Chronic Fluoxetine Treatment Changes S100B Expression During Postnatal Rat Brain Development

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Abstract

Background: Fluoxetine, a selective serotonin reuptake inhibitor, is approved for treatment of childhood depression. In rats, fluoxetine influences neuronal development, but it is unclear whether it also influences glia development. S100B is a glia-derived calcium-binding protein, which may influence the development of serotonergic fibers and, vice versa, serotonin may influence the expression of S100B.

Objectives: The purpose of this study was to investigate whether fluoxetine treatment influences the expression of S100B during postnatal development, and whether potential changes are regionally dependent upon the time frame of drug administration.

Methods: S100B gene expression and S100B protein expression in three different brain regions (frontal cortex, hippocampus, and striatum) were studied by real-time polymerase chain reaction (PCR) and immunohistochemistry, respectively. First, a short-term effect, 24 hours after a 14 day fluoxetine treatment (5 mg/kg/bw s.c.) of rats either from postnatal day (PD) 1 to 15, 21 to 35, or 50 to 64, was investigated. Then, the same treatment was used to analyze S100B gene and protein levels at PD 90 (long-term effect).

Results: At PD 90, a significant increase of gene and protein expression was observed in all regions if rats were treated during PDs 21–35, whereas treatment during other periods had no long-term effects. A short-term effect 24 hours after fluoxetine treatment was found for almost all development stages and regions, demonstrated by a significant increase of S100B.

Conclusions: These results support recent research indicating a highly drug-sensitive period (i.e., periadolescence) of rat brain development. Therefore, further clinical studies should be performed to clarify whether such a sensitive period also exists in children.

Introduction

DEPRESSIVE SYMPTOMS IN CHILDREN AND ADOLESCENTS are increasingly treated with serotonin reuptake inhibitors such as fluoxetine (Usala et al. 2008). Because many studies have revealed neuronal changes induced by fluoxetine and other antidepressants, there is a need to investigate their effects in the postnatal developing brain (Blier and de Montigny 1999; Duman et al. 2001). The S100B protein, a calcium-binding factor, is an interesting target for several reasons.

First, fluoxetine modulates serotonin levels, resulting in S100B changes mediated by the astroglial 5-HT_{1A} receptor (Whitaker-Azmitia et al. 1990). Serotonin receptor 1A (5-HT_{1A}) agonists are able to release S100B from astrocytes. For example, ethanol exposure *in utero* reduces the number of 5-HT neurons and S100B immunopositive glia that are proximal to these neurons. These

effects are prevented by maternal treatment with a 5-HT_{1A} agonist (Eriksen et al. 2002).

Second, fluoxetine seems to be able to stimulate S100B secretion via a serotonin-independent mechanism. Although astrocytes and neurons have similar serotonin transporters, little is known about the sites of action of antidepressants on astrocytes (Tramonita et al. 2008). They proposed an activation of an unknown second messenger cascade followed by binding of fluoxetine at these sites.

Third, increased serum levels of S100B are observed in untreated patients with brain damage, schizophrenia, Down's syndrome, and major depression and are reduced by antidepressive treatment with serotonin reuptake inhibitors (Rothermundt et al. 2001; Schroeter et al. 2002; Rothermundt et al. 2003, 2004; Sarandol et al. 2007). However, in two rat models of depression, the serum S100B levels did not correlate with the regional brain S100B levels (Luo et al. 2010).

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It was previously assumed that the S100B gene was expressed primarily in the nervous system by astrocytes and Schwann cells, as the S100B protein is found in the extracellular fluid of the brain (Shashoua et al. 1984). However, S100B protein is also expressed and released in oligodendrocytes of the rat brain (Richter-Landsberg and Heinrich 1995, Steiner et al. 2008). In astrocytes, the initial expression of S100B defines the stage when glial fibrillary acid protein (GFAP) expressing cells lose their neuronal stem cell (NSC) potential (Raponi et al. 2007). Nevertheless, in general, S100B is a glial-derived protein.

