HIPPOCAMPAL NEUROPLASTICITY AND DEPRESSION

STUDYING AN ANIMAL MODEL, THE CHRONIC PSYCHOSOCIAL STRESS PARADIGM IN MALE TREE SHREWS

DISSERTATION SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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PÉCS, 2006.

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"It is a capital mistake to theorize before you have all the evidence"
Sir Arthur Conan Doyle

PREFACE

The traditional monoamine hypothesis fails to explain sufficiently the neurobiological mechanisms underlying major depressive disorder (MDD). Although MDD indisputably has a strong genetic basis, severe environmental stressors have been associated with a considerable increase in risk for MDD in vulnerable individuals. Recent findings in molecular biology, histopathology and brain imaging revealed that MDD is related with impairments of structural plasticity and cellular resilience in brain areas regulating mood and emotions.

The hippocampal formation is one of the most widely studied brain structures associated to depression. This dissertation focuses on the ***cellular alterations in the hippocampus in an animal model for depression.*** Since histological studies are limited in humans, the ***chronic psychosocial stress paradigm in tree shrews*** (*Tupaia belangeri*), a valid animal model of depression, was used to study the morphological changes as a result of chronic stress and concomitant antidepressant treatment. Following aspects of the cellular plasticity have been investigated:

i. the changes of ***cytogenesis in the dentate gyrus***

ii. the possible involvement of inhibitory ***interneurons***

iii. the role of ***astroglia*** in the structural changes of hippocampus

iv. finally, a novel possible antidepressant agent, a ***non-monoaminergic NK_1 receptor antagonist*** was tested in the chronic psychosocial stress model.

The results show that the number of newborn cells in the dentate gyrus continuously decreases with age, while psychosocial stress significantly worsens this tendency. As a result of chronic social defeat stress, the rate of cytogenesis in the dentate gyrus, as well as the number of

interneurons and astroglia was reduced in the hippocampus; antidepressant treatment, however, had a restorative effect on these cellular changes. Recent theories suggest that adult neurogenesis may play a significant role in the pathogenesis of depression and may contribute to the functional and behavioral changes appearing during antidepressant treatment; until now however, these assumptions have not been unequivocally verified. Nevertheless, cytogenesis in the dentate gyrus can be considered as a sensitive marker of changes of neuroplasticity. Neuroplasticity—in the widest sense—seems to provide a better understanding of the pathophysiology of depressive disorders; and, being a novel approach, it will hopefully help us develop novel therapeutic tools to improve the treatment of depression.

CHAPTER 1

General introduction

Major depressive disorder (MDD) is a common, chronic, and often life-threatening illness. Epidemiological studies have shown that up to 10-25% of the population is getting depressed in their lives. Despite extensive studies on MDD, relatively little is known about its etiology. Neither pathophysiological mechanisms, nor the factors responsible for the therapeutic effects of antidepressant drugs are sufficiently cleared.

Novel theories on the pathophysiology of depression

Although MDD has traditionally been connected to neurochemical alterations, recent neuroimaging studies have demonstrated selective structural and functional changes across various limbic and nonlimbic regions in the brains of depressed individuals. In particular, both metabolism and volume of the prefrontal and cingulate cortex are reduced, while—with further progression of the syndrome—hippocampal volume loss can also occur (Manji et al., 2001; Sheline et al., 2003). Postmortem morphometric studies revealed decreased neuronal size and glial density in some cortical and limbic brain areas (Rajkowska, 2002).

A growing number of clinical and preclinical studies indicate that MDD may be associated with an impairment of structural plasticity and cellular resilience in brain structures associated with regulation of emotions and mood, and that antidepressant medications may act by correcting this dysfunction (Manji and Duman, 2001; Manji et al., 2001, 2003). A number of data suggest that intracellular signaling pathways involved in regulating cell survival and cell death are the long-term targets of antidepressant drugs (Coyle and Duman, 2003).

It has been revealed that antidepressants and lithium seem to have 'neurotrophic'/'neuroprotective' properties, which are mediated by different neurotrophic signaling cascades (most notably cyclic adenosine monophosphate [cAMP] response element binding [CREB] protein, brain-derived neurotrophic factor [BDNF], the protein Bcl-2, and mitogen activated protein [MAP] kinases) (Manji et al., 2001, 2003; Duman and Monteggia, 2006). These intracellular mechanisms may explain the delayed, long-term beneficial effects, detected in patients receiving these agents. Furthermore, there is clinical evidence to suggest that antidepressant

treatment may increase BDNF levels in the human brain (Chen et al., 2001) and can protect against hippocampal volume loss in depressed patients (Sheline et al., 2003).

The main effect of these findings was the modification of the previous monoamine hypotheses on the pathophysiology of major depression. More contemporary theories suggest that disturbed neuroplasticity might form the basis of major depression (Manji and Duman, 2001; Manji et al., 2001, 2003; Castren, 2005). Nevertheless, the neuroplasticity hypothesis does not eliminate the classical monoamine theory; it rather draws the attention from intrasynaptic alterations to intracellular processes and structural changes.

Stress and depression

Stress involves a complex cascade of events too well-known and too little understood at the same time. One of the more recent definitions was presented by McEwen (2000): 'Stress may be defined as a real or interpreted threat to the physiological and psychological integrity of an individual that results in physiological and / or behavioral responses. In biomedicine, stress often refers to situations in which adrenal glucocorticoids (GCs) and catecholamines are elevated because of an experience.'

Stressful life events can lead to the development of MDD in genetically predisposed individuals (Kendler et al., 1999). Stress situations are supposed to trigger episodes of major depression—most probably—via hormonal changes, such as the directly and / or indirectly elevated levels of circulating glucocorticoids (reviewed in Holsboer and Barden, 1996; Holsboer, 2000).

HPA axis dysfunction in depression

In the majority of patients with MDD, some dysfunction of HPA axis regulation has been found which is most easily characterized by elevated circulating levels of cortisol (Carroll et al., 1976). Furthermore, elevated levels of corticotropin-releasing hormone (CRH) in the cerebrospinal fluid (CSF) have also been found in depression (Nemeroff et

al., 1984); and numerous patients with MDD showed decreased ACTH response after CRH injection, suggesting desensitized pituitary CRH receptors due to down-regulation by CRH hypersecretion (Holsboer at al., 1986).

Another indication for the dysfunction of HPA axis regulation is the failed reaction to the dexamethasone suppression test (DST) in depression. Non-response to dexamethasone is seen in around 40-50% of patients with MDD, and characterizes mainly those with severe depression, overwhelmingly with psychotic elements (Rihmer et al., 1984), predicting a high risk of relapse despite continued treatment. Not surprisingly, these patients also show CRH hyperactivity with a blunted ACTH response to exogenous CRH (DEX/CRH-test) (von Bardeleben and Holsboer, 1991). A normalization of the response to the DST or DEX/CRH test, when retesting depressed patients during antidepressant treatment, is associated with clinical improvement (Heuser et al., 1996; Holsboer et al., 1982; Holsboer-Trachsler et al., 1991). Additionally, in individuals who have never had depressive symptoms, but who had family members suffering from depression, an abnormal response to the DEX/CRH could be detected (Munich Vulnerability Study: Lauer et al., 1998).

Convergence between GCs and monoamines in depression

MDD arises from the complex interaction of susceptibility and protective genes and environmental factors (Kessler, 1997; Kendler et al., 1999). Recent genetic studies propose that stress response and monoamine dysregulation interact in the pathogenesis of depression. Caspi and his colleagues demonstrated that the polymorphism, which leads to decreased transcription of the serotonin transporter gene, results in risk for MDD only in individuals who had been exposed to multiple traumatic life events (Caspi at al., 2003). Most recently, these results have been replicated by another cohort study (Kendler et al., 2005). Thus, stressful life events can modify the effect of a genetic variability of the serotonin transporter. Furthermore, Binder and his colleagues described that functional polymorphism in FKBP5, a glucocorticoid receptor chaperone binding protein involved in the stress response by the HPA axis, can be associated with a more rapid

response to antidepressant treatment acting via modulation of monoamine systems (Binder et al., 2004). Hence, a genetic variant of stress response determines how antidepressant treatment affects mood. These data suggest a strong interaction between the monoamine and glucocorticoid system: a genetic variant of one system affects the contribution of the other system to the development of depression.

Animal models of depression

As clinical studies in patients are restricted to non-invasive methods, this limitation has led to the development of animal models for depression. However, mental illnesses are uniquely human conditions; therefore the limitations of animal models must be fully recognized.

Animal models of human depression may be rated according to three criteria: face validity, predictive validity and construct validity (Willner, 1984). High *face validity* is achieved when a model replicates a number of symptoms associated with depression and when chronic administration of antidepressant treatment effectively improves these symptoms. A model fulfills criteria of high *predictive validity* when the treatment of symptoms seen in the animal model brings about identical effects as those seen after treatment in humans suffering from the disorder. The *construct validity* of a model is adequate when the animal model is homologous in etiology and when the model has an empirical and theoretical relation to the disorder it models.

Amongst the most potent factors known to trigger or induce depressive episodes are stressful life events (Kendler et al., 1999; Kessler, 1997; Paykel, 2001). This stress hypothesis of mood disorders has stimulated the development of putative animal models of depression using stress to induce a depressive-like state in animals (Willner, 1991; Nestler et al., 2002). In humans, loss of rank or social status is an example of life events which are associated with a high risk of depression (Brown, 1993). Thus, animal models based on social subordinate status employ realistic conditions which are of particular relevance for psychopathology (Mitchell and Redfern, 2005).

Artificially elevated GC levels can lead to biochemical and behavioral changes seen in animal models of depression (Fernandes et al., 1997) and administration of CRH to healthy animals induces symptoms comparable to those of depressed patients (Owens and Nemeroff, 1991). Considering the facts, that the dysregulation of the HPA axis, leading to high level of circulating GCs, is a predominant feature of chronic stress in animals (Dallman et al., 2004), and that chronic stress may trigger the occurrence of depressive episodes in humans, stress models may be particularly useful in studying the pathophysiological basis of depression.

The chronic psychosocial stress paradigm in adult male tree shrews: a resident-intruder paradigm

Tree shrews (*Tupaia belangeri*) are small, squirrel-like animals, phylogenetically close to primates (Martin, 1990), naturally living in South-East Asia. Males live solitary, exhibiting pronounced territoriality, which can be utilized to establish challenging situations under experimental control. To induce social conflict one naïve male is introduced into the cage of a socially experienced male. This results in active competition for control over the territory, and after a clear dominant–subordinate relationship has been established, the two animals are separated by a wire mesh barrier. The barrier is removed every experimental day for about one hour, thereby allowing physical contact between the two males during this time only. Beyond this time period, the subordinate animal is protected from repeated physical attacks, but it is constantly exposed to olfactory, visual and acoustic cues from the dominant animal.

During periods of daily social stress, subordinate male tree shrews develop symptoms that are very similar to what can be observed in many patients with major depression, such as hyperactivities of the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis, resistance to dexamethasone suppression, disturbances in sleeping patterns, and reduced motor activity (DSM-IV, 1994; Fuchs and Flügge, 2002; Fuchs, 2005). Some of these parameters are renormalized by antidepressant treatment (Fuchs and Flügge, 2002; Fuchs, 2005). Moreover, in the brains of the stressed tree shrews, monoamine receptors show dynamic changes that

reflect adaptation to an initial monoaminergic hyperactivity that is later followed by deficits in the noradrenergic and the dopaminergic system (Flügge et al., 2004). In addition, structural changes of hippocampal neurons can be observed, such as the retraction of the apical dendritic tree of CA3 pyramidal neurons (Magariños et al., 1996) and suppressed dentate neurogenesis (Czéh et al., 2001). These processes are considered as a cause of behavioral alterations that can be counteracted by antidepressants, whereas the anxiolytic diazepam was ineffective (Fuchs et al., 1996; van Kampen et al., 2000). Taken together, the chronic social conflict in the male tree shrew appears to be a valid model for studying stress-related disorders such as depression (for review see Fuchs and Flügge, 2002). Following the definition of Kornetzky (1989), the chronic social stress in tree shrews can be regarded as a '*homologous model*' of depression, because it mimics several aspects of the human disease.

Stress and depression affect hippocampal plasticity

Although multiple brain areas are implicated in the pathogenesis of depression, including not only the hippocampus, but also prefrontal and cingulate cortical regions, as well as the amygdala, thalamic, hypothalamic and several brain stem areas (Nestler et al., 2002; Drevets, 2004), the structural alterations in the hippocampal formation are the ones most widely investigated. The hippocampus has been established to play a definite role in learning and memory, as well as in the regulation of the release of CRH via inhibitory feed-back loop to the hypothalamic paraventricular nucleus. Both cognitive functioning and HPA axis regulation are altered in depression. Depression, however, is not a pure hippocampal disorder, but a part of the symptoms seen in depression, can be explained by the various (if even mild) cognitive impairments caused by the hippocampal dysfunction. This can be best characterized by reduced ability of the hippocampus to cope with novelty and complexity in conjunction with inadequate information processing in brains of depressed patients (Kempermann, 2002), which underlines the key role of this area in the pathophysiology of the MDD. In addition, the hippocampus has reciprocal connections with the

amygdala, and prefrontal cortex, regions that are more directly involved in emotion and cognition.

Data from animal models of depression yielded much evidence for structural and cellular alterations in hippocampus parallel with depression-like symptoms, or during antidepressant treatment. According to animal models, depression induced by chronic stress and antidepressant treatment seems to have opposing effect on both structural and cellular plasticity of the hippocampus (D'Sa and Duman, 2002; Dranovsky and Hen, 2006). Chronic stress leads to decreased transcription of the BDNF and depresses the hippocampal expression of genes important for plasticity, whereas antidepressant treatment has proved itself to have a reversal effect on these alterations (Alfonso et al., 2004; Duman and Monteggia, 2006). In experimental paradigms of chronic psychosocial stress the GC dependent reduction in hippocampal volume, dendritic arborization, and neurogenesis has been reported (Fuchs et al., 2005). Especially, the shrinkage (i.e. shortening and debranching) of apical dendrites of CA3 pyramidal neurons has been described by several authors in various models (Magarinos et al., 1996, Fuchs et al., 2005). Another aspect of the hippocampal plasticity, the adult neurogenesis in the dentate gyrus, is being extensively studied as well. Exposure of animals to various experimental stressors results in decreased cyto- or neurogenesis in the dentate gyrus, while antidepressant treatment have a restorative effect on it (Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006). In animal models, the serotonin reuptake inhibitor fluoxetine (Malberg and Duman, 2003); the monoamine oxidase inhibitor moclobemide (Li et al., 2004); and the serotonin reuptake enhancer tianeptine (Czéh et al., 2001) seem all to restore stress-induced changes both in hippocampal plasticity and in certain behavioral phenotypes.

