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Lasting effects of chronic fluoxetine treatment on the late developing rat brain: Age-dependent changes in the serotonergic neurotransmitter system assessed by pharmacological MRI

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ABSTRACT

Rationale: With the growing prevalence of psychotropic drug prescriptions among children and adolescents, the need for studies on lasting effects of drug exposure on the developing brain rises. Fluoxetine is the only selective serotonin reuptake inhibitor (SSRI) officially registered to treat major depressive disorder in children. Although various (pre)clinical studies have assessed the (long-term) effects of fluoxetine exposure in the perinatal period and in adulthood, limited data is available on its effects on the developing brain later in life, i.e. during adolescence.
Objective: The present study aimed at investigating the effects of age following chronic SSRI treatment on the central serotonin (5-HT) system. To this end, pharmacological MRI (phMRI) was performed in chronic fluoxetine-treated (5 mg/kg, oral gavage for 3 weeks) juvenile (PND25) and adult rats (PND65) after a 1-week washout period, using an acute fluoxetine challenge (5 mg/kg, i.v.) to trigger the 5-HT system.
Results: We observed a diminished brain response to the acute challenge in adult treated animals when compared to control animals, whereas this response was increased in juvenile treated rats. As a result, a significant age by treatment interaction effect was seen in several (subcortical) 5-HT related brain regions.
Conclusion: An opposite effect of chronic fluoxetine treatment was seen in the developing brain compared to that in matured brain, as assessed non-invasively using phMRI. These findings most likely reflect neuronal imprinting effects of juvenile SSRI treatment and may underlie emotional disturbances seen in animals and children treated with this drug. Also, our findings suggest that phMRI might be ideally suited to study this important issue in the pediatric population.

Introduction

Selective serotonin reuptake inhibitors (SSRIs), which induce an increase in extracellular serotonin (5-HT) by blocking the pre-synaptic serotonin transporter (SERT), are increasingly prescribed for treatment of childhood depression and anxiety disorders (Murray et al., 2004; Zito et al., 2002). However, efficacy of these drugs in the pediatric population remains debated (Hetrick et al., 2007). At present, fluoxetine is the only SSRI registered for treatment of major depressive disorder in children over 8 years old. Long-term (side-) effects of this drug are well studied in adulthood (Benmansour et al., 1999; Cipriani et al., 2007; Mourilhe and Stokes, 1998; Racagni and Popoli, 2008; Schule, 2007), as well as in the perinatal period (Alwan and Friedman, 2009; Borue et al., 2007; Morrison et al., 2005; Oberlander et al., 2006; Olivier et al., 2011) in both animals and humans. Yet, limited data exists on its effects on the late developing brain, i.e. during (pre)adolescence. Although the initial mechanisms of action are most probably similar in both the immature and mature brain, the maturational state of the targeted neurotransmitter system is nevertheless vitally important for determining the long term effects of antidepressant exposure (Andersen and Navalta, 2004). Andersen and Navalta postulated the hypothesis that whereas chronic drug exposure in adult animals results mainly in transient compensatory reactions, in juvenile animals these same compensatory reactions might permanently affect the development of the involved neurotransmitter systems.

Adolescence is a critical period for 'neuronal imprinting' effects, in which long-term effects of drug exposure are delayed and expressed only later in (adult) life. Throughout development, widespread reorganizations of brain morphology and function occur. Several neuroimaging studies in humans have shown that in puberty, after an initial period of axonal overgrowth, most circuits in the brain are refined and rewired by a gradual but extensive loss of synapses (as many as 40%) together with strengthening and maturation of

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remaining synaptic connections (Blakemore, 2008; Casey et al., 2008; Paus et al., 1999). This loss of earlier formed synaptic connections, called pruning, peaks during adolescence and accounts for substantial region specific decreases in gray matter density that occur during puberty (Giedd, 2008; Sowell et al., 2001). The 5-HT neurotransmitter system, which is targeted by fluoxetine, is known to play a key role in brain development through its role in the connective organization of the nervous system which includes control of proliferation, differentiation, migration, cell death, synaptogenesis and this earlier mentioned dendritic pruning (Gaspar et al., 2003; Homberg et al., 2010; Whitaker-Azmitia et al., 1996). Animal studies have shown that 5-HT neurotransmission undergoes widespread remodeling from youth through adolescence into adulthood, which is during adolescence most pronounced in the frontal and limbic regions (Crews et al., 2007; Olivier et al., 2011). During this period the number of 5-HT synapses is known to fluctuate, there is a steady increase of SERTs in mainly the frontal cortex, and a clear reorganization of 5-HT receptor expression (Crews et al., 2007; Moll et al., 2000). Hence, the brain is in a critical period of development during adolescence and therefore thought to be more vulnerable to drug exposure in this period (Andersen, 2005).

Indeed, there are clinical indications that SSRI exposure during adolescence may lead to (negative) outcomes that are not seen in adult patients. The most serious side-effects associated with SSRI use in children, adolescents and young adults are higher occurrence of psychiatric and/or behavioral adverse events, such as sleep disturbances and agitation (Wilens et al., 2002) and increased suicide risk (Bridge et al., 2007; Hammad et al., 2006; Wohlfarth et al., 2006) which has led to a black box warning from the Food and Drug Administration (FDA) and European Medicines Agency (EMA) in 2004. Despite these warning signs, the available animal literature on juvenile SSRI exposure is remarkably scarce and focuses mainly on behavioral aspects suggesting anxiogenic and anti-depressant-like effects after juvenile (>PND14) but not adult exposure in rodents (Homberg et al., 2011; Iñiguez et al., 2010; Oh et al., 2009). Strikingly, only very few studies investigated neurobiological changes after juvenile SSRI exposure. A study by Wegerer et al. (1999) reported a significant increase in frontal cortex SERT expression in juvenile (PND25-39), but not in adult rats (PND50-64) after chronic treatment with fluoxetine and that this effect persisted into adulthood, long after discontinuation of treatment (Wegerer et al., 1999). A different study, focusing on dendritic spine proliferation, showed that chronic fluoxetine treatment during adolescence (PND21-42) might arrest spine development in the hippocampus (Norrholm and Ouimet, 2000). On the other hand, fluoxetine treatment during the juvenile period is thought to have a stimulatory effect on adult hippocampal neurogenesis (Navailles et al., 2008) and on neuroplasticity in the visual system following retinal lesions (Bastos et al., 1999).

