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ORIGINAL INVESTIGATION

Age-dependent effects of chronic fluoxetine treatment on the serotonergic system one week following treatment

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Abstract
Rationale Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine are increasingly used for the treatment of depression in children. Limited data are, however, available on their effects on brain development and their efficacy remains debated. Moreover, previous experimental studies are seriously hampered in their clinical relevance.
Objectives The aim of the present study was to investigate putative age-related effects of a chronic treatment with fluoxetine (5 mg/kg, either orally or i.p. for 3 weeks, 1 week washout) using conventional methods (behavioral testing and binding assay using [^{123}I]β-CIT) and a novel magnetic resonance imaging (MRI) approach.
Methods Behavior was assessed, as well as serotonin transporter (SERT) availability and function through ex vivo binding assays and in vivo pharmacological MRI (phMRI) with an acute fluoxetine challenge (10 mg/kg oral or 5 mg/kg i.v.) in adolescent and adult rats.
Results Fluoxetine caused an increase in anxiety-like behavior in treated adult, but not adolescent, rats. On the binding assays, we observed increased SERT densities in most cortical brain regions and hypothalamus in adolescent, but not adult, treated rats. Finally, reductions in brain activation were observed with phMRI following treatment, in both adult and adolescent treated animals.
Conclusion Collectively, our data indicate that the short-term effects of fluoxetine on the 5-HT system may be age-dependent. These findings could reflect structural and functional rearrangements in the developing brain that do not occur in the matured rat brain. phMRI possibly will be well suited to study this important issue in the pediatric population.

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Introduction

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI) is at present the only registered SSRI for the treatment of major depressive disorders (MDD) which affects 2–8% of children and adolescents (Kapornai and Vetró 2008). This drug induces an increase in extracellular serotonin (5-HT) by blocking the serotonin transporter (SERT). The brain in development is sensitive to pharmacological interventions because of its dependence on the emergence of critical developmental processes (i.e., synaptogenesis; Swaab and Boer 2001). As maturation of the brain is not complete until young adulthood, sensitive periods of brain maturation extend into adolescence. The long-term effects of drug exposure may be delayed and expressed once the target system reaches maturation (i.e., typically during adulthood), a phenomenon called neuronal imprinting and occurs when the effects of drug exposure outlast the drug itself (Andersen and Navalta 2004).

In this context, the long-term side effects of fluoxetine are well known in adults but have been sparsely studied in the developing 5-HT system, even with pediatric prescriptions rates for SSRIs increasing (Zito et al. 2006). Since more than 50–90% of drugs prescribed in children have only been tested or licensed in adults, it is potentially dangerous to assume that children will have the same response to SSRIs as adults (Bachrach 2004). In spite of increased number of reported self-harm and aggression among children treated with SSRIs and even a black box warning by the Food and Drug Administration and European Medicines Agency in 2004, fluoxetine is the registered treatment of MDD in children 8 years of age and over.

Neurotransmitters such as 5-HT, are known to play a key role in many aspects of brain development (Whitaker-Azmitia et al. 1996). Given that the 5-HT system of adolescents is still developing (e.g., in rats SERT density increases in the frontal cortex and basal ganglia, and decreases in the midbrain throughout adolescence; Moll et al. 2000), and that treatment with SSRIs can last for years, it is possible that long-lasting manipulation of 5-HT release results in long-lasting and/or delayed adaptations of the 5-HT system. In fact, Wegerer et al. (1999) showed that chronic fluoxetine treatment persistently increases outgrowth of serotonergic projections in the frontal cortex of young but not adult rats. Also, age-dependent effects on hippocampal neurogenesis have been observed (Navailles et al. 2008; Couillard-Despres et al. 2009).

In clinical trials, the most efficient dose of fluoxetine for treatment of depression in all ages including children has been shown to be 20 mg (Beasley et al. 2000) (~0.3–0.9 mg/kg). Drugs are generally administered to rats in about 10-fold higher dosages compared to humans because of the higher hepatic drug metabolism in rodents. A dose of 3–9 mg/kg is therefore a clinically relevant dose of fluoxetine. Although the minimal dose of fluoxetine required for a significant inhibition of SERT and 5-HT was found to be 5 mg/kg (Wegerer et al. 1999; Tordera et al. 2002), most studies in rodents have been performed with much higher drug doses (typically 10–20 mg/kg) which induced acute behavioral impairments. Thus, previous studies are seriously hampered by a dose that is not clinically relevant.

With the purpose to overcome above mentioned shortcomings in previous studies and to investigate the potential of new non invasive imaging modality of the 5-HT system called pharmacological magnetic resonance imaging (phMRI), the aim of the current project was (1) to investigate the putative effects of a chronic treatment with 5 mg/kg fluoxetine on locomotor activity, anxiety-like behavior, working memory and depressive-like behavior (experiment A) as a function of age at the instauration of treatment, and (2) to explore the usefulness of a noninvasive imaging technique that would be able to address this important issue in the pediatric population. In this respect, we conducted explorative studies of ex vivo binding assays in various 5-HT-rich brain regions with a radioligand for SERT ([^{123}I]β-CIT (Reneman et al. 1999) (experiment B), and phMRI with fluoxetine as an index of 5-HT availability and/or SERT functionality. It has been shown that the blood oxygenation level dependent (BOLD) signal is sensitive for changes in 5-HT itself (Preece et al. 2009; McKie et al. 2005; Schwarz et al. 2007) and SERT function (Downey et al. 2010) (experiment C).

Materials and methods

All experiments were carried out in accordance with French, Dutch and UK regulations governing animal welfare and protection. Protocols were also reviewed and approved by a local animal care committee, in accordance with the guidelines of the Principles of Laboratory Animal Care (NIH publication 86–23, revised 1985). All experiments were performed in a randomized order. An overview of the experimental schemes used is presented in Table 1.