In vitro and *in vivo* studies revealed trophic effects of nanomolar dosages of S100B on cells, whereas higher dosages (micromolar) promoted apoptosis (Donato 2003, Rothermundt et al. 2003). Trophic effects of S100B, especially on serotonergic neurons, are reported (Azmitia et al. 1990; Whitaker-Azmitia et al. 1990; Liu and Lauder 1992). Serotonin modulates glial morphology by inducing a release of intracellular S100B (Chang et al. 2005). S100B is also involved in synaptogenesis (Mazer et al. 1997), dendritic development (Yan et al. 1997), and apoptosis (Ahlemeyer et al. 2000).

In conclusion, the interaction between alterations of the serotonergic system (e.g., by drugs) and changes of S100B, may influence brain development either in the short or (more important for child psychiatry) the long term.

Based on the usual postnatal developmental changes of the serotonergic system in rats (Moll et al. 2000), we hypothesize that treatment of these animals with fluoxetine during varying development stages leads to different changes in S100B expression depending upon the time frame of drug exposure. Hence, we investigated short- and long-term effects of 14 days of fluoxetine in three different developmental stages (postnatal day [PD] 1–15, PD 21–35, PD 50–64) of rats.

Materials and Methods

The experimental procedures were performed in accordance with European Community and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animal experiments were performed in accordance with German laws for the care and use of laboratory animals (as approved by the Bezirksregierung Braunschweig, License No. 334250/01-29.05).

Sprague–Dawley rats were obtained from a commercial breeder (Charles River, Sulzfeld, Germany) and used for further breeding in our own environmentally conditioned animal facility under standardized conditions. After mating, the dams were housed in single cages with free access to food and water. Litter size was reduced to six pups per dam. After weaning, the young rats (only males were used in these experiments) were placed in separate cages, two per cage. The first group of rats ($n=24$) received fluoxetine s.c. from PD 1 to 15, a second group ($n=24$) received fluoxetine s.c. from PD 21 to 35 and a third group ($n=24$) received fluoxetine s.c. from PD 50 to 64. The same number of animals received saline as control. The drugs were administered via subcutaneous injection at the same time in the morning. The applied dose of fluoxetine was adjusted to 5 mg/kg/day, based on daily monitoring the amounts of food consumed by the two rats per cage and their body weight. Half of each group ($n=12$) was killed 24 hours after the last injection of fluoxetine or saline and the other half were killed at PD 90. The 12 animals in each group were divided into 6 animals for immunohistochemistry and 6 animals for real-time polymerase chain reaction (PCR) analysis.

Real-time PCR

For real-time PCR analysis, brains were quickly removed and frozen at -80°C until we dissected the frontal cortex, striatum, and hippocampus from corresponding cryostat sections cut in a rostro-caudal extension of $300\ \mu\text{m}$ ($n=36$; PD 16, 36, 65, and 91) each. The total ribonucleic acid (RNA) of homogenized brain tissue was isolated using the Trizol® method according to manufacturer's instructions (GibcoBRL), and its concentration was determined using the nanodrop ND-1000 spectrophotometer, followed by its quality and integrity measurement by electrophoresis on RNA 6000 LabChip® kit (Agilent 2100 Bioanalyzer). The RNA was transcribed into the corresponding deoxyribonucleic acid (cDNA) using the iScript cDNA Synthesis Kit (BioRad).

The following primer pairs were designed by using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi): hypoxanthine guanine phosphoribosyl transferase (*HPRT*, 135 bp; NCBI-Accession No.: NM_012583) F (5'-gtcaagcagtacagcccaaatg-3')/R (5'-gtcaaggccatccaacaacaac-3') and S100 calcium binding protein B (*S100B*, 153 bp; NM_013191.1) F (5'-gggtcacctgtaagaa tcaa-3')/R (5'-gaggacaagcagttgtaa-3').

Gel electrophoresis revealed a single PCR product, and the melting curve analysis showed a single peak for all amplification products. The PCR products were sequenced and blasted to confirm the correct identity of each amplicon. Tenfold serial dilutions generated from cDNA of each sample were used as a reference for the standard curve calculation to determine primer efficiency. Duplicates of all real-time PCR reactions were performed in a $25\ \mu\text{L}$ mixture containing 1/20 volume of the sample cDNA preparation from 250 ng total RNA, 400 nM of each primer, and $1\times$ iQ-SYBR Green Supermix (BioRad, Laboratories, Ltd).