Despite of the growing body of knowledge, there are many unanswered questions regarding the hippocampal neuroplasticity and the way how stress and depression affect it.

Cell proliferation in the hippocampal dentate gyrus: Implications for the genesis of major depression?

A unique feature of the hippocampal dentate gyrus is the presence of adult neurogenesis (Gross, 2000; Lledo et al., 2006). Granule cells proliferate in the subgranular zone, and then migrate into the granular cell layer, where some of them give rise to mature neurons. Cell survival is the functional integration of new neurons into the hippocampal neuronal network within a few weeks (Lledo et al., 2006). Adult hippocampal neurogenesis has been demonstrated in a variety of different species including humans (Eriksson et al., 1998; Gross 2000) and is regulated by a variety of pharmacological, internal, and environmental signals (Eisch, 2002; Fuchs and Czéh, 2005). For example, both acute and chronic stress exposure can suppress the proliferation and survival of the newly generated granule neurons in the adult brain (Gould et al., 1997; Czéh et al., 2002).

Various experimental studies of stress and antidepressant treatment have implicated hippocampal neurogenesis in the etiology of major depression (reviewed by Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006). Santarelli et al. (2003) suggested that neurogenesis might be essential for the therapeutic action of antidepressant drugs. Despite many efforts, however, the direct proof of this hypothesis remains to be established. Further research is required to clarify the factors influencing hippocampal neurogenesis and investigate more thoroughly the impact of antidepressant treatment on it.

A new aspect of structural plasticity: Stress affects hippocampal interneurons

While many studies have investigated the effects of chronic stress on the morphology and number of principal neurons (pyramidal and granule cells) of the hippocampal formation, no one examined the possibility of changes in interneuron numbers so far. About 14% of newly generated neurons in the adult dentate gyrus are apparently GABAergic parvalbumin-positive cells (Liu et al., 2003). Parvalbumin-containing cells represent a subpopulation of GABAergic interneurons regulating the discharge activity of large populations of principal cells (Freund and Buzsáki, 1996). These

parvalbumin-containing cells in the hippocampal dentate gyrus and CA3 region receive most of their excitatory input from granule cells (Seress et al., 2001). Therefore, similarly to CA3 pyramidal cells, that are known to retract their apical dendrites in response to chronic stress, parvalbumin-positive interneurons are also subjected to the deleterious effects of excessive excitatory amino acid release from the mossy fiber terminals during stress exposure (Magariños et al., 1997).

The role of astroglia in the hippocampal plasticity

The role of the astrocytes in the functioning of the central nervous system has been largely underestimated so far (Ransom et al., 2003). However, recent discoveries revealed that beside their well-known housekeeping function, astrocytes are in fact dynamic regulators of synaptic strength, synaptogenesis and neuronal production in the adult dentate gyrus (Ransom et al., 2003). Astrocytes also possess receptors for neurotransmitters and steroid hormones, that, similarly to receptors in neurons, can trigger electrical and biochemical events inside the astrocyte (Haydon, 2001).

Recently, numerous *in vivo* imaging studies revealed that both the hippocampus and prefrontal cortex undergoes selective volume reduction in several stress-related neuropsychiatric illnesses particularly in major depressive disorder, however, the exact cellular basis for this volume decrease has not yet been elucidated (Sheline, 2003; Drevets, 2004). At the same time, analysis of postmortem tissue from patients with affective disorders has revealed decreased number of glial cells in several limbic brain areas (Cotter et al., 2001; Rajkowska, 2002). Not surprisingly, during recent years abnormalities of glial function have been suggested to contribute to the impairments of structural plasticity and overall pathophysiology of mood disorders (Coyle and Schwarcz, 2000; Cotter et al., 2001).

Aims and outline of the dissertation

Findings from animal models and results of human brain imaging and postmortem histopathological studies support the role of structural alterations of hippocampus in MDD. Accordingly, enhancement of structural remodeling can be a promising new way of treating depression. Nevertheless, many aspects of hippocampal neuroplasticity are not yet fully understood.

The neuroplasticity approach focuses on domains beyond the monoamine hypothesis, i.e. on the (sub)cellular and structural changes in depression. This theoretical frame may simulate the search for new classes of antidepressant drugs acting not necessarily on monoamine receptors.

To add some new data to the existing body of knowledge, this dissertation focuses on the following questions:

1. Does age have any impact on hippocampal neurogenesis, and if so, how does stress affect it?
2. Does chronic psychosocial stress affect the number of parvalbumin-immunoreactive interneurons in the hippocampal formation? If so, how can antidepressant treatment influence this?
3. Does long-term psychosocial stress alter the number and morphology of astrocytes in the hippocampus? Does antidepressant treatment have an effect on it?
4. Does SLV-323, a novel neurokinin 1 receptor (NK_1R) antagonist, which might be regarded as a promising, novel, non-monoaminergic antidepressant, exert any effect on the hippocampal cellular plasticity and behavioral changes induced by psychosocial stress?

To answer these questions the following experiments were performed:

1. **Chapter 2** presents a study in which dentate cell proliferation in chronically stressed and non-stressed tree shrews at various ages was compared. To be able to compare data form stressed and non-stressed animals, a mathematical model was necessary to find, which describes

changes in the cell proliferation with age. After finding regression curves, which fit best to the data, parameters in the curves describing stressed and non-stressed groups were compared.

2. In **Chapter 3** an experiment is described in which a group of chronically stressed tree shrews were treated with an NK_1 receptor antagonist or with fluoxetine. The number of parvalbumin-immunoreactive hippocampal interneurons was compared in control, purely stressed, purely treated, and stressed and antidepressant treated animals. The various subregions of the hippocampal formation were investigated separately.
3. In an experiment, presented in **Chapter 4**, the number and morphology of hippocampal astrocytes were studied. Additionally, we examined whether treatment with the antidepressant fluoxetine, a selective serotonin reuptake inhibitor (SSRI), with well-known clinical efficacy offers protection from the possible stress-induced morphological alterations.
4. Finally (in **Chapter 5**), the effect of SLV-323 was tested. In a complex experimental design which provided an extended approach, we focused not only on the effect of stress and treatment on hippocampal neurogenesis, but behavioral effects and cerebral metabolite concentrations were analyzed as well.

CHAPTER 2

Age-dependent susceptibility of adult hippocampal cell proliferation to chronic psychosocial stress

Available online at www.sciencedirect.com
SCIENCE DIRECT
Brain Research 1049 (2005) 244 – 248
BRAIN RESEARCH
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Short Communication

Age-dependent susceptibility of adult hippocampal cell proliferation to chronic psychosocial stress

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Accepted 3 May 2005
Available online 9 June 2005

Abstract

Stress inhibits neurogenesis in the adult dentate gyrus. It is not known, however, whether age has any influence on this process. We subjected adult male tree shrews to 5 weeks of psychosocial stress, after which dentate cytogenesis was determined using BrdU immunohistochemistry. We found that older animals were significantly more vulnerable to the adverse effect of stress on dentate cell proliferation.

Theme: Development and regeneration
Topic: Aging process

Keywords: Neurogenesis; BrdU; Hippocampus; Aging; Social stress; Tree shrew

Adult hippocampal neurogenesis is a form of neuroplasticity that has been shown to persist throughout the entire lifespan of mammals, including humans [7,13]. A number of environmental and endogenous factors have been demonstrated to modulate this process. Two examples are age and stress, both of which are powerful inhibitors. While with increasing age, the proliferation rate and ratio of cells differentiating into neurons rapidly decline, the percentage of surviving newborn cells remains constant [2,6,14,16]. Stress is one of the most potent environmental factors known to suppress both the proliferation and survival rate of newly generated hippocampal granule cells. This has been demonstrated using various stress paradigms in several species [1,3–5,11,12,15,17,18,20,22,23]. It is not known whether dentate cell proliferation in individuals of various ages might be differentially susceptible to stress. Here, we studied tree shrews (*Tupaia belangeri*), a species closely related to primates [19] that exhibits extreme social intolerance. Adult animals (aged 5 to 30 months) were subjected to 5 weeks of daily social confrontation, after which we evaluated the influence of age on the suppressive effect of stress on dentate cell proliferation.

The experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) on the use of laboratory animals and were approved by the Government of Lower Saxony, Germany. Experimentally naive adult male tree shrews (*T. belangeri*) were obtained from the breeding colony at the German Primate Center (Göttingen, Germany). Animals were housed individually on a 12:12 h light/dark cycle, with free access to food and water [8].

Forty-five animals were investigated in this study. These were 5–30 months in age (mean 12.9 ± 0.8 months). Tree shrews reach maturity after about 3–4 months, and under laboratory conditions, their life span is approximately 5–7 years.

Experimental animals were divided into Control (n = 18) and Stress (n = 27) groups. The mean age (±standard deviation) of the Control group was 13.53 ± 5.65 months, and the Stress group 12.75 ± 5.41 months. According to their ages, the animals were assigned to three different

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0006-8993/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2005.05.006

subgroups: <10 months: Control ($n = 6$); Stress ($n = 9$); 11–20 months: Control ($n = 9$); Stress ($n = 15$); 21–30 months: Control ($n = 3$); Stress ($n = 3$). Animals from the Stress group were exposed to daily psychosocial conflict for 35 days, while animals of the Control group remained undisturbed elsewhere in the animal facility. The psychosocial stress procedure followed our standard protocol (for details, see [4,9]).

At the end of the experiment, animals received a single i.p. injection of 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg; Sigma-Aldrich, Germany) to label dividing cells. They were perfused transcardially with 4% paraformaldehyde 24 h later. Serial horizontal 50 μm sections were cut using a freezing microtome throughout the dorso-ventral extent of the left hippocampal formation, and every fifth section was slide-mounted and coded before staining to ensure objectivity.

According to our standard protocol [4], BrdU labeling requires the following pre-treatment steps: DNA denaturation (0.01 M citric acid (pH 6.0), 95 °C, 20 min), membrane permeabilization (0.1% trypsin, Sigma-Aldrich, Germany), and acidification (2 M HCl, 30 min). The primary antibody concentration was mouse anti-BrdU (1:100, DAKO, Germany), and immunocytochemistry was completed by using the avidin–biotin/diaminobenzidine visualization method (Vector Laboratories, Burlingame, CA, USA) followed by counterstaining with hematoxylin.

A modified unbiased stereology protocol that has been reported to be reliable was used to quantify the BrdU labeling [4,18,20]. Every fifth section (an average of 26) through the dorso-ventral extent of the left hippocampal formation was examined. All BrdU-labeled cells in the granule cell layer including the subgranular zone, defined as a two-cell-body-wide zone along the border of the granule cell layer, were counted regardless of size or shape. To enable counting of cell clusters, cells were examined under 400× and 1000× magnification, omitting cells in the outermost focal planes. The total number of BrdU-labeled cells was estimated by multiplying the number of cells counted in every fifth section by five.

Because increased adrenal weight is an indicator of sustained exposure to stress [9,24], the adrenals were removed from all animals and weighed immediately after perfusion. Data are expressed as milligram organ weight per gram average body weight during the last experimental week.

Chronic psychosocial stress resulted in a significant decrease (−46%) of the total number of BrdU-positive cells (Student's t test: $t = 3.47$, $df = 43$, $P = 0.001$, Fig. 1A). In contrast, comparison of the means of the age of the two groups revealed no significant difference ($t = 0.466$, $P = 0.644$). When the animals were divided into three different subgroups according to their ages, an age-dependent vulnerability to stress appeared (Fig. 1B). In the youngest group (<10 months), dentate cytogenesis showed a 41% decrease in response to stress, whereas in the oldest group (21–30 months), this decrement was almost twice as great (76%, Fig. 1B).

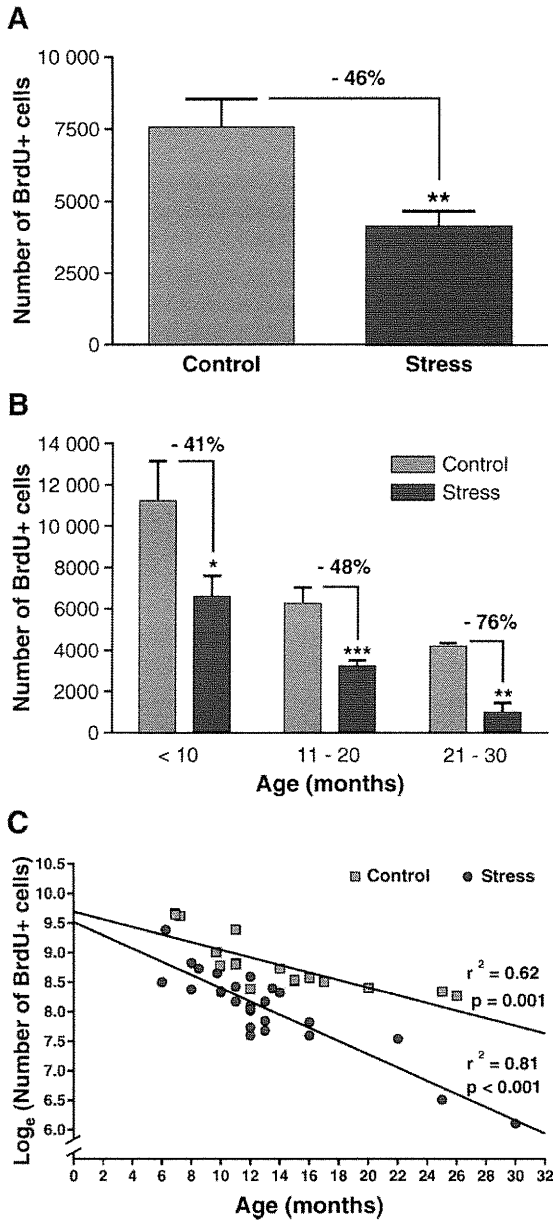

Fig. 1. (A) The number of BrdU-labeled cells was significantly reduced after 5 weeks of daily psychosocial stress ($n = 18$ control; $n = 27$ stressed animals). There was no statistical difference in the mean ages of the Control (13.5 months) and Stress (12.7 months) groups. (B) When animals were divided into subgroups according to their ages, an age-dependent vulnerability to stress appeared. Results are given as the mean ± SEM. Statistics: two-tailed unpaired Student's t test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (C) After logarithmic transformation of the cell proliferation data, linear regression was used to evaluate the relationship between the $\log_e$ (number of BrdU+ cells) and age (expressed in months). We found a significant linear fit in both groups. $b_{control} = -0.06 \pm 0.01$, $b_{stress} = -0.11 \pm 0.01$, and the two slopes are significantly different $F_{1,41} = 7.82$, $P < 0.01$.

