Original Paper

Developmental Neuroscience

Dev Neurosci 2014;36:396–408
DOI: 10.1159/000363102

Received: January 7, 2014
Accepted after revision: April 22, 2014
Published online: August 6, 2014

Increased Aggression, Improved Spatial Memory, and Reduced Anxiety-Like Behaviour in Adult Male Mice Exposed to Fluoxetine Early in Life

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Key Words
Selective serotonin reuptake inhibitor · Fluoxetine · Serotonin · Exposure, perinatal · Pregnancy · Development · Aggression · Anxiety · Mouse · Behaviour

Abstract
Rationale: Fluoxetine (Flx; brand names Prozac, Sarafem, Rapiflux), a selective serotonin reuptake inhibitor, is prescribed for the treatment of depression in pregnant women; however, this commonly prescribed medication could affect fetal brain development as Flx crosses the placenta. The available data concerning the anatomical and behavioural consequences of perinatal exposure to Flx during early development on adult behaviour are limited and often contradictory. ***Objectives:*** To further delineate the long-term behavioural effects of altering 5-HT during development, we examined the effects of perinatal Flx exposure on the behaviour of male mice as adults. ***Methods:*** Dams were treated with approximately 25 mg/kg/day of Flx from embryonic day 15 to postnatal day 12, and the behaviour of the adult offspring was assessed. ***Results:*** We found that perinatal Flx exposure leads to increased aggression, improved spatial memory, and reduced anxiety-like behaviour. This exposure did not cause memory deficits, changes in sensory processing, or changes in gross motor function. ***Conclusions:*** Our results suggest that while perinatal exposure to Flx may have long-term effects on adult behaviour, these effects appear limited and not necessarily detrimental.

Introduction

Women of childbearing age are the population at the highest risk of depression [1]. Selective serotonin reuptake inhibitors (SSRIs) are the medication of choice for the treatment of depression and other psychiatric disorders, including during pregnancy [2]. Fluoxetine (Flx) is the most widely used SSRI, with up to 2.1% of all pregnant women using Flx throughout or at some point during pregnancy [3–5]. Flx inhibits serotonin/5-HT reuptake, thereby increasing the levels of serotonin at the synapse. The high percentage of pregnant women taking SSRIs and Flx is potentially of concern because Flx and its metabolite norfluoxetine (NorFlx) cross the placental barrier and are excreted in breast milk, exposing the fetus and/or newborn to this pharmacological substance [6–9].

In early developmental stages, serotonin acts as a developmental cue. Serotonin regulates neuronal growth cone motility [10, 11] and is involved in various developmental processes such as neuronal and glial proliferation, neuronal differentiation, formation, and migration, synaptogenesis, axon myelination, and the regulation of apoptotic cell death [12–14]. If perinatal Flx exposure alters the activity of the serotonergic system of the fetus, it has the potential to affect normal neurodevelopmental processes and lead to altered brain organization and, consequently, behaviour.

Human studies have not examined the outcomes of individuals prenatally exposed to Flx beyond the pre-

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school age [for a review, see 15]. While more laboratories are beginning to examine the developmental outcomes of perinatal SSRI exposure using animal models, these studies are few [for a review of the subject, see 16]. Following perinatal exposure to Flx, changes observed in adult mice and rats are not always disadvantageous. Flx-exposed rats sometimes perform better than controls, demonstrating better coordination and balance, superior emotional and spatial memory in adolescence [17], and better emotional memory in adulthood [18]. Other findings include decreased anxiety [19], reduced impulsivity [20], and less depressive behaviour [19–21] in adult mice. Rats also show fewer depression-like behaviours [22]. Following early exposure to Flx, adult mice freeze more during the light-dark box test [21], spend more time in thigmotaxis during the open field test [23], and demonstrate an increased latency to feed in the novelty-suppressed feeding test [23, 24]. Similarly, Flx-exposed rats show an increased latency to feed in the novelty-suppressed feeding test as adults [18]. Changes in freezing, thigmotaxis, and feeding are thought to be indicative of increased anxiety-like behaviour. Previous studies of mice have demonstrated decreased sexual motivation following early exposure to Flx [25] and alterations in circadian behaviours [26]. Studies of rats have indicated changes in copulatory behaviours [27, 28], decreased social exploration [18], increased vulnerability to the reinforcing effects of cocaine [29], and increased aggression [30]. Changes in depression-like behaviours were noted in both mice [20, 21] and rats [22].

The present study was carried out to evaluate the effects of perinatal exposure to Flx on the behaviour of mice in adulthood. We examined aggressive behaviour, anxiety-like behaviour, exploration, sensory-motor gating, gross motor function, as well as emotional, spatial, working, and retention memory. All of these behaviours are, at least in part, modulated by serotonin. Both monoamine oxidase A (humans [31]; animals [32–34]) and serotonin transporter dysfunction (humans [35–37]; animals [38, 39]) increase serotonin during development and lead to changes in the aforementioned behaviours, particularly anxiety and aggression.

Animals and Methods

Animals

All experimental procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary. The animals were housed in the colony room under a 12-hour light/12-hour dark cycle. Throughout the entire experiment, the animals were housed in a temperature- and humidity-controlled room, with ad libitum access to food (standard laboratory chow) and water. Animal treatment and husbandry was in accordance with the Canadian Council on Animal Care.

C57BL/6 breeders were obtained from the University of Calgary Biological Sciences breeding colony (Calgary, Alta., Canada; the original colony was supplied by Charles River Laboratories International, Wilmington, Mass., USA). Breeding pairs were housed together until the vaginal plug was observed, or until day 4, whichever came first. The day of seminal plug detection was designated as embryonic day (E) 0. Litter sizes were standardized by culling each litter to 8 pups on the first day after birth. Pups were housed with their mothers and littermates until weaning at postnatal day (P) 21, after which they were separated and housed with 1 or 2 same-sex littermates. For the behavioural analyses, 16 male mice were generated from 4 dams exposed to Flx, and 16 more male mice were generated from 5 dams not exposed to the drug. Eleven additional animals (6 control, 5 Flx exposed) generated for another purpose from 2 control and 2 Flx-exposed dams were used in the elevated plus maze.