However, to investigate the possibility of neuronal imprinting in the living human brain, non-invasive methods that can be easily applied in the pediatric population are needed. Changes in 5-HT system are typically visualized using positron emission tomography (PET) (Aznavour et al., 2006) and single photon emission computed tomography (SPECT) (Hwang et al., 2007). However, PET and SPECT techniques are not considered appropriate research tools in children because of the radiation exposure involved. A relatively new approach for assessing neurotransmitter function is the use of pharmacological MRI (phMRI) (Leslie and James, 2000). This method offers a good spatial and temporal resolution without using ionizing radiations allowing longitudinal studies to follow disease progression and/or treatment efficacy. This technique is based on BOLD signal changes often used in functional MRI (fMRI) to detect drug-evoked changes in brain function (Anderson et al., 2008; Martin and Sibson, 2008). The brain has poor metabolic reserve and, as a result, local increases of neuronal activity are associated by a vascular reaction to supply the energetic substrates needed (glucose and oxygen). This phenomenon is called the cerebro-vascular coupling and allows tracking the central activation by measuring the hemodynamic changes (Gsell et al., 2000). It is thus possible to map patterns of hemodynamic changes across the entire brain in response to stimulation of specific neurotransmitter systems using a pharmacological challenge. Several studies in animals have already demonstrated that drug-induced increases in extracellular levels of e.g. 5-HT (releasing agents, selective re-uptake blockers) evoke region-specific changes in BOLD signal intensity and that these effects can be reversed by treatments that decrease 5-HT availability (Houston et al., 2001; Preece et al., 2009; Sekar et al., 2011; Stark et al., 2006). In adult rats, BOLD signal changes following acute SSRI administration have been described in 5-HT related brain regions, i.e. several cortical areas, hippocampus, hypothalamus and thalamus (Schwarz et al., 2007; Sekar et al., 2011). Thus, stimulation of the 5-HT system and its response to this 'challenge' can be used as a measure of its function (Anderson et al., 2008).

Therefore, the present study aimed at investigating the effects of age following chronic SSRI treatment on the 5-HT system non-invasively using phMRI. To this end, 5-HT phMRI was performed in chronic fluoxetine-treated juvenile and adult rats, as an indication of 5-HT function. In view of the above mentioned literature, we hypothesized to detect a different effect of chronic treatment on brain responses evoked by a 5-HT challenge in juvenile treated rats when compared to adult treated animals, presumably reflecting long-lasting neuronal imprinting effects of chronic SSRI exposure on the developing brain.

Materials and methods

All experiments were carried out in accordance to the guidelines laid out in the Animal Scientific Procedures Act under a project approved by the UK Home Office.

Animals

In total, 4 groups ($n = 8$) of male Wistar rats (HsdRccBrlHan:WIST; Harlan, UK) were investigated. Treatment with either fluoxetine or vehicle started either at PND25 (post-natal day 25: 'periadolescent group', 50–80 g) or at PND65 (post-natal day 65 ± 3 days, 'adult group', 290–320 g). PND25 was selected because it approximates the start of adolescence in humans. In male rats, adolescence lasts from PND28 to PND60 (Spear, 2000), with puberty occurring around PND45 (Engelbregt et al., 2000). All rats were housed (2–5 in each cage) under normal 12 hour light–dark cycle and food and water were available ad libitum.

Drugs and treatment

Fluoxetine hydrochloride (Pinewood Healthcare, Ireland; 5 mg/kg body weight) dissolved in sterile water as a vehicle or vehicle alone was administered once daily to each animal by oral gavage (0.5 ml (young animals) or 1 ml (adults) total volume) for 3 weeks. During the MRI experiment, rats were injected intravenously with fluoxetine hydrochloride (Fagron, Belgium; 5 mg/kg) dissolved in 0.9% saline to challenge the 5-HT system, injected in a volume of 1 ml/kg body weight.

Animal preparation

Following chronic treatment with fluoxetine or vehicle and a washout period of at least one week to ensure total drug clearance, rats were anesthetized with isoflurane (5% induction and then reduced to 1.5–2% for maintenance of anesthesia during animal preparation and scanning) given in a 70:30 mixture of nitrous oxide (N_2O) and oxygen (O_2). Physiological conditions were constantly

monitored to ensure that BOLD signal changes were not caused by peripheral systemic effects unrelated to the 5-HT challenge. The right femoral artery was cannulated for blood gas measurements (RapidLab 348, Siemens Healthcare Diagnostics, Newbury, UK) and blood pressure (Biopac Systems Corp., Goweta, USA) monitoring. The right femoral vein was also cannulated for injection of fluoxetine (5 mg/kg) for the acute 5-HT challenge during the phMRI experiments. Since animals were free-breathing during the whole experiment, respiration rate was constantly monitored as well. Body temperature was checked through a rectal probe and maintained at 37.5 °C by a warm air heating system (SA Instruments, New York, USA).

