugs treatment

Animals were treated with SAL (vehicle) or FLX (a kind gift from Aspen, Port Elizabeth, South Africa) subcutaneously (s.c.) once daily from PnD21 to PnD34. FLX HCl was administered at a dose of 10 mg/kg/day (35,36), similar to previous studies in our laboratory, and as reported in other studies investigating antidepressant-like effects of FLX in rodents (35,37–40). The treatment period from PnD21 to PnD34 represents a neurodevelopmental phase for noradrenergic (41) and dopaminergic (42) neurotransmitter systems, whilst serotonergic neurodevelopment has already matured (41), similar to humans during adolescence (43). After the drug treatment, rats were randomly allocated for use in behavioural, corticosterone or microdialysis experiments. For all microdialysis guide cannula placement surgeries (in the pre-frontal cortex) anaesthesia was induced by intraperitoneal injection (0.1 ml/100 g) of a mixture of xylazine (60 mg/kg) and ketamine (10 mg/kg) (44). After full recovery and in order to facilitate

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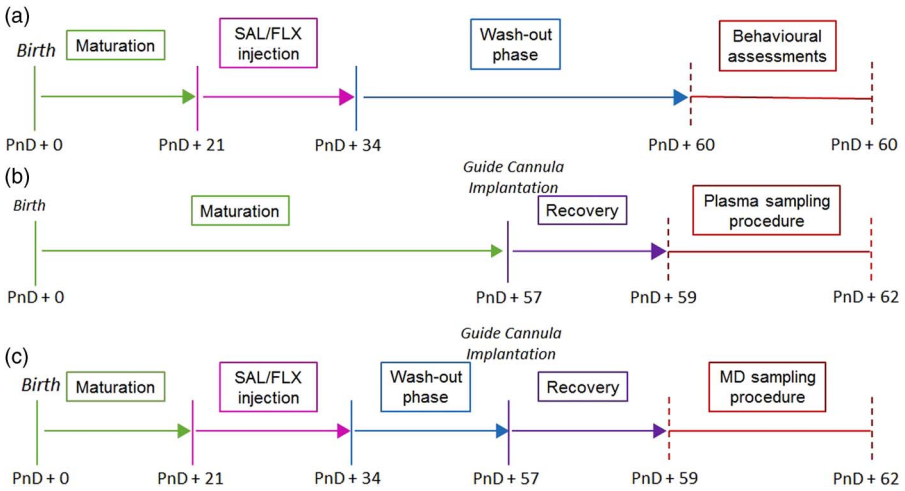

Fig. 1. Schematic representation of the experimental design, depicting (a) the behavioural studies, (b) the corticosterone stress response study and (c) the monoaminergic stress response study. PnD = postnatal day; MD = microdialysis; FLX = fluoxetine; SAL = saline.

microdialysis probe placement on the 3rd day after the surgery, animals were placed in an airtight enclosure, using 5 ml of a 100% halothane solution (Halothane-M & B; Safeline Pharmaceuticals, Johannesburg, Gauteng, South Africa) to render the animal anaesthetised and motionless (45).

Behavioural analyses

On PnD60, following the SAL or FLX treatments from PnD21 to PnD34 described above, all behaviour experiments were performed, video-taped and scored by a researcher blinded to the treatment groups.

FST. The FST, as first described by Porsolt in 1977 (46) and later adjusted by Lucki in 1997 (47) to be sensitive to SSRIs, has been widely used, validated and described to investigate depressive-like behaviour in rodents (48). The test was conducted in clear Perspex cylinders (20 cm diameter and 40 cm high) (49). On the day of the test the cylinders were filled to a depth of 30 cm with 25°C water and the test was conducted under a light intensity of 200 lux (38,48). The typical conditioning swim 24 h before the scoring session is not applicable to FSL rats (39). The rats were placed in the water for 7 min. When scoring, the first and last minutes were ignored which means scoring was only done for a 5 min period (38). The movements of the rats were scored by one person blind to the treatment according to the criteria laid out by Cryan et al. (48), and accordingly immobility was defined as the absence of active movements other than those necessary to keep the animal's head above water. Independent scoring by a second trained and experienced person, also blind to the treatment, yielded comparable results. We implemented manual scoring of animal behaviour from video recordings, utilising custom software (FSTscoreboard). During the 5 min of scoring, the software calculates the time spent immobile (pressing NumPad no. 5), struggling (pressing NumPad no. 8), swimming (pressing NumPad no. 4) or diving (pressing NumPad no. 2). We used only immobility time. The results from FSTscoreboard has been cross-checked with traditional means of scoring (i.e. scoring the main behaviour every 5 s) by different experienced researchers on several sets of data and results have been consistently comparable. After swimming, animals were removed from the water and gently dried with paper towels.