Drug Treatment

Flx hydrochloride (Sigma) was administered to the mice via the drinking water, a method that has a number of advantages over gavage or injection. First, this is one of the least stressful methods of drug administration. Second, Flx is also currently administered orally to humans. Third, while in humans the half-lives of Flx and NorFlx are 1–3 and 7–15 days, respectively, the half-lives in mice are 6 (Flx) and 12.3 h (NorFlx) [40]. Because Flx metabolism is much faster in mice than in humans, continuous drug administration would lead to more stable blood drug levels, which is preferable [41]. Despite the natural fluctuations in water consumption throughout the day, oral drug administration can provide more stable drug blood levels than injection or gavage [41].

In addition to being less stressful and leading to more stable blood drug levels, the oral administration of Flx is an effective delivery method of the drug. Flx administered orally is detected in the blood plasma of the animal [42]. The ability of SSRIs, in particular Flx, to cross the placental barrier is similar in humans and animals [rats: 18; mice: 23; sheep: 43]. Just as in humans, SSRIs readily cross the fetal blood-brain barrier in rodents [18, 24].

In humans, the therapeutic dose of Flx that is prescribed falls between 20 and 80 mg/day (0.25–1.00 mg/kg). A safety factor of 100 is often used to determine the limits of safe exposure in humans, based on animal data. This factor allows for 10-fold interindividual variability and 10-fold interspecies differences [44]. If using a safety factor of 100, the minimal dose that should be used in animals, to be compatible with human Flx exposure, is approximately 25 mg/kg, and this was therefore chosen as the dose for this experiment. The pregnant dams were administered 25 mg/kg/day of Flx from E15 to P12 (an equivalent of the human nervous system development during the 2nd and the 3rd trimester of human gestation [45, 46]). The day of birth was designated as P0. The concentration of Flx in the water was calculated every 48 h for each dam, using the dam's weight and water consumption over the previous 48 h. To remain consistent, the dams from the control litters were also weighed, and their drinking water was changed every 2 days from E15 to P12.

Measurement of Flx, NorFlx, and Serotonin in Pup Brains

Sample Collection

Pup brains were collected from male and female pups at P0 (control: n = 6, from 3 litters; Flx: n = 9, from 4 litters) and P12 (control: n = 6, from 3 litters; Flx: n = 6, from 3 litters). After decapitation, the brain was removed, the spinal cord was severed posterior to the cerebellum, and the olfactory bulbs were removed. Cortical structures were peeled away from the thalamus. For the brains dissected at P0, cortical (cortex and hippocampus) and subcortical structures (thalamus, midbrain, hindbrain, and cerebellum) were individually flash-frozen in 1.5-ml microcentrifuge tubes. For the P12 brains, after the cortical structures were peeled away from the underlying thalamus, the subcortical pieces were bisected between the cerebellum and inferior colliculus, and the three resulting brain areas were separately frozen and analysed separately as (1) cortex and hippocampus, (2) thalamus/midbrain, and (3) hindbrain/cerebellum.

Sample Preparation

The sample preparation method was based on Raap et al. [47]. The samples were homogenized using an ultrasonic probe tissue disruptor (Virtis VirSonic 50) in 10 volumes (1 ml/100 mg tissue) of ice-cold acetone (1 M formic acid, 85:15) containing 100 ng/ml norfluvoxamine as an internal standard. They were then centrifuged at high speed (15,000 rpm, 5 min), and 200 µl of supernatant was transferred to a clean centrifuge tube and dried down at room temperature in a vacuum centrifuge (about 20 min). Then, they were reconstituted in 100 µl of 50% methanol.

Sample Analysis

The concentrations of Flx, NorFlx, and serotonin were determined by high-performance liquid chromatography (HPLC). Quantitative standards were obtained from Dr. Glen Baker. These were serotonin creatinine sulphate (Sigma), Flx hydrochloride (Lilly), NorFlx malate (Lilly), and fluvoxamine malate (Solvay Duphar). The HPLC system used was an HP 1100 device equipped with a refrigerated autosampler, a column heater, a diode array detector, and an LC/mass spectrometry detector. Twenty microlitres of reconstituted sample were injected into the LC instrument. Separation was obtained in a column [Phenomenex Luna 3m C18(2) 100A, 15 × 4.60 mm] equipped with a guard column (SecurityGuard C18). The column heater was set at 40°C. The mobile phase consisted of 5 mM ammonium acetate (pH 3) with formic acid in 18-MΩ water and HPLC-grade acetonitrile. The flow rate was maintained at 1 ml·min^{-1}. The UV absorbance signals were collected at 205 nm (bandwidth 8), 220 nm (bandwidth 16), 250 nm (bandwidth 8), 270 nm (bandwidth 8), and 300 nm (bandwidth 8). The electrospray mass-spectrometric conditions were maintained in positive mode (gas temperature: 350°C; drying gas: 13 l/min; nebulizer pressure: 60 psig; vaporizer: 350 °C; fragmentor: 70; capillary voltage: 3,000 V). Single ions were monitored from 1 to 7 min at 177.2 (MH^+ serotonin), from 7 min onward on SIM channel 1 at 310.1 (MH^+ Flx) and 319.2 (MH^+ fluvoxamine), and from 7 min onward on SIM channel 2 at 296.1 (MH^+ NorFlx). The limits of detection were 5 ng/g for serotonin, 0.009 µg/g for Flx, and 0.005 µg/g for NorFlx.

Maternal Behaviour

The pup-retrieving task was used to evaluate the maternal motivation of Flx-exposed and control dams [48]. This task was conducted when the pups were 6.5 days of age. The mother and the pups were removed to separate cages. Ten minutes later, the pups were returned to their home cage and placed in the corner diagonally opposite to the nest. The test began when the mother was reintroduced to the home cage. The latency to retrieve the first pup into the nest was recorded. The pup was counted as retrieved once completely brought to the nest. The test was terminated after 15 min.

Pup Evaluation

The righting response of the pups, which is often used to assess motor development, was examined at P7 [49]. For this evaluation, all the male pups born to control and experimental dams were used. For this test, the mother was moved to a clean cage. The cage that contained the pups was placed on a heating blanket. One pup was then removed from the nest and placed supine on a smooth horizontal surface and released. The animals were allowed up to 30 s to resume an upright position. The time to resume an upright posture was recorded. At the end of the test, the pup was weighed and placed back in its cage.