Table 1 Experimental schemes used

Rat strain	Age at start of treatment	Treatment dose, duration, and route	Challenge with fluoxetine	*n*
Experiment A				
Male Wistar	PND 25 PND 65	Fluoxetine or saline, 5 mg/kg, 3 weeks oral gavage, 1 week washout	10 mg/kg (oral) or saline	10
Total number of animals used in experiment A: 10 per group × 2 ages × 2 chronic treatments × 2 acute challenges = 160				
Experiment B				
Male Wistar	PND 25 PND 65	Fluoxetine or saline, 5 mg/kg, 3 weeks, i.p., 1 w washout	None	3
Total number of animals used in experiment B: 3 per group × 2 ages × 2 treatments = 12				
Experiment C				
Male Wistar	PND 25 PND 65	Fluoxetine or saline, 5 mg/kg, 3 weeks, i.p., 1 week washout	5 mg/kg (i.v.)	4–6
Total number of animals used in experiment C: 4–6 per group × 2 ages × 2 treatments = 21				

Animals

In all experiments the same strain of male Wistar rats were used, with exactly the same age from the same supplier (Harlan). Rats were aged PND25 (post-natal day 25: adolescent group, 35–49 g) and PND65 (adult group, 275–299 g) at the beginning of the experiments. The assessments were always performed following a 1-week washout at PND53 and PND93, respectively, to ensure total drug clearance (Caccia et al. 1990). PND25-53 was selected because it approximates adolescence in humans. In male rats, adolescence lasts from PND28 to PND60 (Spear 2000), with puberty occurring around PND 45 (Engelbregt et al. 2000). Rats were housed (two to three in each cage) under normal 12-h light–dark cycle (lights on at 8:00 a.m.) and food and water were available ad libitum.

Drugs and treatments

Fluoxetine hydrochloride (5 mg/kg body weight) dissolved in saline as a vehicle or vehicle (saline) alone was either administered by daily oral gavage (0.5–1 ml volume) or i.p. during 3 weeks. The rationale for the 5 mg/kg dose was that a dosage of 5 mg/kg of fluoxetine causes a 40% inhibition of [^{3}H] 5-HT in adult rat (Tordera et al. 2002), and that plasma levels of fluoxetine and its metabolite norfluoxetine do not differ between adolescent or adult rats with this dose (Wegerer et al. 1999). For practical reasons, fluoxetine was injected i.p. in experiments B and C. However, it has been shown that fluoxetine and norfluoxetine plasma levels do not differ between i.p. and oral routes at the 5 mg/kg dose (in contrast to higher doses such as 10 mg/kg) (Bourdeaux et al. 1998). In order to be able to better compare the results from the different experiments, all animals in the three (A–C) experiments underwent the same treatment protocol, as much as possible. Because we had to administer an acute challenge in experiment C (the phMRI study), we also administered an acute challenge (10 mg/kg) prior to experiment A (the behavioral study) in half of the animals tested. The other half did not receive an acute challenge in order to be able to compare the behavioral findings to experiment B (ex vivo binding assay with [^{123}I]β-CIT), in which we were not able to give an acute challenge, otherwise it would have affected the binding of [^{123}I]β-CIT. During experiment C, rats were injected intravenously with fluoxetine (5 mg/kg) dissolved in saline, injected in a volume of 1 ml/kg.

Experiment A behavior

Following chronic treatment with fluoxetine or vehicle, and a 1-week washout, groups of rats (n=10 animals per groups with a total of eight study groups) received an acute challenge with fluoxetine or saline 1 h before the onset of behavioral testing (see below). Each animal underwent four different behavioral experiments in the following order: spontaneous activity, anxiety-like behavior, working memory, depressive-like behavior (inter-test interval: 2 min). Animals were daily handled during 1 month preceding behavioral tests, and habituated to the testing room half an hour before the first test.

Spontaneous activity was assessed in open field (100× 100 cm; wall height: 20 cm) subdivided in 36 equal squares. Number of crossed squares, rearing, fecal boli were collected during 6 min. *Anxiety-like behavior* (Pellow et al. 1985) was analyzed by placing the animal on an elevated plus-maze (EPM, two open arms: 50×10 cm;

closed arms: 50×40×10 cm) for 5 min. Number of entries and time spent in open and closed arms were collected for calculation of % of time and % of entries in each arm. *Working memory* was assessed by recording spontaneous alternation behavior in a single-session Y-maze test (Bouet et al. 2010). To this, a gray painted wooden Y maze (each arm: 50×30×15 cm) was used. Each animal was placed at the end of one arm for free exploration (5 min). The number and sequence of entries (four-paw criterion) and number of rearing were collected. Alternation behavior was defined as consecutive entries into all three arms. The alternation percentage was calculated by the formula: (number of alternation/maximal possible alternation) × 100.

In the three tests described above, the apparatus was cleaned with diluted ethanol (70%) and dried between rats. *Depressive-like behavior* was assessed by a modified version of the forced swimming test (FST) (Castro et al. 2010), initially designed by Porsolt et al. (1977). Briefly, it consisted in placing the rat for one trial in a vase full of water (25°) for 6 min and measuring immobility, swimming, and climbing times from videotapes. Although Porsolt et al. (1977) described the test in two sessions, Castro et al. (2010) performed only one session because (1) previous studies showed that the effects of chronic stress can be measured by only one session; (2) the stress of a pre-test procedure modifies the cell proliferation, whose measurement was in fact the goal of the study. In our conditions, performing FST in one session was more suitable because animals were tested along a battery of tests, consequently performing a pre-test period the day before the assessment of spontaneous activity, anxiety-like behavior, and spontaneous alternation, would have probably increase the stress level and biased the result of the other behavioral tests.

