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Astroglial Plasticity in the Hippocampus is Affected by Chronic Psychosocial Stress and Concomitant Fluoxetine Treatment

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Analysis of post-mortem tissue from patients with affective disorders has revealed a decreased number of glial cells in several brain areas. Here, we examined whether long-term psychosocial stress influences the number and morphology of hippocampal astrocytes in an animal model with high validity for research on the pathophysiology of major depression. Adult male tree shrews were submitted to 5 weeks of psychosocial stress, after which immunocytochemical and quantitative stereological techniques were used to estimate the total number and somal volume of glial fibrillary acidic protein-positive astrocytes in the hippocampal formation. Stress significantly decreased both the number (−25%) and somal volume (−25%) of astroglia, effects that correlated notably with the stress-induced hippocampal volume reduction. Additionally, we examined whether antidepressant treatment with fluoxetine, a selective serotonin reuptake inhibitor, offered protection from these stress-induced effects. Animals were subjected to 7 days of psychosocial stress before the onset of daily oral administration of fluoxetine (15 mg/kg per day), with stress continued throughout the 28-day treatment period. Fluoxetine treatment prevented the stress-induced numerical decrease of astrocytes, but had no counteracting effect on somal volume shrinkage. In nonstressed animals, fluoxetine treatment had no effect on the number of astrocytes, but stress exposure significantly reduced their somal volumes (−20%). These notable changes of astroglial structural plasticity in response to stress and antidepressant treatment support the notion that glial changes may contribute to the pathophysiology of affective disorders as well as to the cellular actions of antidepressants.

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INTRODUCTION

Affective disorders are common and life-threatening illnesses, but despite extensive investigations, little is known about the underlying fundamental biology (Nestler *et al*, 2002). Recently, several *in vivo* imaging studies revealed that both the hippocampus and prefrontal cortex undergo selective volume reduction in several stress-related neuropsychiatric illnesses, particularly in major depressive disorder; the exact cellular basis for this volume decrease, however, has not yet been elucidated (Drevets, 2000; Manji and Duman, 2001; Bremner, 2002; Manji *et al*, 2003; Sheline, 2003). Post-mortem histological analysis of the frontal cortex demonstrated a decreased number of glia in patients suffering from major depressive disorder (Öngür *et al*, 1998; Rajkowska *et al*, 1999; Cotter *et al*, 2001a, 2002), and reduced glial density and glia/neuron ratio has been reported in the amygdala as well (Bowley *et al*, 2002; Hamidi *et al*, 2004). These and other observations led to the hypothesis that glial cell dysfunction may contribute to the pathogenesis of affective disorders (Coyle and Schwarcz, 2000; Cotter *et al*, 2001b).

In the hippocampus, no histopathological studies have so far demonstrated any significant loss of glial cells or neurons in the brains of patients suffering from major depression (Lucassen *et al*, 2001; Müller *et al*, 2001; Stockmeier *et al*, 2004). However, this negative finding might be due to the fact that all these studies were based on a small sample size and none of them used an unbiased counting method, most probably because the investigators did not have access to the complete, intact brain structure of interest (ie the hippocampal formation), an essential requirement for proper stereology. Thus, at this point one cannot rule out the possibility that future more systematic studies may reveal alterations in the number of neurons or glia, especially if specific subpopulation of cells are in focus of the analysis.

Among the most potent factors known to trigger or induce major depressive episodes are stressful life events (Kendler *et al*, 1999). This stress hypothesis of mood

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disorders has stimulated the development of a number of putative animal models that simulate or model aspects of depression by manipulating, for example, social relationships. In humans, loss of rank or social status is an example of the more general class of loss events (which are increasingly recognized as a specific type of 'life event') that are associated with a greater risk of depression (Brown, 1993). Using similar psychosocial perturbations as chronic stressors, powerful animal models for depression have been established. In recent years, our group has provided increasing evidence that chronic psychosocial conflict in the male tree shrew, *Tupaia belangeri*, represents a natural and valid paradigm for studying the behavioral, endocrine, and neurobiological changes that may underlie stress-related disorders such as major depression (Fuchs and Flugge, 2002; Fuchs *et al*, 2004).

To our knowledge, no systematic cell counting study has examined in experimental animals, the possibility of changes in glial cell numbers as a result of chronic stress. To answer this question, we investigated whether long-term psychosocial stress could affect the number and morphology of glial fibrillary acidic protein (GFAP) immunoreactive astrocytes in the hippocampal formation, using the chronic psychosocial stress paradigm in the male tree shrew. We focused on astroglia because of the growing number of data indicating that the role of astrocytes in function of the central nervous system so far has been considerably underestimated (Fields and Stevens-Graham, 2002). Additionally, we examined whether treatment with the antidepressant fluoxetine, a selective serotonin reuptake inhibitor (SSRI) with well-known clinical efficacy (Stokes and Holtz, 1997), offers protection from possible stress-induced morphological alterations. This approach was based on recent clinical and preclinical findings, which suggest that depressive disorders may be associated with an impairment of structural plasticity and cellular resilience, and that antidepressant medications may correct this dysfunction (Manji and Duman, 2001; Manji *et al*, 2000, 2001, 2003). Indeed, several studies have demonstrated that treatment with various classes of antidepressants can reverse both the functional impairments and the structural alterations of the hippocampal formation induced by stress (Watanabe *et al*, 1992; Czéh *et al*, 2001, 2005b; Malberg and Duman, 2003; Vermetten *et al*, 2003; Alonso *et al*, 2004; Lucassen *et al*, 2004).