Behavioural Analysis

The order of tests was as follows: elevated plus maze; open field; resident intruder; horizontal ladder; Morris water test (MWT) spatial, retention and working memory; prepulse inhibition, and fear conditioning. All testing was conducted during the light phase of the cycle, between 10 a.m. and 5 p.m. Behavioural testing commenced at 2 months of age. The tests were conducted 6–7 days apart, except for the resident intruder test, which was conducted 10 days after the preceding behavioural test.

Elevated Plus Maze Test

The elevated plus maze test was conducted as previously described [19, 50]. The following measures were analysed: the number of entries onto open and enclosed arms (a measure of general activity) and the percentage of the total time spent on the open arms (a measure of anxiety-like behaviour). The definition of an arm entry is a mouse placing all four paws on a new arm [51]. Additionally, scanning and risk assessment behaviours were quantified. Scanning was measured by recording the head-dipping behaviour (head over the side of the open arm of the maze). Increased head-dipping behaviour is indicative of higher exploratory activity [52]. Risk assessment was measured by tracking the number of protected stretches, which is characterized by the animal exiting an enclosed arm or the central space of the maze with its head and forepaws only, placing the forepaws onto the open arm and investigating the surroundings [53, 54].

Open Field Test

The open field arena consisted of a white circular Plexiglas surface (1.2 m in diameter) surrounded by a wall (35 cm high) and illuminated by an overhead light (170 ± 30 lux) [19]. The animals were placed in the centre of the arena and allowed to freely explore the area for 5 min, and their activity was recorded with an overhead-mounted video camera and analysed using video tracking software (HVS Image 2020 Plus). The time spent close to the walls in the periphery (thigmotactic behaviour), the travel speed, and the distance travelled were analysed.

Resident Intruder Test

To examine aggressive behaviour, the resident intruder test was used [55, 56]. In this model, the resident mice were separated from their cage mates and isolated in separate cages for 10 days prior to

the experiment. To make the territorial cues more prominent, cage bedding was not changed during the time of isolation. The intruders were male C57BL/6 mice that were obtained from the University of Calgary Biological Sciences breeding colony. They were group housed prior to the test. The residents and intruders were matched by weight and age, allowing for a weight difference of no more than 5 g [57]. An intruder was placed into a resident's cage, and the test session was started immediately. The 10-min encounter was videotaped (Sony DCR-TRV10) and analysed off-line. The latency to the first attack, the total number of attacks, and the duration of each attack were measured [34, 58, 59].

Horizontal Ladder Test

A modified version of the horizontal ladder test was used to assess gross motor skills [60]. This test has been shown to provide a reliable measure of motor skills in rodents and is able to detect motor impairments [61]. The test apparatus consisted of a clear plastic alley 5 cm wide, 100 cm long, and 22 cm elevated from the ground with 100 round, evenly spaced, removable metal rungs. One animal was placed on one end of the alley and required to run to the opposite end, where its home cage was placed. The animal was allowed to cross the alley 3 times, with all the rungs in place. One hour later, the animal's performance was assessed in 2 trials, one with no rungs removed followed by a second trial (30 min later) where 13 out of 50 rungs located in the middle of the alley were removed. The same rungs were removed for all animals. The criteria for rung removal were as follows: the first two and the last two rungs could not be removed, and no two adjacent rungs could be removed. The sessions were digitally recorded (Sony DCR-TRV10) and analysed off-line. The latency to cross the alley and the number of foot faults made while crossing the central half of the alley were measured and analysed. A foot fault was defined as a mouse placing its paw in between the rungs, often visibly breaking the mouse's balance and stride rhythmicity. The latency to escape was calculated as the total time taken to reach the end of the alley minus the time the animal spent grooming, moving backward, or regaining distance lost by moving backward.

MWT Spatial Memory

The MWT procedure was conducted as previously described [62]. The pool (1.2 m in diameter) consisted of a circular plastic tub painted white with no seams or marks on the inside wall. Water (20°C) was filled to a depth of 20 cm, and the square platform was placed in the pool 1.5 cm below the water's surface. The pool was rendered opaque by the addition of 3 cups of skim milk powder. Visual cues consisted of those endogenous to the testing room, including a number of posters of different shapes and colours placed on the walls of the room.

The MWT was conducted every day for 5 days. On the first 4 days, the mice were given 4 trials/day. For each trial, a mouse was placed into the water tank facing the wall at one of the cardinal compass points, chosen in a pseudorandom order. The latency to reach the platform was recorded, and if the animal failed to reach the platform during the trial, a maximum latency of 60 s was recorded. If the mouse failed to find the platform within 60 s, it was placed on it. At the end of each trial, the mouse was left on the platform for 15 s and then returned to its cage. The intertrial interval was approximately 10 min. On the fifth day, the platform was taken out of the pool, and the mouse was placed directly opposite from the previous location of the platform. The mice were given 1 trial where they were allowed to explore the tank for 30 s, and the amount of time spent in each quadrant was analysed. The mouse's swim path was recorded with an overhead-mounted video camera connected to a computer and analysed using a tracking programme (HVS Image 2020 Plus).

MWT Retention Memory

In addition to spatial memory, the MWT was used to test retention memory. To examine retention memory, the average latency to reach the platform on the last MTW spatial memory test day was compared with the average latency to reach the platform 7 days later. The latter test was identical to the test conducted on the last training day of the MWT spatial memory.

MWT Working Memory

The working memory MWT procedure examines the ability of a mouse to integrate new spatial information. This test was similar to the MWT described above, with the exception that the underwater platform was moved at the start of each day. The MWT working memory was conducted over 3 days, with 4 trials/day, where, at the start of each day, the platform was placed in a novel location. In this test, the first trial is called the sample trial, during which the animal has to find the platform by trial and error. The last trial is the test of the memory for the location of the platform learned from the 3 preceding trials. The time to find the platform in the last trial should be shorter if the animal recalls the first trial. The difference in latency to find the platform between the first and the last trial for the last 3 days was analysed.