Locomotor activity. Fatigue and anhedonia are known symptoms of MDD and lead to decreased general activity in the affected individual. To differentiate altered psychomotor from locomotor activity, locomotor activity was assessed in the open-field test (OFT) (50). The apparatus used for the test consisted of an opaque Perspex box, with a 1 m^2 test arena floor, subdivided into 16 smaller squares of 25 × 25 cm (38). In this test, the total number of lines crossed within the 5 min testing session was used as an indication of general locomotor activity.

Stressor response

Forced swim acute stressor. Kirby et al. (51) showed that forced swimming was more effective as an acute stressor than tail-pinch, exposure to cold, immobilisation or forced locomotion on a suspended rotating rod. In this study, we employed 10 min forced swimming as an acute stressor,

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as also employed in previous studies (52,53). Clear Perspex cylinders (20 cm diameter and 40 cm high) (49) were filled with water at 25 ± 4°C to a depth of 30 cm, all under ±200 lux white light (48). After the swim session animals were gently dried with disposable tissue-paper after the swim session.

Confirmation of a significant stressor response was done by measuring plasma corticosterone levels in untreated rats. Guide cannulas were placed in 14 male FSL rats where after they were randomly divided into two groups. One group was exposed to an acute swim stressor described above, whereas the other group served as unstressed controls. Thereafter, rats were decapitated and plasma corticosterone concentrations measured from trunk blood samples collected in 10 ml heparinised blood tubes. Corticosterone concentrations were determined by high-performance liquid chromatography (HPLC) as described below.

Microdialysis

After the pre-pubertal drug treatment, rats for microdialysis experiments underwent surgery for guide cannula placement in the pre-frontal cortex. Anaesthesia was implemented to ensure humane care and optimal wellbeing of the animals at all times. Body temperature was maintained at 37°C with a manually controlled surgical heating pad. A Kopf® stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) was used to place the guide cannula 4.2 mm anterior-posterior, 2.4 mm lateral, 2.4 mm ventral relative to bregma in the pre-frontal cortex (54). Three jeweller screws, 0.9 × 1.92 mm, were placed shallowly into the bone to provide extra support and anchorage for the cannula (45,55). An intraperitoneal diclofenac injection, dose 2 mg/kg, was given after completion of the surgery for pain control (45). The rat was then transferred to the microdialysis home cage and left to recover for 3 days before implantation of the microdialysis probe and commencement of the microdialysis procedure (45). The subjects were monitored for signs of pain and infection during the recovery phase and immediately euthanised if any such symptoms and signs became manifest (45).

On the day before the exposure to the forced swim stress (18:00 h) the rat was placed in an airtight cage with a halothane-soaked cotton swab. As soon as the rat became immobile, it was removed and the probe was placed into the guide cannula before the subject recovered full consciousness (45). The rat was then placed into the microdialysis enclosure and perfusion was initiated at 1.0 µl/min to allow equilibrium to be achieved prior to the microdialysis sampling procedure at 08:00 the next day. The probe was placed on the evening prior to the sampling procedure (55) to limit acute halothane-mediated changes in sample monoamine concentrations (56). Perfusion with artificial cerebrospinal fluid (aCSF) was initiated immediately after probe placement at a flow rate of 1.0 µl/min (45).

Sampling commenced at 08:00 h the next morning over a timeframe of 7 h and 20 min. Samples were collected in 20 min intervals yielding a sample volume of 20 µl eluent/sample. The first three samples were used to determine baseline levels of the monoamines. At the start of the fourth sampling interval the rat was placed in a FST cylinder filled with water (acute swim stress) for 10 min. The rat was removed from the FST cylinder and returned to the microdialysis enclosure. A further 18 samples were then collected over a period of 6 h. The first sample collected in each hour after the swim stress was analysed, yielding six samples to investigate changes in monoamine concentrations for an extended time after the forced swim stress.

Isotonic aCSF, kept at room temperature, was used as perfusion fluid in this study. Each sample was removed from the microfraction collector and immediately placed in an amber vial and placed in the auto-injector of the HPLC for analysis. Relative recovery was not determined in this qualitative study as the aim was to compare the results obtained in FSL rats treated with either FLX or SAL. The aCSF was prepared by adding the following salts to ultra-pure water: NaCl (145 mM), KCl (3.0 mM), $CaCl_2$ (1.2 mM), and $MgCl_2$ (1.0 mM) (55). The apparatus used included microdialysis probes (acquired from TSE Systems, Chesterfield, MO, USA) with 4 mm membrane working length and 6 kDa cut-off. The syringe pump used was a MAB 40 Dual Channel Microdialysis Pump (by Microbiotech, Stockholm, Södermanland and Uppland, Sweden). The refrigerated microfraction collector was an 820 Microsampler (by Univentor, Zejtun, South Eastern Region, Malta). Guide cannula placement surgeries were done with the aid of a Kopf® stereotaxic frame.