Data acquisition

PhMRI experiments were acquired on a Varian direct drive 4.7T animal MRI scanner (Varian Medical Systems, Inc., Oxford, UK). A cylindrical quadrature RF coil with 72 mm inner diameter (m2m Imaging Corp., Cleveland OH, USA) placed around the head of the animal was used to transmit and receive the signal. For each animal, a T2-weighted anatomical image volume was acquired using a turbo spin echo sequence with an echo train length of 8, matrix = 256 × 256, FOV = 50 mm, 30 contiguous 1 mm coronal slices, centered 8 mm caudal to the posterior edge of the olfactory bulb, 4 averages, TReff = 5112 ms, and TEeff = 60 ms. The time series acquisition used the same sequence with an echo train length of 16; 20 slices of 1 mm thickness centered to the same position as before with TReff = 4915 ms, TEeff = 60 ms and a matrix size of 128 × 128. Thirty two time points (acquisition time per time series volume was 158 s; total scan time of 84 min.) were acquired with injection of 5 mg/kg fluoxetine via the intravenous cannula during the acquisition of 9th time series volume.

Data preprocessing

Obtained anatomical and time series data were converted to 4D Analyze format using ImageJ (Abramoff et al., 2004). 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 of $3.91 \times 3.91 \times 10\ mm^3$ for the time series data. Preprocessing included brain extraction with FSL/BET v. 2.1 (Brain Extraction Tool, part of FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl) with an adjusted fractional intensity threshold and threshold gradient to correct for use with rat data and motion correction using FSL/MCFLIRT. phMRI scans that were of too poor quality due to extensive movement were excluded at this point (6% of cases). Data for each remaining animal was spatially normalized to a stereotactic rat brain template (Schwarz et al., 2006) by computing a six degree-of freedom rigid transform for the phMRI time series to the accompanying anatomical images and a six degree-of freedom rigid transform for the anatomical images to the rat brain template. Finally, these two transformations were combined into a third, and this transformation matrix was used to directly register the low resolution time series into the template space during group analysis (FSL/FEAT v 5.98) (Smith et al., 2004; Woolrich et al., 2009).

Voxel-based analysis

Explorative image-based time series analysis of the BOLD response in individual animals was carried out using Stimulate software (Strupp, 1996). All voxels that showed more than 1% change in signal intensity after the challenge were identified by performing a *t*-test. The average signal time course in all these activated voxels was then determined in order to decide upon the shape of brain signal change per animal. All individual time courses were then averaged to obtain the basic shape of the to-be-modeled waveform. This waveform was used to setup the framework of the general linear model (GLM) using FSL/FEAT v. 5.98 (FMRI Expert Analysis Tool), part of FMRIB's Software Library, (www.fmrib.ox.ac.uk/fsl) with 8 mm FWHM spatial smoothing, FILM prewhitening and a high pass filter of 5060 s. The first time series volume was deleted from further analysis to ensure steady-state imaging has been reached. The design matrix consisted of the custom-made waveform explained above with an initial off period of 1106 s (7 volumes of baseline recordings), followed by a gradual onset of 790 s (starting with the volume during which the 5-HT challenge was injected) and then a steady-state on period of another 3002 s (19 volumes). A temporal derivative was added to the waveform and no HRF convolution or temporal filtering was applied.

Statistical maps of mean response to the acute challenge were calculated within the GLM framework by means of higher-level (group) analysis using the FSL-toolbox FEAT v. 5.98. Z (Gaussianised T/F) statistical maps were corrected for multiple comparisons using GRF theory-based cluster correction within FSL/FEAT (Worsley, 2001) with FWE 0.05 and a Z-threshold of $Z > 3.6$ (all animals) or $Z > 2.3$ (per group).

Also within FEAT, a 2-factors 2-levels ANOVA design was used to determine the interaction effects of age and treatment within the response to the acute challenge. Subsequently, the effect of age in untreated animals only and the effect of treatment in both age groups separately were determined as posthoc tests using a two-sample *T*-test design. F- and T-values above threshold ($p = 0.05$) are automatically transformed into Z-values by FEAT, again thresholded ($Z > 2.3$) and then mapped onto a Z-statistic image. Thus, only voxels that survived in the ANOVA/*T*-test at $p = 0.05$ and had a Z-value of above 2.3 are shown in the resulting statistical maps. Due to small sample size and thus lower power in these subanalyses, correction for multiple comparisons was only carried out when investigating the main effect of the acute challenge, in the subanalyses, we report clusters with a minimal size of 20 voxels instead.

A 3D digital reconstruction of the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1986) co-registered with the rat brain template (Schwarz et al., 2006) was used to identify the location of significantly activated clusters.

Results

Physiological parameters

Blood gas measurements (pCO_2: 32.0–45.0 mm Hg; pO_2: 75.0–100.0 mm Hg; pH: 7.35–7.45), mean artery blood pressure (100–150 mm Hg), respiration rate (50–70 breaths/min) and temperature (37 ± 1.5 °C) were all within the here given normal ranges before start of scanning. Animals that showed unstable physiological responses during scanning that were 1) clearly outside of the above mentioned ranges and that 2) could have affected reliable interpretation of the BOLD signal, but were 3) undoubtedly unrelated to the intravenous challenge were excluded from further analysis (12% of cases). All animals showed a distinctive drop in blood pressure signal of about 20% and a short rise in respiration rate upon administration of the intravenous 5-HT challenge. Blood pressure returned to pre-challenge values within 5–10 min in all cases included in the analyses. These physiological responses to the challenge appeared to be unrelated to age or previous treatment.