MATERIALS AND METHODS

Animals, Experimental Procedure, and Fluoxetine Treatment

For the experiments, we used the tree shrew, *Tupaia belangeri*, a species phylogenetically close to primates (Martin, 1990). We decided to investigate these animals because they are well suited for a chronic stress paradigm (Fuchs and Flugge, 2002). Experimentally naive adult male tree shrews ($n = 22$; mean age 9 ± 1 months) were obtained from the breeding colony at the German Primate Center (Göttingen, Germany). Animals were housed individually on a 12 h light 12 h dark cycle with *ad libitum* access to food and water (Fuchs, 1999). All animal experiments were in accordance with the European Communities Council Directive of 24 November 1986, (86/EEC), with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and were approved by the Government of Lower Saxony, Germany. The minimum number of animals required to obtain consistent data was used.

Experimental design. As depicted in Figure 1a, animals were divided into four experimental groups: *Control* ($n = 6$), *Control + Fluoxetine* ($n = 6$), *Stress* ($n = 5$), and *Stress + Fluoxetine* ($n = 5$). The experiment consisted of two phases and lasted for 5 weeks (35 days). The first experimental phase ('Stress') lasted 7 days, during which the animals of the *Stress* and the *Stress + Fluoxetine* group were submitted to daily psychosocial conflict. The psychosocial stress procedure was carried out according to our standard protocol (for details see Czéh *et al*, 2001, 2005a, b). The second experimental phase consisted of the fluoxetine treatment for 4 weeks (28 days), while animals remained in the psychosocial conflict situation.

Animals of the *Control + Fluoxetine* and *Stress + Fluoxetine* groups received the compound (15 mg/kg body weight

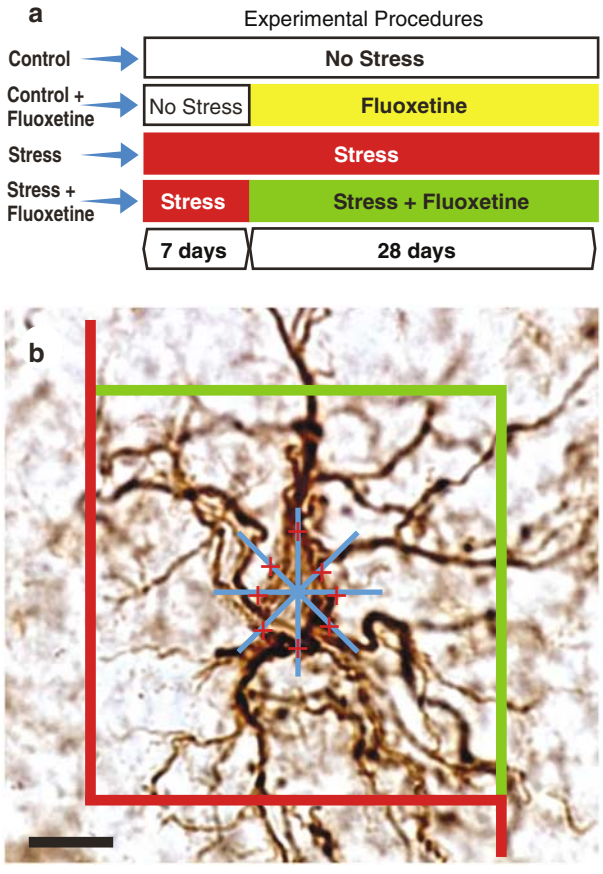

Figure 1 (a) Experimental groups and design. For details, see the Materials and methods. (b) Light micrograph illustrating the stereological counting frame. In the center is one astrocyte from a control CA1 stratum radiatum area. Only cells that lay within the volume ($50 \times 50 \times 20\,\mu m$) of the frame or touched the green lines were counted; those that crossed the red lines were excluded from the analysis. Estimates of cell size were generated using the nucleator probe (blue lines). For each cell, eight isotropic lines converged on the nucleus and intersected the somal boundary. Scale bar, 10 μm.

(BW) per day) orally in the morning between 0800 and 0815. The drug, Fluoxetin ratiopharm (4 mg/ml oral solution; Ratiopharm GmbH, Ulm, Germany), was administered via a bulb-headed cannula into the buccal cavity, and the animals were allowed to swallow the solution. Animals of the *Control* and *Stress* groups were treated with vehicle only. Animals received the drug orally because this is the most common route of antidepressant administration in psychiatric patients.

We conducted a pilot study to establish the necessary dosage of fluoxetine. Adult male tree shrews ($n = 9$, three per dosage group) received 5, 10, or 30 mg/kg BW fluoxetine per day orally for five consecutive days. Blood samples were collected 2 and 8 h after the first application, and then every 24 h after the daily application. Serum concentrations of fluoxetine and norfluoxetine were determined. Based on these results, we decided to treat the animals with 15 mg/kg BW fluoxetine per day, which resulted in a mean ($\pm$SEM) plasma concentration of 380 ± 111 ng/ml (range 81–634 ng/ml) of norfluoxetine 24 h after the last treatment of the 4-week treatment period; a similar range has been reported for patients under fluoxetine treatment (Baumann *et al*, 2004).