Prepulse Inhibition

Acoustic startle response and prepulse inhibition were evaluated using the SM100SP Startle Monitor system (Hamilton-Kinder LLC, San Diego, Calif., USA). This system consists of a high-quality laminate and medium-density fibreboard sound-attenuated chamber (27.6 × 35.6 × 49.5 cm) supplied with a sensory monitor that measures the displacement of an animal's body, providing a measure of the startle response. The mice were restrained in a clear Plexiglas chamber (10 × 3.8 cm). A piezoelectric device located below the container transformed whole-body startle response movements into an analogue signal. The analogue signal was then transformed into a digital signal and analysed by the Startle Monitor software. The experimental session consisted of a 3-min acclimatization period to a steady 65-dB background ambient noise followed by a habituation phase in which every 5–20 s a noise elevation from 65 to up to 120 dB (duration of 40 ms) was delivered 10 times. The habituation phase was followed by a test session. During the test session, three types of trials were presented 10 times each at 5- to 20-second intervals: no pulse (40 ms, 65 dB), pulse alone (40 ms, 120 dB), and prepulse stimuli (a 40-ms 120-dB pulse preceded by a 20-ms 80-dB pulse with 100 ms in between). The average startle response was calculated by analysing the response to the pulse alone. Prepulse inhibition was calculated by the formula 100% × (pulse alone – prepulse plus pulse)/pulse alone.

Fear Conditioning

The fear conditioning paradigm, which operates on principles of Pavlovian conditioning, was used to examine emotional memory. The fear conditioning test was conducted as previously described in McAllister et al. [19], except that testing during day 3

was modified. On day 3, the mice were returned to the conditioning chamber for 2 min in the absence of both tone and foot shock. They were taped with an overhead digital camera (Sony DCR-TRV10). The freezing behaviour (absence of movement except that necessary for breathing) in response to the original conditioning context was manually scored during the entire session.

Data Analysis

For tasks that involved repeated testing of the animals (i.e., serotonin levels, water consumption, MWT, fear conditioning, and horizontal ladder test), a repeated-measures ANOVA was computed. If Mauchly's test of sphericity was violated, it was corrected with the Greenhouse-Geisser correction. Protected t tests using the Bonferroni correction were used for all multiple comparisons among group means. Tests that did not involve repeated testing (i.e., litter size and latency to retrieve the first pup) were analysed using an independent-sample t test. If Levene's test for equality of variances was violated, homogeneity of variance was not assumed in the statistical calculations. To examine whether litter effects contributed to any of the significant findings, all significant treatment effects were followed up ($p \leq 0.05$) with a nested ANOVA; this assessed the effect of litter nested within group. We failed to find any significant litter effects ($p > 0.10$; data not shown). If two categorical variables had to be analysed [i.e., righting response and attack behaviour (attacked or not)], a χ^2 analysis was computed. Unless otherwise indicated, all statistics were two-tailed. Values of $p \leq 0.05$ were considered significant. All data and values on the figures are reported as means ± SEM.

Results

Flx, NorFlx, and Serotonin Levels in the Pup Brains

At P0, the average concentration of Flx in the whole brain of Flx-exposed pups was 3.13 ± 0.67 μg/g; however, the concentration in the subcortical structures (3.44 ± 0.71 μg/g) was higher than in the cortical structures (2.84 ± 0.64 μg/g; $F_{1,8} = 9.98$, $p = 0.013$). The average brain concentration of NorFlx was 8.33 ± 0.36 μg/g, but the concentration in the subcortical structures (9.34 ± 0.40 μg/g) was also higher than in the cortical structures (7.09 ± 0.31 μg/g; $F_{1,8} = 253.44$, $p < 0.001$). Flx and NorFlx were not detected in any of the control mice at P0 or P12.

In the P0 animals, the concentration of serotonin in the subcortical structures (147.23 ± 6.01 ng/g) was higher than in the cortical structures (64.92 ± 2.91 ng/g) regardless of the treatment condition ($F_{1,13} = 302.35$, $p < 0.001$). There was no effect of treatment or treatment-by-brain-region interaction on serotonin concentrations in the brain.

In the P12 animals, the average concentration of Flx in pup brains was 0.515 ± 0.13 μg/g, and that of NorFlx was 4.15 ± 0.14 μg/g. The Flx and NorFlx concentrations did not differ between brain areas.

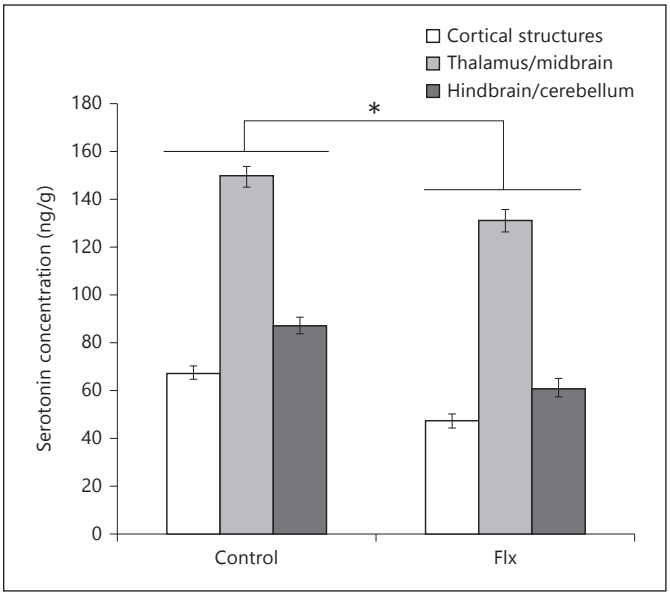

Fig. 1. Serotonin concentrations in the cortex, thalamus/midbrain, and hindbrain/cerebellar regions of P12 control and Flx-exposed mice. * $p < 0.05$, significant difference between the groups.