The technique described by Bert et al. (57) was used to verify the probe position. Computer software is used to superimpose a digital photograph of a coronal section of the harvested brain (of the experimental animal) onto a digital image of an appropriate stereotaxic atlas representation of a coronal section of the animal brain. The track of the dialysis probe is visible and is used to verify the correct position of the probe against the atlas. This technique was modified by combining it with a conventional staining procedure in order to enhance the ease and accuracy with which the investigator could determine the probe position.

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Immediately after the sampling procedure the rat was disconnected from the sampling apparatus and placed in the airtight halothane enclosure until immobile. Cresyl violet acetate (Sigma-Aldrich, Johannesburg, Gauteng, South Africa) obtained from Sigma-Aldrich (CAS no: 10510-54-0, molecular formula: $C_{18}H_{15}N_3O_3$) was used. It was prepared by adding 0.1 g of the cresyl violet acetate to 100 ml distilled water. Just before use 10 drops of glacial acetic acid was added and the solution was filtered (58). The 0.1% cresyl violet solution was injected via the inlet of the microdialysis probe, the rat was then placed back in the halothane enclosure for 7 min (recommended times 5–10 min) to allow the cresyl violet to stain the area of the brain adjacent to the tip of the microdialysis probe. Hereafter, the rat was decapitated and the brain was removed in order to determine the probe position. Immediately after removal, the brain was placed in ice-cold SAL. Ice was added to the SAL; the brain was left in this environment for 3 min. The brain was then transferred to a pre-chilled Kopf® PA 001 brain blocker (David Kopf Instruments, Tujunga, CA, USA) and placed in a freezer (−20°C) overnight to freeze. This brain blocker was developed to be used with the stereotaxic atlas of the rat brain developed by Paxinos and Watson (54). On the next day successive sections were made (starting anteriorly moving in a posterior direction) with a single-sided razor blade in the brain blocker until the stain could be identified (see Fig. 2 for an example of the cresyl violet stain of the probe tract).

HPLC

Measurement of plasma corticosterone levels. Plasma corticosterone levels were measured to verify that the swim stress indeed induced measurable physiological response. Following the swim stressor or unstressed controls described above, rats were decapitated and plasma corticosterone concentrations measured from trunk blood samples collected in 10 ml heparinised blood tubes. These samples were prepared and analysed (via HPLC) according to the method described by Viljoen et al. (59). The constituents of the mobile phase were: distilled water; acetonitrile and glacial acetic acid (65 : 35 : 0.05, v/v), with the pH of the mobile phase ranging from 4.10 to 4.20. The sample injection volume was 100 µl and a flow rate of 1.0 ml/min was implemented in a temperature controlled room (24°C). The eluent was monitored at a wavelength of 245 nm by the diode array detector, with a run time of ~15 min evident for each sample.

Measurement of monoamine levels in the pre-frontal cortex. Following drug treatments (SAL or FLX) on PnD21 to PnD34 described above, and thereafter following normal housing until PnD57, rats were moved to the microdialysis laboratory. Rats were subjected to microdialysis surgical implants, recovery, guide cannula placement and the acute swim stress. Pre-frontal cortical

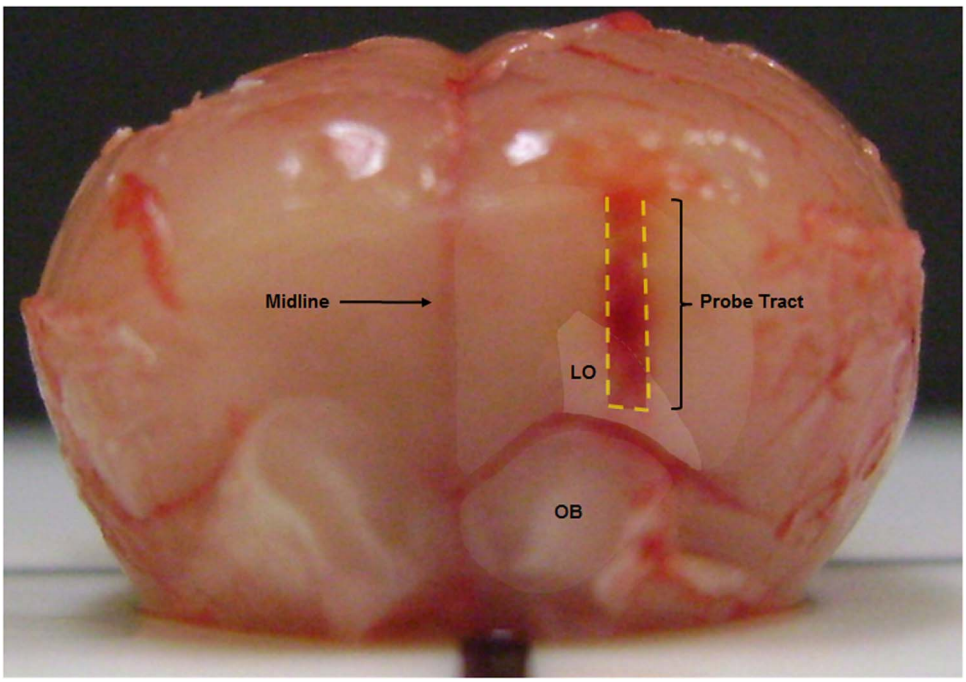

Fig. 2. Verification of the probe position by means of cresyl violet staining of the probe tract. LO = lateral orbital cortex and OB = olfactory bulb.