Experiment B ex vivo binding assay with [^{123}I]β-CIT

Following chronic treatment with fluoxetine or vehicle, and a 1-week washout, groups of rats (n=3) were injected i.v. with approximately 1.85 MBq [^{123}I]β-CIT. Three hours after injection of [^{123}I]β-CIT (Reneman et al. 1999), animals were killed by bleeding via heart puncture under isoflurane anesthesia. The brains were quickly removed and dissected into the following regions: prefrontal cortex, cingulated cortex, occipital cortex, hippocampus, hypothalamus, striatum, raphe area and cerebellum and weighed. The striatum was included as a negative, since [^{123}I]β-CIT uptake in this region reflects primarily binding to dopamine transporters (Reneman et al. 1999). The ^{123}I radio activity of [^{123}I]β-CIT in each region was assayed with a gamma counter. The data were corrected for radioactivity decay back to the time of preparation of the injection syringes in order to compare relative concentrations in the tissues taken and to relate the results to the injected dose. The amount of radioactivity was expressed as a percentage of the injected dose, multiplied by the body weight per gram tissue weight (% ID×kg/g tissue), as described previously (Rijks et al. 1996). Uptake in the cerebellum, presumed low in SERT, was used as a reference for background radioactivity. Then, specific (total minus cerebellar uptake) to nonspecific (cerebellar activity) ratios were calculated.

Experiment C phMRI study

Following chronic treatment with fluoxetine or vehicle, and a 1-week washout, groups of rats (four groups of rats were studied (adults control: n=6, adults treated: n=5, adolescent control: n=6 and adolescent treated: n=4). were anaesthetized with isoflurane (5% induction and then reduced to 1.5–2% for maintenance of anesthesia during surgery and scanning) given in a mixture of nitrous oxide (1.2–1.5 l/min) and oxygen (0.5 l/min). Animals were free breathing during the whole experiment. To monitor physiological conditions, the right femoral artery was cannulated for blood gas (AVL, Roswell, GA, USA) and blood pressure (Biopac Systems Corp. Goweta, USA) monitoring. The right femoral vein was also cannulated for injection of the fluoxetine (5 mg/kg) for the acute challenge during the phMRI experiments. Intravenous injection was chosen in order to see a rapid change of brain activity. However, because of the systemic effect due to the intravenous injection of fluoxetine, we decided to reduce the dose to 5 mg/kg with respect to the 10 mg/kg used in experiment A, to prevent blood pressure change outside the range of the autoregulation.

PhMRI experiments were acquired on a Varian direct drive 4.7T (Varian, Palo Alto, CA, USA). A cylindrical quadrature coil placed around the head of the animal was used to transmit and receive the signal. Temperature was monitored through a rectal probe and maintained at 37.5±0.5°C by a warm air heating system (SA Instruments, New York, USA). For each subject, we acquired a T2-weighted anatomical image volume using a fast spin–echo sequence with 16 echo train, matrix= 256×256, FOV=50 mm, 30 contiguous 1-mm coronal slices, centered 8 mm caudal to the posterior edge of the olfactory bulb, four averages, TR_{eff}=5,112 ms, and TE_{eff}=60 ms. The time series acquisition used the same sequence with an echo train of 8, 16 slices of 1-mm thickness centered to the same position as before with TR_{eff}=4,500 ms, TE_{eff}=60 ms and a matrix size of 128×28. Thirty-two time points per subject (acquisition time per time series volume was 150 s; total scan time of 80 min.) were acquired with injection of 5 mg/kg fluoxetine during the acquisition of ninth time series volume. Anatomical and time series data were converted to 4D Analyze format. All image processing was performed with the physical pixel dimensions scaled up by a factor of 10, in order to ensure compatibility with analysis algorithms designed for use with human data. This resulted in a voxel

size 3.91×3.91×10 mm^3 for the time series data. Preprocessing included brain extraction with FSL/BET v. 2.1 with an adjusted fractional intensity threshold and threshold gradient and motion correction using FSL/MCFLIRT. Data for each animal were spatially normalized to a stereotactic rat brain template (Schwarz et al. 2007) by computing a 6 degrees-of freedom affine transform for the anatomical image and applying the resulting transformation matrix to the accompanying phMRI time series (FSL/FEAT v 5.98).

Statistics

Experiment A behavior and experiment B ex vivo binding assay with [^{123}I]β-CIT

Data were analyzed with a two-way analysis of variance (ANOVA) with age and treatment as independent factors, followed by post hoc multiple comparisons tests (Fischer's PLSD) (Statview®). In addition, we analyzed the age× treatment interaction term. Univariate *t*-test was used to compare the working memory performances to a reference value (50% alternation corresponding to a random exploration of the Y-maze).

Data are presented as mean ± standard deviation (SD). The chance of a type I error (α) was set at 0.05 using two-tailed tests of significance, unless stated otherwise. All data were analyzed using SPSS version 16.0.