At P12, Flx treatment led to a significant reduction in the average concentration of serotonin (control: 101.10 ± 2.45 ng/g; Flx exposed: 79.61 ± 2.69 ng/g; $F_{1,9} = 34.94$, $p < 0.001$; fig. 1). There also was a significant effect of brain region on serotonin concentration ($F_{2,18} = 339.61$, $p < 0.001$; fig. 1). Post hoc comparisons revealed that the serotonin concentration differed significantly between all the brain regions examined ($p \leq 0.003$), such that the serotonin concentration was highest in the thalamus/midbrain region (140.00 ± 2.04 ng/g), second highest in the hindbrain/cerebellum (73.92 ± 2.71 ng/g), and lowest in the cortical structures (57.15 ± 2.04 ng/g). There was no treatment by brain region interaction on serotonin concentrations in the brain of P12 pups.

Flx Dose and Water Consumption

The water consumption of the dams was monitored from E15 to P12. There was a main effect of day indicating that the control and Flx-treated dams increased their water consumption between E15 and P12 ($F_{16,112} = 35.79$, $p < 0.001$). Notably, there was no significant difference in water consumption between the control and Flx-treated animals, and there was no treatment-by-day interaction. While the intended Flx dose was 25 mg/kg, due to increased water consumption throughout pregnancy and lactation, the actual mean Flx dose consumed by each dam was 28.60 ± 2.05 mg/kg/day.

Number of Pups Born and Offspring Weight

There were no differences in the mean number of pups born from control and Flx-exposed dams. At P7 and P21, the weights of all the male and female pups born to 4 Flx-exposed and 5 control dams were analysed. At P7, the Flx-exposed male (control: 3.78 ± 0.19 g; Flx-exposed: 4.40 ± 0.14 g) and female pups (control: 3.92 ± 0.12 g; Flx-exposed: 4.50 ± 0.21 g) weighed more than the pups not exposed to Flx (male: $t_{34} = 2.58$, $p = 0.014$; female: $t_{19} = 2.53$, $p = 0.020$). There was no weight difference between control and Flx-exposed male and female pups at weaning.

Because the animals were marked at week 8 and could, therefore, be identified individually after that point, a repeated-measures ANOVA was conducted to examine the difference in weight between control and Flx-exposed male offspring that were used for behavioural testing. Weight was measured at week 8 (beginning of behavioural tests) and week 16 (close to the completion of behavioural tests). As expected, there was a significant effect of age on the weight of male mice ($F_{1, 30} = 207.51$, $p < 0.001$), with the mice having become heavier as they aged (week 8: 24.15 ± 0.36 g; week 16: 32.69 ± 0.59 g). There was no significant effect of group or group-by-age interaction on the weight of male offspring at week 8 or 16.

Maternal Behaviour

In the pup-retrieving test, all dams were able to retrieve their first pup within the first 15 min. The latency to retrieve the first pup did not differ between control and Flx-exposed dams.

Pup Evaluation

To determine whether there was a relationship between righting response and treatment group, a 2 (treatment: control, Flx treated) × 2 (righting: yes, no) χ^2 analysis was conducted. A similar proportion of control and Flx-exposed male pups resumed an upright position. If evaluating only the pups that did resume an upright position, there was no difference in time to resume an upright position between control and Flx-exposed animals.

Behavioural Evaluation of Adult Animals

We found no differences in the behaviour of control and Flx-exposed mice in the open field, horizontal ladder, MWT retention and working memory, prepulse inhibition, and fear conditioning tests (table 1). Differences were found only in the elevated plus maze, resident intruder, and MWT spatial memory tests.

Elevated Plus Maze Test

In the elevated plus maze test, the control and Flx-exposed mice did not differ in the number of total entries into the open and closed arms of the apparatus, indicating no difference in exploratory behaviour. The Flx-exposed mice entered open arms for significantly more time (in percent) than the control mice ($t_{41} = 2.32$, $p = 0.025$; table 1; fig. 2a). However, the control and Flx-exposed mice had no difference in their mean number of entries to open arms. Additionally, no difference in risk assessment behaviour was observed, as the number of protected stretches did not differ between the groups. However, the Flx-exposed mice showed more head-dipping behaviour ($t_{41} = 2.18$, $p = 0.035$; table 1; fig. 2b).

Resident Intruder Test

The proportion of Flx-exposed resident mice that attacked the intruder was significantly greater than the proportion of control resident mice that did so [χ^2 (1, n = 32) = 6.35, $p = 0.029$; Cramér's V = 0.45]. When examining only the resident mice that attacked, no differences were found for attack latency time, the number of attacks, and attack duration (table 1; fig. 3).

MWT Spatial Memory

The analysis of the latency to reach the platform in the MWT spatial memory indicated that there was a significant main effect of treatment group ($F_{1, 30} = 6.38$, $p = 0.017$; fig. 4a). A significant main effect of test day on the latency to reach the platform was also observed ($F_{2.25, 67.6} = 11.79$, $p < 0.001$). Independently of the treatment group, the latency to find the platform was significantly shorter on day 4 than on day 1, indicating that both groups were able to learn the task. The control and Flx-exposed groups did not differ in swimming velocity (fig. 4b). Averaged over all trials, Flx-exposed mice spent less percent of the time at the edge of the pool (in thigmotaxis) than control mice ($t_{30} = 2.35$, $p = 0.026$), indicating either decreased anxiety-like behaviour or a difference in platform search strategy (fig. 4c). There was no significant difference in the latency to find the platform or in thigmotactic behaviour on training day 1; therefore, reported differences were not present from the start of testing and are not responsible for group differences in the time to find the platform. There was no significant interaction between testing day and treatment group. There was no effect of treatment group on the amount of time the mice spent in the target quadrant during the probe trial (table 1).