The PCR reactions were performed as follows: *initial denaturation* at 95°C for 10 minutes, 40 cycles of *denaturation* $95^{\circ}\text{C}/15$ seconds, *annealing* $55^{\circ}\text{C}/15$ seconds, and *extension* $72^{\circ}\text{C}/30$ seconds, and a final gradual increase of 0.5°C in temperature from 55°C to 95°C .

All real-time quantifications were performed with the iCycler iQ system (BioRad) and were analyzed by using a two tailed paired *t* test following normalization to a *HPRT* control. For methodical reasons, duplicates of only three animals per group could be analyzed on one plate by real-time PCR; therefore, we used a two factorial ANOVA followed by two tailed post-hoc *t* test (Bonferroni) to compare corresponding plates. Results were considered significant for *p* values ≤ 0.05 .

Immunohistochemistry

For immunohistochemistry, Sprague–Dawley rats were deeply anesthetized with isoflurane (1-Chloro-2,2,2-trifluoroethyl-di-fluoromethylether, Abbott, Wiesbaden, Germany). After thoracotomy, animals were transcardially perfused with 50 mL 0.9% sodium chloride followed by 200 mL 4% phosphate-buffered formaldehyde ($10\ \text{mL}\times\text{min}^{-1}$). The brain was removed and post-fixed for 4 hours with the same fixative at 4°C . Tissues were cryoprotected in 10% sucrose for 2 hours followed by 30% sucrose in 0.1 M phosphate buffer overnight at 4°C and frozen at -25°C afterwards. Series of $40\ \mu\text{m}$ thick transverse brain sections were cut using a cryostat (Frigocut, Reichert-Jung, Germany). Before any immunohistochemical treatment, free-floating sections were rinsed three times in phosphate-buffered saline (PBS) (pH 7.4) for 15 minutes each.

S100B-positive cells were visualized as follows: The intrinsic peroxidase activity was blocked with hydrogen peroxide-methanol

(1:100) for 45 min at room temperature (RT) in the dark. After washing, sections were permeabilized with 0.2% Triton X-100 for 30 minutes and directly transferred into PBS containing 5% bovine serum albumin (BSA) for 1 hour at RT to block nonspecific binding sites. Sections were incubated at 4°C in primary antibody solution at a dilution of 1:5000 overnight, and subsequently washed three times for 10 minutes each. Secondary horseradish peroxidase (HRP)-conjugated antibodies (diluted 1:1000) were applied for 2 hours at RT and rinsed with PBS three times for 10 minutes each. After incubation, sections were extensively washed and pre-incubated with freshly prepared filtered diaminobenzidine (DAB)-solution (120 μ L DAB [75 mg DAB dissolved in 1.5 mL 0.1 M phosphate buffer] diluted in 30 mL PBS) for 3 minutes at RT. The enzymatic reaction was started by adding 10 μ L of 35% H₂O₂ to 10 mL DAB solution and stopped with PBS after 1–5 minutes. DAB-stained sections were washed in PBS, mounted onto gelatin-coated microscope-slides, dehydrated (2 $\times$ 50% ethanol, 2 $\times$ 80% ethanol, and 2 $\times$ 99.9% ethanol, 5 minutes each), cleared with four changes of xylene, cover-slipped with mounting medium (DePeX from Serva, Heidelberg, Germany), and finally analyzed with a digital microscope (Coolscope Nikon, Melville, USA). Images were taken at 2048 $\times$ 2048 dpi and were imported into Adobe Photoshop CS5, were digitally adjusted if necessary for brightness and contrast, and were assembled into plates. Five sections per animal were counted for quantification of S100B-positive cells for each region. The values were denoted as cells/area. The statistical significance of differences between means of the value of the cell number/area counted in the different brain areas in fluoxetine-treated animals versus controls was tested by one way analysis of variance (ANOVA) followed by two tailed post-hoc *t* test (Bonferroni). Results were considered significant for *p* values ≤ 0.05 .

Results

Very early treatment (VET) period (PD 1–15)

In the frontal cortex, there was a significant increase of the S100B mRNA expression measured 24 hours after the last injection in the fluoxetine-treated group (PD 1–15) (see Table 1 and Fig. 1) and, in addition, a significant increase of S100B immunopositive cells (see Table 2 and Fig. 2). No long-term effects measured at day 90 were detectable with both real-time PCR and immunohistochemistry (see Tables 1 and 2, and Figs. 1 and 2).

