Antidepressants and the adolescent brain: Changing the course of neurodevelopment?

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CHAPTER 7

LASTING AND DIVERGENT EFFECTS
OF FLUOXETINE TREATMENT ON NEUROGENESIS
IN ADOLESCENT AND ADULT RATS

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In preparation
Abstract

Over the years, the antidepressant drug fluoxetine (Prozac®) is increasingly being prescribed to children and adolescents with a depressive disorder, despite ongoing debate on its efficacy in the pediatric population and a lack of detailed knowledge on its putative neurodevelopmental effects. Here, we investigated the effects of chronic fluoxetine on adult hippocampal neurogenesis, a structural readout relevant for antidepressant action, in the rat brain and whether this differed from treatment during adolescence or at an adult age. Stereological quantification of the immunoreactivity of the proliferation marker Ki-67 and the neuronal differentiation marker doublecortin, revealed a significant age-by-treatment interaction effect, which indicates that fluoxetine differently affects adult neurogenesis in adolescent-treated animals than in those treated as adults. Fluoxetine enhanced proliferation in mainly the dorsal parts of the hippocampus and differentiation in both the suprapyramidal and infrapyramidal blades of the dentate gyrus in adolescent-treated animals, while such an effect was not seen in the adult-treated animals. Thus, fluoxetine exerts divergent effects on structural plasticity in adolescent versus adult-treated animals, possibly due to the neurodevelopmental state of the adolescent brain. These preliminary data warrant further research into behavioral and translational aspects of our findings and, together with related recent findings, call for caution in prescribing these drugs to the adolescent population.
Introduction

Adolescence is a sensitive developmental period during which several important brain systems and structures mature. As such, it is highly susceptible to interference by environmental factors such as stress or psychotropic drugs, that can influence this brain maturation, often in a lasting manner (Andersen and Navalta, 2004). The SSRI fluoxetine (FLX; Prozac®) is currently the only approved drug available for treatment of pediatric depression. despite ongoing debate (Bridge et al., 2007; Hetrick et al., 2007) on increased risks for suicidal thinking in the pediatric population (Gibbons et al., 2006; Jureidini et al., 2004) and despite earlier warnings by FDA and EMEA, discouraging practitioners from prescribing antidepressants to children (Lancet Editorial Note, 2006). SSRIs selectively block the 5-HT transporter (SERT) and thereby increase levels of 5-HT in the synaptic cleft. Stable 5-HT concentrations are established through negative feedback control mediated through 5-HT autoreceptors located on the presynaptic cell. Upon chronic treatment, 5-HT receptor desensitization is believed to be instrumental in alleviating depressive symptoms (Pineyro and Blier, 1999; Sharp, 2010).

5-HT plays a key role in many aspects of early brain development, including cell proliferation and differentiation, and is crucial for proper wiring of the brain (Gaspar et al., 2003; Lauder, 1990; Whitaker-Azmitia et al., 1996). Although most of the 5-HT system is already fully functional around birth (Murrin et al., 2007), it was 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). In view of this ongoing development of 5-HT transmission in adolescence and the fact that 5-HT also influences hippocampal neurogenesis (Migliarini et al., 2012; Whitaker-Azmitia et al., 1996), it is likely that exposure to SSRIs during periods of DG development may exert lasting consequences on the adult 5-HT system, which may influence the vulnerability to neuropsychiatric disorders with developmental origins such as schizophrenia, depression, autism and mental retardation (Migliarini et al., 2012).

The neurogenesis theory of depression builds on rodent data showing that chronic stress not only induces depressive-like symptoms, but also decreases the incorporation and/or survival of newborn cells in the hippocampus. Chronic treatment with FLX e.g. stimulates neurogenesis in naive rats (Encinas et al., 2006; Huang and Herbert, 2006; Kodama et al., 2004; Malberg et al., 2000; Marcussen et al., 2008; Pinnock et al., 2009; Sui et al., 2009), and
can rescue chronic stress-induced reductions in neurogenesis (Czeh and Lucassen, 2007; Hanson et al., 2011b; Lucassen et al., 2010a; Oomen et al., 2007; Sahay and Hen, 2007; Sass and Wortwein, 2012; Su et al., 2009). Neurogenesis is further needed for FLX to exert its behavioral effects (Santarelli et al., 2003), and can be influenced by stress (David et al., 2009) and age (Couillard-Despres et al., 2009; Navailles et al., 2008). FLX further modulates structural plasticity in human depression (Boldrini et al., 2012; Lucassen et al., 2010b), but does not alleviate depressive symptoms until after several weeks of treatment. This time-to-effect matches the time required for new cells to integrate into a network, further supporting a role for neurogenesis in antidepressant action (Taupin, 2010).