Table 1. Summary of the results of the behavioural testing battery demonstrating effects of perinatal Flx exposure on the behaviour of adult male mice

	Group		Statistics	p
	Control	Flx		
Elevated plus maze test				
Mice, n	22	21		
Time on open arms, %	20.6 (4.74)	36.2 (4.75)	$t_{41} = 2.32$	0.025[a]
Open and closed arm entry, n	14.2 (0.92)	12.2 (0.87)	$t_{41} = 1.61$	0.114
Open arm entry, n	3.5 (0.39)	4.4 (0.45)	$t_{41} = 1.54$	0.132
Protected stretches, n	12.0 (1.29)	12.3 (1.39)	$t_{41} = 0.13$	0.900
Head dipping, n	9.7 (1.70)	15.0 (1.70)	$t_{41} = 2.18$	0.035[a]
Open field test				
Mice, n	16	16		
Distance, m	30.7 (2.5)	26.9 (2.23)	$t_{30} = 1.11$	0.274
Speed, m/s	0.10 (0.01)	0.09 (0.01)	$t_{30} = 1.16$	0.256
Thigmotaxis time, %	68.5 (2.68)	64.7 (2.38)	$t_{30} = 1.06$	0.299
Resident intruder test				
Mice, n	16	16		
Proportion attacking	0.38	0.81	χ^2 (1, n = 32) = 6.35; Cramér's V = 0.45	0.029[a]
Attack latency, s	286.7 (42.9)	258.3 (42.7)	$t_{17} = 0.34$	0.741
Attacks, n	2.6 (0.5)	4.0 (0.6)	$t_{30} = 1.30$	0.211
Attack duration, s	57.8 (16.0)	40.9 (6.2)	$t_{5.71} = 0.63$	0.555
Horizontal ladder test				
Mice, n	16	15		
Time to cross (all rungs in), s	7.6 (0.7)	7.1 (0.7)	group: $F_{1, 29} = 0.04$	0.845
Time to cross (13 rungs removed), s	8.6 (0.6)	8.7 (0.9)	rungs in/out: $F_{1, 29} = 4.18$	0.050
			interaction: $F_{1, 29} = 0.21$	0.649
Foot faults (all rungs in), n	1.68 (0.4)	1.2 (0.2)	group: $F_{1, 29} = 0.019$	0.891
Foot faults (13 rungs removed), n	2.3 (0.4)	2.7 (0.8)	rungs in/out: $F_{1, 29} = 3.67$	0.065
			interaction: $F_{1, 29} = 0.60$	0.447
MWT (spatial memory)				
Mice, n	16	16		
Latency day 1, s	34.7 (3.6)	31.2 (2.1)	group: $F_{1, 30} = 6.38$	0.017[a]
Latency day 2, s	26.0 (3.7)	20.5 (2.9)	day: $F_{2.25, 67.6} = 11.79$	<0.001[a]
Latency day 3, s	26.5 (0.3)	15.5 (2.5)	interaction: $F_{2.25, 67.6} = 0.63$	0.555
Latency day 4, s	20.0 (3.6)	13.9 (1.2)		
Speed, m/s	0.15 (0.01)	0.15 (0.003)	$t_{30} = 0.24$	0.808
Thigmotaxis time, %	9.25 (1.5)	5.51 (0.4)	$t_{30} = 2.35$	0.026[a]
MWT (retention memory)				
Mice, n	16	16		
Latency (MWT spatial memory day 4), s	20.0 (3.6)	13.9 (1.2)	group: $F_{1, 30} = 1.37$	0.252
Latency (7 days later), s	16.0 (3.4)	13.6 (2.1)	day: $F_{1, 30} = 2.75$	0.107
			interaction: $F_{1, 30} = 2.12$	0.156
MWT (working memory)				
Mice, n	16	16		
Latency (first trial), s	34.9 (4.8)	44.9 (4.0)	group: $F_{1, 30} = 0.83$	0.369
Latency (last trial), s	33.3 (5.0)	29.2 (5.1)	trial: $F_{1, 30} = 21.69$	<0.001[a]
			interaction: $F_{1, 30} = 1.13$	0.297
Speed, m/s	0.15 (0.01)	0.15 (0.02)	$t_{30} = 0.24$	0.808
Thigmotaxis time, %	9.25 (1.5)	5.51 (0.4)	$t_{30} = 1.49$	0.144

Table 1. (continued)

	Group		Statistics	p
	Control	Flx		
Prepulse inhibition				
Mice, n	16	16		
Startle response	0.47 (0.04)	0.5 (0.05)	$t_{30} = 1.00$	0.323
Prepulse inhibition, %	60.6 (3.7)	61.6 (2.8)	$t_{30} = 0.19$	0.851
Fear conditioning				
Mice, n	16	16		
Cued (freezing before tone), %	37.0 (5.0)	38.4 (4.7)	group: $F_{1, 30} = 1.52$	0.227
Cued (freezing during tone), %	40.3 (3.8)	29.5 (2.9)	time: $F_{1, 30} = 0.366$	0.550
			interaction: $F_{1, 30} = 1.78$	0.192
Context (freezing at minute 1), %	27.4 (3.2)	28.1 (2.9)	group: $F_{1, 30} = 0.03$	0.896
Context (freezing at minute 2), %	43.9 (4.3)	41.5 (5.0)	time: $F_{1, 30} = 30.69$	<0.001[a]
			interaction: $F_{1, 30} = 0.34$	0.563

All data are reported as means with SEM in parentheses unless specified otherwise.
[a] Significant results.

Discussion

The present study evaluated the behavioural outcomes of male mice perinatally exposed to Flx. Flx was administered at 25 mg/kg/day to dams between E15 and P12 and led to detectable concentrations of Flx and NorFlx in pup brains at P0 and P12. In adulthood, male offspring behaviour was evaluated. The behavioural evaluation demonstrated that perinatal exposure to a therapeutically relevant dose of Flx leads to minor behavioural alterations in adulthood.

Unlike the authors of many earlier studies, we observed few effects of early exposure to Flx on the behaviour of the mice as adults despite a higher Flx dose than that used in other studies. The difference in findings could come from administering Flx via the less stressful method of dissolving it in drinking water. In most of the earlier studies, Flx was delivered either through oral gavage to the dam [18, 20], intraperitoneal injection to the dam [23, 30], intraperitoneal injection to the pup [21, 24, 63], or subcutaneous injection to the pup [22]. Differences in the pharmacokinetic profiles of these delivery methods could lead to different availability of Flx [64], potentially leading to disparate offspring behavioural outcomes. Also, oral gavage and injection methods are invasive and are known to be stressful, sometimes even harmful, to rodents [65–68]. Perinatal stress leads to behavioural and neurophysiological changes that last into adulthood [69–72]. More importantly, the combination