Experiment C phMRI study

Explorative image-based time series analysis of the hemodynamic response in individual subjects was carried out in the framework of the general linear model (GLM) using FSL/FEAT v. 5.98 with 8 mm FWHM spatial smoothing, FILM prewhitening and a high pass filter of 4,800 s. The first time series volume was deleted from further analysis to ensure steady-state imaging has been reached. The design matrix consisted of a square basic waveform with an initial off period of 1,050 s (seven volumes of baseline recordings corresponding to the eight time points before the injection from which we removed the first time point) followed by an on-period of 3,600 s (recordings of functional activity induced by fluoxetine) with a gamma convolution and an added temporal derivative in order to match the time course profile expected form a first analysis step looking at all significant changes in the brain (*t*-test with *p* threshold of 0.05 using Stimulate; Strupp 1996). Maps of the group mean responses and positive and negative group mean differences (two-sample unpaired *t*-test) were calculated within a GLM framework at the higher level using FSL/FEAT v. 5.98 with ordinary least squares simple mixed effects inference. *Z* (Gaussianised T/F) statistic images were thresholded using clusters determined by $Z>2.3$ in case of group means and $Z>1.8$ in case of group differences and a (corrected) cluster significance threshold of $p=0.05$ (see Figs. 3 and 4).

Results

Experiment A behavior

Spontaneous activity

Horizontal activity was not affected by fluoxetine treatment or by age as attested by an absence of significant difference in number of crossed squares in open field (Fig. 1a). There was no significant difference in the vertical activity (number of rearing) neither. The percentage of time spent in the central area was not modified by treatment neither by age (interaction age×chronic treatment: $p=0.07$). PND25 animals chronically treated with fluoxetine showed a trend to a decrease in the time spent in the center of the open field ($p=0.06$). Acute challenge in itself had no effect on these parameters.

Anxiety-like behavior

Global activity in the plus maze tended to decrease with age (age effect $p=0.06$) (Fig. 1b). There was no significant effect of chronic treatment or age×chronic treatment. Overall, % of open arm entries was not significantly modified by age or chronic treatment. There was a trend for an interaction between age×chronic treatment ($p=0.06$). However, when taken separately, the data from adult PND65 group displayed a significant effect of treatment ($p=0.03$) suggesting an increase in anxiety level in adult animals. The % of time spent in open arms did not differ with age of chronic treatment, and there was also no significant age × chronic treatment interaction, neither any effect of acute challenge.

Working memory

There was no significant effect of treatment, age or age × treatment on the number of arm entries or number of rearing. Alternation percentages were not affected either by age or treatment and there was no significant age × treatment effect. Concerning spontaneous alternation, ANOVA also failed to show significant effects of age, treatment or age × chronic treatment. In contrast to the control group, which displayed an alternation percentage significantly different from the chance level ($p<0.05$, univariate *t*-test), PND25 animals chronically treated with fluoxetine but without challenge displayed significantly impaired spontaneous alternation (not different from chance: $p=0.63$). Such an impairment was also observed in PND65 chronically treated and receiving the challenge

(comparison with 50%, $p=0.09$) (Fig. 1c). Again, the acute challenge had no effect on these parameters.

Depressive-like behavior

Immobility and swimming time during the forced swimming test were affected by age ($p=0.03$ and $p<0.001$, respectively), but not modified by chronic treatment, and there was no significant age × chronic treatment interaction (Fig. 1d). Acute treatment did not significantly decreased immobility time ($p=0.09$). Climbing time was not modified by age or chronic treatment and there was no significant age × chronic treatment interaction.

Experiment B ex vivo binding assay with [^{123}I]β-CIT

The mean binding ratios for the four different groups are outlined in Table 2. As expected, [^{123}I]β-CIT binding ratios were higher in the raphe area and hypothalamus compared to the three cortical regions, with the lowest binding ratio in the occipital cortex. A significant effect of age was observed in the prefrontal cortex, cingulate cortex and raphe nuclei. [^{123}I]β-CIT binding ratios were statistically significantly higher in control adult rats when compared to control adolescent animals, but significantly lower in the raphe nuclei. An effect of treatment was observed in both adolescent and adult animals in the hypothalamus (+5% in adults vs. 24% in adolescent animals) and raphe nuclei (both groups +20%). In the prefrontal cortex, only an effect was observed in the adolescent group (+19%), together with the hippocampus (+10%). Only in adult animals we observed a statistically significant reduction in [^{123}I]β-CIT binding ratios following fluoxetine treatment in the occipital (−13%) and cingulate cortex (−23%). Most importantly, a significant age × treatment effect was observed in the hypothalamus ($p=0.01$), and all cortical regions studied: the