Previously, we found differential, age-dependent effects of FLX treatment on 5-HT related brain activity using pharmacological MRI (Klomp et al., 2012b). This suggested that in terms of sensitivity to FLX, the brain of adolescent animals differs from adults, which may bear relevance for neuronal imprinting (Andersen and Navalta, 2004). Although many studies have looked into the effects of e.g. maternal FLX exposure on structural plasticity, studies on adolescent exposure are less abundant with generally mixed results (Cowen et al., 2008; Hodes et al., 2009; Navailles et al., 2008). Here we investigated whether FLX induces age-dependent effects on adult neurogenesis in the hippocampus after either adolescent or adult chronic exposure. For this, Ki-67 and doublecortin (DCX) immunohistochemistry was used to respectively study changes in cell proliferation and to determine if FLX had influenced survival and neuronal differentiation of progenitor cells.

**Experimental procedures**

All experiments were carried out according to the guidelines set forth by Dutch regulations governing animal welfare and protection. Protocols were reviewed and approved by the local Animal Welfare Committee for Animal Experiments at the Academic Medical Centre in Amsterdam, the Netherlands. All efforts were made to ensure that animal suffering and the number of animals was kept to a minimum.

**Animals**

In total, 32 male Wistar rats (n=8 per group) were obtained from Harlan (Venray, The Netherlands). The adolescent-treated animals arrived on postnatal day 21 (PND21) and treatment started at PND25 (body weight of 50-80g) with either FLX or vehicle. Since the peripubescent period begins around PND24 (Spear, 2000), PND25 lent itself to model the clinical situation where FLX is administered to children and adolescents from the age of 8 years onwards. A second group of young adult animals began treatment at PND65 ±4
days (body weight of 290-320g). Henceforth, the group representing the rats treated in adolescence will be referred to as PND25 and those treated in adulthood as PND65. All animals were group-housed four per cage, weighed and handled daily and provided with food and water ad libitum. A normal 12:12 hour light/dark cycle was applied with lights on at 7:00 am. Laboratory conditions were kept at a constant 20 ± 1°C with normal humidity.

**Drugs and treatment protocol**

Fluoxetine hydrochloride (Fagron, Belgium) was dissolved in sterile water (vehicle) and administered at a final dose of 5 mg/kg/day per os via oral gavage with a total volume of 0.5 ml in young animals and 1 ml in adult animals. Control animals received the same total volumes of vehicle only. Treatments were administered daily before lights out for 3 weeks, followed by a washout period of one week to allow the pharmacological agent to be removed from the animals’ system (Caccia et al., 1990). See also Figure 1 for the time line of this study.

**Animal and Tissue Preparation**

At the end of the washout period, animals were sacrificed at PND53 for the adolescent group, and at PND93 ±4 days for the adult group. Animals were anaesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M Phosphate Buffered Saline (PBS), pH 7.4. Brains were post-fixed in the skull overnight at 4°C before careful removal and storage in PBS azide pH 7.4. Subsequently, brains were cryoprotected by saturation in 15% sucrose that was later replaced by a 30% sucrose solution in PB. Sections were cut at 40 μm with a freezing microtome. Tissue sections were collected in a one-in-10 series in 0.1M phosphate buffer and ranged from the prefrontal cortex (Bregma 5.2) through to the dorsal raphe nucleus (Bregma -10.30, (Paxinos and Watson, 1986). Sections were washed in chilled PB to extract the cryoprotectant and stored at 4°C in PB + 0.01% sodium azide prior to immunohistochemistry.