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monoamines were microdialysed and samples collected 60 min before, during and up to 3 h after the acute stressor.

Samples were collected in 100 µl HPLC glass inserts (Agilent, Santa Clara, CA, USA) placed in the refrigerated microfraction collector. 5 µl of a solution containing sodium metabisulphite (0.5 mM), ethylenediaminetetraacetic acid (EDTA) disodium salt (0.3 mM), 60% perchloric acid solution (0.25 M) and a 1500 ng/ml solution of isoproterenol hydrochloride obtained from Merck South Africa, Midrand, was added to each vial before sampling was initiated (60). Isoproterenol hydrochloride served as the internal standard. The glass inserts were placed into an amber vial, which was transferred to the HPLC autosampler rack. The MDTM mobile phase (ESA Inc., Chelmsford, MA, USA) was used [75 mM sodium dehydrogenate phosphate (monohydrate), 1.7 mM 1-octanesulfonic acid (sodium salt), 100 ml/l triethylamine, 25 µM EDTA, 10% acetonitrile, with pH adjusted to 3.00 with phosphoric acid]. The HPLC instrument used was an Agilent 1200 series HPLC equipped with an isocratic pump and autosampler set to a flow rate of 0.1 ml/min and injection volume of 20 µl, and coupled with a Coulochem III Electrochemical detector with a coulometric (5014B Dionex Microdialysis Cell by Thermo Scientific, Sunnyvale, CA, USA) flow cell. The software was programmed to inject 20 µl onto a Kinetix 2.6 µm C18, 100 Å, 150 × 4.6 mm (Phenomenex, Torrance, CA, USA) column. NE, DA, the DA end-stage metabolite homovanillic acid (HVA) and the 5-HT metabolite [5-hydroxyindole-3-acetic acid (5-HIAA)] chromatographs were identified by comparison with elution times of standards, with the second electrode set at +220 mV for measuring of the analytes. The 5-HT and the DA metabolite 3,4-dihydroxy phenylacetic acid levels were below the limit of detection. Results were expressed as ng/ml and converted to nM before data analysis.

Data analysis

The data are represented as means and standard error of the mean (SEM). First, independent two-tailed Student's *t*-tests were performed to compare the immobility data in the SAL control versus FLX-treated groups as well as the plasma corticosterone levels in stressed versus unstressed groups of FSL rats, and in general a *p*-value of <0.05 was considered statistically significant. GraphPad Prism® version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphical presentations of the corticosterone and behaviour data. Thereafter, hierarchical linear model (HLM) analyses were performed to examine the effect of FLX versus SAL-treated FSL rats over time on the monoamine concentrations in the pre-frontal cortex. Because different measurements over time were taken in the FSL rats, the dependency of measurements on the same rat had to be taken into account, where the rat was considered as primary unit of measurement and an autoregressive covariance structure, AR(1), was assumed. These analyses were performed on the microdialysis data of the monoamine levels between −60 and 0 min before the swim stress and again between the indicated time intervals (20–240 min for NE and DA and 60–300 min for 5-HIAA, as indicated in Fig 5) after the swim stress. For the latter analyses we used SAS System for Windows Release 9.3 TS Level 1M0, Copyright© by SAS Institute Inc. 2015, Cary, NC, USA.

Results

Behaviour

Figure 3 depicts the behaviour of FSL rats in the FST and OFT. It can be seen in Fig 3a that pre-pubertal SAL-and FLX-treated rats did not show any significant differences in scored behaviours in the FST.