Voxel-based analysis

Effect of acute 5-HT challenge

Areas that were significantly activated by the acute 5-HT challenge averaged over all animals are depicted in Fig. 1A. Cortical areas that showed significant activation (Z-value > 3.6 with GRF-based cluster correction at $p < 0.05$ (Worsley, 2001)) include large parts of the sensory and motor cortices and the (pre)frontal cortex. Subcortical areas that were significantly activated by the 5-HT challenge were the

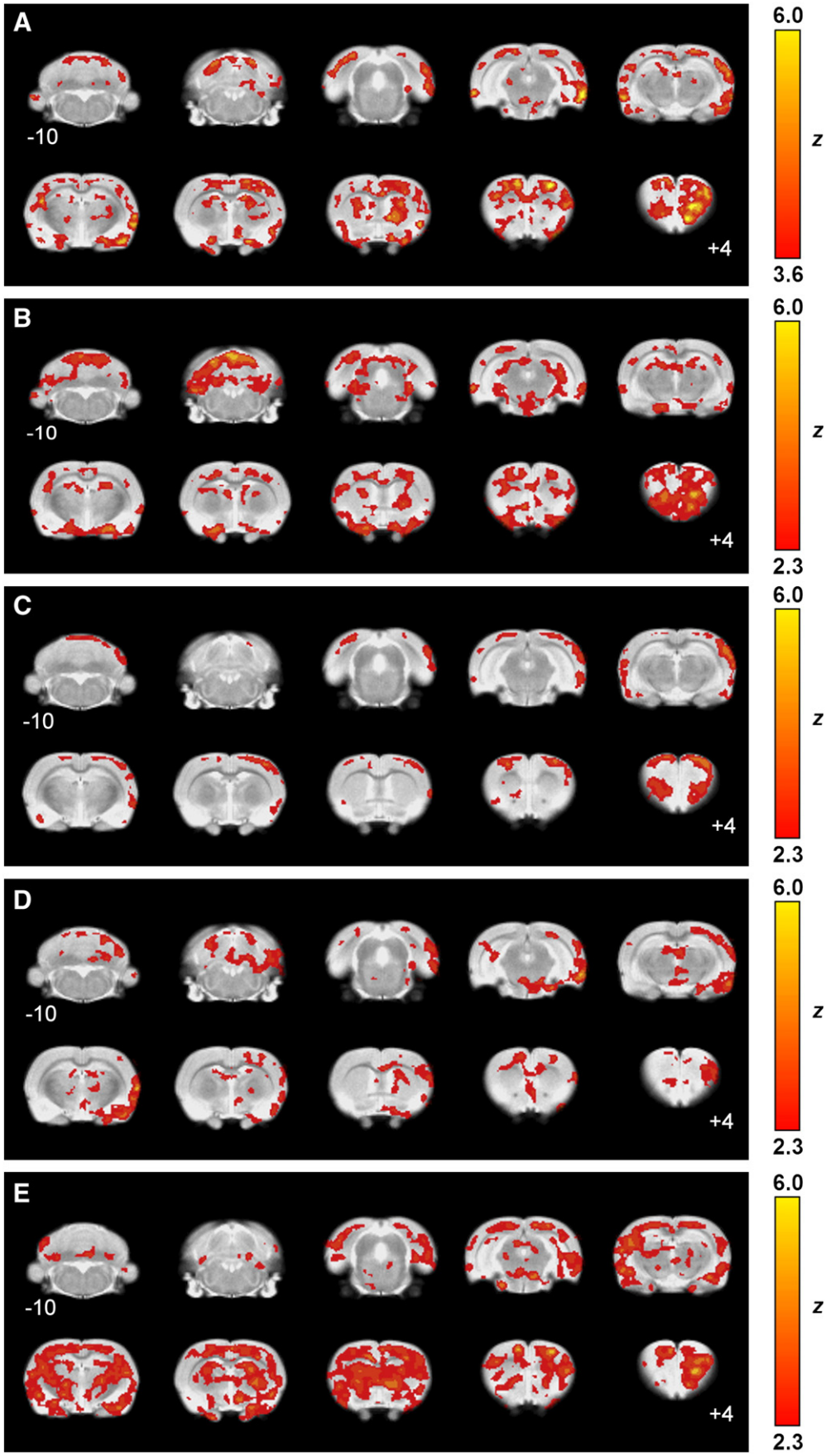

Fig. 1. Group mean effects following acute 5-HT challenge with fluoxetine. A) Mean effect in all animals (n = 26), B) Mean effect in the adult control group (n = 8), C) adult-treated animals (n = 8), D) young control group (n = 5), and E) juvenile-treated animals (n = 5). All statistical parametric maps were thresholded using clusters determined at Z>3.6 (Fig. 1A) or Z>2.3 (Fig. 1B t/m E) and GRF-based cluster correction at p<0.05 (Worsley, 2001). Range: bregma −10 mm to bregma +4 mm.

amygdala, hippocampus, dorsolateral thalamus and caudate putamen. On average, there were no significant areas of deactivation following the challenge. In Fig. 1B up to E, the average effects of the challenge are presented per group (Z-value>2.3 with GRF-based cluster correction at $p<0.05$).

Age by treatment interaction effect

Explorative ANOVA analysis, used to determine the effects of age and treatment within the effect of the acute 5-HT challenge, showed an age by treatment interaction effect ($P=0.05$ (uncorrected), Z-value >2.3, cluster size >20 voxels) on the response to the acute 5-HT challenge in voxels in several cortical and subcortical areas, including the amygdala, caudate putamen, hypothalamus, hippocampus, inferior colliculus, substantia nigra, orbitofrontal cortex and the parts of the somatosensory cortex (See Table 1 and Fig. 2A). In most brain regions, except for the somatosensory cortex, juvenile-treated rats showed an increased activation evoked by the 5-HT challenge following chronic fluoxetine treatment when compared to adult-treated animals. In order to determine what caused this interaction effect to occur, post-hoc analyses were performed on the effect of age in the untreated animals only and on the effect of treatment in both age categories separately, using a two-sample *T*-test design.