Analysis of Fluoxetine

Fluoxetine and its pharmacologically active metabolite norfluoxetine were determined in blood plasma using a high-performance liquid chromatography (HPLC) method with column switching and spectrophotometric detection, as described previously for the antipsychotic drug amisulpride (Sachse *et al*, 2003), with slight modifications. Serum (0.1 ml) was injected into the HPLC system. For online sample clean-up on a column (10×4.0 mm i.d.) filled with LiChrospher CN material of 20 μm particle size (MZ-Analysentechnik, Mainz, Germany), the column was washed with deionized water containing 8% (v/v) acetonitrile to remove proteins and other interfering compounds. Drugs were eluted and separated on LiChrospher CN material (5 μm; column size 250×4.6 mm i.d., MZ-Analysentechnik) using phosphate buffer (8 mM, pH 6.4) containing 500 ml/l acetonitrile, and were quantified by ultraviolet (UV) spectroscopy at 210 nm. HPLC analysis was completed within 20 min. Each analytical series included at least two control samples containing low (50 ng/ml) or high (400 ng/ml) concentrations of fluoxetine and norfluoxetine, respectively. There was a linear correlation between drug concentration and UV signal from 10 ng/ml to at least 600 ng/ml. The limit of quantification was 10 ng/ml. Intra- and interassay reproducibility of quality control samples was within 15%.

Perfusion and Brain Tissue Preparation

Animals were anesthetized with an overdose of xylazine/ketamine and perfused transcardially with 100 ml of saline followed by 200 ml of fixative containing 4% 0.1 M sodium phosphate-buffered paraformaldehyde (pH 7.4). The heads were postfixed in fresh fixative and, on the following day, the brains were gently removed from the skull. After cryoprotection with 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 30% sucrose and 10% glycerol, a freezing microtome was used to collect serial horizontal 50-μm-thick sections throughout the dorsoventral extent of the left hippocampal formation. A stereotaxic brain atlas of the tree shrew (Tigges and Shantha, 1969) was used for reference during the cryosectioning procedures.

GFAP Immunocytochemistry

Samples from each treatment group were always processed in parallel to avoid any nonspecific effect of the staining procedure. Free-floating sections were washed in 0.1 M PBS and then treated with 1% H_2O_2 for 20 min. After washing, nonspecific binding of antibodies was prevented by incubating the sections for 1 h with 3% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS containing 0.5% Triton X-100. The sections were subsequently incubated overnight with a mouse monoclonal antibody against GFAP (1 : 10 000; Chemicon, Hofheim, Germany) at 4°C in 0.1 M PBS containing 0.5% Triton X-100 and 1% NGS. The next day, the sections were rinsed several times in 0.1 M PBS, incubated in a biotinylated mouse secondary antibody (1 : 200; Vector Laboratories, Burlingame, CA, USA) for 1 h, rinsed, incubated in avidin–biotin–horseradish peroxidase (1 : 200; Vectastaine *Elite* ABC Kit, Vector) for 1 h, rinsed again, and developed for 5 min in diaminobenzidine (1 : 200; DAB Peroxidase Substrate Kit, Vector), and then thoroughly rinsed. The sections were then mounted on glass slides in a 0.1% gelatin solution and dried overnight, after which they were dehydrated through stepped alcohol washes, cleared in xylene for 30 min and finally coverslipped under Eukitt.

Stereological Estimates of Astrocyte Number and Size

A single examiner who was blind to the group identification of each animal performed the data collection. After randomly selecting a starting point, every tenth section along the dorsoventral extent of the hippocampus was selected, yielding an average of 14 sections per animal for analysis. First, the boundaries of the hippocampal formation (hippocampus proper together with the dentate gyrus) were demarcated for the stereological analysis on the GFAP-stained sections using a low power magnification lens ($\times 6.3$, 0.16 NA). The exact boundaries of the hippocampal formation in the tree shrew has been described in detail in an earlier study from our laboratory (Keuker *et al*, 2003), and these boundaries were easily recognizable in the GFAP stained sections as well. The total number (N) of astrocytes was estimated with the optical disector following fractionator rules (Gundersen, 1998; West, 1999) and a semiautomated system (StereoInvestigator, version 4.04, Microbrightfield, Williston, VT, USA). Video images of GFAP-immunoreactive astrocytes were acquired with a $\times 100$ oil objective (1.3 NA) on a Zeiss III RS microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CCD camera output to a high-resolution computer monitor and a Ludl X–Y–Z motorized stage (Ludl Electronics Products, Hawthorn, NY, USA). An astrocyte was counted only if it had a clearly defined nucleus within the disector area, did not intersect forbidden lines (Figure 1b), and came into focus as the optical plane moved through the height of the disector. After having counted all cells fulfilling the criteria

of sampling, the total number of cells was estimated as:

$$N_{\text{total}} = \Sigma Q^{-} \times 1/\text{ssf} \times A(x, y\,\text{step})/a(\text{frame}) \times t/h$$

where ΣQ^{-} is the number of counted astrocytes; ssf is section sampling fraction (1/10); $A(x,y$ step) is the area associated with each x, y movement (sampling area), which was 500 μm × 500 μm; a(frame) is the area of the counting frame, which was 50 μm × 50 μm; t is the thickness of the section (24.3 μm); and h is the height of the of the disector (20 μm). The average postprocessing section thickness was 24.3 μm and the guard height was 2 μm. Section thickness was determined in the hilus of each section by differential focusing using a ×100 oil objective (1.3 NA). A single observer focused between the first and last sharp DAB-positive profile (which were processes of the GFAP-positive astrocytes), while the vertical movement of the microscope stage was measured by the StereoInvestigator software (Microbrightfield). The precision of each cell number estimate was expressed as the coefficient of error (CE; Table 1).

The somal volume of the astrocyte cell body was calculated in every fifth counted glial cell using the nucleator probe (Möller *et al*, 1990, see Figure 1b).