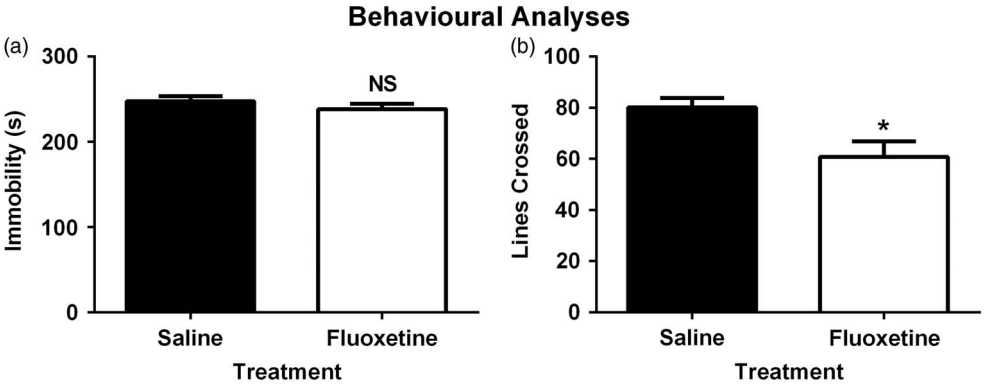

Fig. 3. (a) Immobility data of Flinders sensitive line (FSL) rats in the forced swim test (FST) after treatment with saline or fluoxetine. (b) Locomotor data of FSL rats in the open field test after treatment with saline or fluoxetine. Data points represent the mean and SEM, $n = 15$ rats/group (Student's *t*-test). s = seconds, NS = not significant, $^*p < 0.05$ (Student's *t*-test).

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Fig 3b shows a significant decrease in locomotor activity in FLX-treated FSL rats compared with SAL-treated rats (60.80 ± 5.962 vs. 80.13 ± 3.664 lines crossed, $p < 0.05$). No changes were, however, observed in the total time spent in the centre of the open field in FLX-treated FSL rats compared with SAL-treated rats (34.28 ± 4.92 vs. 26.83 ± 5.76 s, $p > 0.05$ – data not shown).

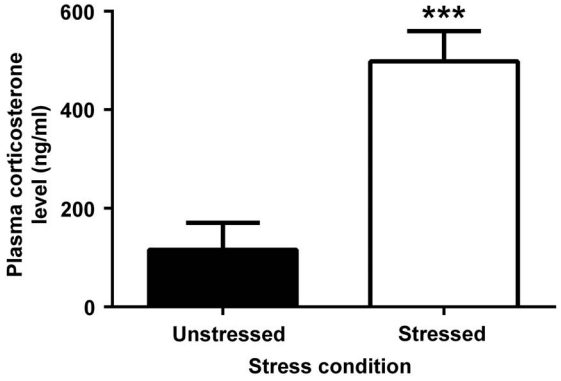

Fig. 4. Plasma corticosterone levels in Flinders sensitive line rats on PnD60 before and after the 10 min forced swim stressor, as measured with high-performance liquid chromatography. Data points represent the mean ± SEM, with $n = 7$ rats/group and $^{***}p < 0.001$ (Student's *t*-test).

Corticosterone stress response

Figure 4 depicts the plasma corticosterone levels in FSL rats on PnD60 before following a 10 min forced swim stressor. It can be seen in Fig 4 that exposure to the acute forced swim stress caused a significant increase (more than fourfold) in plasma corticosterone concentrations (115.6 ± 55.3 vs. 498.1 ± 61.5 ng/ml, $p < 0.001$).

Monoaminergic stress response

Figure 5 depicts the indicated monoamine concentrations in the pre-frontal cortex of FSL rats on PnD60 following pre-pubertal SAL or FLX treatment before, during and after the 10 min forced swim stressor. HLM analyses with group factor and time factor as repeated measures were performed on the data of the indicated monoamine levels (after SAL or FLX treatment) between −60 and 0 min before the swim (represented by shaded areas a0–d0) and again for different time intervals between 20 and 300 min after the swim (represented by shaded areas a1–d1).

In Fig 5a for SAL-treated control animals, the data from before the swim stress (−60–0 min; that is all