We tried to find the best-fit mathematical model to describe the age-related declines in both groups in order to verify that stress has a significantly greater effect on the age-related decline of dentate cell proliferation. We then

compared the parameters of these models. The relationship between age and cell proliferation was examined using linear and nonlinear regression models (Prizm 4, GraphPad Software Inc.). Some simple nonlinear models can be converted into linear models by transforming one or both of the variables and the covariates. After plotting the BrdU+ cell number data against age and evaluating the scatterplot visually, we hypothesized that the exponential decay model may describe the relationship between age and cell proliferation. The function for the simple exponential decay model is

$$Y = ae^{-bx},$$

where Y is the number of the BrdU-positive cells, and x is the age (expressed in months). For $Y > 0$, this can be transformed into the linear model

$$\log_e(Y) = \log_e(a) - bx,$$

which we used to analyze the number of BrdU-positive cells in both groups. Because the variation in the $\log_e$-transformed data was more symmetric than for the raw data, we tried linear regression with $\log_e$-transformed BrdU data against age. This analysis provided better estimates for the parameters a and b than the simple exponential decay model.

We therefore evaluated linear, linear with $\log_e$-transformed number of BrdU-positive cells, and nonlinear (such as rational, polynomial, second order exponential decay) models. Regressions were performed separately for both groups. We selected the linear model with $\log_e$-transformed number of BrdU-positive cells, based on regression statistics and plots of residuals, as the best model for the regression analysis of both groups. We found a significant linear fit in both groups ($b_{control} = -0.06 \pm 0.01$, adjusted $r^2_{control} = 0.62$, $F_{1,17} = 25.96$, $P_{control} = 0.001$; $b_{stress} = -0.11 \pm 0.01$, adjusted $r^2_{stress} = 0.81$, $F_{1,26} = 100.1$, $P_{stress} < 0.001$). As is shown in Fig. 1C, the slopes (b values in the linear functions) of the two linear fits were remarkably different; the rate of the decrease in cell proliferation being more pronounced in the stressed animals. We used the global fitting method and an F test to compare the shared model with the separate models. We found that the slopes of the two linear fits were significantly different ($F_{1,41} = 7.82$, $P < 0.01$), indicating that chronic psychosocial stress resulted in a significantly steeper decline in cell proliferation with advancing age (Fig. 1C).

In addition, repeated territorial conflict had a substantial effect on the physiology of the animals as indicated by the significantly increased adrenal weights in the subordinate tree shrews (Fig. 2A). Analysis with two-way ANOVA (age × stress) of the three different age groups (Fig. 2B) revealed a highly significant main effect of stress [$F_{1,39} = 19.94$; $P < 0.001$] but no effect of age nor an interaction between the two factors.

We report that stress significantly suppressed dentate cell proliferation, in agreement with previous studies [1,3–5,11,12,15,17,18,20,22,23]. However, to our knowledge, this is the first study demonstrating that older animals are significantly more vulnerable to the adverse effect of chronic stress on dentate cytogenesis. This observation was somewhat surprising to us, especially since the animals used in this study were all relatively young (5–30 months), compared with the life span of 5–7 years for tree shrews living under laboratory conditions. One may assume that this difference may be even more pronounced in older animals. However, the predictive relationships found between age and other age-related variables can, with care, be extrapolated beyond the observed age period.

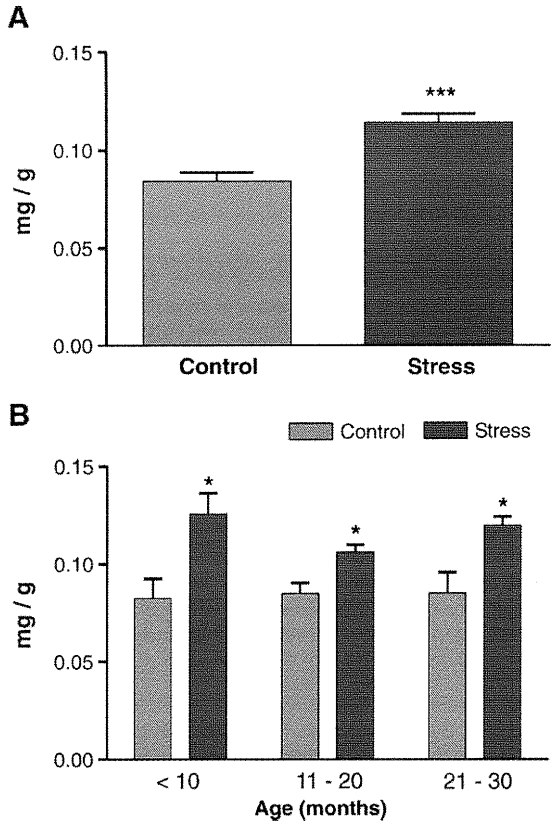

Fig. 2. (A) Chronic psychosocial stress resulted in significantly increased relative adrenal weights. (B) The different age subgroups were equally stressed, as confirmed by the fact that all animals showed a similar degree of relative adrenal weight increase. Results are given as adrenal weight in mg/g average body weight during the last experimental week (mean ± SEM). Statistics: two-tailed unpaired Student's t test. *$P < 0.05$, ***$P < 0.001$.

Looking at Fig. 1C, one may assume that the slope of regression in the stressed group is determined for a large part only by one 30-month-old animal, and thus the group differences on which we report here depend on this one animal solely. In fact, there is a statistically significant group difference between the control and stressed animals even if we analyze only those animals that were younger than 21 months ($P < 0.05$). This implies that an age-related vulnerability of adult dentate cell proliferation to chronic stress is already present at a relatively early age.

Our observation that all age groups showed a similar increase in adrenal weights (see Fig. 2B) suggests that they had experienced equal levels of stress. This excludes the possibility that animals at different ages were somehow differentially stressed.

Regulation of neurogenesis occurs on several levels such as cell proliferation, differentiation, migration, and survival. In an earlier study, we demonstrated that 3 weeks after BrdU injection, about 80% of BrdU-labeled cells expressed the neuronal marker neuron specific enolase and were incorporated into the granule cell layer [11]. Animals of the present study were sacrificed 24 h after the BrdU injection, thus histological analysis could evaluate only the rate of cell proliferation. Survival and future cellular phenotype of these newly generated cells was not investigated here. We can only speculate that the minority of these cells would later express glial phenotype, whereas the majority of them would have finally differentiated into neurons and by that have an impact on neuronal plasticity.

The present experiment was not designed to be an aging study. Initially, we wanted to examine the correlation between inter-individual differences in stress response and adult dentate cytogenesis. This also explains the major limitation of this report, namely that no aged animals are included. In previous experiments, we noticed individual differences in the total number of newly generated cells in response to chronic stressful situations [4,11]. Therefore, we decided to collect and analyze individual data from a relatively large group of animals. Our results reveal that age is indeed an important factor, and even relatively small age differences can significantly influence stress-reactivity on the level of dentate cell proliferation. In this context, it is of interest that a recent preliminary report demonstrated that the ability of the dentate gyrus to increase neurogenesis in response to hippocampal injury also declines with age [21]. Thus, it appears that the mechanisms regulating adult dentate cytogenesis are significantly influenced by age in both intact and injured hippocampi. The observation that the expressions of the glucocorticoid and mineralocorticoid receptors on neural precursor cell populations in the adult hippocampus increase with age [10] may explain the underlying mechanism for these results.

In summary, these observations provide new information on the age-related dynamics of adult dentate cell proliferation, showing that older animals are more vulnerable to the adverse effects of chronic stress, but such a difference is already present at early age.

Acknowledgments

The presented study has been partly supported by GSK, Verona, Italy. We are grateful to A. Bartolomucci, G. de Biurrun, M.G.C. van der Hart, and B. Schmelting for their help during the animal experiments. We thank S. Bauch, S. Donath, and A. Heutz for their excellent technical assistance. B. Czéh is supported by the DFG Research Center Molecular Physiology of the Brain (CMPB).

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CHAPTER 3

Chronic stress decreases the number of parvalbumin-immunoreactive interneurons in the hippocampus: Prevention by treatment with a substance P receptor (NK_1) antagonist

Neuropsychopharmacology (2005) 30, 67–79

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Chronic Stress Decreases the Number of Parvalbumin-Immunoreactive Interneurons in the Hippocampus: Prevention by Treatment with a Substance P Receptor (NK_1) Antagonist

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Previous studies have demonstrated that stress may affect the hippocampal GABAergic system. Here, we examined whether long-term psychosocial stress influenced the number of parvalbumin-containing GABAergic cells, known to provide the most powerful inhibitory input to the perisomatic region of principal cells. Adult male tree shrews were submitted to 5 weeks of stress, after which immunocytochemical and quantitative stereological techniques were used to estimate the total number of hippocampal parvalbumin-immunoreactive (PV-IR) neurons. Stress significantly decreased the number of PV-IR cells in the dentate gyrus (DG) (−33%), CA2 (−28%), and CA3 (−29%), whereas the CA1 was not affected. Additionally, we examined whether antidepressant treatment offered protection from this stress-induced effect. We administered fluoxetine (15 mg/kg per day) and SLV-323 (20 mg/kg per day), a novel neurokinin 1 receptor (NK_1R) antagonist, because the NK_1R has been proposed as a possible target for novel antidepressant therapies. Animals were subjected to a 7-day period of psychosocial stress before the onset of daily oral administration of the drugs, with stress continued throughout the 28-day treatment period. NK_1R antagonist administration completely prevented the stress-induced reduction of the number of PV-IR interneurons, whereas fluoxetine attenuated this decrement in the DG, without affecting the CA2 and CA3. The effect of stress on interneuron numbers may reflect real cell loss; alternatively, parvalbumin concentration is diminished in the neurons, which might indicate a compensatory attempt. In either case, antidepressant treatment offered protection from the effect of stress and appears to modulate the hippocampal GABAergic system. Furthermore, the NK_1R antagonist SLV-323 showed neurobiological efficacy similar to that of fluoxetine.

Neuropsychopharmacology (2005) **30,** 67–79, advance online publication, 6 October 2004; doi:10.1038/sj.npp.1300581

Keywords: neuroplasticity; antidepressant; interneuron; cell loss; stereology; fluoxetine

INTRODUCTION

Depressive disorders are common and life-threatening illnesses, but little is known about the underlying fundamental biology (Wong and Licinio, 2001; Nestler *et al*, 2002). Focus on the monoaminergic system considerably deepened our understanding of the pathophysiology of mood disorders, but examination of the contribution of other neurotransmitter systems to the neurobiology and treatment of depression is required. Several lines of evidence originating from both animal and human studies suggest the involvement of the GABAergic system in the pathophysiology of depressive disorders (Sanacora *et al*, 1999; Krystal *et al*, 2002; Brambilla *et al*, 2003).

One brain structure that has been extensively studied with regard to the actions of stress, depression, and antidepressant treatment is the hippocampal formation (McEwen, 1999). In humans, numerous imaging studies revealed that the hippocampus undergoes selective volume reduction in several stress-related neuropsychiatric illnesses (Shenton *et al*, 2001; Bremner, 2002; MacQueen *et al*, 2003; Sheline, 2003), whereas exposing experimental animals to stress results in structural alterations such as remodeling of the apical dendrites of CA3 pyramidal cells (Magarinos *et al*, 1996; Kole *et al*, 2004), marked ultrastructural alterations at the synaptic terminals of the mossy fiber bundle (Magarinos

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Received 20 January 2004; revised 13 July 2004; accepted 19 August 2004
Online publication: 1 September 2004 at http://www.acnp.org/citations/NPP09010404025/default.pdf

et al, 1997), suppression of adult neurogenesis in the dentate gyrus (DG) (Gould et al, 1997), and reduced hippocampal volume (van der Hart et al, 2002). Furthermore, it has been argued that prolonged stress may induce loss of CA3 pyramidal cells, which could contribute to hippocampal atrophy (Sapolsky et al, 1985; Uno et al, 1989), although recent stereological studies questioned the evidence of principal cell loss (Vollmann-Honsdorf et al, 1997; Sousa et al, 1998). Although many studies have investigated the effects of chronic stress on the morphology and number of principal neurons of the hippocampus, few data about possible changes in interneuron numbers are available.

Parvalbumin-containing cells represent a subpopulation of GABAergic interneurons, most prominently chandelier (or axo-axonic) cells and a subset of basket-type interneurons. They selectively innervate the axon initial segments and the somata of cells, and consequently regulate the discharge activity of large populations of principal cells (Kosaka et al, 1987; Ribak et al, 1990; Freund and Buzsaki, 1996). A further important characteristic of these interneurons is that they are fast spiking neurons creating dual networks. Besides, being connected by mutual synaptic contacts, they form a syncytium throughout the hippocampus by dendro-dendritic gap junctions, which is implicated in mediating synchronization of oscillatory activities (Fukuda and Kosaka, 2000). These parvalbumin-containing cells in the hippocampal DG and CA3 region receive most of their excitatory input from granule cells (Seress et al, 2001). Therefore, similarly to CA3 pyramidal cells, parvalbumin-positive interneurons are subjected to the deleterious effect of excessive excitatory amino acid release from the mossy fiber terminals during stress exposure (Magarinos et al, 1997).