Determination of Hippocampal Volume

Hippocampal volumes were assessed in the same sections as those used for counting astrocytes. The volumes were estimated according to the formula based on the Cavalieri principle (Gundersen *et al*, 1998):

$$V = \Sigma A \times t_{\text{nom}} \times 1/\text{ssf}$$

where ΣA is the cross-sectional hippocampal area, measured by tracing the borders of the hippocampus (Ammon's horn together with the dentate gyrus) using the Stereo-Investigator 4.04 software; t_{nom} is the nominal section thickness of 50 μm; and ssf is the sampling section fraction (1/10).

Adrenal and Testes Weight

Increased adrenal and decreased testes weights are indicators of sustained stress exposure. Therefore, these organs were removed from the animal immediately after perfusion and weighed. Data are expressed in organ weight in milligrams per gram average body weight of the preceding week.

Analysis of Serum Testosterone

Before perfusing the animals, blood samples were collected. For determination of testosterone, 100 μl serum was two times extracted with 1 ml of diethylether by vortexing for 10 min. Following extraction, the combined ether phases were evaporated under a stream of N_2 and dried extracts reconstituted in 300 μl assay buffer (PBS, containing 0.1% BSA, pH 7.0). Aliquots (50 μl) of reconstituted extracts were then measured for concentrations of testosterone by

Table 1 Detailed Individual Figures of Astrocyte Number and Somal Volume

Animal number	Treatment	Total number of astrocytes N ($\times 10^5$)	CE	Somal volume (μm^3)[a]	Hippocampal volume (mm^3)
12284	Control	8.427	0.04	605±43	40.46
12288	Control	9.287	0.05	428±26	41.98
12290	Control	9.652	0.04	707±24	41.25
12161	Control	7.086	0.05	631±33	39.04
12159	Control	7.130	0.06	595±20	38.43
12153	Control	8.194	0.04	634±30	41.78
12283	Control+Fluoxetine	9.798	0.04	697±27	41.93
12289	Control+Fluoxetine	8.675	0.05	505±18	39.72
12294	Control+Fluoxetine	8.719	0.05	451±20	39.70
12160	Control+Fluoxetine	8.223	0.05	426±14	39.28
12157	Control+Fluoxetine	8.048	0.05	402±19	36.36
12154	Control+Fluoxetine	7.873	0.05	444±18	39.27
11519	Stress	6.882	0.05	428±26	38.39
11703	Stress	4.505	0.06	368±36	34.84
11724	Stress	7.173	0.05	498±18	39.67
11734	Stress	5.963	0.06	468±20	39.76
11831	Stress	6.678	0.05	503±30	40.82
11520	Stress+Fluoxetine	6.911	0.05	350±23	39.14
11704	Stress+Fluoxetine	6.823	0.06	378±18	38.39
11725	Stress+Fluoxetine	7.611	0.05	378±10	40.65
11655	Stress+Fluoxetine	8.500	0.05	480±18	42.60
11832	Stress+Fluoxetine	7.655	0.05	446±17	37.14

CE: coefficient of error, mean CE was calculated as $\sqrt{\text{mean}(\text{CE})^2}$.
[a]Results of somal volumes are presented as mean±SEM.

enzyme immunoassay, as described in detail by Kraus *et al* (1999).

Statistical Analysis

Results are expressed as the mean ± SEM. Overall treatment effects were assessed with two-way analysis of variance (ANOVA, treatment × stress), followed by Student–Newman–Keuls *post hoc* analysis for further examination of group differences. Two-tailed Pearson test was used for correlation analysis.

RESULTS

Changes of Organ Weights and Serum Testosterone as an Indicator of Stress

Activation of the sympathoadrenomedullary system is an important and reliable indicator by which tree shrews can be classified as subordinates (Fuchs and Flugge, 2002). In animals from the *Stress* and *Stress + Fluoxetine* groups, adrenal weights were significantly increased (Figure 2a): two-way ANOVA (treatment × stress) revealed significant main effect of stress ($P = 0.001$), but not of treatment ($P = 0.6$). Moreover, we measured testes weights because gonadal hypotrophy is an indicator of sustained stress exposure (Fischer *et al*, 1985). Chronic stress exposure significantly reduced testes weight in the animals of the *Stress* and *Stress + Fluoxetine* groups (Figure 2b): two-way ANOVA (treatment × stress) revealed a highly significant main effect of stress ($P < 0.001$), but not of drug treatment ($P = 0.2$).

Not only testes weighs but serum testosterone concentrations were also significantly reduced by the repeated territorial conflict in the stressed animals, and this was not normalized by fluoxetine administration (Figure 2c). Two-way ANOVA (treatment × stress) revealed a significant main effect of stress ($P < 0.01$), but not of drug treatment ($P = 0.5$).