**Immunohistochemistry**

**Proliferation (Ki-67)**  
Ki-67 is an endogenous protein that is expressed during all stages of the cell cycle, except G0. Free-floating coronal sections were mounted on glass slides, dried and then randomized. Sections were boiled in citrate buffer (0.01M, pH 6.0) in a microwave oven (Samsung, M6235 800W) for antigen retrieval and further processed according to standard protocols described before (Oomen et al., 2010) using antibodies against Ki-67 (polyclonal rabbit anti-Ki67p; 1:2000; Novocastra), biotinylated goat anti-rabbit (Vector, 1:200), amplification with Avidin-Biotin Complex (ABC-elite (Vector) 1:800
Part 2: Age-related effects of fluoxetine in the rat’s brain

in TBS) and biotinylated tyramide (1:500 in 0.01% H₂O₂ in TBS). Chromogen development was in 3,3’-Diaminobenzidine (DAB; Sigma D-5637; 0.01g/ml DAB, 0.01% H₂O₂) followed by counterstaining in Haematoxylin (1g/l), dehydration and coverslipping with Entellan (Merck).

Neuronal differentiation (DCX) DCX is a microtubule-associated protein expressed in young migratory neurons. Free-floating hippocampal sections were processed according to protocols described in detail before (Oomen et al., 2010) using anti-doublecortin (Santa-Cruz 1:800), biotinylated donkey anti-goat (Jackson, 1:500) and amplification as described above.

Data acquisition and stereological quantification

Ki-67+ cells in the granule cell layer (GCL), subgranular zone SGZ) and hilus were quantified by an observer (LV) unaware of the conditions of the material, using a light microscope (Figure 2A and 2B). The SGZ was defined as a two-three cell-body wide band running along the base of the GCL and facing the hilus. The hilus was defined by drawing a virtual line from the caudal tip of the suprapyramidal blade to the tip of the CA3-4 that ended within the DG, and then to the caudal end of the infrapyramidal blade. Ki-67+ cells were quantified in a 1-in-10 series (11 ±1 hippocampal sections per animal) in the SGZ of the infrapyramidal and suprapyramidal blade, in the GCL of both infra- and supra-pyramidal blades, and in the hilus, in both hemispheres. Dorsal and ventral regions were defined as the first 6 and final 4-6 hippocampal sections, respectively, along the entire extent of the rostro-caudal axis.

DCX+ cells were too numerous to be counted by hand (Figure 3A and 3B), and a design-based stereological procedure was applied based on every tenth serial section along the rostro-caudal extent of the hippocampus (12 sections per animal) as described before (Oomen et al., 2007) including the infra- and supra-pyramidal blades as well as the dorsal and ventral hippocampus as described above. DCX+ cells were quantified using a systematic random sampling technique with an optical fractionator method in the StereoInvestigator software (MicroBrightField, Germany). The following settings were employed: grid size 140 x 80, counting frame 50 x 50, which resulted in counts ranging from 300 to 800.

Statistical Analysis

All data are expressed as group means ± S.E.M. Data were analyzed using SPSS.20 (IBM) by analysis of variance (2-by-2 ANOVA) with ‘age’ and ‘treatment’ as factors, after log-transformation of the data where necessary. In case of significant interaction effects, post-hoc
analyses were performed. A Paired Student’s t-test was used to evaluate interhemispheric differences. P-values of ≤ 0.05 were considered statistically significant.

Figure 1. Chronological timeline representing the experimental protocol
Two age groups were used: the adolescent-treated group (referred to as PND25) and the adult-treated group (referred to as PND65). After an acclimatization period of 4 days, chronic treatment with either FLX (5 mg/kg) or vehicle started at the age of PND25 or PND65. After 21 days of treatment and one week of drug washout, brains were extracted at either PND53 or PND93.