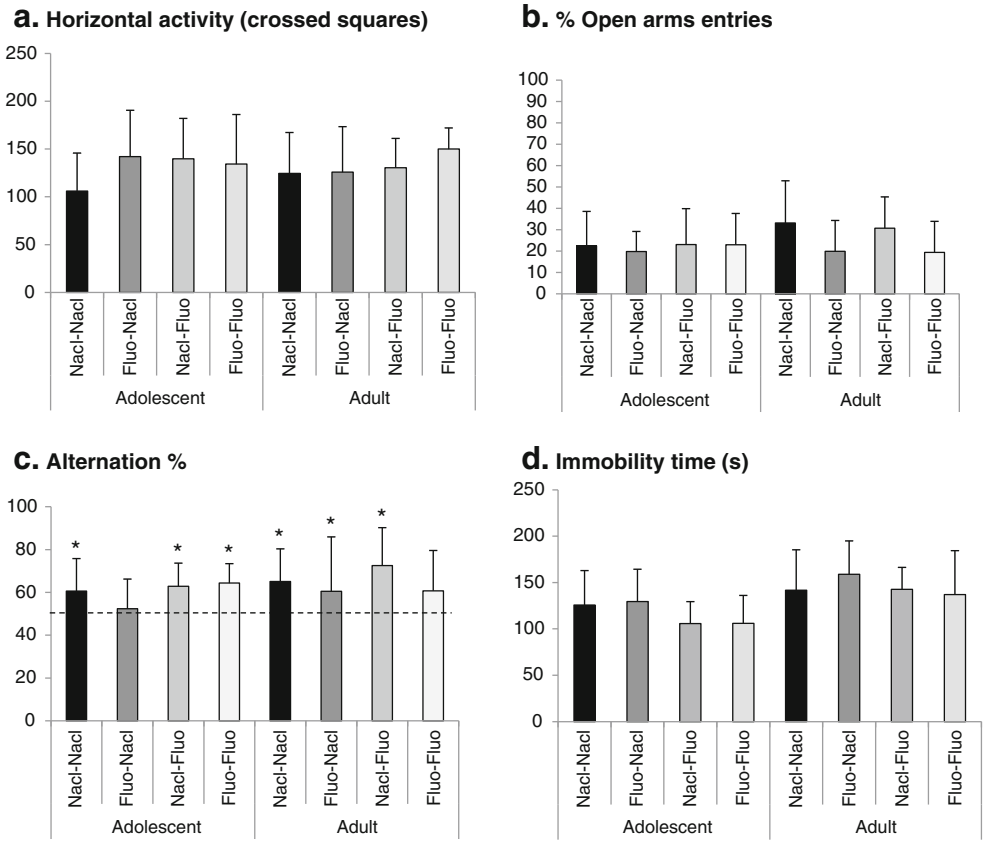

Fig. 1 Effects of chronic treatment with fluoxetine (5 mg/kg) on behavior in adolescent and adult rats. **a** Horizontal activity in open field displayed by the number of crossed squares. No effect of treatment, age or age×treatment. **b** Percentages of open arms entries in elevated-plus maze. Overall, there was no effect of treatment, age or age×treatment, but when analyzed separately, the adult group displayed a significant effect of chronic treatment ($p=0.03$). **c** Alternation percentages calculated from Y-maze exploration behavior (see Material and methods section). No effect of treatment, age or age×treatment. Comparison with chance level (univariate *t*-test; *asterisk* indicates a significant difference with 50% chance level) showed a deficit in adolescent Fluo–NaCl and adult Fluo–Fluo groups. **d** Immobility time (s) in the forced swimming test shows an increase with age but not according to the treatments. NaCl–NaCl: control group receiving chronic and challenge saline administration; Fluo–NaCl: animals chronically treated with fluoxetine (5 mg/kg) and receiving a saline challenge (10 mg/kg); NaCl–Fluo: animals receiving chronic saline administration and a fluoxetine challenge (10 mg/kg); Fluo–Fluo: animals receiving chronic fluoxetine administration (5 mg/kg) and fluoxetine challenge (10 mg/kg)

Table 2 Effects of chronic treatment with fluoxetine on [^{123}I]β-CIT Binding ratios in various brain regions in adolescent (PND53) and adult rats (PND93)

	Adolescent			Adult		
	Control	Fluoxetine	Delta (%)	Control	Fluoxetine	Delta (%)
Prefrontal cortex	3.67±0.27	4.54±0.42	+19*	4.60±0.03$^{\perp}$	4.81±0.13	+4
Occipital cortex	1.22±0.32	1.53±0.17	+20	1.65±0.02	1.43±0.06	−13*
Cingulate cortex	3.59±0.95	4.16±0.35	+14	6.02±0.50$^{\int}$	4.63±0.40	−23*
Hypothalamus	5.49±0.74	7.25±0.11	+24*	6.51±0.01	6.87±0.01	+5
Hipocampus	3.11±0.09	3.44±0.12	+10*	3.34±0.18	3.33±0.22	0
Raphe nuclei	6.24±0.35	7.78±0.31	+20*	5.70±0.45§	7.09±0.31	+20
Striatum	13.47±2.00	13.76±1.91	+2	16.23±0.04	16.53±1.13	+2

$^{\perp,\int}p<0.01$ vs. adolescent control (age effect)

$^{\S}p=0.019$ vs. adolescent control (age effect)

*Significantly different from control group $p<0.05$ (treatment effect)

occipital cortex ($p=0.04$), cingulate cortex ($p=0.02$) and a trend in the prefrontal cortex ($p=0.06$). No significant effect of age, treatment nor their interaction term was observed in the striatum.

Experiment C phMRI study

Recording was started only when blood gas values (arterial pCO_2 mmHg, pO_2 mmHg and pH) were within normal range and stable. Other physiological parameters (body temperature [37.5±0.5°C], respiration rate, level of anesthesia) were maintained within normal ranges throughout the duration of each experiment. Administration of fluoxetine caused a sharp transient (1–2 min) decrease in blood pressure (Fig. 2), followed by a gradual decrease of the blood pressure over the subsequent 15 min. The blood pressure, then reached a new lower level that partially recovered by 50 min after injection.

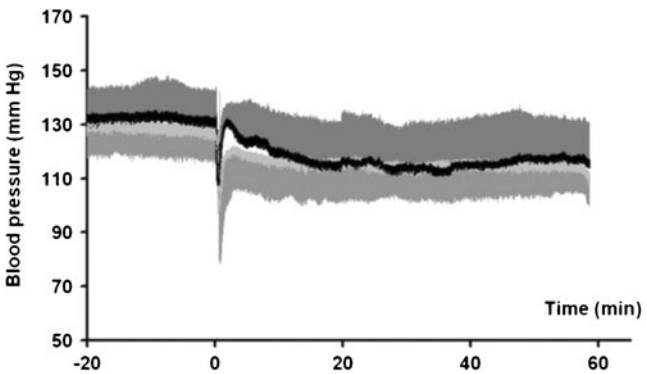

Fig. 2 Blood pressure changes induced by i.v. injection of 5 mg/kg fluoxetine. The *black curve* represents the average time course for the adult animals and the *gray curve* the average for the adolescent treated group. Administration of fluoxetine caused a sharp transient (1–2 min) decrease in blood pressure following the administration of the challenge (*gray rectangle*). This was followed by a gradual decrease of the blood pressure over the subsequent 15 min. The blood pressure, then reached a new lower level that partially recovers by 50 min after injection