In the striatum, the S100B mRNA expression and the S100B immunopositive cells were significantly increased in very early fluoxetine-treated animals compared with the controls 24 hours after the last injection. Measured at PD 90, no significant increase could be observed in the group treated during PD 1–15 only, compared with controls (see Tables 1 and 2, and Figs. 1 and 3). No long-term effects measured at day 90 were detectable using both real-time PCR and immunohistochemistry (see Tables 1 and 2, and Figs. 1 and 3).

In the hippocampus, S100B mRNA expression was increased in the very early treated group compared with controls, and similarly, the S100B immunopositive cells were significantly increased in the CA1 region and the gyrus dentatus in comparison with controls (See Table 1 and 2, and Figs. 1, 4 and 5). In the hippocampal CA3 region, no significant change in the S100B immunopositive cells could be detected (see Table 2 and Fig. 6).

No long-term effects measured at day 90 were observed at the RNA and protein levels (see Tables 1 and 2, and Figs. 1 and 4, 5, and 6).

TABLE 1. REAL-TIME POLYMERASE CHAIN REACTION (PCR) ANALYSIS DURING DIFFERENT DEVELOPMENT STAGES AND 14 DAY FLUOXETINE TREATMENT

Treatment (day of investigation)	1–15 (16)		21–35 (36)		50–64 (65)		1–15 (90)		21–35 (90)		50–64 (90)	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
Frontal cortex (Plate 1)	2.41 $\pm$ 0.28	3.36* $\pm$ 0.51	1.01 $\pm$ 0.06	1.83*** $\pm$ 0.12	0.73 $\pm$ 0.20	0.44 $\pm$ 0.15	1.136 $\pm$ 0.32	1.59 $\pm$ 0.32	1.12 $\pm$ 0.07	1.87*** $\pm$ 0.29	1.15 $\pm$ 0.35	1.51 $\pm$ 0.37
Frontal cortex (Plate 2)	2.23 $\pm$ 0.42	2.89* $\pm$ 0.20	1.4 $\pm$ 0.36	2.17*** $\pm$ 0.48	0.74 $\pm$ 0.09	0.66 $\pm$ 0.33	1.22 $\pm$ 0.27	1.18 $\pm$ 0.11	0.99 $\pm$ 0.26	1.5*** $\pm$ 0.4	0.96 $\pm$ 0.14	0.93 $\pm$ 0.16
Striatum (Putamen) (Plate 1)	2.03 $\pm$ 0.19	2.6* $\pm$ 0.38	1.35 $\pm$ 0.40	2.18*** $\pm$ 0.45	0.64 $\pm$ 0.19	1.35* $\pm$ 0.07	1.39 $\pm$ 0.23	1.512 $\pm$ 0.15	1.14 $\pm$ 0.19	1.99*** $\pm$ 0.15	1.35 $\pm$ 0.06	1.26 $\pm$ 0.05
Striatum (Putamen) (Plate 2)	1.57 $\pm$ 0.09	2.47* $\pm$ 0.24	0.84 $\pm$ 0.09	1.44*** $\pm$ 0.36	0.35 $\pm$ 0.08	0.95* $\pm$ 0.32	0.69 $\pm$ 0.16	0.67 $\pm$ 0.16	0.73 $\pm$ 0.18	1.35*** $\pm$ 0.15	0.81 $\pm$ 0.16	0.91 $\pm$ 0.14
Hippocampus (Plate 1)	5.61 $\pm$ 0.43	7.42*** $\pm$ 0.43	3.48 $\pm$ 0.15	5.21*** $\pm$ 1.03	1.91 $\pm$ 0.28	2.67*** $\pm$ 0.26	3.06 $\pm$ 0.23	3.54 $\pm$ 0.33	3.62 $\pm$ 0.33	6.2*** $\pm$ 0.25	3.1 $\pm$ 0.13	3.78 $\pm$ 0.42
Hippocampus (Plate 2)	3.94 $\pm$ 0.27	5.84*** $\pm$ 0.41	2.62 $\pm$ 0.68	4.34*** $\pm$ 0.66	1.2 $\pm$ 0.14	2.14*** $\pm$ 0.3	2.66 $\pm$ 0.39	2.1 $\pm$ 0.22	2.25 $\pm$ 0.26	3.81*** $\pm$ 0.39	2.9 $\pm$ 0.57	2.83 $\pm$ 0.62

Relative S100B expression data are presented as mean $\pm$ SD.

p* ≤ 0.05 ; *p* ≤ 0.01 ; ****p* ≤ 0.001 .