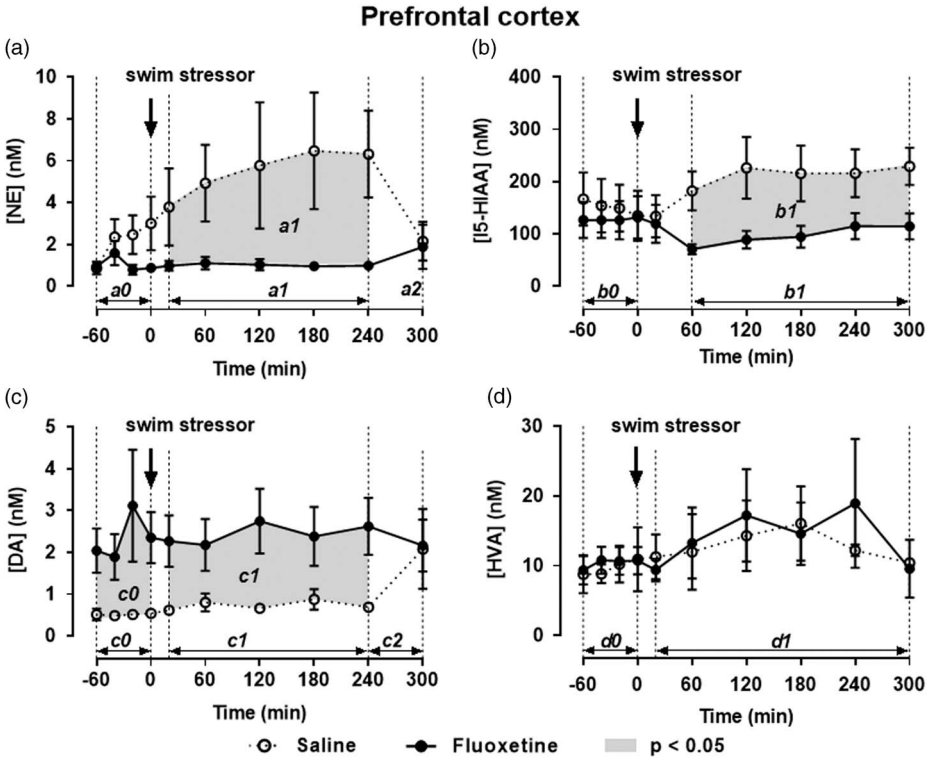

Fig. 5. Monoamine concentrations in the pre-frontal cortex of fluoxetine-treated versus saline Flinders sensitive line (FSL) rats. (a) [NE] = concentration of norepinephrine, (b) [5-HIAA] = concentration of 5-hydroxyindole-3-acetic acid, (c) [DA] = concentration of dopamine, (d) [HVA] = concentration of homovanillic acid. Data points represent the mean ± SEM, with $n = 5$ rats in the saline group and $n = 7$ in the fluoxetine group. Shaded areas indicate statistically significant differences ($p < 0.05$) as analysed with hierarchical linear model analyses with group factor and time factor as repeated measure.

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data points collected during the a0 timeframe before the stress) and after the swim stress (20–240 min; that is all data points collected during the a1 timeframe after the stress), indicate that the stressor significantly increase pre-frontal cortical NE concentrations (1.96 ± 1.21 vs. 5.68 ± 1.32 nM, $p = 0.0145$; HLM). In area a0, there was no significant interaction effect over time between SAL- and FLX-treated animals [$F(2, 23) = 1.42$, $p = 0.2613$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was no significant group effect [$F(1, 23) = 2.62$, $p = 0.1194$], indicating that the base-line NE levels of SAL- (1.93 ± 0.39) and FLX-treated (1.19 ± 0.33) animals were not statistically significantly different before the swim stressor. In Fig 5a, area a1 there was no significant interaction over time between SAL- and FLX-treated animals [$F(4, 37) = 0.45$, $p = 0.7732$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was a significant group effect [$F(1, 37) = 13.67$, $p = 0.0007$], indicating that NE levels in the pre-frontal cortex were significantly higher in SAL-treated animals (5.27 ± 0.98 nM) than in FLX-treated animals (1.33 ± 0.81 nM).

In Fig 5b for SAL-treated control animals, the data from before the swim stress (−60–0 min; that is all data points collected during the b0 timeframe before the stress) and after the swim stress (60–300 min; that is all data points collected during the b1 timeframe after the stress) in control animals, indicate that the stressor significantly increase pre-frontal cortical 5-HIAA concentrations (150.5 ± 43.6 vs. 196.7 ± 44.23 nM, $p = 0.0149$; HLM). In area b0, there was no significant interaction over time between SAL- and FLX-treated animals [$F(2, 24) = 0.14$, $p = 0.8700$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was no significant group effect [$F(1, 24) = 2.75$, $p = 0.1105$], indicating that the base-line 5-HIAA levels of SAL- (126.62 ± 36.53) and FLX- (178.10 ± 39.28) treated animals were not statistically significantly different before the swim stressor. In Fig 5b, area b1 there was no significant interaction over time between SAL- and FLX-treated animals [$F(4, 34) = 0.43$, $p = 0.7864$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was a significant group effect [$F(1, 34) = 8.76$, $p = 0.0056$], indicating that 5-HIAA levels in the pre-frontal cortex were significantly higher in SAL-treated animals (217.12 ± 36.90 nM) than in FLX-treated animals (126.06 ± 32.85 nM) after the swim stressor.

In Fig 5c, area c0 shows there to be no significant interaction over time between SAL- and FLX-treated animals [$F(2, 24) = 0.45$, $p = 0.6435$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was a significant group effect [$F(1, 24) = 8.83$, $p = 0.0066$], indicating that base-line DA levels in the pre-frontal cortex were significantly higher in FLX-treated animals (2.35 ± 0.49 nM) than in SAL-treated animals (0.40 ± 0.57 nM). In Fig 5c, area c1 shows there to be no significant interaction over time between SAL- and FLX-treated animals [$F(4, 34) = 0.97$, $p = 0.4388$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was a significant group effect [$F(1, 34) = 4.31$, $p = 0.0455$], indicating that DA levels in the pre-frontal cortex were significantly higher in FLX-treated animals (2.41 ± 0.54 nM) than in SAL-treated animals (0.90 ± 0.66 nM) after the swim stressor. In Fig 5c, area c2, it can be seen that the cortical DA levels of SAL- and FLX-treated animals merged, blunting the original differences in base-line DA levels.