The challenge with fluoxetine caused a number of regional changes (Fig. 3a), which were most prominent in the cortical regions, with additional foci in hypothalamus and hippocampus. These region specific effects with a predominant cortical effect are in line with a previous study (Schwarz et al. 2007), although in that study activation was also observed in the thalamus but with a higher challenge dose than used in the current study (10 vs. 5 mg/kg in this study). Overall, there were no differences in levels of activation in response to fluoxetine challenge between adult and adolescent animals (age effect: Fig. 3b and c). However, on average, control animals (Fig. 4a) showed much higher levels of activation in response to the 5-HT challenge than treated animals (Fig. 4b). This treatment effect was statistically significant in several subcortical areas, such as the hypothalamus and (left) caudate putamen and in the (left) primary motor cortex (Fig. 4c). When analyzing the effect of treatment within the adolescent and adult groups separately, we observed no significant age by treatment interaction effects. The effect of the treatment (decrease of activation after 5-HT challenge) was similar in both age groups.

Discussion

In this explorative study, chronic treatment with fluoxetine caused an increase in anxiety-like behavior only in adult treated rats. Only in adolescent rats, we observed increases in SERT densities in most cortical brain regions studied, in addition to the hypothalamus. In most cases, SERT densities approached or were even higher (hypothalamus) than adult control levels. Finally, we observed reduced brain activation on the phMRI in response to the acute 5-HT challenge, in treated rats compared to control rats.

Chronic fluoxetine did not induce age-related behavioral changes since we did not observe an interaction between age and treatment. We did, however, observe an increase in anxiety-like behavior in adult treated rats, and not adolescent rats. To our knowledge, only two studies reported previously on behavioral changes induced by fluoxetine during *adoles-*

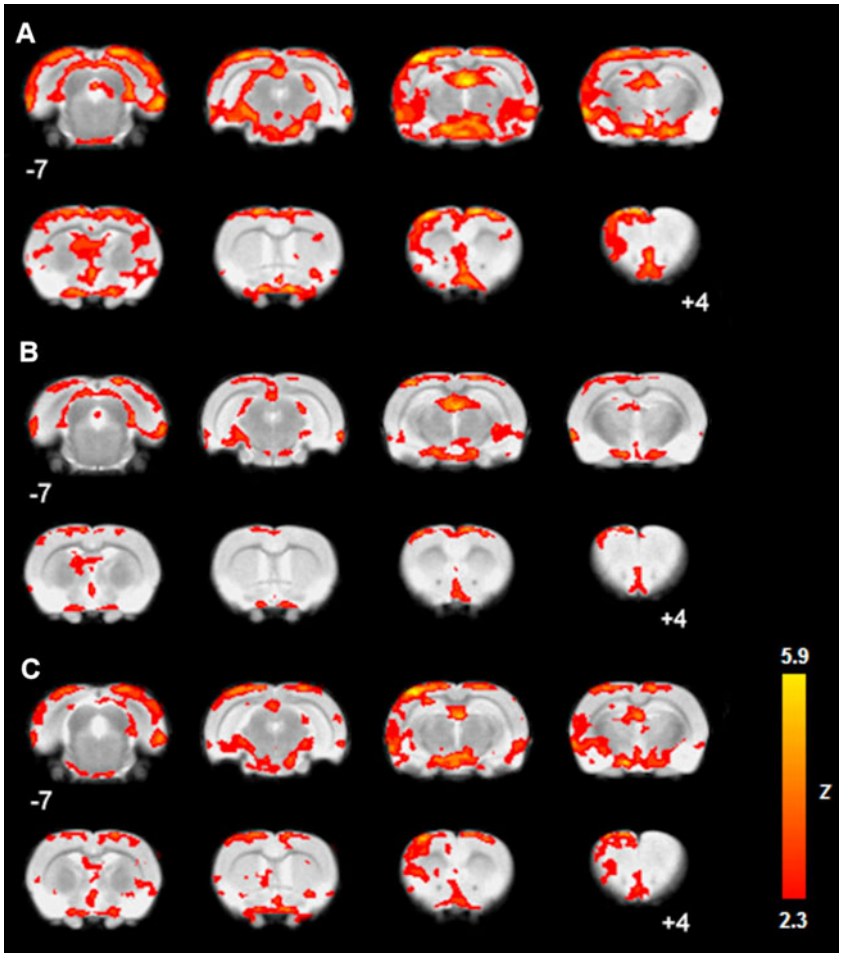

Fig. 3 Effect of age on brain activation following fluoxetine (5 mg/kg, i.v.) challenge averaged for all animals. Areas of significant activation following fluoxetine (5 mg/kg, i.v.) challenge, averaged for all animals (n=21) (**a**). No (significant) difference in activation between adult (n=11) (**b**) and adolescent animals (n=10) (**c**). FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). Z (Gaussianised T/F) statistic images were thresholded using clusters determined by Z>2.3 and a (corrected) cluster significance threshold of p=0.05

cence in male rats (LaRoche and Morgan 2007; Iñiguez et al.2010). LaRoche and Morgan (2007) showed an alteration in attention-related performances with chronic 5 and 10 mg/kg fluoxetine treatment. Unfortunately, mood parameters were not investigated, impeding comparison with our data. Iñiguez et al. (2010) observed an increase in anxiety-like behavior in the elevated plus maze 24 h or 3 weeks after treatment end. However, these effects were obtained with a much higher dose than the one used in the present study (20 vs. 5 mg/kg daily). These findings are in line with the adult literature in which the 5 mg/kg dose exerts an effect on anxiety-like behavior when administered for at least 3 weeks (Griebel et al. 1999; Lifschytz et al. 2006). Also, Shishkina et al. (2007) showed a significant effect of fluoxetine (7.5 mg/kg) only after a 4-week daily treatment, and not a 2-week-long treatment. Thus, overall our findings are in line with the literature, taking into account differences in dose and duration of treatment. The absence of an effect on anxiety-like behavior in adolescent animals suggests the existence of a minimal active dose of fluoxetine, which apparently is higher in adolescent rats than adult rats, presumably reflecting increased neuroplasticity in the younger animals (see also below).