Effect of age in control animals

The effect of age on the BOLD change evoked by the acute challenge was determined in the non-treated control animals (Fig. 1B vs. D). Voxels with a significant ($P=0.05$ (uncorrected), Z-value >2.3, cluster size >20 voxels) higher response to the challenge in young (±PND55 at time of scanning) versus older (±PND95 at time of scanning) animals were found only in small areas of the left primary and secondary cortex, piriform cortex, and in the dorsal thalamus (see Fig. 2B). No voxels with lower activation in young versus older animals were found.

Effect of treatment within different age categories

Chronic fluoxetine treatment caused a diminished response to the acute challenge in the adult animals compared to the adult controls (Fig. 1B vs. C), in voxels in the hypothalamus, substantia nigra, amygdala, inferior colliculus, hippocampus and in voxels in several areas of the sensory cortex and only an increased response in voxels in the secondary auditory part of the sensory cortex (see Table 2 and Fig. 2C). In the juvenile-treated animals (Fig. 1D versus E), chronic treatment mainly increased the response to the acute challenge, compared to juvenile control animals. This increase was seen in voxels in the caudate putamen, orbitofrontal cortex and the secondary auditory part of the sensory cortex. A diminished response was only seen in the lateral entorhinal part of the piriform cortex (see Table 2 and Fig. 2D).

Table 1
Clusters of voxels with a significant age by treatment interaction effect.

Region	Left–right	Size	Z-value	X	Y	Z	Activation
Subcortical areas							
Inferior colliculus		65	3.57	50	56	9	↑
Caudate putamen	R	44	3.09	34	49	21	↑
	R	28	2.82	35	39	22	↑
Hypothalamus	R	40	2.96	40	21	18	↑
Hippocampus	L	21	2.70	72	44	13	↑
Substantia nigra	L	27	3.18	57	30	13	↑
Amygdala	L	20	3.40	64	26	18	↑
Cortical areas							
Orbital frontal cortex	L	63	3.96	58	49	26	↑
Sensory cortex							
Primary somatosensory cortex	R	62	3.01	24	59	17	↑
	R	35	3.37	33	62	17	↑
	R	29	3.09	33	59	20	↑
	L	28	2.95	76	62	17	↓
Secondary somatosensory cortex	L	23	3.11	85	44	19	↓

Voxels thresholded by Z>2.3. Only clusters with a size of >20 voxels were included. Size is the number of voxels above threshold in the cluster; Z-value is the Z-value (converted from F-value) of the most activated voxel in the corresponding cluster. X-, Y-, and Z are MRI rat brain template coordinates of the most activated voxel (Schwarz et al., 2006); ↓ = negative interaction effect; significantly more decrease in activity related to the 5-HT challenge after treatment in adult animals than in juveniles; ↑ = positive interaction effect; significantly more increase in activity related to the 5-HT challenge after treatment in juvenile animals than in adults.

Discussion

Chronic treatment with an SSRI caused an opposite effect of the acute 5-HT challenge on brain activation in adult- versus adolescent treated rats after a one-week washout period. In juvenile animals, fluoxetine treatment caused an increase in brain activation, whereas this response was reduced in adult treated animals.

Effect of acute challenge

We observed an overall increase in brain activation in response to the acute fluoxetine challenge, mainly in the cortical brain regions (Fig. 1A), which are known to be highly innervated by 5-HT projections and are rich in 5-HT receptors (D'Amato et al., 1987). These observations are in correspondence with the results of Schwarz et al. (2007), who also observed a predominantly cortical effect with fluoxetine-induced phMRI. Although in our study the cortical activation pattern is more widespread than in the Schwarz study and includes more ventral cortical regions as well, we believe that these differences could be attributed to the fact that Schwarz et al. (2007) used a surface coil to receive the MR signal. This could have lead to a drop of the signal to noise ratio for deeper lying regions. Studies that used another SSRI (citalopram) as acute 5-HT challenge, as well report extensive cortical activation in both animals (Sekar et al., 2011) and humans (McKie et al., 2005) as a result of this challenge. In addition to these cortical regions, we observed statistically significant subcortical effects in the hippocampus, dorsolateral thalamus, amygdala, and caudate. These findings are in agreement also with earlier 5-HT phMRI studies using either a fluoxetine (Schwarz et al., 2007) or a citalopram challenge (McKie et al., 2005; Sekar et al., 2011). These regions are all 5-HT related as well, either having a high 5-HT and/or SERT density (thalamus, amygdala, and caudate) or being abundant in 5-HT receptors (hippocampus and caudate). Although the juvenile-treated animals had reached (young) adulthood by the time scanning took place, there was still a small but significant age effect within the effect of the acute challenge in some cortical areas, the hippocampus and thalamus.

Effect of chronic SSRI treatment

In the adult animals, chronic treatment with fluoxetine caused a general diminished response to the challenge, when compared to the effect on brain activation in untreated adult animals.