In the present study, we investigated whether long-term psychosocial stress could affect the number of parvalbumin-immunoreactive (PV-IR) interneurons in the hippocampal formation. In the first experiment, we examined this in chronically stressed tree shrews, an animal model with high validity for research on the pathophysiology of depression (Fuchs and Flugge, 2002; van Kampen et al, 2002). Additionally, as we did find that stress can affect the number of PV-IR cells, we conducted a second experiment to examine whether treatment with antidepressant drugs offered protection from stress-induced morphological alterations. This experiment 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 (Duman et al, 1999; Manji et al, 2000). Indeed, several studies demonstrated that treatment with various classes of antidepressants could reverse both the functional impairments and the structural alterations of the hippocampal formation induced by stress (Watanabe et al, 1992; Czeh et al, 2001; van der Hart et al, 2002; Herman et al, 2003; Malberg and Duman, 2003; Vermetten et al, 2003; Lucassen et al, 2004). To mimic a realistic situation of antidepressant intervention, we administered the drugs for the clinically relevant period of 4 weeks. We treated animals with fluoxetine, a well-known serotonin selective reuptake inhibitor. Furthermore, we tested a novel neurokinin 1 receptor (NK_1R) antagonist, SLV-323 (Czeh et al, 2003; Hesselink et al, 2003), because the inhibition of the neurokinin substance P (SP) and its preferred NK_1R pathway is a promising novel approach to antidepressant treatment (Kramer et al, 1998; Rupniak and Kramer, 1999; Stout et al, 2001). Finally, we preformed an experiment to evaluate whether chronic fluoxetine or SLV-323 treatment of unchallenged animals could affect the number of hippocampal parvalbumin-IR neurons.

MATERIALS AND METHODS

Animals and Antidepressant Treatment

For the experiments we used tree shrews (*Tupaia belangeri*), which are considered to be phylogenetically closely related to primates (Martin, 1990). Experimentally naive adult male tree shrews (mean age 9 ± 3 months; $n = 46$) were obtained from the breeding colony at the German Primate Center (Göttingen, Germany). Animals were housed individually with a 12 h light/12 h dark cycle and had *ad libitum* access to food and water (for details see Fuchs, 1999). All animal experiments were in accordance with the European Communities Council Directive of November 24, 1986, (86/EEC) and with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and were approved by the Government of Lower Saxony, Germany.

Animals received fluoxetine (Ratiopharm, Ulm, Germany) and the highly brain-penetrant NK_1 receptor antagonist SLV-323 (Solvay Pharmaceuticals, Weesp, The Netherlands). Drugs were administered orally because this is the most common route of antidepressant administration in psychiatric patients. Moreover, we aimed to minimize uncontrollable stress effects caused by daily injections. We conducted a pilot study to establish the dose of SLV-323 that blocks NK_1 receptors in the tree shrew brain. The methodology used is based on the ability of NK_1 receptor antagonists to block nicotine-induced vomiting in musk shrews (Tattersall et al, 1995). Adult male tree shrews ($n = 4$) received either vehicle or SLV-323 in different dosage orally followed 30 min later by subcutaneous administration of (−) nicotine (4 mg/kg; Sigma-Aldrich), and the number of emetic episodes occurring during the following 30 min was recorded. Each animal received each treatment in a crossover design, with 10 days washout period between studies. Emetic episodes were abolished by treatment with 20 mg/kg per day SLV-323, whereas lower doses, that is, 5 or 10 mg/kg per day and vehicle were ineffective. These results showed that treatment with 20 mg/kg per day SLV-323 could effectively block central NK_1 receptors. In another pilot study, the necessary dosage of fluoxetine was determined. Accordingly, we treated the animals with 15 mg/kg per day fluoxetine, which resulted in a 81–634 ng/ml plasma concentration of norfluoxetine 24 h after the last dose in the 4-week treatment period; a similar range is reported for patients under fluoxetine treatment (Laboratory Corporation of America database).

The experimental designs are shown in Figure 1. In the first experiment, we examined whether chronic stress affected the number of PV-IR neurons in the hippocampal formation. A *Control* ($n = 6$) and a *Stress* ($n = 6$) group were compared. Animals of the *Control* group remained singly housed in their cages, while animals of the *Stress* group were

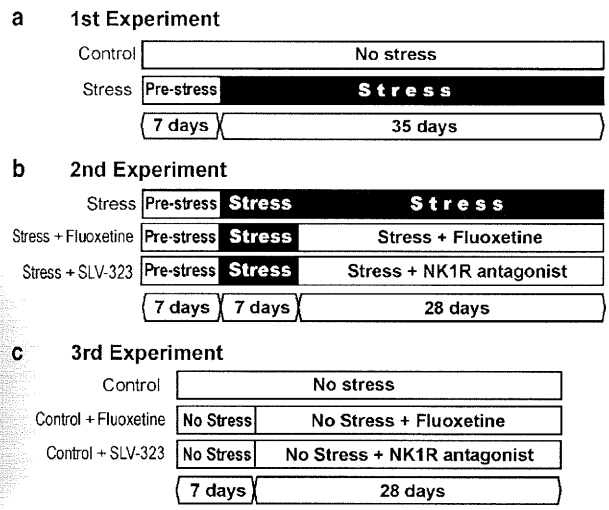

Figure 1 Experimental design. (a) In the first experiment a *Control* ($n=6$) and a *Stress* ($n=6$) group were compared, where animals of the *Stress* group were submitted to daily psychosocial conflict for 5 weeks and the *Control* group remained undisturbed. (b) In the second experiment a *Stress* group ($n=5$), a *Stress + Fluoxetine* ($n=5$) and a *Stress + SLV-323* (or *Stress + NK_1R antagonist*) ($n=6$) group were used. The first experimental phase consisted of a 7-day Pre-Stress period. During the second phase, which also lasted 7 days, the animals of the three stress groups (*Stress*, *Stress + Fluoxetine*, *Stress-SLV-323*) were submitted to daily psychosocial conflict, whereas animals of the control group (*Control*) remained undisturbed. The third experimental phase lasted 28 days. Stressed animals remained in the psychosocial conflict situation and received the drugs (*Stress + Fluoxetine*, 15 mg/kg per day; *Stress + SLV-323*, 20 mg/kg per day) or vehicle (*Stress*) orally. In total, the psychosocial stress exposure lasted 35 days. (c) Finally, in the third experiment we treated control unchallenged animals either with fluoxetine (*Control + Fluoxetine*, 15 mg/kg per day; $n=6$ animals) or with SLV-323 (*Control + SLV-323*, 20 mg/kg per day; $n=6$ animals) or with vehicle (*Control*; $n=6$ animals) orally for 28 days.

submitted to daily psychosocial conflict for 5 weeks (Figure 1a). Urine samples were collected on a daily basis throughout the whole experiment to monitor the neurosympathetic tone by measuring free norepinephrine in the morning urine.

The induction of psychosocial conflict was carried out according to standard procedures (Fuchs and Flugge, 2002). Briefly, one naive male was introduced into the cage of a socially experienced male. This resulted in active competition for control over the territory, and when a clear dominant–subordinate relationship had been established, the two animals were separated by a wire mesh barrier. The barrier was removed every day for about 1 h, thereby allowing physical contact between the two males during this time only. Using this procedure, the subordinate animal was protected from repeated attacks, but it was constantly exposed to olfactory, visual, and acoustic cues from the dominant animal. Under these conditions, subordinate animals displayed characteristic subordination behavior.

In the second experiment, we examined whether concomitant antidepressant treatment counteracted the chronic stress-induced changes. For this purpose, three experimental groups were used: a second *Stress* group ($n=5$), a *Stress + Fluoxetine* group ($n=5$) and a *Stress + NK_1R antagonist* ($n=6$) group (Figure 1b). The first phase of this experiment (Pre-Stress) lasted for 7 days, during which all animals remained undisturbed (Figure 1b). The second phase was a 7-day period (Stress) during which the animals were submitted to daily psychosocial conflict. The third experimental phase consisted of the antidepressant treatments, which lasted for a clinically relevant period of 4 weeks. During this time, the animals remained in the psychosocial conflict situation and each morning received oral administration of the compounds between 0800 and 0815. Animals of the *Stress* group were submitted to daily psychosocial conflict for 5 weeks. Urine samples were collected from all animals daily throughout the whole experiment.

Finally, we performed an experiment, to investigate whether treatment of unchallenged animals with either fluoxetine or SLV-323 influences the number of PV-IR cells. This third experiment consisted of a *Control* ($n=6$), a *Control + Fluoxetine* ($n=6$) group, and a *Control + SLV-323* ($n=6$) group (Figure 1c). The drug-exposed animals underwent the same treatment protocol as the animals in experiment 2, that is, they received 15 mg/kg fluoxetine per day, or 20 mg/kg SLV-323 per day, orally for 28 days.

Perfusion and Brain Tissue Preparation

Animals were anesthetized with an overdose of xylazin/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 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 dorso-ventral 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. Every tenth section was selected and processed for parvalbumin immunostaining. Samples from each treatment group were always processed in parallel to avoid any unspecific 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 in a mouse monoclonal antibody against parvalbumin (1 : 3000; 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 2 h, rinsed, incubated in avidin–biotin–horseradish peroxidase (1 : 200; Vectastaine *Elite* ABC Kit, Vector) for 2 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 alcohols, cleared in xylene for 30 min, and finally coverslipped under Eukitt.

We also evaluated whether using different titers of the primary antibody might affect the number of parvalbumin-positive cells. For this purpose, we used four animals of the stress group and processed three series of serial sections for immunohistochemistry with different titers of the primary antibody (ie 1 : 1000, 1 : 2000, 1 : 3000). Using this approach, higher concentrations of the antibody should mark cells that express lower levels of PV and result in a higher total number of cells.

Quantification of Parvalbumin-Immunoreactive Cells

Neuron numbers were estimated with the modified optical fractionator technique (West *et al*, 1991; West, 1999). The optical fractionator is an unbiased counting method, which is independent of the size, shape, and orientation of the cells to be counted, and combines the optical disector (Sterio, 1984) with the fractionator-sampling scheme (Gundersen *et al*, 1988). The parameters of the fractionator-sampling scheme were established in a pilot experiment, and were uniformly applied to all animals. For examination, every tenth section (an average of 14 sections per animal) was systematically sampled ($f_1 = 10$) through the dorso-ventral extent of the left hippocampus. Before quantitative analysis, slides were coded, and the code was not broken until the analysis was completed. Cell counting was conducted using a Zeiss III RS microscope with the aid of the Stereo-investigator 3.16 software (Microbrightfield, Colchester, VT, USA; for details of the setup see Keuker *et al*, 2001). The border of the region was outlined using a $\times 6.3$ objective (NA 0.16), and for counting cells a $\times 16$ (NA 0.16) objective was used. The optical disector frame area, a(frame), and the sampling area, $A(x,y$ step), were selected such that 1–3 PV-IR neurons per optical disector were counted on average. The size of the disector frame area, a(frame) was 200 μm × 300 μm, and the sampling area, $A(x,y$ step) was 300 μm × 300 μm, yielding: $f_2 = a(\text{frame}) \times 1/A(x,y \text{ step}) = 1.5$. We used the same a(frame) and $A(x,y$ step) for analysis of the DG, CA3 and CA1 regions. Proper optical disector rules require guard zones both at the upper and lower surfaces of the section, but here we applied the modified optical disector method, which means that we did not use guard zones. Thus, the height of the optical disector, h, equals the actual section thickness, t; hence, the thickness sampling fraction equals 1 ($f_3 = 1$). After having counted all cells ($\sum Q^-$) fulfilling the criteria of sampling, the total number of cells was estimated: $N_{\text{total}} = \sum Q^- \times f_1 \times f_2 \times f_3$.

To verify that the PV antibody penetrated the full thickness of the section, we analyzed several sections from each experimental animal, focusing with a $\times 100$ lens through the entire section thickness. We found that the penetration of the antibody was complete, because labeled cells were detectable in all deeper layers within a given section.

The distinction of subfields and laminae of the hippocampal formation after PV staining is relatively simple (Figure 2) and well described by Keuker *et al* (2003). Quantification was carried out in the DG and in the three subfields of Ammon's horn (CA1-3). To be able to fulfill the requirements of the stereological approach, namely that there should be 1–2 cells counted on average in each sampling area, we decided to exclude certain layers from the

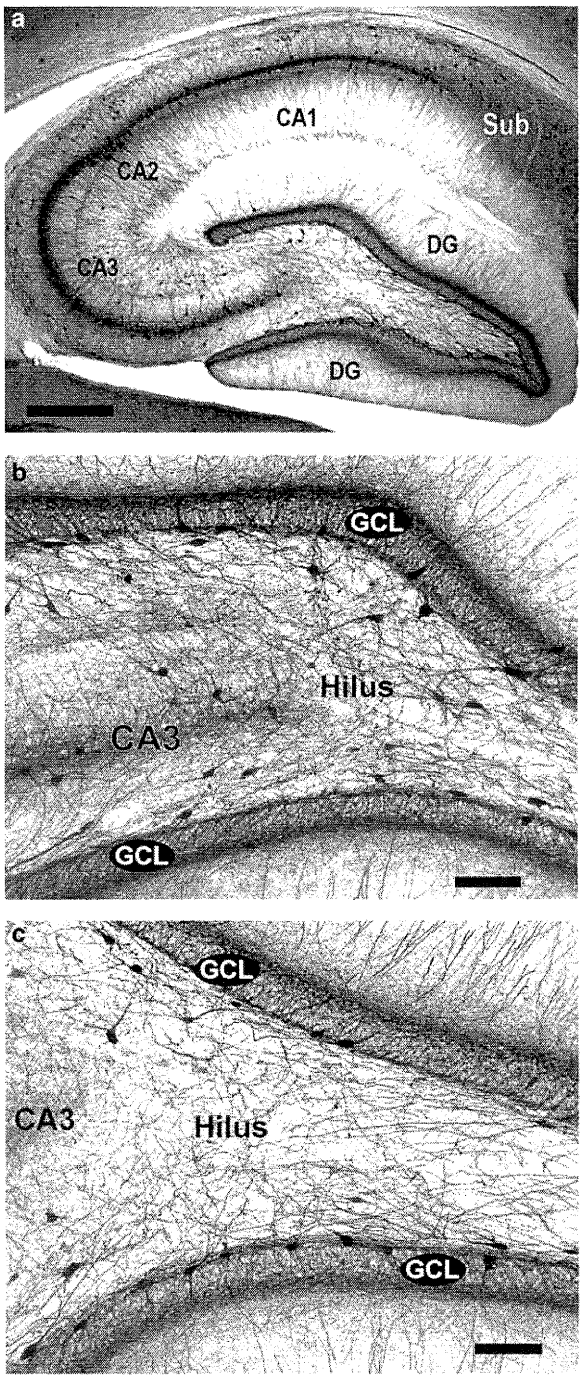

Figure 2 Representative example of a parvalbumin stained horizontal section of the tree shrew hippocampal formation (a). Parvalbumin-immunoreactive neurons were present exclusively as nonprincipal cells. Note that all subregions and layers are clearly distinguishable. Detailed images of the dentate gyrus from a representative control (b) and chronically stressed animal (c). In the dentate gyrus, most of the PV-positive interneurons were aligned at the border of the granule cell layer and the hilus. Furthermore, numerous PV-IR cells were distributed throughout the hilus, and located within the granule cell layer, but such cells in the stratum moleculare were extremely rare. Note the smaller density of PV-positive cells after the long-term stress (c). DG, dentate gyrus; GCL, granule cell layer; SUB, subiculum. Scale bars: 500 μm (a); 100 μm (b, c).

analysis, namely those where PV-containing cells are extremely rare. Within the DG, most of the PV-IR interneurons are located either in the hilus or in the granule cell layer, and therefore only these two laminae were investigated, excluding the molecular layer from the analysis. In the case of the CA3 area, PV-IR cells were quantified in all strata except the stratum lacunosum moleculare. Within the CA1, PV-positive interneurons are mostly located just above and below the pyramidal cell layer, as well as in the stratum oriens; thus, only these two laminae were analyzed during the quantification and we excluded the stratum radiatum and stratum lacunosum moleculare. As the CA2 region is a very small area, it was presently impossible to apply the essential fractionator-sampling rules. Instead, all PV-IR cells were counted and finally their total number was estimated by multiplying the number of cells counted in every tenth section by 10.