FIG. 1. Developmental S100B-mRNA expression under control versus fluoxetine treated conditions. (A) Frontal cortex, (B) striatum (putamen), and (C) hippocampus. The bar diagrams represent short- (24 hours) and long-term (postnatal day [PD] 90) effects as mean $\pm$ SD after very early treatment (VET) from pd 1 - pd 15, early treatment (ET) from PD 21 to PD 35, or late treatment (LT) from PD 50 to PD 64. Asterisks represent statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Early, periadolescent treatment (ET) period (PD 21–35)

In the frontal cortex, there was a significant increase of the S100B mRNA expression measured 24 hours after the last injection in the fluoxetine-treated group (PD 21–35) (see Table 1 and Fig. 1). A significant increase of S100B immunoreactivity was found 24 hours after the last drug injection in the animals treated with fluoxetine (see Table 2 and Fig. 2).

Interestingly, in this treatment period, we found a significant long-lasting increase of S100B mRNA expression and immunopositive cells measured at day 90, compared with controls (see Tables 1 and 2, and Fig. 2).

In the striatum, the S100B mRNA expression and the S100B immunopositive cells were significantly increased in fluoxetine-

TABLE 2. IMMUNOHISTOCHEMISTRY DURING DIFFERENT DEVELOPMENT STAGES AND 14-DAY FLUOXETINE TREATMENT

Treatment (day of investigation)	1–15 (16)		21–35 (36)		50–64 (65)		1–15 (90)		21–35 (90)		50–64 (90)	
	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine	Control	Fluoxetine
Frontal cortex	6.57 $\pm$ 0.22	7.32*** $\pm$ 0.45	2.09 $\pm$ 0.13	3.12*** $\pm$ 0.17	1.52 $\pm$ 0.08	1.62 $\pm$ 0.19	3.6 $\pm$ 0.51	3.29 $\pm$ 0.34	3.97 $\pm$ 0.19	4.91*** $\pm$ 0.18	4.09 $\pm$ 0.35	4.13 $\pm$ 0.31
Striatum	7.71 $\pm$ 0.27	9.14*** $\pm$ 0.41	2.6 $\pm$ 0.12	3.77*** $\pm$ 0.3	1.52 $\pm$ 0.167	2.38*** $\pm$ 0.14	4.99 $\pm$ 0.75	4.99 $\pm$ 0.43	4.97 $\pm$ 0.71	7.32*** $\pm$ 0.19	4.89 $\pm$ 0.33	4.77 $\pm$ 0.32
CA1 of hippocampus	7.81 $\pm$ 0.11	9.76*** $\pm$ 0.17	3.95 $\pm$ 0.2	5.21*** $\pm$ 0.2	2.7 $\pm$ 0.08	3.06 $\pm$ 0.21	3.79 $\pm$ 0.25	4.35 $\pm$ 0.64	4.51 $\pm$ 0.32	5.62*** $\pm$ 0.57	4.83 $\pm$ 0.28	4.38 $\pm$ 0.26
CA3 of hippocampus	10.01 $\pm$ 0.55	10.67 $\pm$ 0.88	4.21 $\pm$ 0.54	5.27*** $\pm$ 0.11	1.97 $\pm$ 0.11	2.94*** $\pm$ 0.18	4.20 $\pm$ 0.24	4.15 $\pm$ 0.19	4.53 $\pm$ 0.37	5.49*** $\pm$ 0.44	4.63 $\pm$ 0.32	4.57 $\pm$ 0.09
Gyrus dentatus	8.16 $\pm$ 0.79	10.58*** $\pm$ 0.93	5.31 $\pm$ 0.98	7.93*** $\pm$ 0.48	3.7 $\pm$ 0.32	6.09*** $\pm$ 0.23	4.65 $\pm$ 0.35	4.78 $\pm$ 0.25	5.13 $\pm$ 0.58	6.4* $\pm$ 0.41	5.38 $\pm$ 0.37	5.05 $\pm$ 0.43