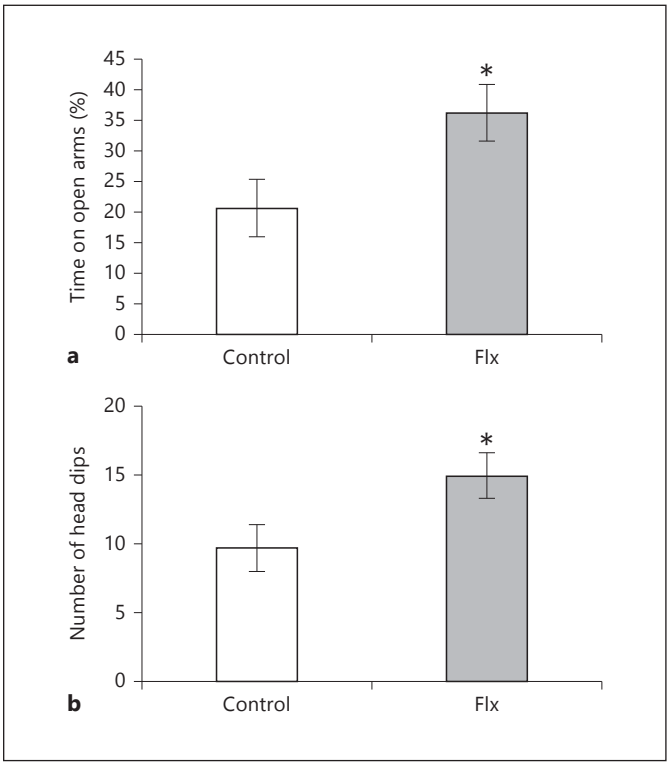

Fig. 2. a Performance of control (n = 22) and perinatally Flx-exposed (n = 21) mice in the elevated plus test as measured by the time spent on the open arms of the apparatus. **b** Number of head dips. * $p < 0.05$, significant difference between the groups.

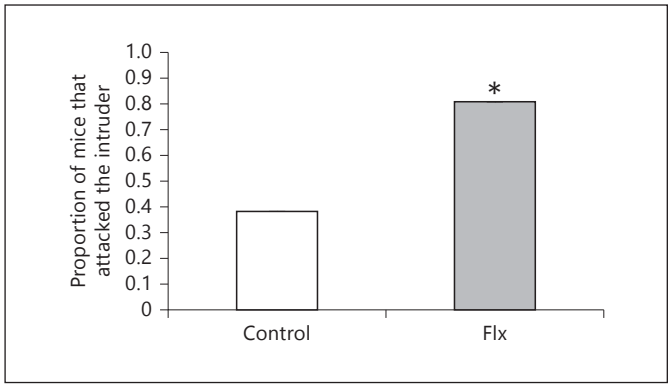

Fig. 3. Effects of perinatal Flx exposure on isolation-induced aggressive behaviour in adult mice (control: n = 16; Flx exposed: n = 16). * $p < 0.05$, significant difference between the groups.

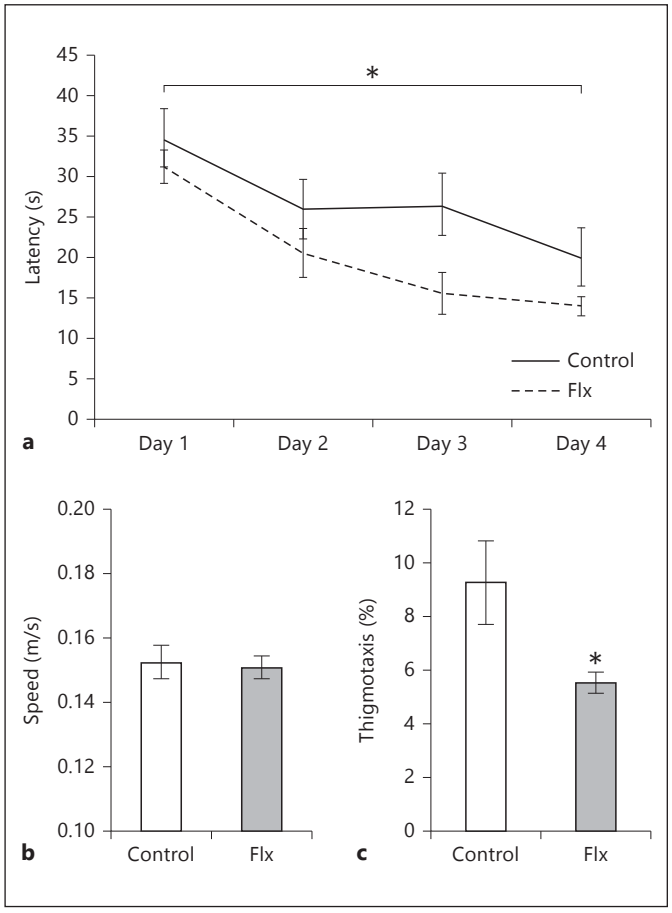

Fig. 4. a Performance of control (n = 16) and perinatally Flx-exposed (n = 16) mice in the MWT spatial memory as measured by the latency to find the platform. * $p < 0.05$, significant difference between the groups across the 4 test days. **b** Swim speed. **c** Thigmotaxis. * $p < 0.05$, significant difference between the groups.

of stress and Flx affects offspring outcomes differently than either one alone [73–75]. Therefore, behavioural changes previously reported after perinatal Flx administration could result from the interaction of stress and Flx exposure rather than as a consequence of Flx administration alone.

Our study shows that Flx has few effects on the adult behaviour of offspring, paralleling most human studies. Currently, no human study shows that prenatal Flx exposure affects mental, cognitive, or affective outcomes of children between 2 months and 7 years of age [15, 76]. However, adults who were prenatally exposed to Flx have yet to be examined.

In this study, a number of behavioural differences were observed between mice exposed to Flx early in life and control mice. Perinatal Flx exposure leads to an increase in aggressive behaviour in adult animals. Previous studies found that altered monoamine metabolism can lead to an increase in aggressive behaviour in both humans and animals [77]. Brunner syndrome, for example, is the result of a nonsense mutation in the MAOA gene, causing a deficiency in MAOA enzymatic activity and a subsequent reduction in the breakdown of serotonin and other monoamines [78]. People with Brunner syndrome exhibit frequent impulsive bursts of aggression [31]. Similarly, MAOA-deficient mice exhibit higher levels of aggression than control mice [32–34]. However, studies examining the effect of perinatal Flx exposure on aggression in rodents are scarce. While Lisboa et al. [20] reported no enduring effects of prenatal Flx exposure on aggressive behaviour in mice, Singh et al. [30] reported an increase in the number of fighting bouts during a foot shock-induced aggression task using rats prenatally exposed to Flx.