Only one previous study reported on SERT densities following chronic fluoxetine treatment in periadolescent rats (Wegerer et al. 1999). They also observed an increase in SERT densities in the frontal cortex in the order of 20%, only in adolescent but not adult rats, treated orally with 5 mg/kg fluoxetine. We, however, not only observed an effect in the frontal cortex, but also in the parietal or occipital cortices. Although we used the same age (PND 25), gender (male), and species (Wistar), the discrepancies between the two studies are best explained by the longer duration of treatment in the present study (3 vs. 2 weeks in the Wegerer study) and route of administration (oral vs. i.p.). Because treatment for MDD with an SSRI can last for years (Bhagwagar and Cowen 2008), a longer duration of treatment better simulates clinical practice. It has been postulated by Wegerer et al. (1999) that increased SERT densities following early fluoxetine treatment most likely reflects enhanced outgrowth of the 5-HT system, due to stimulation of 5-HT axonal growth and synaptogenesis by high levels of 5-HT following fluoxetine treatment. It is thought that release of a growth factor from astroglial cells in response to stimulation by 5-HT of postsynaptic 5-HT_{1A} receptors in the developing brain causes

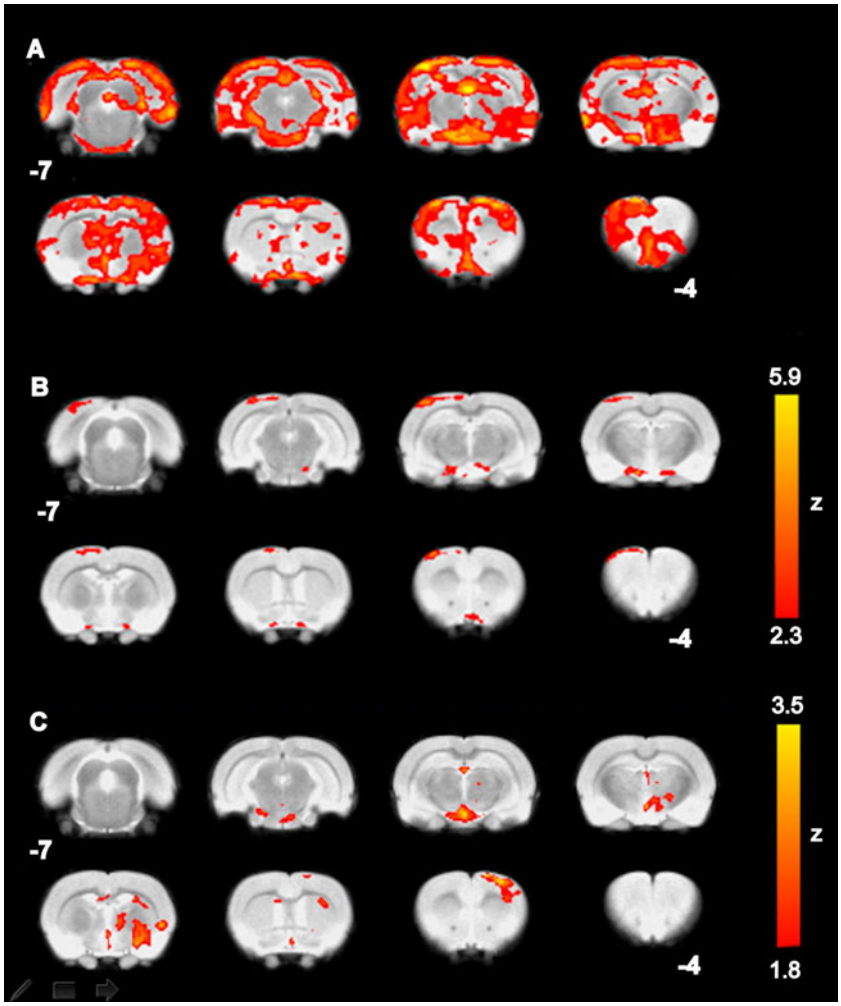

Fig. 4 Effect of treatment on brain activation following fluoxetine (5 mg/kg, i.v.) challenge. Areas of significant activation following fluoxetine (5 mg/kg, i.v.) challenge, averaged for all control animals (n=12) (**a**) and all treated animals (n=9) (**b**) show a clear reduction of signal in the treated animals. This effect of treatment was statistically significant (two-sample unpaired t-test) in several subcortical areas (hypothalamus, (*left*) caudate putamen) and in the (*left*) motor cortex (**c**). FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). Z (Gaussianised T/F) statistic images were thresholded using clusters determined by Z>2.3 (**a** and **b**) or Z>1.8 (**c**) and a (corrected) cluster significance threshold of p=0.05

this 5-HT hyperinnervation (Whitaker-Azmitia and Azmitia 1989; Wegerer et al. 1999).