Cells/area data are presented as mean $\pm$ SD.
* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

FIG. 2. Developmental S100B expression after fluoxetine treatment in the frontal cortex. (A) Schematic representation of the frontal cortex analyzed. (B) S100B expression under control conditions at postnatal day (PD) 16. (C) S100B expression after very early treatment (VET) from PD 1 to PD 15 with fluoxetine at PD 16. (D) S100B expression under control conditions at PD 90. (E) S100B expression after early treatment (ET) with fluoxetine from PD 21 to PD 35 at PD 90. Scale bars = 20 μ m. (F) Bar diagrams represent short- and long-term effects of S100B-labeled cells per area during different development stages under control versus fluoxetine-treated conditions. Asterisks represent statistical significance (***) $p \leq 0.001$.

treated animals compared with the controls 24 hours after the last injection, and measured at day 90 (see Tables 1 and 2, and Figs. 1 and 3).

In the hippocampus, there was a significant increase of the S100B mRNA expression measured 24 hours after the last injection in the fluoxetine-treated group (see Table 1, and Fig. 1). A significant increase of S100B immunoreactivity was found 24 hours after the last drug injection in the animals treated with fluoxetine in all examined hippocampal regions (see Table 2, and Figs. 4–6).

At day 90, there was a significant increase of the S100B mRNA expression in the fluoxetine-treated group compared with controls (see Table 1, and Fig. 1).

FIG. 3. Developmental S100B expression after fluoxetine treatment in the putamen. (A) Schematic representation of the putamen analyzed. (B) S100B expression under control conditions at postnatal day (PD) 16. (C) S100B expression after fluoxetine treatment (very early treatment [VET] from PD 1 to PD 15) at PD 16. (D) S100B expression under control conditions at PD 90. (E) S100B expression after fluoxetine treatment (ET from PD 21 to PD 35) at PD 90. Scale bars = 20 μ m. (F) Bar diagrams represent short- and long-term effects of S100B-labeled cells per area during different development stages under control versus fluoxetine-treated conditions. Asterisks represent statistical significance (***) $p \leq 0.001$.

Similarly, in all investigated hippocampal regions, a significant increase in S100B immunopositive cells was observed (see Table 2, and Figs. 4–6).

Late, postadolescent treatment (LT) period (PD 50–65)

In the frontal cortex, there were no changes of S100B mRNA expression or S100B immunopositive cells measured 24 hours after the last injection in the fluoxetine-treated group (PD 50–64)

FIG. 4. Developmental S100B expression after fluoxetine treatment in the CA1 region of the hippocampus. (A) Schematic representation of the CA1 region of the hippocampal formation analyzed. (B) S100B expression under control conditions at postnatal day (PD) 16. (C) S100B expression after fluoxetine treatment (very early treatment [VET] from PD 1 to PD 15) at PD 16. (D) S100B expression under control conditions at PD 90. (E) S100B expression after fluoxetine treatment (ET from PD 21 to PD 35) at PD 90. Scale bars = 20 μm. (F) Bar diagrams represent short- and long-term effects of S100B-labeled cells per area during different development stages under control versus fluoxetine-treated conditions. Asterisks represent statistical significance (***) $p \leq 0.001$.

compared with controls (see Tables 1 and 2, and Figs. 1 and 2). No long-term effects were detected at day 90 either by real-time PCR analysis or by immunohistochemistry (see Tables 1 and 2, and Figs. 1 and 2).

In the striatum, the S100B mRNA expression and the S100B immunopositive cells were significantly increased in fluoxetine-treated animals compared with the controls 24 hours after the last injection (see Tables 1 and 2, and Figs. 1 and 3).