Analysis of Urine Samples and Testis Weight Measurement

Analysis of urine levels of norepinephrine and creatinine was performed at KCL Bioanalysis b.v., Leeuwarden, The Netherlands. In brief, urinary norepinephrine was quantified by LC-MS (analytical column; Alure Basics, Restek, 50 mm, 2 mm ID) with electrospray ionization (5500 V, 200°C) after liquid–liquid extraction. To correct for physiological alteration in urine dilutions, the resulting concentrations were related to creatinine concentrations, which were determined with a Roche Modular P800 clinical chemistry analyzer with creatinine reagents (Jaffe method).

As decreased adrenal and testis weights are indicators of sustained stress exposure, these organs were removed from the animals immediately after perfusion and weighed. Data are expressed as milligrams organ weight per gram average body weight of the preceding week.

Statistical Analysis

Results are presented as the mean ± SEM. Treatment effects were assessed with two-tailed unpaired Student's *t*-test or one-way ANOVA, followed by either Student–Newman–Keuls or Tukey's *post hoc* analysis for further examination of group differences. Importantly, because results of the two *Stress* groups did not differ significantly in any parameter between the first and second experiments, their values were pooled and presented throughout the article as a single *Stress* group.

RESULTS

Activation of the sympatho-adrenomedullary system is an important and reliable indicator by which tree shrews can be classified as subordinates (Fuchs and Flugge, 2002). Animals from the *Stress*, *Stress + Fluoxetine* and *Stress + SLV-323* groups all displayed significantly elevated urinary norepinephrine levels after 5 weeks of social encounters (Figure 3a). Moreover, we measured testis weights because gonadal hypotrophy is an indicator of sustained stress exposure (Fischer *et al*, 1985). Chronic confrontations significantly reduced testis weights in the animals of the *Stress* and *Stress + Fluoxetine* groups, whereas SLV-323 treatment resulted in a highly significant increase in testis weights both in the control and stressed animals (Figure 3b).

Parvalbumin-immunoreactive neurons were present exclusively as nongranule cells of the DG and nonpyramidal cells of the CA1-3 of the Cornu Ammonis (Figure 2). The morphology and distribution of these PV-positive interneurons was similar to what has been described in the tree shrew (Keuker *et al*, 2003) and rat hippocampal formation (Kosaka *et al*, 1987).

The first experiment was designed to evaluate whether long-term stress affected the number of PV-IR interneurons in the hippocampal formation (Figure 4). Exposure to chronic psychosocial stress resulted in a significant decrease in the total number of PV-IR interneurons in the DG (−33%, $t_{17} = 3.88$, $p = 0.001$; Table 1), and in the CA3

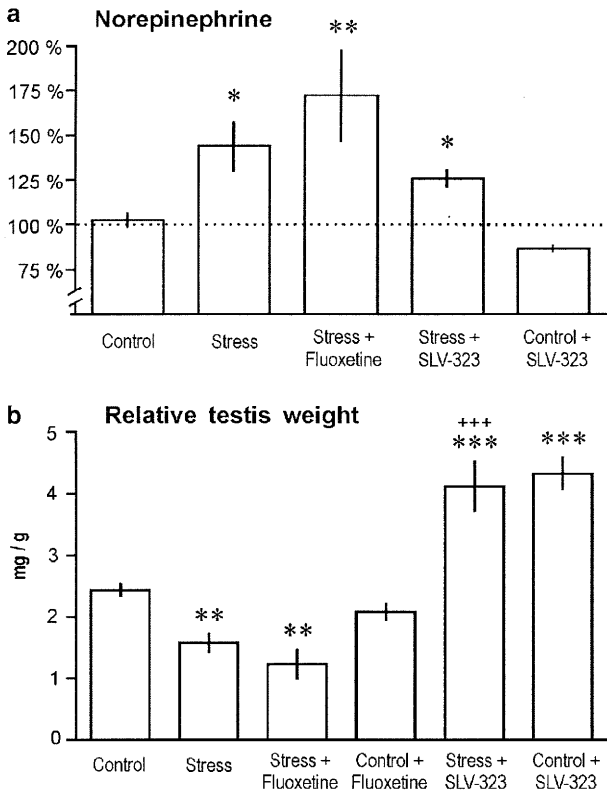

Figure 3 (a) Concentration of urinary norepinephrine. Long-term stress resulted in the sustained activation of the sympatho-adrenomedullary system, as indicated by the significant elevation of urinary norepinephrine levels in all stressed groups after the 5 weeks of social encounters. Horizontal dashed line indicates the mean baseline urinary norepinephrine excretion according to the measurement during the Pre-Stress week. Data are expressed as percent of the Pre-Stress week. (b) Effects of chronic psychosocial stress and concomitant drug treatment on testis weight. Long-term stress resulted in a significantly reduced relative testis weight in the animals from the *Stress* and *Stress + Fluoxetine* groups, while treatment with the NK_1R antagonist SLV-323 significantly increased the weight of the organ. Note that SLV-323 treatment resulted in significantly increased testis weight both in control and stressed animals. Data are expressed as mg testis weight per g average body weight of the last week (mean ± SEM). Statistics: One-way ANOVA followed by Student–Newman–Keuls *post hoc* analysis. * < 0.05, ** < 0.01, *** < 0.001 *vs* Control; +++ < 0.001 *vs* Stress.

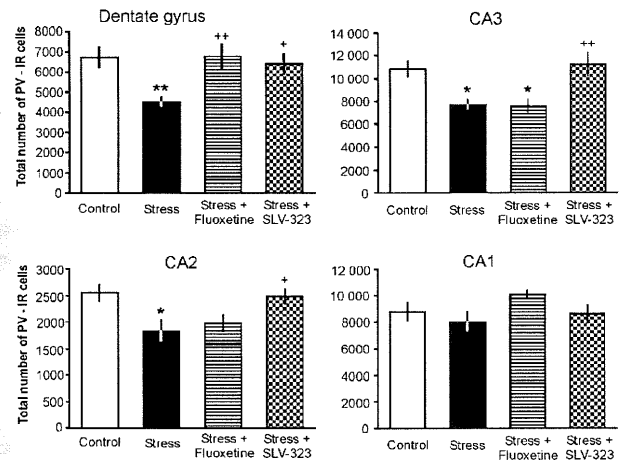

Figure 4 Effects of chronic psychosocial stress and concomitant antidepressant drug treatment on the total number of parvalbumin-immunoreactive (PV-IR) cells in the different hippocampal subregions. Stress significantly decreased the number of PV-IR cells in the dentate gyrus, CA2, and CA3, whereas the CA1 was not affected. The NK_1R antagonist SLV-323 prevented the stress-induced effect in all hippocampal subregions, whereas fluoxetine had a beneficial effect in the dentate gyrus, but not in the CA2 and CA3 subfields. Results are shown as mean ± SEM. Importantly, because comparison of the results of the two *Stress* groups from the first and second experiment (Figure 1) did not reveal a statistically significant difference, their values were combined and presented here as one group. *Statistics*: one-way ANOVA, followed by Tukey's *post hoc* analysis. * <0.05, ** <0.01 *vs* Control; + <0.05, ++ <0.01 *vs* Stress.

(−29%, $t_{17} = 3.43$, $p < 0.01$; Table 1). A minor, nonsignificant difference (−9%) was observed in the total number of PV-positive cells in the CA1 area (Table 1). In the CA2 area, total PV-IR interneuron numbers were also significantly decreased (−28%, $t_{17} = 2.85$, $p < 0.05$; *Stress*: 1833 ± 218 PV-IR cells) compared with the unstressed controls (*Control*: 2547 ± 162 PV-IR cells) (Figure 4). Importantly, because there was no statistical difference between the two *Stress* groups from the first and second experiment, their values were pooled, and presented here as one group.

The second experiment was designed to examine whether the chronic stress-induced decrease in the number of PV-IR interneurons was blocked by concomitant application of antidepressant drugs (Figure 4). Cell quantification revealed that treatment with both fluoxetine and the NK_1R antagonist prevented the effect of stress in the DG (Table 1). One-way ANOVA revealed a significant difference between the groups ($F_{(3,24)} = 6.84$, $p < 0.01$) and Tukey's *post hoc* comparisons showed a significant difference between the *Control* and *Stress* groups ($q = 5.05$, $p < 0.01$). Treatment of stressed animals with the NK_1R antagonist SLV-323 offered protection from the effect of stress, resulting in the preservation of PV-IR interneurons and a statistically significant difference compared with the *Stress* group ($q = 4.36$, $p < 0.05$). A similar effect was observed in animals from the *Stress + Fluoxetine* group ($q = 4.89$, $p = 0.01$ *vs Stress*), whereas values of the two drug-treated groups were similar to those for the *Controls*.

Analysis of the CA3 area revealed that administration of the NK_1R antagonist prevented the stress-induced changes, whereas fluoxetine treatment presently had no beneficial effect (Table 1). One-way ANOVA revealed a significant difference between the groups ($F_{(3,24)} = 6.93$, $p = 0.01$) and *post hoc* comparisons showed significant difference between the *Control* and *Stress* groups ($q = 4.50$, $p < 0.05$). Treatment of stressed animals with the NK_1R antagonist SLV-323 resulted in normalization of the total number of PV-IR interneurons, yielding a significant difference compared with the *Stress* group ($q = 5.07$, $p = 0.01$). In contrast, results of the *Stress + Fluoxetine* group did not differ from the *Stress* group; however, the total number of PV-IR interneurons was significantly lower than in the *Control* group ($q = 3.91$, $p < 0.05$).

In the CA2 subregion, chronic treatment with the NK_1R antagonist resulted in a normalized total number of PV-IR cells (*Stress + NK_1R antagonist*: 2480 ± 140 PV-IR cells), indicating that SLV-323 counteracted the effect of stress ($q = 4.01$, $p < 0.05$ *vs Stress*). In contrast, results of the *Stress + Fluoxetine* (1974 ± 175 PV-IR neurons) were in the same range as those from the *Stress* group; statistically, however, they were not different from either the *Control* or the *Stress* group (Figure 4).

In the CA1 subregion of the Ammon's horn, neither stress nor antidepressant treatment had a significant effect on the number of PV-labeled neurons (ANOVA ($F_{(3,24)} = 1.63$, $p = 0.21$), Table 1).

Using the experimental parameters for the optical fractionator that were established during a pilot experiment, the average number of counted neurons varied between 197 and 582 in the DG, between 334 and 965 in the CA3, and between 389 and 860 in the CA1, which is well beyond the recommendation of Gundersen and Jensen (1987). Furthermore, according to the rules of the optical fractionator technique, to make justified group comparisons, the biological variance (BCV^2) should contribute more than 50% to the total observed variance (CV^2), where $CV^2 = CE^2 + BCV^2$ (Gundersen, 1986). As shown in Table 1, our stereological sampling fulfilled this criterion in all cases, except in the CA1 region of the *Stress + Fluoxetine* group, where the biological variance was unusually low.

The third experiment was designed to test whether treatment of unchallenged animals with the two compounds might affect the number of parvalbumin-IR cells. As shown in Figure 5, neither fluoxetine nor SLV-323 influenced the number of hippocampal PV-IR interneurons.

Finally, we evaluated whether using higher titers of the primary antibody might pick up cells that express lower levels of parvalbumin. For that, we selected four animals of the *Stress* group, which had the lowest number of PV-IR cells, and processed three series of every tenth serial sections for immunohistochemistry with different titers of the primary antibody (ie 1 : 1000, 1 : 2000, 1 : 3000). Quantification of the parvalbumin-positive cells revealed that different titers of the primary antibody had no effect on the incidence of labeled cells (data not shown).

DISCUSSION

The present study is the first to quantify the absolute number parvalbumin-immunoreactive interneurons in the hippocampal formation; furthermore, this is the first observation showing that long-term psychosocial stress

Table 1 Stereological Results: Mean Estimated Total PV-IR Interneuron Numbers in the Three Major Hippocampal Subregions after Chronic Psychosocial Stress and Concomitant Antidepressant Treatment with Fluoxetine or SLV-323, a Novel NK_1 Receptor Antagonist

	Control	Stress[a]	Stress+Fluoxetine	Stress+SLV-323
Dentate Gyrus				
Mean *N*	6700	4475**	6762##	6393#
Mean CE	0.082	0.137	0.089	0.059
SD	1298	1033	1436	1323
CV = SD/mean	0.1937	0.2307	0.2123	0.2069
BCV^2 (in % of CV^2)	82%	65%	82%	92%
Hippocampal CA3				
Mean *N*	10848	7696*	7575*	11248##
Mean CE	0.057	0.080	0.079	0.059
SD	1822	1802	1388	2634
CV = SD/mean	0.1680	0.2341	0.1833	0.2342
BCV^2 (in % of CV^2)	88%	88%	81%	94%
Hippocampal CA1				
Mean *N*	8778	8004	10 104	8615
Mean CE	0.069	0.076	0.058	0.069
SD	1886	2003	757	1697
CV = SD/mean	0.2148	0.2503	0.0749	0.1970
BCV^2 (in % of CV^2)	90%	91%	40%	79%

[a]Results of the two *Stress* groups were pooled and presented here as one group.
CE, coefficient of error, mean CE was calculated $\sqrt{\text{mean}(CE)^2}$; SD, standard deviation; BCV, biological variance, where $CV^2 = CE^2 + BCV^2$.
Statistics: one-way ANOVA, followed by Tukey's *post hoc* analysis.
*<0.05, **<0.01 *vs* Control; #<0.05, ##<0.01 *vs* Stress.