To our knowledge, there is only one other 5-HT phMRI study available that looked at effects of chronic SSRI treatment in rats. Sekar et al. (2011) recently compared the acute and chronic effects of the SSRI citalopram on brain activity in adult rats. Although there is an essential methodological difference with the present study in that these authors did not introduce a washout period after chronic treatment and thus did not look at lasting effects of chronic treatment, our results are partially in line with this study. Sekar et al. also report a diminished response after chronic treatment, although this was only visible in the midbrain areas, whereas in our study the effect is more widespread. Moreover, in contrast to Sekar et al., we extended these chronic studies by comparing effects of age. Interestingly, we found an opposite treatment effect in the

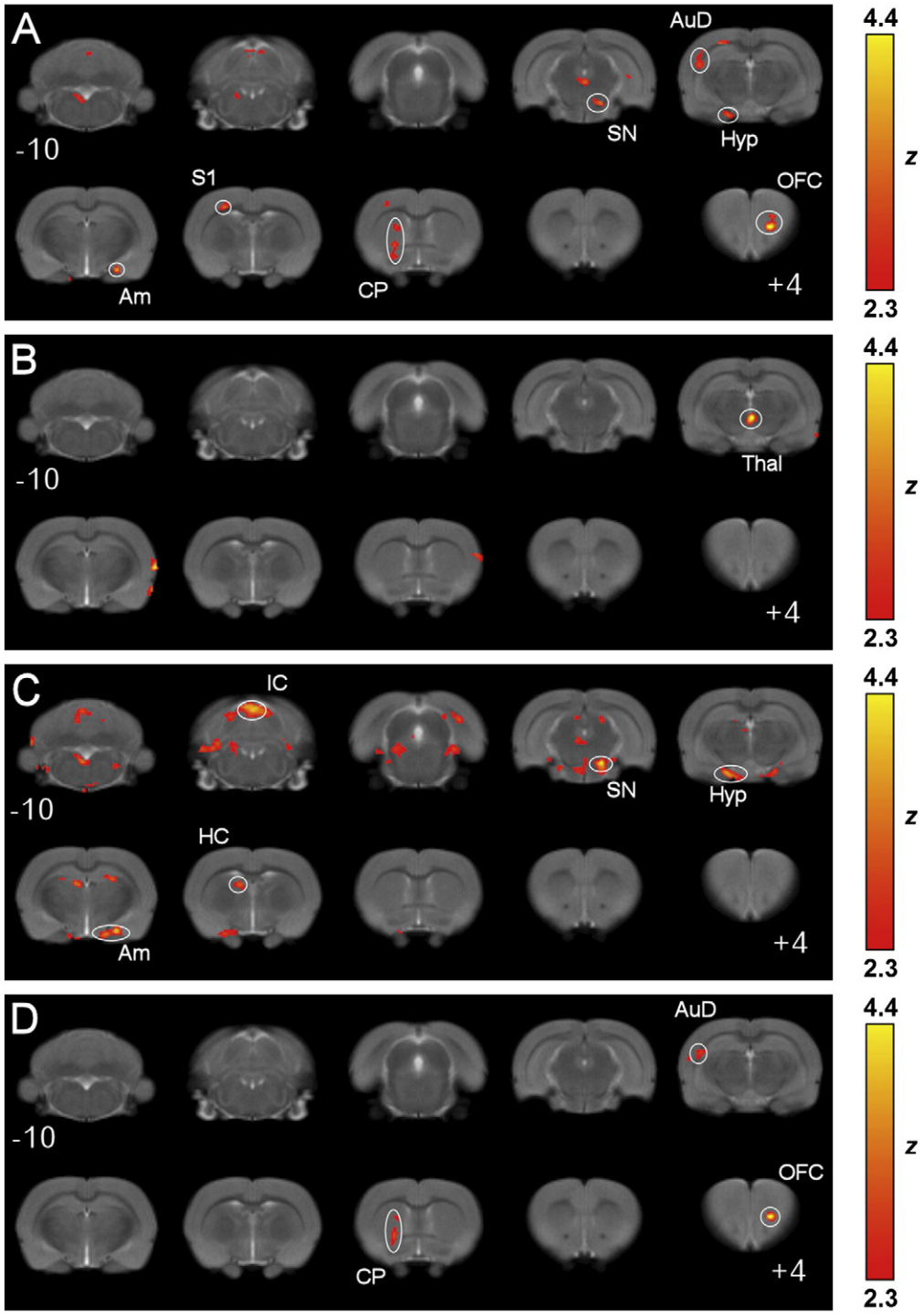

Fig. 2. Age, treatment and age by treatment effects following 5-HT challenge with fluoxetine. A) Voxels with significant age by treatment interaction effect; B) Voxels with significant effect of age (juvenile animals higher activation than adult animals) in control animals only; C) Voxels with significant effect of treatment (control animals HIGHER activation than fluoxetine-treated animals) in the adult animals; D) Voxels with significant effect of treatment (control animals LOWER activation than fluoxetine-treated animals) in the juvenile animals. All maps were thresholded at $p = 0.05$ (uncorrected), $Z > 2.3$ and cluster size >20. Range: bregma −10 mm to bregma +4 mm. Am = amygdala; AuD = secondary auditory cortex; CP = caudate putamen; HC = hippocampus; Hyp = hypothalamus; IC = inferior colliculus; OFC = orbital frontal cortex; S1 = primary sensory cortex; SN = substantia nigra; Thal = dorsal thalamus.

juvenile-treated animals. Instead of a decrease, an increased response to the fluoxetine challenge was observed in this group, compared to untreated juvenile animals. In support of this, there was a significant age by treatment interaction effect in several voxels in 5-HT related brain regions such as the amygdala, hippocampus, caudate putamen, hypothalamus, and orbitofrontal cortex. Note that these are mainly limbic and frontal areas and, as mentioned before, remodeling of the 5-HT system during adolescence is most prominent in the frontal and limbic regions as well (Crews et al., 2007). The findings are also in concordance with our hypothesis, in which we predicted a differential effect of chronic treatment on brain responses evoked by a 5-HT challenge in juvenile animals compared to adult animals.

Table 2
Clusters of voxels with significant in- or decreased activity after fluoxetine treatment related to the acute 5-HT challenge, compared to untreated animals.