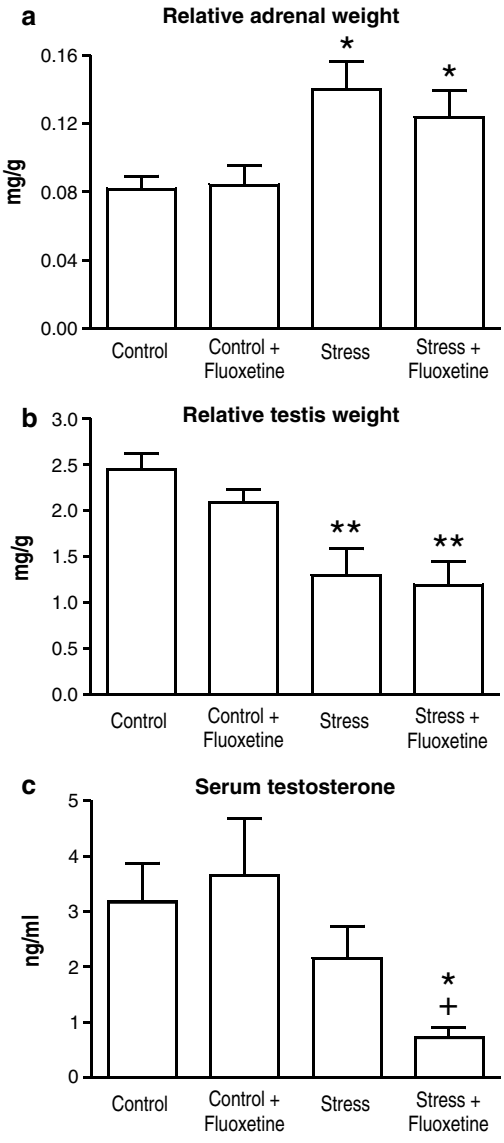

Figure 2 The physiological consequences of the repeated territorial conflict were expressed in the significant changes in relative organ weights and testosterone concentrations. Stress significantly increased relative adrenal weights (a), and reduced relative testes weights (b) and serum testosterone concentrations (c). Results are given as organ weight in milligrams per gram average body weight during the last experimental week (mean ± SEM). Serum testosterone levels were measured from blood samples collected before perfusing the animals, and presented here in nanograms testosterone per milliliter serum (mean ± SEM). Statistics: Two-way ANOVA followed by Student–Newman–Keuls *post hoc* analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs Control*, $^{+}P < 0.05$ *vs Contrtol + Fluoxetine*.

Stereological Estimates of Astrocyte Number and Somal Volume

Immunolabeling of the hippocampal formation for GFAP revealed numerous stellate structures distributed in a pattern similar to that observed in previous studies using rats (Schmidt-Kastner and Szymas, 1990). However, as shown in Figure 3, we observed a marked difference in GFAP staining intensity of the hippocampal tissue between control (*Control*, *Control + Fluoxetine*) and stressed animals (*Stress* and *Stress + Fluoxetine*).

Stereological estimates showed that exposure to chronic stress significantly decreased the total number of GFAP-immunoreactive cells by 25% (Figure 4a, Table 1). Two-way ANOVA (treatment × stress) revealed a highly significant main effect of stress ($P < 0.001$), and the effect of fluoxetine treatment was close to the level of significance ($P = 0.06$). Student–Newman–Keuls *post hoc* comparisons showed a significant difference between the *Control* and *Stress* groups ($q = 5.35$, $P < 0.01$). Treatment of stressed animals with fluoxetine offered protection from the effects of stress, resulting in an almost complete preservation of GFAP-immunoreactive astroglia and a statistically significant difference was revealed when the *Stress + Fluoxetine* group was compared with the *Stress* group ($q = 3.14$, $P < 0.05$). In contrast, no difference was found between the *Stress + Fluoxetine* group and controls ($P = 0.6$). In control animals, fluoxetine treatment alone had no effect on GFAP-positive astrocyte numbers (Figure 4a, Table 1).

Cell bodies immunopositive for GFAP were significantly decreased both by chronic stress and by fluoxetine treatment (Figure 4b, Table 1). Two-way ANOVA (treatment × stress) revealed a significant main effect both of

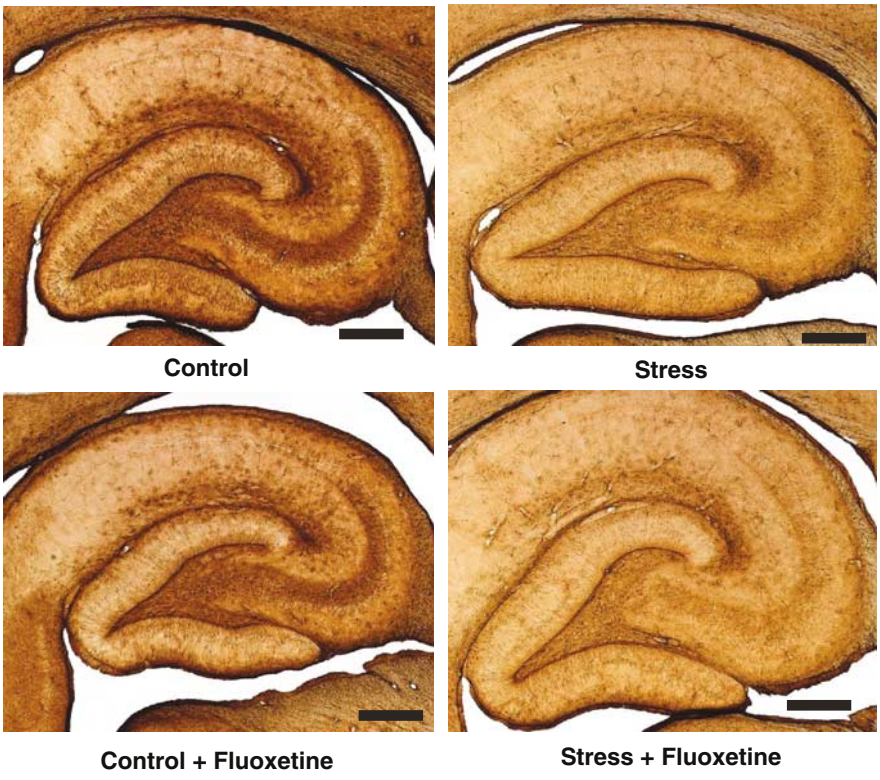

Figure 3 Representative examples of a glial fibrillary acidic protein (GFAP) stained horizontal section of the tree shrew hippocampal formation. Note the marked difference in GFAP staining intensity between the control (*Control*, *Control + Fluoxetine*) and stressed groups (*Stress* and *Stress + Fluoxetine*). Scale bars, 500 μm.