Results

Cell Proliferation
Quantification of the numbers of Ki-67+ cells in the bilateral SGZ, GCL and hilus revealed no significant difference between left and right hemispheres in any group (P65SAL: t(4) = 0.456, p=0.672; P65FLX: t(4) = 0.706, p =0.519; P25SAL: t(6) = -0.721, p=0.498; and P25FLX: t(6)=1.801, p=0.122, paired student’s t-test). In the SGZ, a significant main effect of age was found (2-by-2 ANOVA; F(1,28) = 57.140, p<0.001) regardless of FLX treatment, and a significant age-by-treatment interaction effect (F(1,28) = 5.286, p=0.029), indicating that FLX exerts a different effect on cell proliferation depending on age-at-treatment (Figure 2C). Post-hoc analysis showed a clear trend towards decreased cell proliferation due to FLX treatment in the adult-treated animals (t(14) = -1.956, p=0.071), but in the adolescent-treated animals, FLX did not significantly increase cell proliferation in the SGZ (t(14) = 1.411 p=0.180). There was also a significant age-by-treatment interaction effect detected in the hilus (F(1,28) = 8.122, p=0.008) and a significant main effect of age (F(1,28) = 58.316, p <0.001) such that it reflected the overall effects seen in proliferation.

The infrapyramidal blade of the dorsal hippocampus (dorsal infra), the suprapyramidal blade of the dorsal hippocampus (dorsal supra) and those of the ventral hippocampus (ventral infra and ventral supra) were analyzed separately. The highest numbers of Ki-67+ cells were observed in the infrapyramidal blade of all groups, relative to the suprapyramidal
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blade (Figure 2D). A 2-by-2 ANOVA revealed significant age effects, irrespective of treatment, in all sub-regions (dorsal infra: F(1,28) = 45.041, $p<0.001$; dorsal supra: F(1,28) = 18.197, $p<0.001$; ventral infra: F(1,28) = 40.77, $p<0.001$; ventral supra: F(1,28) = 41.414, $p<0.001$). Furthermore, there was a significant age-by-treatment interaction in the dorsal infra (F(1,28) = 7.810, $p=0.009$) and in the dorsal supra (F(1,28) = 6.434, $p=0.017$). This suggests that FLX primarily exerts effects in the dorsal hippocampus, and that these effects of FLX cell proliferation are dependent on the age-at-treatment.

Neuronal Differentiation

Stereological quantification of DCX+ (doublecortin-positive) cell numbers revealed a significant main effect of age, irrespective of treatment, when the DG was taken as a whole (2-by-2 ANOVA F(1,28) = 92.172, $p<0.001$) and a significant age-by-treatment interaction effect (F(1,28) = 4.852, $p=0.036$), such that adolescent-treated animals had higher numbers of DCX+ cells and adult-treated animals lower numbers of DCX+ cells (Figure 3C), indicating that FLX had an age-dependent, divergent effect on neuronal differentiation. Post-hoc analysis showed that there was a clear trend towards increased cell differentiation due to FLX treatment in the adolescent-treated animals ($t(14) = 2.039$, $p=0.061$), but that in the adult-treated animals, FLX did not significantly decrease cell differentiation ($t(14) = -1.010$, $p=0.330$). When investigating the regional effects of FLX, a 2-by-2 ANOVA revealed significant age effects, irrespective of treatment in all sub-regions (dorsal infra: F(1,28) = 55.837, $p<0.001$; dorsal supra: F(1,28) = 80.664, $p<0.001$; ventral infra: F(1,28) = 61.084, $p<0.001$; and ventral supra: F(1,28) = 40.455, $p<0.001$) indicating an age-dependent decline in cell differentiation. Additionally, a significant main effect of treatment was detected in the ventral infra (F(1,28) = 5.969, $p=0.021$) such that FLX increased DCX+ cells in both treatment groups. Furthermore, there was a significant age-by-treatment interaction effect in the dorsal infra (F(1,28) = 5.369, $p=0.028$) such that FLX age-dependently influenced DCX+ cells (Figure 3D). Noteworthy is that there was a borderline significant age-by-treatment interaction effect in the dorsal supra (F(1,28) = 3.660, $p=0.066$) such that FLX again influenced cell differentiation in an age-dependent manner. Together, these results indicate that FLX exerts its effects on neuronal differentiation primarily in the infrapyramidal blade of both the dorsal and ventral hippocampus. Analysis of the DG volume divulged an age effect (F(1,28) = 6.461, $p=0.017$) with adolescents having slightly but significantly larger DG volumes than adults, and a significant treatment effect (F(1,28) = 4.418, $p=0.045$) such that FLX age-dependently influenced the total volume of the DG (Figure 3E).
Figure 2. Age-related effects of fluoxetine and regional differences in cell proliferation
An example of Ki-67 expression in the hippocampus is shown for (A) adult-treated and (B) adolescent-treated rats. C) Significant age-by-treatment interaction effect (p = 0.029) as well as a significant effect of age (p < 0.001) in the expression of Ki-67+ cells; D) Regional differences in proliferation exist in various sub-regions of the hippocampus (divided into the infrapyramidal and suprapyramidal blades of the dentate gyrus in either the dorsal or ventral portion of the hippocampus). There was a significant effect of age in all sub-regions (p < 0.001), and a significant age-by-treatment interaction effect in both the dorsal infrapyramidal (p = 0.009) as the dorsal suprapyramidal blade (p = 0.017). Also note the higher cell numbers in the infrapyramidal blade overall compared with the suprapyramidal blade, regardless of being in the dorsal or ventral portion. * = main effect of age; $ = main effect of treatment; # = age-by-treatment effect. P-values below 0.050 were considered statistically significant. Error bars indicate ± 1 S.E.M.
Part 2: Age-related effects of fluoxetine in the rat's brain