Region	Left-right	Size	Z-value	X	Y	Z	Activation
Adult							
Hypothalamus	R	540	3.65	36	25	16	↓
Substantia Nigra[a]	L	364	4.26	57	31	14	↓
Amygdala[a]	L	364	3.89	63	26	18	↓
Inferior Colliculus		266	3.91	48	58	10	↓
Hippocampus	R	137	4.43	43	49	19	↓
	L	43	3.22	61	53	18	↓
	L	24	2.88	57	53	14	↓
Sensory cortex							
Secondary visual cortex	L	23	3.03	69	54	12	↓
Primary somatosensory cortex	R	22	3.27	20	55	15	↓
Secondary auditory cortex	L	33	2.79	80	56	16	↑
Young							
Lateral entorhinal cortex	L	31	3.78	82	31	15	↓
Secondary auditory cortex	R	58	2.93	17	48	17	↑
Orbitofrontal cortex	L	51	3.70	59	49	26	↑
Caudate putamen	R	46	3.37	34	49	21	↑
	R	33	2.79	35	39	22	↑

Voxels thresholded by Z>2.3. Only clusters with a size of >20 voxels were included. Size is the number of voxels above threshold in the cluster; Z-value is the Z-value (converted from T-value) of the most activated voxel in the corresponding cluster. X-, Y-, and Z are MRI rat brain template coordinates of the most activated voxel (Schwarz et al., 2006); ↓ = activation attenuated compared to untreated animals; ↑ = activation enhanced compared to untreated animals.
[a] Two local maxima in one cluster.

Different effect on phMRI in adolescent- versus adult-treated animals

The increased phMRI signal induced by acute SERT blockade seen in all animals is most likely caused by increased extracellular levels of 5-HT, due to the inhibition of reuptake. The role of extracellular 5-HT on BOLD response was previously demonstrated by Preece et al. (2009) who showed that 5-HT release by the 5-HT releasing agent fenfluramine evokes effects on BOLD signal which are attenuated by 5-HT depletion with p-chlorophenylalanine. This does however not fully explain why chronically treated animals respond differently to the SSRI challenge when compared to non-treated animals; although chronic treatment with SSRIs in adult animals causes increased concentrations of extracellular 5-HT at the terminal fields, microdialysis studies have shown that these concentrations normalize again shortly after treatment discontinuation (Invernizzi et al., 1996). One week washout should have abolished the effects of chronic treatment if only extracellular 5-HT concentrations were of importance. However, the chronically elevated extracellular 5-HT concentrations are well-known to induce numerous adaptive responses in the expression of receptors and intracellular signaling mechanisms (Blier and de Montigny, 1999; Faure et al., 2006; Tardito et al., 2006).

Sekar et al. (2011) recently showed that also the 5-HT_{1A} (auto) receptor is involved in the brain response evoked by 5-HT challenge with an SSRI. They demonstrated that blockage of these receptors with a selective antagonist influenced the BOLD response to a citalopram challenge markedly. Although it is unknown how long it takes for the desensitization of 5-HT_{1A} presynaptic autoreceptors to normalize again after discontinuation of fluoxetine treatment, it is known that postsynaptic 5-HT_{1A} receptor function takes at least 14 days to return to normal values (Raap et al., 1999). Thus, an initial increase in extracellular 5-HT levels with subsequent longer-lasting desensitization of 5-HT_{1A} autoreceptors might be an explanation for our findings of reduced brain activation in adult treated animals. However, it should be kept in mind that separating different contributors affecting the BOLD signal is difficult due to the complex interactions within the neurotransmitter system. Hemodynamic measurements are an indirect index for 5-HT function as they measure the system as a whole, including function from other receptor subtypes (e.g., 5-HT_{2A}, 5-HT_{2C} and 5-HT_{1B}; (Gozzi et al., 2010; Houston et al., 2001; Scanley et al., 2001; Stark et al., 2006)) and their impact on other neurotransmitters as well.

Unfortunately, much less is known about these effects in adolescent animals. To our knowledge, there are no studies documenting extracellular 5-HT levels after SSRI treatment in young animals. In a recent study, no changes in 5-HT_{1A} receptor immunoreactivity following chronic fluoxetine treatment were observed (Homberg et al., 2011). As pointed out by the authors, this is somewhat surprising given the role of the 5-HT_{1A} receptor in the antidepressant effects of SSRIs (see above). Yet, these observations do not exclude involvement of the 5-HT_{1A} receptor in our study, because 5-HT_{1A} receptor function was not assessed in the study of Homberg et al. We can therefore only conclude that whatever the precise underlying mechanisms may be, they apparently affect the developing brain differently when compared to the matured brain. Future studies focusing on 5-HT levels and 5-HT (auto)receptor functionality in juvenile SSRI treated rats are clearly of great interest.