In Fig 5d, area d0 shows there to be no significant interaction over time between SAL- and FLX-treated animals [$F(2, 24) = 0.65$, $p = 0.5330$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was no significant group effect [$F(1, 24) = 0.84$, $p = 0.3683$], indicating that base-line HVA levels in the pre-frontal cortex were not significantly lower in SAL-treated animals (9.36 ± 8.46 nM) than in FLX-treated animals (19.55 ± 7.15 nM) before the swim stressor. In Fig 5d, area d1 shows there to be no significant interaction over time between SAL- and FLX-treated animals [$F(4, 32) = 0.17$, $p = 0.9543$], indicating that SAL- and FLX-treated animals did not respond differently over time. There was no significant group effect [$F(1, 32) = 0.06$, $p = 0.8121$], indicating that HVA levels in the pre-frontal cortex were not significantly different in SAL-treated animals (15.17 ± 3.56 nM) than in FLX-treated animals (15.88 ± 3.11 nM) after the swim stressor.

Discussion

Corticosterone stress response

The significant increase in plasma corticosterone levels in rats exposed to the acute forced swim stressor, as compared with the unstressed controls (Fig 4), suggest that the stressor induces a significant biological stress response. Furthermore, the data also confirms that the physiological stressor response via the hypothalamic-pituitary-adrenal (HPA) axis remained intact in rats after guide cannula placement surgery and exposure to halothane for microdialysis probe placement. Corticosterone has been shown to facilitate FLX-induced neuroplasticity (61), however the current study did not investigate the effect of

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FLX on the cortisosterone levels, so that this warrants further investigation.

Developmental effects of pre-pubertal FLX administration on behaviour

Depressive-like behaviour. No significant differences were seen between FLX and SAL-treated FSL rats with regard to time spent immobile in the FST. The data would suggest that pre-pubertal FLX does not affect typical depressive-like behaviour of FSL rats in early adulthood. However, immobility data should be interpreted together with locomotor data, as discussed below.

Locomotor activity. Pre-pubertal FLX significantly reduced locomotor activity in FSL rats, relative to SAL-treated animals. The activation of 5-HT_{1A} (62,63) and 5-HT_{2A} (64,65) receptors is known to increase locomotor activity, whereas 5-HT_{2C} receptor activation has been shown to decrease locomotor activity (64–66). Developmental changes induced by FLX in either or both of these systems may underlie the reduced locomotor activity seen in the open-field. In particular, pre-adolescent FLX may have altered relative expression of serotonergic receptors and an increased 5-HT_{2C} receptor number or a decrease in 5-HT_{1A} and/or 5-HT_{2A} receptor number relative to each other may explain these effects. So although we did not investigate receptor expression in this regard, suffice to say that pre-pubertal FLX exposure *does* engender late-life neurodevelopmental effects, which warrants further investigation, especially any resulting effects on neuroreceptor expression.

Reduced locomotor activity in FLX-treated animals would be expected to enhance immobility in the FST. However, this was not observed. We postulate as working hypothesis for further evaluation that pre-pubertal FLX may have induced marginal long-term anti-depressant-like behaviour into early adulthood, but that this psychomotor response may be blunted (masked) by the simultaneous reduction in locomotor activity.

Anxiety-related behaviour. No FLX-induced change in anxiety-related behaviour (i.e. time spent in the centre of the open field) was observed. These findings are in keeping with the fact that increased anxiety-like behaviour is not a feature typically/consistently observed in FSL rats (67,68), at least not using the current method of behavioural analysis, although we recently demonstrated that desipramine and escitalopram reduce anxiety-like behaviour in FSL rats in a different treatment scenario (69).

Developmental effects of pre-pubertal FLX administration on monoaminergic stress response

The interdependence of monoaminergic neurotransmission and the HPA-axis has been well described and acute stress may induce brief activation of the HPA-axis, resulting from the stress response involving the catecholaminergic (CA) systems, such as of NE and DA neurons in the brain (25). After cessation of the stressor, homoeostasis is usually restored, although the nature and intensity of the stressor may determine whether normal CA functioning will in fact be restored. During stress sustained over long periods of time the synthesis of CAs may not meet the demand placed on the system, resulting in reduced CA levels available for release at the synapse (25). Reuter et al. reviewed the evidence of the effect of stressors of various types on extracellular 5-HT concentrations and found that 5-HT concentrations increase by 30–100% following the introduction of acute stress in virtually all the brain areas studied (27). Indeed, our microdialysis data indicate that acute swim stress on PnD60 produced a significant increase in NE and 5-HIAA, where the latter relates to serotonergic neurotransmission.