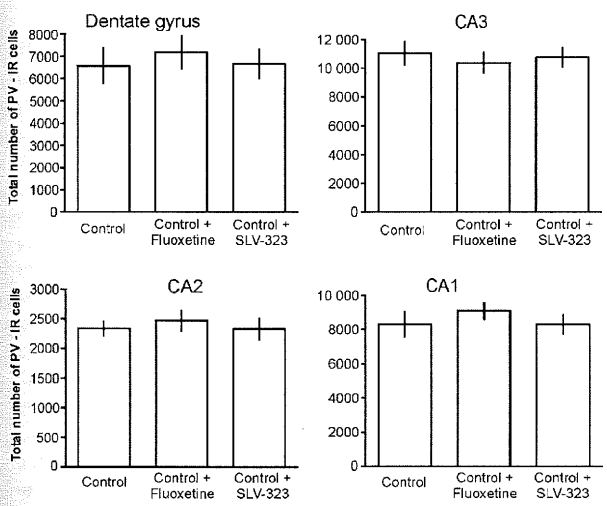

Figure 5 Chronic treatment with fluoxetine or the NK_1R antagonist SLV-323 did not affect the total number of parvalbumin-IR neurons in the hippocampi of control animals. Results are presented as mean ± SEM.

may affect the number of nonprincipal cells in the hippocampus. We demonstrated that chronic psychosocial stress resulted in a significantly fewer PV-IR cells in the DG and in the CA2 and CA3 regions of the Ammon's horn, whereas the CA1 subfield was not affected. Additionally, we found that this effect of long-term stress was prevented by concomitant treatment with the NK_1 receptor antagonist SLV-323. Treatment with fluoxetine partially counteracted the stress-induced changes: it normalized the number of PV-IR interneurons in the DG, but had no effect in the CA2 and CA3 areas. This suggests that antidepressant drugs directly or indirectly modulate the hippocampal GABAergic network. Furthermore, we demonstrated that, in this context, blockade of NK_1 receptors with the selective antagonist SLV-323 had a similar, or even a better, protective profile than the established antidepressant fluoxetine. SLV-323 treatment had a further interesting effect on the periphery; namely, it blocked the stress-induced decrease of testis weight. In this respect, SLV-323 may devoid the common and unwanted side effect of many SSRIs, namely sexual dysfunction.

Effect of Stress on the Hippocampal GABAergic System

To our knowledge, this is the first report demonstrating that the number of hippocampal parvalbumin-containing GABAergic interneurons is modulated by long-term stress and antidepressant treatment, and our data thereby provide further support for theories that link stress or depression with epilepsy (Magarinos *et al*, 1997; Kanner and Balaba-

nov, 2002). Disturbances in the anatomy and function of the GABAergic system have been implicated in connection with various stress-related psychiatric disorders (Sanacora *et al*, 1999; Benes and Berretta, 2001; Krystal *et al*, 2002; Brambilla *et al*, 2003). Changes in the number of local inhibitory neurons have been reported, especially in the anterior cingulate and prefrontal cortices, and in the hippocampi of schizophrenic and bipolar patients (Benes, 1999; Benes and Berretta, 2001; Reynolds *et al*, 2001; Beasley *et al*, 2002; Cotter *et al*, 2002). These observations are often interpreted as a consequence of altered neurodevelopment, but the contribution of stressful experiences, especially in the perinatal period, should be also taken into account (Vaid *et al*, 1997; Benes *et al*, 1998). A recent post-mortem study comparing densities of different subpopulations of GABAergic interneurons revealed a significant and profound deficit in the relative density of PV-immunoreactive neurons in all hippocampal subfields of schizophrenic patients, but they did not reveal any changes in patients with a depressive disorder (Zhang and Reynolds, 2002). Another post-mortem study evaluating the density of hippocampal nonprincipal neurons in Nissl stained sections found significantly decreased density of interneurons in the CA2 subregion of both schizophrenic and bipolar subjects (Benes *et al*, 1998). Furthermore, in both disorders, the number of nonprincipal cells was substantially decreased in the CA3 area, approaching the level of significance, whereas the CA1 was not affected (the DG was not analyzed) (Benes *et al*, 1998). As the authors pointed out, this histopathological change seems to be related more to an environmental factor such as stress, which occurs to an equivalent extent in both disorders, as this decrease of interneuron number in the hippocampus and elsewhere in the cortex appears to be a feature of both schizophrenia and bipolar disorder (Benes *et al*, 1998).

There are many reports of chronic stress-induced structural alterations within the hippocampal formation, including remodeling of the apical dendrites of CA3 pyramidal cells (Magarinos *et al*, 1996; Kole *et al*, 2004), suppression of adult neurogenesis in the DG (Czéh *et al*, 2002; Pham *et al*, 2003) and reduced hippocampal volume (van der Hart *et al*, 2002). The CA3 pyramidal cells are considered especially vulnerable to excitotoxic injury (McEwen, 1999) because they are subjected to the elevated excitatory amino-acid release from the mossy fiber terminals (Magarinos *et al*, 1997). As PV-containing interneurons receive similarly robust, or probably an even stronger, excitatory input from the mossy fibers (Acsady *et al*, 1998; Seress *et al*, 2001), one may assume that they might eventually die from excitotoxic injury, similar to that following epileptic seizures (Buckmaster and Dudek, 1997). Our group examined the possibility of cell loss within the same chronic psychosocial paradigm, but stereological cell counting of pyramidal and granule cells of the hippocampal formation failed to reveal any loss of cells (Vollmann-Honsdorf *et al*, 1997; Sousa *et al*, 1998; Keuker *et al*, 2001). It should be noted though that possible changes in the number of interneurons or hilar cells were not specifically addressed in these studies. Recently, we also quantified the incidence of apoptosis in this chronic stress paradigm, and we found a significant increase in the number of apoptotic cells in the hilus and a nonsignificant increase in the granule cell layer, whereas in the CA3 the occurrence of apoptosis was decreased (Lucassen *et al*, 2001). Considering the results of apoptotic cell number, excitotoxic cell death may explain the decreased number of PV-containing interneurons in the dentate hilus, but it conflicts with the present data observed in the CA3 area. Altogether, we cannot rule out the possibility that long-term stress may induce excitotoxic interneuron loss.

More importantly, our data indicate that this effect of stress can be prevented by antidepressant treatment. The fact that the number of parvalbumin-containing hippocampal interneurons were not increased by treatment with either of fluoxetine or the SLV-323 in the control animals suggest that the effect of stress were blocked by the drug treatments and not *vice versa*. Currently, antidepressants are believed to exert their primary biochemical effects by readjusting aberrant intrasynaptic concentrations of serotonin and norepinephrine. However, a rapidly growing number of clinical and preclinical studies indicate that major depressive disorders may be associated with an impairment of structural plasticity and cellular resilience, and that antidepressant medications may act by correcting this dysfunction (Duman *et al*, 1999; Manji *et al*, 2000, 2001, 2003). Recent preclinical data suggest that the common cellular mechanism underlying the effects of different types of antidepressant compounds may be their neurotrophic/neuroprotective properties, which are mediated by different neurotrophic signaling cascades (most notably cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein, brain-derived neurotrophic factor (BDNF), bcl-2, and mitogen-activated protein (MAP) kinases) (Duman *et al*, 1999; Manji *et al*, 2000, 2001, 2003). Furthermore, there is clinical evidence to suggest that antidepressant treatment may increase BDNF levels in human brain (Chen *et al*, 2001) and can protect against hippocampal volume loss in human patients (Sheline *et al*, 2003). Suppose, the reduced number of PV-positive neurons we observed in stressed animals is indicative for cell death, one might suggest that fluoxetine or SLV-323 treatment could protect against excitotoxic cell loss. Indeed, we recently demonstrated in the same experimental paradigm that treatment with the antidepressant tianeptine can protect against neural cell death (Lucassen *et al*, 2004).

Another possible explanation for our results is that, because of the stress insult, the perikaryal PV content falls bellow levels detectable by immunohistochemistry, this might be the case even though processing sections with a much higher concentrations of the primary antibody could not label more parvalbumin-IR cells. A reduction of such a calcium-buffering system within the cells may potentially reflect a functional impairment of the PV-containing interneurons, but it could also be a compensatory mechanism (see below). There is experimental evidence that, in animals subjected to either acute or chronic stress, the expression of the GABA synthesizing enzyme GAD67 mRNA is significantly enhanced within the hippocampal formation (Bowers *et al*, 1998). Notably, after 2 weeks of stress, the most pronounced increase of GAD67 mRNA expression was detected in the DG and CA3 region (Bowers *et al*, 1998), and the same subregions were affected in our experiment. Very similar results were observed after combination of pre- and postnatal corticosterone exposure:

5 days after the final corticosterone treatment, GAD67 mRNA expression was increased in the cells located on the border of the hilus and granule cell layer, in the stratum pyramidale of CA3, and in the stratum oriens and pyramidale of the CA1 (Stone *et al*, 2001); these are the areas where basket and chandelier cells are located most densely. The authors pointed out a remarkable fact that the largest increase (two-fold) in GAD mRNA expression was found in the GABAergic basket cells of the DG, whereas GAD mRNA expression was not altered in adjacent GABAergic interneurons projecting to the granule cell dendrites (Stone *et al*, 2001). Increased GAD67 mRNA expression may reflect a general cellular response to injury; alternatively, it may indicate a compensatory attempt to increase the production and release of GABA, which in turn would suppress neuronal firing within the overexcited circuits. Similar explanations may account for the alterations of parvalbumin content within the cells.

The exact physiological role of the calcium-binding protein parvalbumin is not yet clear, but results of studies on PV-deficient mice show that low levels of parvalbumin in the axon terminals result in increased GABA release, so PV is likely to modulate the Ca^{2+}-dependent release of GABA (Vreugdenhil *et al*, 2003). It has also been demonstrated that, after repeated seizures, the density of hippocampal PV-immunoreactive neurons rapidly decreases, without changing the density of GABA-immunoreactive neurons in the same area. This indicates that in response to seizure activity, the perikaryal PV content falls below levels detectable by immunohistochemistry (Scotti *et al*, 1997). A reduction of such a calcium-buffering system within the cells may potentially reflect a functional impairment of the PV-containing basket and chandelier cells, but it might also be a compensatory mechanism. Hypothetically, this might reflect a kind of plastic change; perisomatic inhibitory cells might be able to downregulate their parvalbumin content to facilitate GABA release. Such an adaptive change may help the GABA system prevent excessive firing of the principal cells caused by either repeated stress or epileptic seizures. This explanation may resolve the seemingly controversial observations of decreased numbers of PV-IR cells (present data) and enhanced expression of GAD67 mRNA levels after chronic stress (Bowers *et al*, 1998) or repeated corticosterone treatment (Stone *et al*, 2001). Moreover, this may also explain the observation that *in vivo* high-frequency stimulation of the mossy fiber inputs to CA3 produced epileptic after-discharges in 56% of acutely stressed animals, whereas this happened in only 29% of chronically stressed animals (Pavlides *et al*, 2002). Of course, we cannot exclude another possibility, namely that the remaining GABA neurons upregulate their GAD production to compensate the loss of their vulnerable subpopulation (Esclapez and Houser, 1999).

Antidepressant Treatment can Counteract the Effect of Stress

Remarkably, the above-mentioned chronic stress-induced enhancement of GAD67 mRNA expression in the hippocampus can be blocked by treatment with an antidepressant, the highly selective norepinephrine reuptake inhibitor reboxetine (Herman *et al*, 2003). In our experiment, treatment with fluoxetine, a serotonin (5-HT) selective reuptake inhibitor (SSRI) with well-known clinical efficacy (Stokes and Holtz, 1997) prevented the chronic stress-induced effect. The serotonergic innervation of the hippocampus originates from the dorsal and median raphe nuclei, but only 21% of serotonin-containing varicosities make synaptic contacts, mostly with dendrites and somata of GABAergic interneurons, and it has been suggested that those varicosities without synaptic contact release 5-HT at nonsynaptic sites for long distance diffusion (Vizi and Kiss, 1998). Experimental serotonin treatment hyperpolarizes both pyramidal and granule cells, with the effect possibly occurring directly on principal cells, or mediated by excitation of interneurons (Freund and Buzsaki, 1996). Accordingly, treatment with the SSRI fluoxetine can lead to a general inhibition of hippocampal network activity and, indeed, animal experiments suggest that SSRIs at therapeutic doses are able to decrease seizure susceptibility of the hippocampus (Wada *et al*, 1995; Hernandez *et al*, 2002). Thus, in our case, fluoxetine treatment may provide sufficient counterbalancing inhibition to the overexcited hippocampal circuitry, and thereby prevent the compensatory downregulation of parvalbumin to enhance GABA release. Alternatively, the beneficial effect of fluoxetine treatment could be explained by its potential neurotrophic/neuroprotective properties, which results in an enhancement of neuronal viability.

The Effect of the NK_1 Receptor Antagonist

In the present study, in addition to fluoxetine, we used the NK_1 receptor antagonist SLV-323 (Czeh *et al*, 2003; Hesselink *et al*, 2003), because inhibition of substance P and its preferred NK_1R pathway is a promising novel approach to antidepressant treatment (Rupniak and Kramer, 1999; Stout *et al*, 2001). Functional studies indicate that pharmacological blockade or deletion of the NK_1R might be as effective as currently used antidepressants in the suppression of psychological and behavioral stress responses (Rupniak, 2002). In humans, the first clinical study using an NK_1R antagonist for the treatment of depression reported promising results regarding the therapeutic efficacy of an NK_1R antagonist (Kramer *et al*, 1998, 2004). It has been suggested that the potential therapeutic effects of NK_1R antagonists are mediated via the dorsal raphe nucleus, a major source of forebrain serotonin that has been implicated in affective disorders. According to this explanation, treatment with an NK_1 receptor antagonist treatment results in desensitization of the autoinhibitory $5\text{-}HT_{1A}$ receptor and enhanced serotonergic neurotransmission (Santarelli *et al*, 2001). However, it should be noted that, in rats treated with the NK_1 antagonist GR205171, the efflux of 5-HT in the hippocampus was not increased (Millan *et al*, 2001).