stress ($P<0.01$) and of fluoxetine treatment ($P<0.05$). Student–Newman–Keuls *post hoc* comparisons showed significant differences between the *Control* and *Stress* groups ($q=4.10$, $P<0.05$), between the *Control* and *Stress + Fluoxetine* groups ($q=5.40$, $P<0.01$), and between the *Control* and *Control + Fluoxetine* groups ($q=3.29$, $P<0.05$), see Figure 4b.

Hippocampal Volume

A mild decrease (−5%) of hippocampal volume was observed in the chronically stressed animals, but neither stress nor fluoxetine treatment had a statistically significant effect on hippocampal volume (Figure 5a). Interestingly, correlation analysis revealed significant correlations between the hippocampal volume and the total number of astrocytes (Pearson $r=0.6528$, $P=0.001$), as well as between hippocampal volume and the somal volumes of the astrocytes (Pearson $r=0.4828$, $P<0.05$) (Figure 5b and c).

DISCUSSION

To our knowledge, this study is the first to examine numerical and morphological changes of astrocytes after long-term psychosocial stress and antidepressant treatment. We demonstrated that chronic psychosocial conflict can result in significantly fewer astroglia, and concomitant treatment with fluoxetine can block this effect of long-term stress. Both chronic stress and fluoxetine treatment reduced the somal volumes of astrocytes; moreover, this treatment's effect was additive. Furthermore, we found that hippocampal volume correlated with the number and somal size of astrocytes.

Effect of Stress and Fluoxetine Treatment on the Endocrine System

Chronic psychosocial stress activates the hypothalamic–pituitary adrenal (HPA) system, increases the neurosympathetic tone, and suppresses gonadal activity. As demonstrated in previous studies (Fuchs and Flugge, 2002), repeated confrontations significantly increased the adrenal weights, but in contrast reduced testis weights and serum testosterone concentrations. Similar to earlier findings, fluoxetine administration did not influence these organ weight changes and had no effect on serum testosterone levels (Taylor *et al*, 1996).

Structural Changes of Astrocytes—Implication for Psychiatry

Abnormalities of glial function are likely to contribute to the impairments of structural plasticity and overall pathophysiology of mood disorders (Coyle and Schwarcz, 2000; Cotter *et al*, 2001b; Manji *et al*, 2003; Fuchs *et al*, 2004). A number of post-mortem histological analyses demonstrated a decreased number of glia in specific areas of the prefrontal, orbitofrontal, and cingulate cortex (Öngür

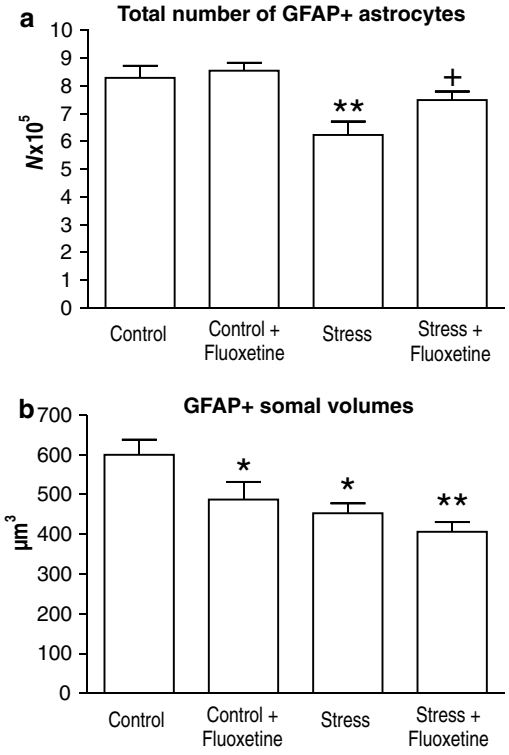

Figure 4 Effects of chronic psychosocial stress and concomitant fluoxetine treatment on the total number of GFAP-immunoreactive astrocytes in the hippocampal formation (a) and on the somal volumes of these GFAP-positive cells (b). (a) Stress significantly decreased the number of GFAP-IR astrocytes, whereas fluoxetine treatment resulted in a partial normalization of GFAP-positive glial numbers. Results are given as mean total numbers ($\times 10^5$) $\pm$ SEM. (b) Somal volumes were significantly reduced by both stress and fluoxetine treatment. Results are presented as mean somal volume (cubic micrometers) $\pm$ SEM. *Statistics*: Two-way ANOVA followed by Student–Newman–Keuls *post hoc* analysis. $^*P<0.05$, $^{**}P<0.01$ *vs Control*; $^+P<0.05$ *vs Stress*.