Figure 3. Age-related effects of fluoxetine and regional differences in cell differentiation

An example of doublecortin (DCX) expression along the subgranular zone is shown for adult-treated (A) and adolescent-treated rats (B). C) There was both a significant age-by-treatment interaction effect (p = 0.036) and a significant effect of age (p < 0.001) in the expression of DCX+ cells. D) There were regional differences in the amount of DCX+ cells. There was a significant effect of age in all sub-regions (p < 0.001), a significant treatment effect in the ventral infrapyramidal blade of the dentate gyrus (p = 0.021), and a significant age-by-treatment interaction effect in the dorsal infrapyramidal blade (p = 0.028). E) There was both a significant effect of age (p = 0.017) as well as an effect of treatment (p = 0.045) on dentate gyrus volume. * = main effect of age; $ = main effect of treatment; # = age-by-treatment effect. P-values below 0.050 were considered statistically significant. Error bars indicate ± 1 S.E.M.
Discussion

The present study investigated the age-specific changes in structural plasticity in the hippocampus after chronic FLX treatment, administered either during adolescence or adulthood. One week after drug cessation, adult cell proliferation and neuronal differentiation were increased in the adolescent-treated animals, whereas a reduction or no effect was found in animals treated as adults, resulting in a significant age-by-treatment interaction effect on adult neurogenesis. Adult neurogenesis is a commonly used measure for structural plasticity in rodents. It originates from stem cells present in the SGZ that differentiate into new neurons throughout life. Neurogenesis is extensive in the early life period and slows down with age (Korosi et al., 2012). Consistent with the significant age effect in Ki67+ and DCX+ numbers, neurogenesis is thus reduced in adults as compared to adolescent animals. In young rats, the DG is formed during the first 2-3 weeks of life and approximately 80% of its granule neurons are born postnatal. After PND14, the rate of neurogenesis starts to slow down, but the neurons born during this period continue to differentiate until PND60, when neurogenesis has reached relatively low levels and most granule neurons are considered mature (Rahimi and Claiborne, 2007). In male rats, adolescence lasts from PND28 to PND60 (Spear, 2000), with puberty occurring around PND45 (Engelbregt et al., 2000). Hence, our experimental design in which FLX treatment was started at PND25 and lasted for 3 weeks, not only approximated a comparable period of adolescence in humans, but also coincided with the sensitive period of delayed DG development. As such, it was optimally suited to detect putative effects on its structural organization.

For human depression, the most efficient dose of FLX is 20 mg (Beasley et al., 2000) (appr. 0.3–0.9 mg/kg). In rats, however, drugs are generally administered at 10-fold higher dosages because of the difference in the rate of liver metabolism and a clinically relevant dose for FLX would be 3–9 mg/kg. The minimal dose of FLX required for a significant inhibition of SERT and 5-HT is 5 mg/kg, which indeed is in the same range (Tordera et al., 2002; Wegerer et al., 1999). Despite this, most rodent studies used higher FLX concentrations, typically 10–20 mg/kg, which are not only clinically less relevant, but also induce behavioral impairments including impairments in water-maze probe trial performance and/or higher levels of anxiety after doses of FLX up to 10 mg/kg/day from PND28-60, notably without changes in proliferation (Hansen et al., 2011; Sass and Wortwein, 2012).