Administration of an NK_1R antagonist is likely to exert an effect directly on hippocampal neurons. Nevertheless, the exact physiological role of substance P in the hippocampus is still disputed, partly because of a general mismatch between the extent of fibers containing substance P and the quantity of NK_1 receptors (Nakaya *et al*, 1994; Ribeiro-da-Silva and Hökfelt, 2000). Anatomical data indicate that, in the hippocampal formation, the highest density of

immunoreactive substance P receptors is present on GABAergic inhibitory interneurons, including PV-containing cells (Sloviter *et al*, 2001). It is possible that in the hippocampus, as in other CNS areas, substance P may be reaching its target receptors via nonsynaptic diffusion (Mantyh *et al*, 1995). Diffusion of substance P from its release sites onto dentate granule cells thus may provide a strong excitatory influence on these cells, by prolonging the time that the NMDA channel spends in the open state (Lieberman and Mody, 1998). As granule cells of the DG are thought to gate the amount of excitatory input to the hippocampus, administering an NK_1R antagonist may result in an overall suppression of hippocampal network activity and, indeed, NK_1R antagonists are suggested to constitute a novel category of drugs in antiepileptic therapy (Liu *et al*, 1999). Therefore, similarly to fluoxetine treatment, SLV-323 may supply a sufficient amount of compensatory inhibition to prevent the excessive firing of the principal cells, so the downregulation of parvalbumin is not necessary. Alternatively, the protective effect of SLV-323 could be due to its potential neurotrophic/neuroprotective properties.

Stress and Antidepressant Treatment can Affect Adult Hippocampal Neurogenesis

Another remarkable feature of PV-positive interneurons has been demonstrated recently—about 14% of newly generated neurons in the adult DG are apparently GABAergic parvalbumin-positive cells (Liu *et al*, 2003). This is especially interesting in light of the fact that both acute and chronic stress can affect the production of new neurons in the dentate subgranular zone by suppressing both the proliferation rate of precursors and the survival rate of the daughter cells (Gould *et al*, 1997; Czeh *et al*, 2001, 2002; Pham *et al*, 2003). Furthermore, this stress-induced suppression of cytogenesis is counteracted by various classes of antidepressant drug treatment, including NK_1R antagonists (Czeh *et al*, 2001; van der Hart *et al*, 2002; Malberg and Duman, 2003). This form of neuroplasticity may provide an alternative explanation to our present observations. Accordingly, in the DG the reduced number of PV-IR interneurons may be a consequence of the stress-induced suppression of adult neurogenesis, whereas the neurotrophic effect of antidepressant treatment could normalize the number of PV-containing cells. However, this explanation cannot account for the alterations observed in the CA2 and CA3 regions.

Methodical Considerations

To our knowledge, the present study is the first that quantified the total number of PV-positive cells in the hippocampus; thus, no comparison to other results from any species is available. It may appear that the number of PV-IR cells are extraordinarily low, but it should be emphasized that the number of nonprincipal neurons in the hippocampus is about 10–20 times smaller than of principal cells (Freund and Buzsaki, 1996; Benes *et al*, 1998). Furthermore, immunohistochemistry staining using the parvalbumin antibody visualizes only a fraction (~20–40%) of all GABAergic neurons in the hippocampus (Freund and Buzsaki, 1996).

Due to their low number, quantifying hippocampal interneurons using the unbiased stereological technique is challenging (Benes *et al*, 1998). To be able to fulfill the requirements of the stereological approach, we had to exclude certain hippocampal layers from the analysis, where the occurrence of PV-containing neurons is extremely low. Furthermore, we applied the modified optical disector method, which could have caused a potential bias. In contrast to the unbiased optical disector technique, we did not use guard zones at the top and bottom of the section. However, results from studies working similarly to us on 50 μm cryosections suggest that the outcome of the unbiased and the modified optical disector approach is the same (Harding *et al*, 1994; Keuker *et al*, 2004). Altogether, the presented values here may not precisely reflect the absolute number of PV-containing interneurons in the tree shrew hippocampal formation, but given the fact that we applied the same cell counting protocol for each group, it is very unlikely that the observed group differences are artifacts due to any potential biases caused by the method.

CONCLUSION

In summary, long-term stress results in a decreased number of PV-containing GABAergic interneurons in the hippocampal formation. This may indicate either a real excitotoxic cell loss or that the intracellular parvalbumin content is reduced below levels detectable by the immunocytochemical method. A reduction of such a calcium-buffering system within the cells may reflect a functional impairment of the basket and chandelier cells. Alternatively, it could hypothetically be a kind of compensatory mechanism, a form of neuroplasticity, to facilitate GABA release, as an attempt to counterbalance the enhanced excitatory activity of the principal cells caused by repeated stress. Importantly, concomitant antidepressant treatment prevented the effect of stress either by enhancing neuronal viability or probably by providing sufficient compensatory inhibition to the overexcited hippocampal circuitry, so the downregulation of the calcium-buffering parvalbumin is prevented by the drug treatment. Notably, neither fluoxetine nor the SLV-323 had any effect on the number of parvalbumin-containing hippocampal interneurons in control animals. Furthermore, we demonstrated that, in this experimental setting, the NK_1R antagonist SLV-323 showed even greater neurobiological efficacy than fluoxetine.

ACKNOWLEDGEMENTS

The presented study has been partially supported by Solvay Pharmaceuticals, Weesp, The Netherlands and Glaxo-SmithKline, Verona, Italy. We are grateful to JI Keuker and MHP Kole for the stimulating discussions and for critically reading the manuscript. We thank Professor C Hiemke for determining fluoxetine plasma concentrations, and S Donath and A Heutz for their excellent technical assistance. Analysis of urine levels of norepinephrine and creatinine was performed at KCL Bioanalysis b.v., Leeuwarden, The Netherlands. We thank Jan Berk for his help.

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CHAPTER 4

Astroglial plasticity in the hippocampus is affected by chronic psychosocial stress and concomitant fluoxetine treatment

Neuropsychopharmacology (2006) 31, 1616–1626

www.neuropsychopharmacology.org

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.

Neuropsychopharmacology (2006) **31,** 1616–1626. doi:10.1038/sj.npp.1300982; published online 14 December 2005

Keywords: glia; astrocyte; cell number; stereology; antidepressant; hippocampal volume

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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Received 27 June 2005; revised 21 September 2005; accepted 18 October 2005
Online publication: 25 October 2005 at http://www.acnp.org/citations/Npp102505050420/default.pdf

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

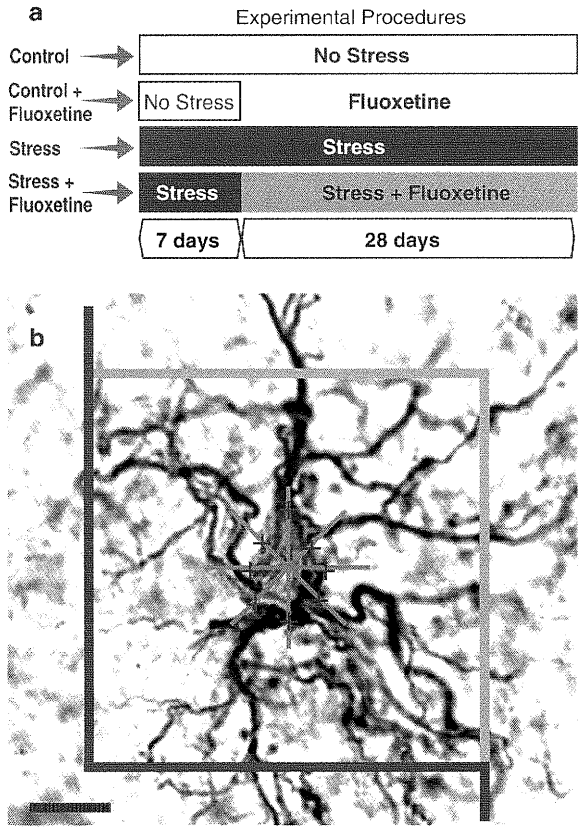

Figure 1 (a) Experimental groups and design. For details, see the Materials and methods. (b) Light micrograph illustrating the stereological counting frame. In the center is one astrocyte from a control CA1 stratum radiatum area. Only cells that lay within the volume ($50 \times 50 \times 20\,\mu m$) 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, $10\,\mu m$.

(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 ($P = 0.5$).

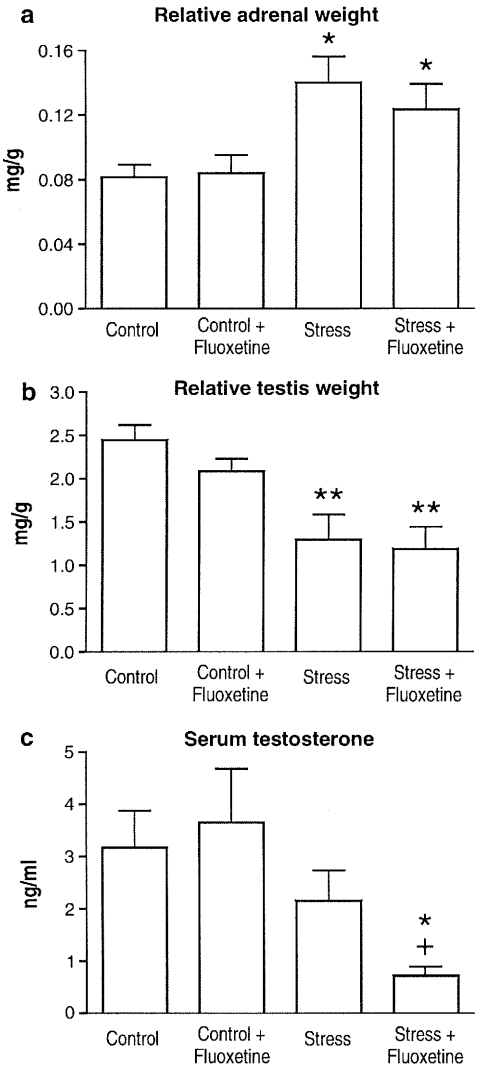

Figure 2 The physiological consequences of the repeated territorial conflict were expressed in the significant changes in relative organ weights and testosterone concentrations. Stress significantly increased relative adrenal weights (a), and reduced relative testes weights (b) and serum testosterone 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

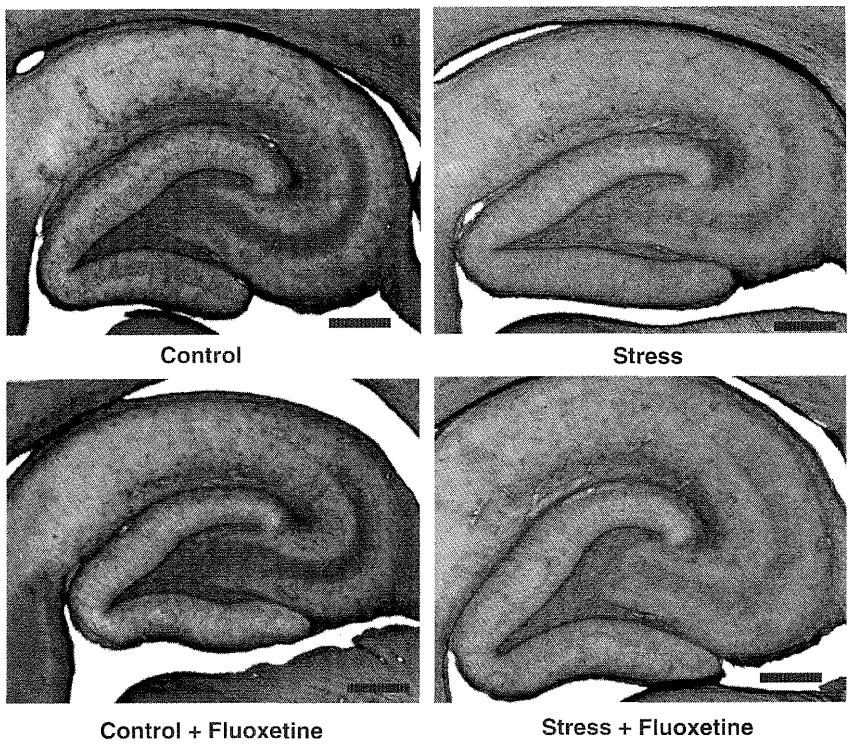

Figure 3 Representative examples of a glial fibrillary acidic protein (GFAP) stained horizontal section of the tree shrew hippocampal formation. Note the marked difference in GFAP staining intensity between the control (*Control*, *Control + Fluoxetine*) and stressed groups (*Stress* and *Stress + Fluoxetine*).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

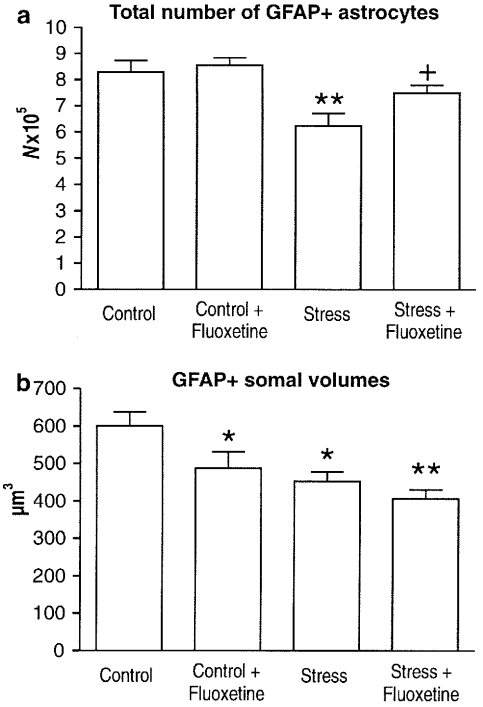

Figure 4 Effects of chronic psychosocial stress and concomitant fluoxetine treatment on the total number of GFAP-immunoreactive astrocytes in the hippocampal formation (a) and on the somal volumes of these GFAP-positive cells (b). (a) Stress significantly decreased the number of GFAP-IR astrocytes, whereas 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*.