Ascending 5-HT projections of the 5-HT neurons in the raphe nuclei have an enormous degree of structural plasticity, even in the adult brain: non-human primates with documented proximal denervated 5-HT projection fields following treatment with the 5-HT neurotoxin 3,4-methylenediomethamphetamine (MDMA) such as the thalamus and amygdala, completely reinnervated or hyperinnervated (Hatzidimitriou et al. 1999), 7 years post-drug survival. The degree of structural plasticity of ascending 5-HT projections is assumed to be higher during brain development than at later stages in life. Therefore, the increase in SERT densities we observed (predominantly in cortical regions), is best explained by enhanced 5-HT axonal outgrowth resulting from early fluoxetine treatment (Wegerer et al. 1999). The fact that we observed an opposite effect in adult animals (reduction in SERT densities in occipital and cingulate cortices) further supports this hypothesis. This finding is in line with previous studies in adult rats. Following treatment with SSRIs, either no effects or a reduction in the amount of SERT mRNA or SERT densities were observed, but never an increase (Lesch et al. 1993; Piñeyro et al. 1994). These effects are long-lasting, since Wegerer et al. (1999) observed that increased SERT densities in the frontal cortex following early treatment persisted until adulthood (Wegerer et al. 1999).

In the present study, we also explored the consequences of the presumed 5-HT hyperinnervation from early fluoxetine treatment on brain activation using phMRI. Overall, we observed a reduction of brain activation in response to the fluoxetine challenge between control animals and treated animals, but no statistically significant difference between adult and adolescent animals. It has previously been shown that (BOLD) signal is sensitive to changes in 5-HT: the SSRI citalopram was shown to cause region specific changes in MRI signal in human volunteers (McKie et al. 2005). In rats, the potent 5-HT releaser fenfluramine also caused region specific changes in brain activation, which were attenuated by the 5-HT synthesis inhibitor p-chlorophenylalanine (pCPA; Preece et al. 2009). Furthermore, Downey et al. (2010) demonstrated that citalopram phMRI can be used as a

probe of SERT function, since they observed significant and bilateral reductions in BOLD responses in the caudate nucleus, mid-cingulate gyrus and parietal cortex of carriers of the short (s) allelic variant of a functional polymorphism in the promoter region of SERT (the 5-HTTLPR), when compared to carries of the long (l) form. This is of particular interest to the current study as the s-allelic variant is associated with reduced SERT availability and/or function compared to the long (l) form (Heils et al. 1996, 1997; Canli and Lesch 2007). Then, the reduced brain activation in response to the 5-HT challenge following treatment observed in the current study could also reflect reduced SERT functionality. However, using SERT binding assays, we observed a treatment-induced reduction in (mainly cortical) SERT density in adult animals while an increase in SERT densities occurred in adolescent rats treated with fluoxetine. Nevertheless, these findings do not inform us about possible changes in the functionality of the SERT in (adolescent) rats due to treatment with fluoxetine. It may be that the functionality of these SERT densities in adolescent rats has been reduced along to their increased density.

Unfortunately, it is not possible to perform the presently used ex vivo binding assays and phMRI studies in the same animals at the same age (due to blockade of the transporter by the pharmacological challenge). Although we used the same treatment protocol in all experiments, the routes of administration differed between the behavioral experiments (oral gavage) and the binding assays and phMRI studies (i.p administration). It has been shown by Bourdeaux et al. (1998) that fluoxetine and norfluoxetine plasma levels do not differ between i.p. and oral routes at the 5 mg/kg dose. However, since we did not measure drug plasma concentrations we cannot be certain that differences in plasma concentrations underlie the different findings in the three experiments: "only" a treatment effect on behavioral studies and phMRI, whereas an interacting effect of age and treatment on the binding assays. In line with this, at higher oral concentrations of 12 mg/kg fluoxetine age-related treatment effects have been noted on behavior (Homberg et al. 2011).

Although we used the same treatment protocol in the binding assay and phMRI studies, the reason why we presently only observed an age × treatment effect in the binding assays and not the phMRI studies, most likely reflects our relatively low statistical power in the phMRI studies. Future phMRI studies with larger sample sizes are therefore needed, to further investigate this interesting issue. Although sample sizes were small, the observed age and treatment effects in the binding assay are in line with previous studies by Wegerer et al. (1999) and Moll et al. (2000). Finally, it is possible that the increase in SERT densities does not reflect an increase in serotonergic outgrowth or number of neuronal processes/terminals. Future studies with more focused methods, such as immunohistochemistry, should be conducted and correlated to phMRI data obtained in the same animals in larger sample sizes.

Collectively, the present data suggest age-dependent changes in 5-HT rich brain regions following chronic treatment with fluoxetine, which reflect altered SERT density (5-HT hyperinnervation) in addition to SERT hypofunctionality in treated animals, but without functional deficits in behavior. It is well known from the literature that there is a certain threshold before functional changes become apparent (e.g., reduction of >50% of striatal dopamine transporter is needed to induce motor signs of Parkinson's disease (Booij et al. 2001)). Apparently, the developing central 5-HT system is capable of structural rearrangements that are different from those seen in the mature brain, without influencing brain function.

The significance of our observations for children and adolescents that are being treated with SSRIs is difficult to predict. However, it is conceivable that, also in humans, the degree of plasticity of ascending 5-HT projections is higher in children and adolescents than in the matured brain, and that pharmacological manipulations of 5-HT (release) would then also affect children differently from adults. To what extent our findings in rats are associated with observations in humans that the younger the child treated with fluoxetine, the greater the risk of suicidal thoughts or attempts (Olfson et al. 2006), is a subject for future studies. However, our study supports the notion that it is potentially dangerous to assume that children will have the same response to SSRIs as adults, as has already been pointed out for other medicines used in the pediatric population (Bachrach 2004). The current results therefore raise serious concern and call for further investigations in humans of possible long-term effects in children and adolescents treated with SSRIs.

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