Effects of adult FLX exposure on neurogenesis

Even though neurogenesis is reduced with age, it can still be stimulated by environmental factors (Knoth et al., 2010; Marlatt et al., 2010; Marlatt et al., 2012), in depression models
(Czeh and Lucassen, 2007; Malberg and Duman, 2003; Oomen et al., 2007; Rainer et al., 2012), and with various antidepressants including FLX, which stimulate proliferation in young, naive rodents (Kodama et al., 2004; Madsen et al., 2005; Malberg et al., 2000; Sahay and Hen, 2007; Su et al., 2009). The lack of effect or even reduction in proliferation and DCX+ numbers in the adult-treated animals that we find differs from the earlier, mostly stimulatory, reports on FLX (Malberg et al., 2000; Perera et al., 2011; Sahay and Hen, 2007). However, these studies often used different experimental designs, different markers or studied shorter survival times. Moreover, several groups have now demonstrated that the neurogenic effects of antidepressants like FLX, strongly depend on strain, age and brain region of the animal (Balu, 2010), and its stress history (Couillard-Despres et al., 2009; David et al., 2009; Marlatt et al., 2010; Rayen et al., 2011; Surget et al., 2011; Suri et al., 2013). Consistent with our present results, several studies failed to find stimulatory effects of FLX or other antidepressants on neurogenesis in rats of comparable ages, or they report decreases in neurogenesis (Cowen et al., 2008; Hanson et al., 2011a; Navailles et al., 2008; Paizanis et al., 2010) or even neurogenesis-independent effects (David et al., 2009; Nollet et al., 2012).

Another difference with these previous studies is the washout period of 1 week that we introduced to avoid acute effects of the drug on neurogenesis and TPH readouts. Although this design enables us to study lasting effects, it could also have allowed compensatory responses to develop once treatment was stopped, that could have masked initial differences between groups by the time of sacrifice. Similar dynamic responses are known from studies in which neurogenesis was initially reduced by radiation, chemotherapy or stress but showed later recovery (Fike et al., 2007; Heine et al., 2004; Marlatt et al., 2011; Nokia et al., 2012). The current results may then suggest that neurogenesis is stimulated by chronic FLX treatment, but later reduced when the drug is no longer present. If this is true, it means the drug should remain continuously present in order to propagate its positive effect on neurogenesis. Another option is that exhaustion, or depletion, of the initial neurogenic pool has occurred that might be due to forced increases in division of the progenitor cells by FLX treatment, which may have slowed down subsequent stages of neurogenesis at later time points (Encinas and Sierra, 2012).

Effects of adolescent FLX exposure on neurogenesis

In contrast to the adult-treated animals, we found statistically significant stimulatory effects of FLX on both neurogenesis markers in young adult animals treated as adolescents. This is consistent with an earlier study performed in mice (Navailles et al., 2008) that also reported stimulatory effects of FLX (16 mg/kg/day in drinking water for 14 days) on neurogenesis, only when treatment was initiated during adolescence and not in adulthood. However,
two other studies failed to find effects of adolescent FLX treatment, both acutely and after several weeks of treatment discontinuation (Cowen et al., 2008; Hodes et al., 2009). Although studied in rats of comparable ages and with similar FLX doses, i.e. 5 mg/kg (i.p.), Cowen et al. (2008) reported no effect of FLX whatsoever whereas Hodes et al. (2009) only found FLX to increase cell proliferation in adult male rats but not in the peri-pubescent male, or female, rats. Also treatment duration in our study was comparable to that of Navailles et al. (2008), i.e. between 14 and 25 days.