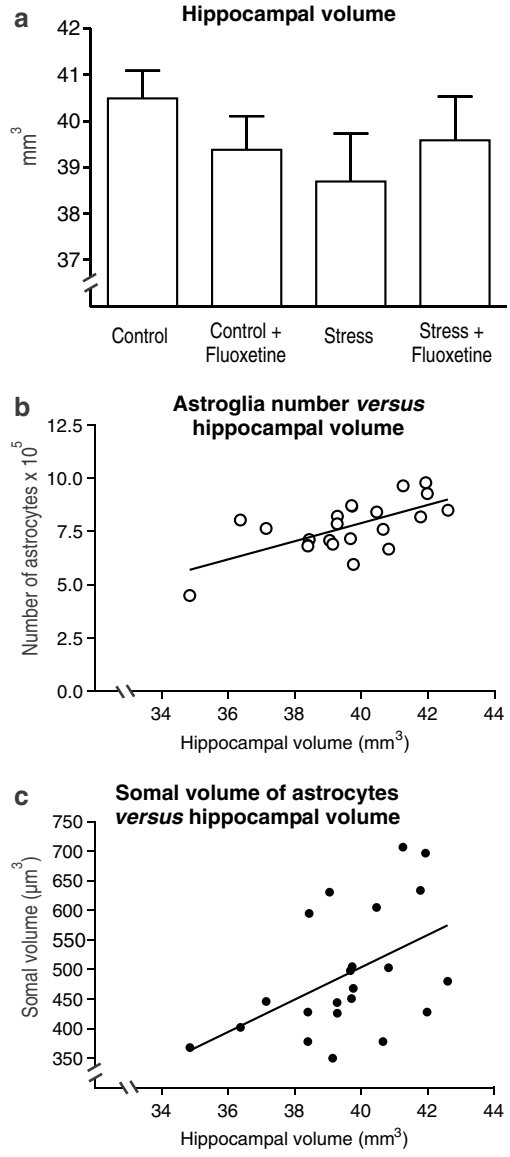

Figure 5 (a) There was a minor (−5%) nonsignificant decrease of the hippocampal volume in the *Stress* group, but neither stress nor drug treatment had a statistically significant effect on hippocampal volume. (b and c) Correlation analysis revealed that individual hippocampal volumes significantly correlated both with the total number of astrocytes ($r=0.65$, $P=0.001$), as well as the somal volumes of the astrocytes ($r=0.48$, $P<0.05$).

et al, 1998; Rajkowska *et al*, 1999; Cotter *et al*, 2001a, 2002; Miguel-Hidalgo and Rajkowska, 2002), and in the amygdala of depressed patients (Bowley *et al*, 2002; Hamidi *et al*, 2004). Despite the *in vivo* documented hippocampal volume reduction in patients suffering from depression, post-mortem histopathological analysis so far has not revealed any significant reduction of neuronal or glial cells in hippocampal samples from patients (Lucassen *et al*, 2001; Müller *et al*, 2001; Stockmeier *et al*, 2004). However, reduced GFAP staining was reported in the hippocampi of steroid-treated and depressed patients (Müller *et al*, 2001). It should be noted that, in most of these clinical studies, the depressed patients were not free of antidepressant medication, and our present results indicate this could be a confounding factor. Furthermore, all of these studies examined only a few sections from the hippocampal formation, and thus these negative findings might be due to the small sample size. Future and more systematic studies that examine the entire structure will be able to answer the question of possible changes in neuronal/glial cell numbers in the hippocampus of depressed patients. It might also be the case that anatomical examinations should focus on specific subpopulation of neuronal/glial cells, as we did in this study, to reveal changes in cell number. This is important, because there might be a significant reduction in the number of a certain type of cells, for example, specific interneurons or particular glial cell types. One has to consider that this numerical change—which affects only the portion of the cells—is not great enough to yield a significant change in the overall number of neurons or glial cells.

Using a chronic psychosocial stress paradigm, we found a significantly reduced number of astroglia in response to stress. More importantly, this stress effect was prevented by concomitant antidepressant treatment. The fact that the number of GFAP-labeled astroglia was not affected by treatment with fluoxetine in nonstressed animals suggests that the effects of stress were blocked by the SSRI and not

vice versa. These findings support current theories proposing that stress-related disorders such as major depression may be associated with an impairment of structural plasticity, and that antidepressants may act by correcting this dysfunction (Manji *et al*, 2003).

Functional Consequences

Recent studies have revealed that, beside their housekeeping functions, astrocytes are dynamic regulators of synaptogenesis and synaptic strength and control neuronal production in the adult dentate gyrus (Goldman, 2003; Horner and Palmer, 2003; Nedergaard *et al*, 2003; Newman, 2003; Slezak and Pfrieger, 2003). Morphological changes of the astrocytes must have functional significance on the neuron–glia and finally on neuron–neuron communication. The reduced number or weakened activity of astrocytes may lead to impairment reducing the levels of extracellular glutamate, and this may result in too much glutamate in the synaptic cleft, and in consequence to excitotoxic cell damage. Upregulation of the glial glutamate transporter (GLT-1) in the hippocampus has been reported after chronic stress, and it has been suggested that this might be a compensatory mechanism to control the increased extracellular concentrations of glutamate observed during stress. Interestingly, antidepressant treatment with tianeptine can block the stress-induced upregulation of GLT-1 (Reagan *et al*, 2004).

Another possible mechanism by which the altered activity of astrocytes can induce functional impairments of neuronal activity is the production of neurotrophic factors. Astrocytes synthesize and release many neurotrophic factors vital for neuronal health such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and neurotrophins 3 and 4/5 (Friedman *et al*, 1998; Althaus and Richter-Landsberg, 2000). These neurotrophic factors regulate neuronal growth, maintenance, and plasticity, and their reduced availability can result in increased cellular vulnerability or even cell death. Stress can reduce the expression of BDNF in the hippocampus, which in turn can be prevented by long-term chronic antidepressant treatment (reviewed by Duman *et al*, 1997; Russo-Neustadt and Chen, 2005). It is likely that astrocytes contribute to the enhancement in neurotrophic support and associated augmentation in synaptic plasticity that may form the basis for antidepressant efficacy.