Other studies on age-related effects

Although few in number, other animal studies support our findings of age-related effects following chronic fluoxetine treatment. A number of behavioral studies reported differential effects of chronic fluoxetine treatment with age on behavior in both rats and mice. In rats, Iñiguez et al. (2010) recently showed that fluoxetine treatment during adolescence (PND35-49) results in long-lasting decreases in behavioral reactivity to forced swimming stress, while LaRoche and Morgan (LaRoche and Morgan, 2007) observed age and sex-specific alterations of visual discrimination and attention (PND25-49). Homberg et al. (2011) recently reported increased behavioral despair in the forced swim test and an increased number of amygdala PSA-NCAM (marker for synaptic remodeling) immunoreactive neurons only in juvenile (PND25-46) but not adult fluoxetine treated rats (PND67-88). In mice, Mason et al. (Mason et al., 2009) showed age-related differences (5 weeks vs. 12–13 weeks) in depression-like behavioral effects, although Norcross et al. (Norcross et al., 2008) did not find any anxiety-, fear- or stress-related behavioral differences or differences in brain serotonin levels between adolescent (3–7 weeks) and adult (8–12 weeks) treated mice. On a more neurobiological level, Wegerer et al. (1999) observed an increase in frontal cortex SERT densities in the order of 20%, only in adolescent rats (PND25-39). In addition, Norrholm and Ouimet (2000) showed that juvenile chronic fluoxetine treatment (PND21-42) might arrest spine development in the hippocampus and Navailles et al. (2008) observed a stimulatory effect of chronic fluoxetine treatment during adolescence (PND32-56) on adult hippocampal neurogenesis. It should be noted however, that all above mentioned studies used different dosages, durations and routes of administration, different washout periods, and are not uniform in the age at which treatment started (although all animals were older than PND14 at start of treatment), which makes comparisons between them difficult. We however believe that the currently used low oral dosage of 5 mg/kg has a high clinical relevance, as noted by Wegerer et al. (1999) as well. The human therapeutic dose of fluoxetine is approximately 0.3–1.0 mg/kg/day, and because rodents metabolize fluoxetine about ten times faster than humans (Caccia et al., 1990; Hiemke and Härtter, 2000), the currently used dose is highly comparable to the human therapeutic dose.

Neuronal imprinting

The present finding of different and even opposite effects evoked by chronic treatment with SSRI in adult versus juvenile rats, as assessed using phMRI, is in support of the “equal but opposite”

hypothesis (Andersen, 2003). This hypothesis proposes that chronic drug exposure in adult animals will result in adjustment to the drug effects by a series of compensatory mechanisms, as shown by the reduced response to an acute challenge after chronic pretreatment in the current study, whereas in juvenile animals it will lead to more permanent developmental alterations of the involved neurotransmitter system. Adolescence is a particularly critical period for such 'imprinting' effects, due to changes in neurotransmitter function, and changes in functional connectivity. It is thought that altering neurotransmitter levels will have its greatest impact during childhood to adolescence, when the synaptic selection process reached its peak. Therefore, we propose that the age-associated effects of chronic treatment with an SSRI on brain 5-HT function observed in this study most likely reflect a series of (transient) compensatory mechanisms in the adult animals, in contrast to more permanent developmental alterations to the 5-HT system in the juvenile animals. In order to unravel the neurobiological mechanisms underlying these alterations, it is necessary to first deciphering the underlying mechanism of the phMRI findings to have an idea which mechanisms are likely to be involved.

Limitations

As pointed out by Martin and Sibson (2008), a potential confound of all 5-HT phMRI studies is that it is assumed that the changes in brain activity evoked by the 5-HT challenge reflect changes in neuronal activity rather than peripheral systemic effects. In our study, there are several observations that support this assumption: the general pattern of activation was region specific and restricted to areas with a high 5-HT innervation, and not as much a general vascular response, as the strongest hemodynamic changes were observed in cortical regions and subcortical gray matter. Also, a different temporal profile between vascular and hemodynamic changes was observed; whereas blood pressure returned to pre-challenge values within 5–10 min in all cases, brain signal changes did not return to baseline within the scanning frame, which lasted until about 60 min after administration of the challenge. Therefore, we have no reason to believe that the age-dependent differences in the effects of chronic fluoxetine treatment found in this study are due to differences in basal cerebral blood flow. Also, the physiological responses to the acute challenge where similar in all animals, so no differences between age or treatment groups were observed on these parameters. Nevertheless, it is also known that a neurogenic regulation of the local blood flow by 5-HT exist (Cohen et al., 1996). Therefore it cannot be excluded that local changes of BOLD signal could have attributed to vascular changes due to release of 5-HT at the proximity of vessels. Although we can consider these effects as false positive results since they are not associated to local neuronal activation, it is also an index of the overall specific function of the serotonergic system. Finally, due to the explorative character and low statistical power of the ANOVA and subsequent posthoc subanalyses, we chose to report all clusters bigger than 20 significant voxels, instead of correcting for multiple comparisons using the standard GRF-based cluster correction method. The complexity of this type of study does not allow for the large sample sizes needed to obtain sufficient statistical power for these subanalyses, in which we looked at the effects of treatment and age *within* the acute effect of the challenge. Using standard GRF-based cluster correction at $p = 0.05$ and $Z > 2.3$, this would require clusters of at least 217 voxels. As can been seen in Table 2 only the effects of treatment in the hypothalamus, substantia nigra, amygdala, and inferior colliculus in the adult-treated animals would survive in this case. We did correct for multiple comparisons when examining the effects of the acute challenge on BOLD signal. Despite these limitations, we believe that, considering the overlapping results with earlier 5-HT phMRI studies in rats (Schwarz et al., 2007; Sekar et al., 2011) and humans (McKie et al., 2005), 5-HT phMRI is a well suited method to assess 5-HT function. One of the major strengths of the present study is the easy clinical translation of the technique used and its non-invasive character, which suggests that this technique can and should be used to study neuronal imprinting effects of early SSRI treatment in children suffering from depression or anxiety disorders.

Conclusions

In line with our hypothesis, we observed age-dependent effects of chronic fluoxetine treatment on brain responses evoked by an acute 5-HT challenge with phMRI in juvenile-treated rats compared to adult-treated rats after a one-week washout period. Although future studies directed towards the underlying mechanism of this observation are warranted, our opposite findings in juvenile-treated animals compared to adult-treated animals most likely reflect neuronal imprinting effects of early SSRI treatment. These findings of age-dependent alterations in 5-HT system function after chronic SSRI treatment during late development support the 'equal but opposite' theory of Andersen (2003). In comparison to previous animal studies on this subject, our study has a strong clinical relevance because of the non-invasive nature of the technique used and the highly clinical relevant dosage and route of administration, and calls for further investigations in the pediatric population.

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