Explanation of these inconsistent findings might lie in known effects of social stress that can inhibit proliferation whereas FLX can reverse reductions in neurogenesis (Czeh and Lucassen, 2007). On the other hand, the stimulatory effect of adolescent FLX exposure on neurogenesis can be abolished by early life stress (Navailles et al., 2008). In both studies (Cowen et al., 2008; Hodes et al., 2009), animals were individually housed just before start of the treatment. This represents a serious social stressor for juvenile animals that are particularly sensitive to environmental stress as brain areas targeted by stress such as the prefrontal cortex and hippocampus develop relatively late. Also, social play behavior is known to be disrupted after earlier individual housing (von Frijtag et al., 2002) while social isolation can also preclude the positive effects of running on adult neurogenesis (Stranahan et al., 2006). It is well possible that the social isolation just before and during treatment has masked effects of FLX on neurogenesis in the studies by Hodes and Cowen.

While changes in neurogenesis after a specific stimulus are generally transient during adulthood (Madsen et al., 2005; Malberg and Duman, 2003), this is different in the adolescent period where FLX may exert lasting ‘imprinting’ effects on DG development (Andersen and Navalta, 2004). It would be very interesting to study whether the increased numbers of proliferating and differentiating cells in our current study do survive and alter DG structure or function at later ages, as was found before e.g. after early stress exposure (Oomen et al., 2011; Suri et al., 2013), but this awaits further research.

Possible mechanisms of action underlying the age-dependent effects on neurogenesis

Although it is still not clear how 5-HT transmission precisely regulates adult neurogenesis, as is demonstrated by the recent findings that neurogenesis is not affected in SERT knockout animals (Benninghoff et al., 2012; Schmitt et al., 2007), it is known that adult neurogenesis is mediated by a variety of 5-HT (auto)receptors. For instance, 5-HT1A receptor activation is known to have a proliferative effect (Klem pin et al., 2010; Soumier et al., 2010), and also 5-HT1B, 5-HT2A, 5-HT2C and 5-HT4 receptor-mediated signaling have been described to affect proliferation, differentiation and survival of newborn neurons, partly selective of each neurogenic zone (Banasr et al., 2004; Jha et al., 2008; Klem pin et al., 2010; Kobayashi et
FLX is thought to exert its effect on neurogenesis by primarily targeting early progenitor cells in the DG (Encinas et al., 2006). So far, it remains largely unknown which 5-HT receptors are present on individual progenitor cells, or whether similar changes in their receptor subtype composition contribute to the sensitivity changes during different ages. Similarly, it remains elusive whether FLX alters glucocorticoid hormone receptor expression, or NMDA receptor expression on the new cells (Garcia et al., 2004; Huang and Herbert, 2006; Nacher and McEwen, 2006).

Another important player in the regulation of neurogenesis is BDNF (brain derived neurotrophic factor). The actions of FLX on neurogenesis are believed to depend on BDNF activity (Pinnock et al., 2010). In turn, BDNF expression is thought to be regulated by 5-HT transmission. As suggested by Migliarini et al. (2012), effects of 5-HT depletion on BDNF expression propose a possible regulatory feedback mechanism through which 5-HT itself might regulate the formation of the 5-HTergic neuronal circuitry in the hippocampus. A reduction in 5-HT levels may trigger endogenous BDNF signaling that would then exert a neurotrophic effect on 5-HTergic axons, which in turn, could restore original BDNF levels. Ongoing plasticity within this regulatory feedback loop during adolescence could be accountable for age-dependent effects of chronic FLX on adult neurogenesis.

**Implications; relevance for human treatment**

The present preliminary observations provide a first step towards an improved understanding of the consequences of FLX exposure on young patient populations. In future studies, it would be interesting to investigate the long-term survival and behavioral effects of the newborn cells. Although the clinical significance of our observations for children and adolescents treated with SSRIs is difficult to predict, it is conceivable that, also in humans, the plasticity of the 5-HT system and of the newborn neurons is higher in children and adolescents than in the mature brain. Pharmacological manipulations of 5-HT would then likely also affect children differently from adults.

**Conclusions**

We conclude that the widely prescribed antidepressant FLX has divergent actions on brain measures relevant for depression in adolescent or adult individuals. Our results thus support the notion that adult neurogenesis is influenced by SSRI administration, however only during critical developmental time periods. As these drugs, for obvious reasons, have not been thoroughly tested in the adolescent population, the present data call for further research into the underlying mechanisms and long-term consequences of juvenile FLX.
treatment in particular, and emphasize the need for continued appropriate use of FLX and other antidepressants to adolescents in general.

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