A number of mechanisms could be responsible for the increase in aggression observed in Flx-exposed mice. Serotonin transporter and vesicular monoamine transporter 2 are transiently expressed in the limbic system during development [79, 80]. This transient expression can allow for the uptake, storage, and release of serotonin by non-serotonergic neurons of the limbic system (including the amygdala), making these structures potentially vulnerable to Flx exposure [81]. As such, perinatal Flx exposure could affect any of the neurodevelopmental processes typically regulated by serotonin. In fact, serotonin transporter knockout mice show alterations in the neuronal morphology of the limbic system [38, 39].

Our study showed that Flx-exposed mice reach the platform faster than control mice in the MWT spatial memory, indicating better spatial memory in Flx-exposed

mice. This differs from the previously reported lack of spatial memory changes in females [19], indicating early effects of Flx may differ depending on the sex of the offspring. No previous studies have examined the effect of perinatal Flx exposure on spatial memory in adolescent or adult male mice. However, Bairy et al. [17] have demonstrated a dose- and time-dependent improvement in spatial learning and memory in the MWT with adolescent rats exposed to 8 and 12 mg/kg of Flx during the 2nd and the 3rd week of gestation. Conversely, no change in performance was demonstrated in a number of other tests of spatial memory, such as the Cincinnati maze or the T maze, in either adolescent or adult rats prenatally exposed to Flx as compared with controls [82].

Flx-exposed mice appear to be less anxious than their control counterparts as they spend more time on the open arms of the elevated plus maze. We reported a similar change in female offspring following early exposure to Flx [19]. Such a decrease in anxiety mirrors the findings in MAOA-deficient mice, which also exhibit reduced anxiety-like behaviour [32, 34, 77]. Previous examinations of rodents in the elevated plus maze show no change in open arm time between control animals and animals treated with Flx prenatally [18, 23], postnatally [24, 63], or perinatally [20, 29]. However, different species/strains of mice, treatment periods, routes of administration, and dosages were used in the aforementioned studies, potentially explaining our conflicting reports.

This study is the first to report drug concentrations in pup brains following maternal exposure to Flx in mice. In humans, Flx and NorFlx cross the placental barrier [6] and are excreted in breast milk, resulting in plasma concentrations that can reach therapeutic levels in breastfed infants [9]. The dose selected in this study was therapeutically relevant; the concentrations of Flx and NorFlx recorded in the pup brains were within the range observed in postmortem human brain tissue [83]. The dose selected for this study was higher than doses used before. The highest dose previously used in mice was 10 mg/kg/day, administered orally to a dam [84] or intraperitoneally to a pup [21, 24, 63, 85]; the highest dose given to rat dams was 17.2 mg/kg/day, delivered via the drinking water [86].

One potential reason for the very limited behavioural changes observed in the present study is a homoeostatic mechanism that is activated by Flx exposure. By blocking presynaptic serotonin transporters, Flx leads to an increase in serotonergic tone in the synapse. It is possible that this could increase the stimulation of serotonin autoreceptors, downregulating serotonin production. Such conjecture is supported by the finding of this study that Flx exposure leads to a global decrease in brain serotonin. Thus, the homoeostatic downregulation of serotonin may counterbalance the initial serotonergic increase at the synapse, resulting in serotonergic system functioning that is close to normal.

This study suggests that perinatal Flx exposure may not be harmful. While the increased aggression of male mice may put the animals at higher risk of injury and death, it also enhances their ability to establish social dominance, thereby enhancing access to females and mating success [87]. Furthermore, spatial memory is crucial for rodent foraging behaviour [88], another advantage for Flx-exposed animals. Other studies have also found perinatal Flx exposure to have positive outcomes for mice [19–21] and rats [17, 22].

A number of potential limitations of the present study need to be acknowledged. First, maternal behaviour was assessed only with one test (pup retrieval). While we and a number of other researchers [73, 86, 89, 90] have not found differences in maternal behaviour between Flx-exposed and control dams, Pawluski et al. [91] demonstrated an increase in arched-back nursing in rat dams exposed to Flx. Because maternal behaviour is known to affect offspring outcomes, a more thorough investigation following Flx administration is crucial. Second, it is important to point out that this study examined the effects of Flx in isolation of maternal stress. In a clinical situation, depression may be associated with high levels of stress. Maternal stress alone is known to be able to affect fetal development and the child's health as well as longer-term behavioural outcomes (for a review see [92] and [93]). Furthermore, studies that examine long-term outcomes of early Flx exposure have shown that perinatal stress and Flx exposure interact and significantly affect the animals as adolescents and adults [73–75, 89, 94]. While beyond the scope of this study, the long-term consequences of maternal stress and Flx exposure on the offspring, separately and in combination, should be examined.

As evident from the present study, approximately 25 mg/kg/day of Flx administered to dams from E15 to P12 leads to decreased anxiety-like behaviour, better spatial memory, and increased aggression but causes neither memory deficits nor changes in sensory processing or gross motor function. It is important to know whether adult humans who are perinatally exposed to Flx will experience changes similar to those seen in this study. If decreased anxiety were also evident in humans after perinatal exposure to Flx, this finding would be encouraging.

Reduced anxiety in highly anxious individuals may lead to better health, higher quality of life, and improved personal relationships and work performance [95–101]. Enhanced spatial memory could also be a positive outcome. Nevertheless, the literature reporting negative outcomes following perinatal Flx exposure cannot be ignored, particularly rodent studies. More research is needed to determine other beneficial or detrimental effects of early exposure to Flx, especially in combination with maternal stress.

Acknowledgements

This study was supported by an NSERC Discovery Grant to R.H.D. and an NSERC graduate scholarship to V.K. The authors thank Brendan McAllister, Sara Iablokova, and Simon Spanswick for their assistance in editing this manuscript.

Disclosure Statement

The authors declare that no competing interests exist.

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