FIG. 5. Developmental S100B expression after fluoxetine treatment in the dentate gyrus region of the hippocampus. (A) Schematic representation of the polymorph layer of the hippocampal dentate gyrus region (PoGD) analyzed. (B) S100B expression under control conditions at postnatal day (PD) 16. (C) S100B expression after fluoxetine treatment (very early treatment [VET] from PD 1 to PD 15) at PD 16. (D) S100B expression under control conditions at PD 90. (E) S100B expression after fluoxetine treatment (ET from PD 21 to PD 35) at PD 90. Scale bars = 20 μm. (F) Bar diagrams represent short- and long-term effects of S100B-labeled cells per area during different development stages under control versus fluoxetine-treated conditions. Asterisks represent statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$).

However, no long-term effects were detected at day 90 either at the RNA or at the protein level (see Tables 1 and 2, and Figs. 1 and 3).

In the hippocampus, short-term effects were visible in the postadolescent treated group compared with controls: Significant increase of S100B mRNA expression as well as a significant increase of S100B immunopositive cells in the hippocampal CA3 region and the gyrus dentatus (see Tables 1 and 2, and Figs. 4–6).

FIG. 6. Developmental S100B expression after fluoxetine treatment in the CA3 region of the hippocampus. (A) Schematic representation of the CA3 region of the hippocampal formation analyzed. (B) S100B expression under control conditions at postnatal day (PD) 16. (C) S100B expression after fluoxetine treatment (very early treatment [VET] from PD 1 to PD 15) at PD 16. (D) S100B expression under control conditions at PD 90. (E) S100B expression after fluoxetine treatment (ET from PD 21 to PD 35) at PD 90. Scale bars = 20 μm . (F) Bar diagrams represent short- and long-term effects of S100B-labeled cells per area during different development stages under control versus fluoxetine-treated conditions. Asterisks represent statistical significance (** $p \leq 0.01$).

In contrast, no long-term effects were detected at day 90 either by real-time PCR or by immunohistochemistry in any examined hippocampal regions (see Tables 1 and 2, and Figs. 4–6).

Discussion

This study was performed to detect brain developmental periods that are potentially sensitive to fluoxetine treatment. It was assumed

that fluoxetine is able to induce detectable short- and long-term changes of the glial-derived calcium binding protein S100B. This protein seems to be involved in neuronal and glial growth processes and, moreover, it seems to play a yet uncertain role in the pathogenesis of certain psychiatric diseases, for example, major depression.

Regarding the developmental profile of S100B immunopositive cells, this study revealed a constant developmental profile of S100B in all investigated regions (frontal cortex, striatum, hippocampus). The highest levels of S100B mRNA expression and S100B immunopositive cells were observed at PD 16. This was followed by a decrease until PD 64, and a slight increase at PD 90. Postnatal developmental changes in S100B were not only found in rats. In the human frontal lobe and hippocampus (CA 1,2,3,4), the number of S100B immunopositive cells increased during early infancy, reached a plateau, and then gradually declined (Mito and Becker 1993). An age-related decrease of S100B and serotonergic fibers was also described in the dentate gyrus of rats, and the authors suggest that the age-related decrease of S100B is responsible for the serotonergic fiber degeneration (Nishimura et al. 1995; Frey et al. 2006).

These described profiles agree with the normal developmental profile in our study.

VET period (PD 1–15)

With the exception of the CA3 region, the S100B mRNA expression and the number of S100B immunopositive cells were increased 24 hours after the last fluoxetine injection.

These findings are not surprising, as fluoxetine elevates the serotonin level after 2 weeks, which stimulates S100B expression via the 5-HT_{1A} receptor. In addition, fluoxetine can potentially stimulate S100B expression via an unknown, formerly described mechanism (Tramonita et al. 2008). Whether fluoxetine has a direct effect or whether the effect is mediated via serotonin and/or additional factors such as brain-derived neurotrophic factor (BDNF) (discussed later) still needs to be elucidated. VET has no long-term consequences. No differences were found between the VET group and the control group at day 90.

ET period (PD 21–35)

After fluoxetine treatment during the periadolescent period, the S100B mRNA expression and S100B immunopositive cells were increased 24 hours after the last fluoxetine injection, similar to the VET group. In addition, this developmental stage was the only stage when fluoxetine treatment caused long-lasting changes as measured at day 90. Recently, we described a long-lasting increase of GFAP in animals treated with fluoxetine from PD 21 to 35 (Bock et al. 2012). These results support our hypothesis that fluoxetine treatment during a certain time frame of development (i.e., periadolescence) may lead to long-lasting brain changes. Also, the reported increase of S100B and GFAP may reflect an astroglial response. Chang et al. (2005) found that injections of *para*-chloroamphetamine (PCA), a potent serotonin releaser, increased the number of GFAP immunoreactive cells only in wild type mice but not in S100B knockout mice, suggesting a direct connection between S100B and GFAP increase. Transgenic mice with elevated S100B levels revealed equal alterations in astrocytes, such as astrocytosis and axonal sprouting, especially in the dentate gyrus (Reeves et al. 1994).