The microdialysis data of the current study indicate that pre-pubertal FLX administration caused a statistically significant and lasting attenuation of the NE release in response to an acute stressor when compared with untreated rats (Fig 5a). Such a developmental effect may be desirable, as it suggests that pre-pubertal FLX prevents an excessive noradrenergic stress response. However, the data also suggest the potential to induce other long-lasting effects on noradrenergic function and it is not clear whether this may represent other long-term maladaptive noradrenergic stress responses, potentially impairing an appropriate response to environmental stressors later in life.

The microdialysis data indicates a statistically significant blunting of the serotonergic response (as represented by the 5-HT metabolite 5-HIAA) in FLX-treated animals (Fig 5b) 1 h after the forced swim stressor was applied, which lasts for the duration of the experiment (i.e. 5 h). This response was delayed for >20 min, different from the noradrenergic response. The serotonergic stress response data suggests a similar adaptation has occurred in the serotonergic system as described for the noradrenergic system, with similar implications for the positive or maladaptive nature of these developmental changes. 5-HIAA levels have previously been correlated with 5-HT function (70) and was therefore deemed adequate as an indicator of 5-HT-ergic function in the current study.

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Pre-pubertal FLX treatment appears to have significantly increased baseline DA release when compared with SAL control rats in the absence of acute stressor (Fig 5c). The long-lasting FLX-induced increase in baseline DA levels, as observed in our study, may represent normalisation of DA neurotransmission. In fact, this is in agreement with previously reported reduction in DA D_2 receptor expression in FSL rats (33) and reduced DA baseline extracellular levels in the nucleus accumbens in FSL versus control Sprague–Dawley rats (71–73). In addition, DA levels are increased in FSL rats following sub-chronic antidepressant treatment (72,73). The dopaminergic stressor response also appears to be attenuated in these rats, consistent with previous studies demonstrating that 15 min forced swimming increases DA levels in the nucleus accumbens of Sprague–Dawley, but not FSL rats (74). In SAL-treated animals no significant dopaminergic stressor response was apparent, although at 300 min after the swim stress SAL-treated rats and FLX-treated rats displayed the same concentrations of DA released in the pre-frontal cortex. No dopaminergic stressor response was apparent in FLX-treated animals. Impaired DA neurotransmission has been associated with depression, stress and anhedonia (75), albeit without direct evidence of impaired DA release. In the current study the increase in DA levels in the pre-frontal cortex of FSL rats treated with FLX during pre-puberty may therefore represent a positive developmental adaptation with regard to depressive-like behaviour. The lack of a dopaminergic stress response is however concerning as it may also potentially represent, as is the case for noradrenergic and serotonergic neurotransmission, a maladaptive response conceivably associated with inhibition of appropriate dopaminergic responses to environmental stressors later in life. The lack of significant corresponding differences in HVA concentrations (Fig 5d) suggests that the developmental change may have occurred within the re-uptake machinery of the dopaminergic neurons. In fact, altered HVA levels have been shown in humans and in rats, including FSL rats, not necessarily to mirror altered DA levels (76–78). It has been shown before that NET and DA transporters (DAT) have the ability to transport both NE and DA (79). In fact, any changes in the expression and distribution of DAT or NET in the brain may contribute to altered DA concentrations in the pre-frontal cortex, as seen with FLX-treated FSL rats. Non-specific transporters known as organic cation transporters (OCTs) are also present in the brain (80,81). Three subtypes of OCTs (i.e. OCT1, OCT2 and OCT3) are found in the brain (82–84), the third of which has been shown to be inhibited by corticosterone and may therefore result in increased brain monoamine concentrations under conditions of stress (81,82). As the corticosterone concentrations were shown to be significantly elevated in FSL rats exposed to a 10 min forced swim stress, OCTs may also have been affected to reduce DA reuptake and increasing DA concentration in the pre-frontal cortex of FLX-treated FSL rats.

Conclusions

To our knowledge, the data presented here is the first evidence of significant developmental effects of pre-pubertal FLX administration on acute monoaminergic stress responses later in life. Although results hinted towards positive (desired), rather than negative long-term effects, it cannot be excluded that the neurodevelopmental changes may also affect the individual's ability to cope successfully with acute environmental stressors later in life. The clinical implication of the data should therefore be interpreted with caution. Significant changes were also observed in locomotor activity between FLX-treated FSL rats and SAL control rats, supporting the notion of long-term neurodevelopmental changes. Prospective studies should investigate the role of FLX in the expression and distribution of the serotonergic receptor subtypes (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}), the development and distribution of monoamine transporters (DAT, NET and OCTs), as well as the mechanisms by which 5-HT might influence and regulate the development of the noradrenergic and dopaminergic neurodevelopment.

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Conflicts of Interest

Brian Harvey has participated in advisory boards and received honoraria from Servier®, and has received research funding from Servier® and Lundbeck®.

The authors declare that no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional services, other than the MRC, NRF and that noted above, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. The remaining authors have no conflicts of interest to declare.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. All animals were maintained and all procedures performed in studies involving animals were in accordance to the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation. This article does not contain any studies with human participants performed by any of the authors.

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