Effect of Fluoxetine Treatment on Astroglial Plasticity

Recently, several groups proposed that glial cells should receive much greater attention when we attempt to understand the underlying biological mechanisms of psychiatric disorders, or the action of antidepressant therapy (Coyle and Schwarcz, 2000; Cotter *et al*, 2001b; Öngür and Heckers, 2004). The exact cellular mechanism by which fluoxetine exerts its therapeutic effect is not fully understood; its curative effect is attributed to its capacity to inhibit the neuronal reuptake of serotonin. However, fluoxetine exerts a direct effect on astrocytes as well (Chen *et al*, 1995; Kong *et al*, 2002) and this mechanism cannot be ignored when attempting to elucidate its mechanisms of action. Astrocytes, as part of the blood–brain barrier, form close connections with capillaries and thus are the primary target of any molecule entering the brain. In mammals, astrocytes can take up serotonin by a sodium-dependent, high affinity system (Kimelberg and Katz, 1985) and they express several different 5-HT receptor subtypes, for example, 5-HT_{1A}, 5-HT_{2A} (Azmitia, 2001; Azmitia *et al*, 1996). Especially the 5-HT_{1A} receptors, activation of which is suggested to be a critical component in the mechanism of action of SSRIs (Santarelli *et al*, 2003), occur in high abundance on hippocampal astroglia (Azmitia *et al*, 1996).

There are a few reports that beside fluoxetine, other drugs can also modulate the structural plasticity of astrocytes. After chronic lithium and antipsychotic medication, increased numbers of glia have been reported in the hippocampus and prefrontal cortex of rats and nonhuman primates (Rocha *et al*, 1998; Selemon *et al*, 1999). In contrast to that in our study, fluoxetine had no effect on hippocampal astrocyte numbers in control animals, but could counteract the stress-induced decrease of astrocyte numbers. Chronic treatment with lithium upregulates GFAP expression and modifies the morphology (orientation) of astrocytes (Rocha and Rodnight, 1994; Rocha *et al*, 1998). We report here that fluoxetine can reduce the somal volumes of astrocytes. Altogether our data demonstrate that fluoxetine, a prominent member of the SSRI family (Hiemke and Härtter, 2000), can significantly modify the structural plasticity of astrocytes, and it is very likely that these morphological alterations either reflect or induce functional changes within the glial–neuronal interaction.

Morphological Changes of Astroglia may Contribute to Hippocampal Volume Decrease

Recent imaging studies in humans revealed that the hippocampus undergoes selective volume reduction in several stress-related psychiatric illnesses such as major depressive disorder (reviewed by Manji and Duman, 2001; Manji *et al*, 2003; Bremner, 2002; Drevets, 2000; Sheline, 2003). Similar findings have been reported in animals submitted to chronic stress (Ohl *et al*, 2000; van der Hart *et al*, 2002; Alonso *et al*, 2004; Czéh *et al*, 2005a). The exact mechanisms responsible for this hippocampal volume loss have not yet been identified. Massive neuronal loss following exposure to repeated episodes of hypercortisolemia can be excluded, because, in human post-mortem brain tissue of severely depressed patients or of steroid hormone treated human or nonhuman primate subjects, no major cell loss was apparent, nor was any neuropathology present (Leverenz *et al*, 1999; Lucassen *et al*, 2001; Müller *et al*, 2001; Stockmeier *et al*, 2004). This is consistent with findings of preclinical studies that failed to reveal any loss of principal neurons in the hippocampal formation after chronic stress exposure (Vollmann-Honsdorf *et al*, 1997; Sousa *et al*, 1998; Keuker *et al*, 2001). Stress-induced dendritic retraction of CA3 pyramidal neurons and suppression of dentate neurogenesis have been implicated as mechanisms contributing to hippocampal shrinkage (McEwen, 2000). In the present study we found that the hippocampal volume correlated both with the total number of astrocytes as well as their somal volumes. Based on the present findings, it appears that the stress-induced reduc-

tion of astroglia number and size can contribute to the hippocampal volume changes.

Limitations of the Study

There are certain limitations to our study that should be kept in mind when interpreting these data. First, the visualization of astrocyte morphology by immunostaining for cytoskeletal proteins, such as GFAP, delineates only about 15% of the cell's total volume (Bushong *et al*, 2002). Because of this limitation of the labeling technique, it is probable that the somal volume values reported here do not reflect the real somal volumes; however, it is unlikely that this could significantly influence the results of the comparisons between the groups. Our experiment does not answer the question whether the stress-induced decrease of astrocytic cell number represents true cell loss, or is due to expression of the cytoskeletal GFAP protein being down-regulated to a level undetectable by immunohistochemistry. Note that the GFAP staining intensity was markedly reduced in the stressed animals (see Figure 3). Stressed tree shrews have significantly increased cortisol levels (Fuchs and Flugge, 2002) and it is well known that glucocorticoids can reduce GFAP mRNA and protein expression in the hippocampus (Laping *et al*, 1994). Further animal studies are required to determine whether other stress paradigms and other types of antidepressant have similar effects on astrocytes in the hippocampus and prefrontal brain areas in order elucidate results from human studies.

CONCLUSION

The pronounced astroglial structural plasticity as a result of chronic psychosocial stress and the antagonizing effects by fluoxetine treatment provides further support for the notion that glial changes may contribute to the pathophysiology of stress-related disorders as well as to the biological actions of antidepressants.

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