In addition, the involvement of other factors responsible for these long-lasting changes of S100B, for example, growth factors

such as BDNF, can be assumed for varying reasons. The survival and maturation of serotonergic neurons is not only promoted by S100B, but also by serotonin itself and by BDNF. BDNF, in turn, is required for the local expression of S100B. Therefore, BDNF may indirectly influence the development of the serotonergic system by stimulating the expression of S100B in astrocytes (Djalali et al. 2005). In S100B knockout mice, a significant increase of hippocampal BDNF was found, which may represent an endogenous attempt to compensate trophic effects of S100B protein (Schulte-Herbruggen et al. 2008).

Deviations of S100B during development lead to cognitive and behavioral consequences. Removal of serotonin between PD 10 and PD 20 causes a loss of S100B. This leads to a loss of the synaptic markers synaptophysin and microtubule-associated protein 2 (MAP2) in the hippocampus, and the animals revealed long-term consequences such as cognitive deficits (Mazer et al. 1997).

Moreover, overexpression of S100B leads to behavioral consequences as well, as was demonstrated by Bell and coworkers (2003), showing that S100B transgenic mice were slower to habituate to novelty than were control animals. Behavioral consequences depending on a special time frame of fluoxetine treatment were also found in mice. If the application period was between PD 4 and 21, the application was followed by abnormal emotional behavior of the mice; later treatment did not result in visible behavioral alterations (Ansorge et al. 2004, 2008).

Such a "special time frame of action" was not only demonstrated for fluoxetine but also for methylphenidate. Methylphenidate treatment from day 21 to 35 reduced dopamine transporter density in the striatum of adult rats, but treatment at the post-adolescent period had no consequences (Roessner et al. 2010).

LT period (PD 50-65)

Twenty-four hours after the last fluoxetine injection, S100B expression was increased in the striatum and hippocampus of the animals of the postadolescent group. These findings are in line with previous studies using adult rats, which described that a 21 day treatment with fluoxetine (5 mg/kg/bw), once daily, resulted in an increase of S100B in the hippocampus (Manev et al. 2001). The authors concluded that S100B might be one of several factors that could be responsible for the observed neurogenesis in the hippocampal formation (Manev et al. 2001) after fluoxetine treatment. Upregulation of neurogenesis can be observed with different classes of antidepressants, including 5-HT and norepinephrine (NE) selective reuptake inhibitors, monoamine oxidase inhibitors, lithium, and electroconvulsive seizure; for review see Duman et al. (2001). The postadolescent treatment has no long-term consequences.

Conclusions

The reported results revealed a certain period of postnatal brain development (i.e., periadolescence), when chronic fluoxetine treatment led to long-lasting changes in S100B expression. This periadolescent period is a very important period during brain development, because a wave of overproduction and elimination of synapses and receptors occurs. This developmental phenomenon has been observed in rats (Teicher et al. 2003), primates (Lidow et al. 1991), and humans (Huttenlocher 1979).

Moreover, behavioral and structural changes were observed caused by drug treatment and behavioral interventions during certain postnatal brain developmental periods; for a review see Andersen (2003) and Andersen and Navalta (2011). Therefore, pre-

clinical studies such as the present study have an impact on further clinical studies to improve the safety of psychopharmacological treatment in children and adolescents.

Disclosures

Nathalie Bock designed the study and wrote the protocol and the first draft of the manuscript. Hannah Alter and Emre Koc managed the analyses of the study. Veit Roessner managed the literature search. Andreas Becker performed the statistical analysis. Aribert Rothenberger was involved in writing the manuscript. Till Manzke designed the study and wrote the protocol. All authors contributed to and have approved the final manuscript. The authors declare that there are no conflicts of interest concerning the contents of this study.

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