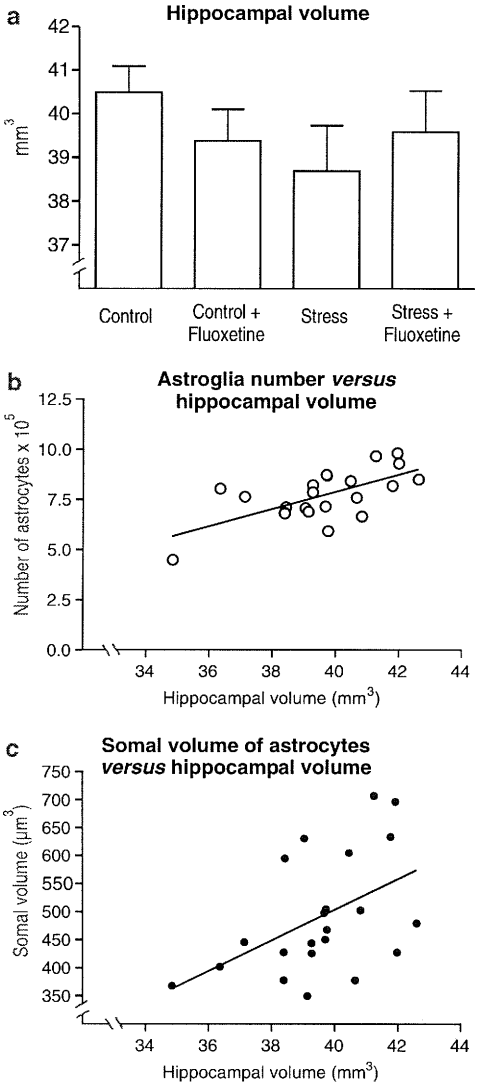

Figure 5 (a) There was a minor (−5%) nonsignificant decrease of the hippocampal volume in the *Stress* group, but neither stress nor drug treatment had a statistically significant effect on hippocampal volume. (b and c) Correlation analysis revealed that individual hippocampal volumes significantly correlated both with the total number of astrocytes ($r=0.65$, $P=0.0001$), 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.

ACKNOWLEDGEMENTS

This study was partially supported by GlaxoSmithKline, Verona, Italy. We thank S Donath and A Heutz for their excellent technical assistance and S Heller and A Rieger-Gies for the analysis of fluoxetine and norfluoxetine. B Czéh is supported by the DFG Research Center Molecular Physiology of the Brain (CMPB).

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CHAPTER 5

Examining SLV-323, a novel NK_1 receptor antagonist, in a chronic psychosocial stress model for depression

Psychopharmacology (2005) 180: 548–557
DOI 10.1007/s00213-005-2184-8

ORIGINAL INVESTIGATION

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Examining SLV-323, a novel NK_1 receptor antagonist, in a chronic psychosocial stress model for depression

Received: 1 November 2004 / Accepted: 4 January 2005 / Published online: 22 February 2005

Abstract *Rationale:* Substance P antagonists have been proposed as candidates for a new class of antidepressant compounds. *Objectives:* We examined the effects of SLV-323, a novel neurokinin 1 receptor (NK_1R) antagonist, in the chronic psychosocial stress paradigm of adult male tree shrews. *Methods:* Animals were subjected to a 7 day period of psychosocial stress before being treated daily with SLV-323 (20 mg kg^{-1} day^{-1}). The psychosocial stress continued throughout the treatment period of 28 days. Brain metabolite concentrations were determined in vivo by proton magnetic resonance spectroscopy. Norepinephrine excretion was monitored from daily urine samples, and serum testosterone concentrations were measured at the end of the experiment. All animals were videotaped daily to analyze scent-marking behavior and locomotor activity. Cell proliferation in the dentate gyrus and hippocampal volume were measured postmortem. *Results:* Stress significantly decreased cerebral concentrations of *N*-acetyl-aspartate, total creatine, and choline-containing compounds in vivo and resulted in an increase of urinary norepinephrine and decrease of serum testosterone concentrations. Moreover, stressed animals displayed decreased scent-marking behavior and locomotor activity. The proliferation rate of the granule precursor cells in the dentate gyrus was reduced, and hippocampal volume was mildly decreased. The stress-induced alterations in the central nervous system were partially prevented by concomitant administration of SLV-323, while drug treatment had only a minor effect on the stress-induced behavioral changes. *Conclusions:* The novel NK_1R antagonist SLV-323 has certain antidepressant-like effects in a valid animal model of depression.

Keywords Substance P · Mood disorder · Antidepressant · Neurogenesis · Hippocampus · Proton magnetic resonance spectroscopy · Testosterone · Behavior · Tree shrew

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Introduction

A promising novel antidepressant treatment approach is the inhibition of substance P (SP) and its preferred neurokinin 1 receptor (NK_1R) pathway (Rupniak and Kramer 1999; Stout et al. 2001). Several lines of evidence suggest that the SP–NK_1R system plays an important role in the regulation of emotional behavior. Neuroanatomical studies demonstrate that SP-containing neurons are widely distributed in brain structures that are involved in the regulation of the stress response and affective behavior, such as depression and anxiety (Caberlotto et al. 2003). Functional studies indicate that the pharmacological blockade of the NK_1R might be as effective as currently used antidepressants to suppress psychological and behavioral stress responses (Rupniak 2002). Importantly, the first human studies, testing NK_1R antagonists in the treatment of depression, reported promising results about the therapeutic efficacy of the NK_1R antagonists MK-869 and L-759,274 (Kramer et al. 1998, 2004). Despite these encouraging findings, the development of NK_1R antagonists for the treatment of depression has been abruptly terminated, e.g. by Merck. On the other hand, other companies are still continuing their NK_1R antagonist program for the treatment of depression, and they are developing new compounds, which should

similarly go through the preclinical evaluation. Efforts are ongoing, not only to determine their pharmacological profile, but also to eventually test them in animal models for depression as well.

One major difficulty in studying SP antagonists is the species variability of the NK_1 receptor (Beresford et al. 1991). Tree shrews (*Tupaia belangeri*) are closely related to primates (Martin 1990), and there is indication that their central NK_1R shows homology with the human NK_1R (van der Hart et al. 2002). Thus, these animals are particularly suitable for the preclinical evaluation of novel NK_1R antagonists. Recently, we demonstrated that the NK_1R antagonist L-760,735 has an antidepressant-like efficacy similar to that of a tricyclic antidepressant such as clomipramine (van der Hart et al. 2002). The study was performed using a chronic psychosocial stress paradigm in male tree shrews, which is an established animal model for researching the pathophysiology of major depression (Fuchs and Flügge 2002; van Kampen et al. 2002; Fuchs et al. 2004).

Here, we investigated the effect of SLV-323 as a novel NK_1R antagonist in a similar experimental design, which mimics the realistic situation of antidepressant medication in patients. Animals were treated with SLV-323 for the clinically relevant period of 4 weeks. Oral drug application started after the stress-induced bio-behavioral alterations had been established. Psychosocial stress continued during the whole treatment period and neuroendocrine and behavioral parameters were frequently monitored. After the stress period, brain metabolite concentrations were determined in vivo by localized proton magnetic resonance spectroscopy (MRS). Finally, we quantified cell proliferation in the dentate gyrus and measured hippocampal volume postmortem.

Materials and methods

Animals, experimental procedure, and drug treatment

Experimentally naive adult male tree shrews were obtained from the breeding colony at the German Primate Center (Göttingen, Germany). Animals were housed individually on a 12/12 h light/dark cycle with free access to food and water (Fuchs 1999). All treatments were performed during the day (activity period, lights on). Animal experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC), and were approved by the Government of Lower Saxony, Germany. The minimum number of animals required to obtain consistent data was used.

Animals received the NK_1R antagonist SLV-323 (Solvay Pharmaceuticals, Weesp, the Netherlands) orally. This route of administration was chosen because oral application is the most common route of administration for antidepressants in psychiatric patients. Moreover, we aimed to minimize uncontrollable stress effects caused by daily injections. We conducted a pilot study to establish the dose of SLV-323 that blocks NK_1 receptors in the tree shrew brain. The methodology used is based on the ability of NK_1 receptor antagonists to block nicotine-induced vomiting in musk shrews (Tattersall et al. 1995). Adult male tree shrews (n=4) received either vehicle or SLV-323 in different dosage orally followed 30 min later by subcutaneous administration of (−) nicotine (4 mg kg^{-1}; Sigma-Aldrich), and the number of emetic episodes occurring during the following 30 min was recorded. Each animal received each treatment in a crossover design, with a 10 day washout period between studies. Emetic episodes were abolished by treatment with 20 mg kg^{-1} day^{-1} SLV-323, whereas lower doses, i.e. 5 or 10 mg kg^{-1} day^{-1}, and vehicle were ineffective. These results showed that treatment with 20 mg kg^{-1} day^{-1} SLV-323 could effectively block central NK_1 receptors.

The experimental design is shown in Fig. 1. Animals were divided into three experimental groups: Control, Stress, and Stress+SLV-323, each consisting of six tree shrews. The experiment consisted of three different phases and altogether lasted for 6 weeks (42 days). The first experimental phase ('No Stress') lasted 7 days, during which all animals remained undisturbed. The second phase was a further 7 day period, during which the animals of the Stress and the Stress+SLV-323 groups were submitted to daily psychosocial conflict ('Stress'). The psychosocial stress procedure was carried out according to our standard protocol (for details, see Czéh et al. 2001; van der Hart et al. 2002). The third experimental phase consisted of the SLV-323 treatment lasting for 28 days, while animals remained in the psychosocial conflict situation. Animals of the Stress+SLV-323 group received the compound (20 mg kg^{-1} day^{-1}) orally in the morning between 8:00 and 8:15. The drug was administered via a bulb-headed cannula into the bucal cavity, and the animals were allowed to swallow the solution. Animals of the Stress group were treated according to the same experimental schedule but received vehicle only. Animals of the Control group remained undisturbed in separate quarters elsewhere in the animal facility. Urine samples were collected on a daily basis throughout the whole experiment to monitor the neurosympathetic tone by measuring free norepinephrine.

During the entire experiment, the daily routine was as follows: every morning between 7:30 and 8:00, before the lights were turned on, urine samples were collected from the animals by applying a slight massage over the hypogastrium, as tree shrews usually start to urinate after waking up. This was immediately followed by oral drug application. Later, between 9:00 and 14:00, at an unpredictable time, the psychosocial confrontations took place for about 1 h. Finally, on certain weeks (Fig. 1), every evening all animals were videotaped for behavioral assessment. This time point was chosen to avoid any unwanted confounding factors (e.g., human activity around the animals).

Localized proton magnetic resonance spectroscopy

During experimental days 37–40, all animals underwent localized proton magnetic resonance spectroscopy (MRS) in vivo to ascertain the concentrations of major brain metabolites, as described previously (Michaelis et al. 2001). In brief, anesthetized animals (70:30 N_2O:O_2, 0.5–1.0% halo-

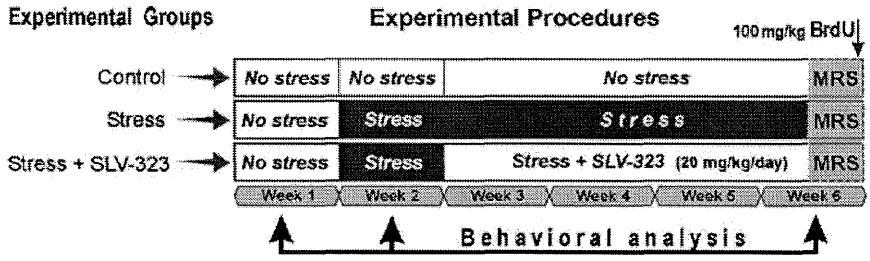

Fig. 1 Experimental design and animal groups: Control, Stress, Stress+SLV-323. For details, see text

thane) were measured at 2.35 T using a MRBR 4.7/400 mm magnet (Magnex Scientific, Abingdon, England) equipped with a DBX system (Bruker BioSpin, Ettlingen, Germany). Radiofrequency excitation and signal reception were accomplished by a 14 cm Helmholtz coil and a 2 cm surface coil, respectively. The volume-of-interest ($7\times5\times7$ mm^3) for proton MRS (STEAM, TR/TE/TM=6,000/20/10 ms, 64 averages) was carefully selected from multislice sagittal and coronal T1-weighted gradient-echo images (FLASH, TR/TE=150/5 ms, 20° flip angle, 50 mm field-of-view, 256×256 data matrix, 1 mm sections) and centrally placed in the forebrain, including parasagittal neocortex, subjacent white matter and portions of subcortical forebrain structures (caudate–putamen, hippocampus, thalamus, ventricles). Metabolite quantification involved fully automated and user-independent spectral evaluation by an LCModel (Provencher 1993) and calibration with respect to the brain water concentration (Michaelis et al. 1999).

Behavioral analysis

During the first, second and sixth weeks of the experiment (Fig. 1), the animals were videotaped from 19:00 until 19:15 daily, an hour before the lights were turned off and before the animals went to sleep. The videotapes were coded so that the observer was blind to the experimental treatment. Each daily recording was analyzed using the Observer 5.0 software (Noldus Information Technology, Wageningen, the Netherlands), and the duration of scent-marking (with abdominal gland, sternal gland, and urine) was recorded. Locomotor activity was scored using Ethovision 2.1 (Noldus Information Technology, Wageningen, the Netherlands). The home cage of the animal was divided into six zones, and each crossing of these zones was counted during the trials.

Bromodeoxyuridine injection and immunocytochemistry

At the end of the experiment, animals received a single i.p. injection of 5-bromo-2′-deoxyuridine (BrdU; 100 mg kg^{-1}; Sigma) and were perfused 24 h later (Fig. 1). The deeply anesthetized animals were perfused transcardially with 4% paraformaldehyde. Serial horizontal sections of 50 μm thickness were collected with a freezing microtome throughout the dorso-ventral extent of the left hippocampal formation. Every fifth section was slide-mounted and coded before processing for immunocytochemistry to ensure objectivity. According to our standard protocol (Czéh et al. 2001), BrdU labeling requires the following pretreatment steps: DNA denaturation (0.01 M citric acid, pH 6.0, 95°C, 20 min), membrane permeabilization (0.1% trypsin, 10 min), and acidification (2 M HCl, 30 min). Primary antibody concentration was mouse anti-BrdU (DAKO, 1:100), and immunocytochemistry was completed using the avidin–biotin/diaminobenzidine visualization method (Vector Laboratories) followed by counterstaining with hematoxylin.

Quantification of BrdU-labeled cells

We used a modified unbiased stereology protocol, which has been reported to successfully quantify BrdU labeling (Malberg et al. 2000; Czéh et al. 2001). Every fifth section (an average of 27) through the dorso-ventral extent of the left hippocampus was examined. All BrdU-labeled cells in the granule cell layer together with the subgranular zone, defined as a two-cell-body-wide zone along the border of the granule cell layer, were counted regardless of size or shape. To enable counting of cell clusters, cells were examined under ×400 and ×1,000 magnification, omitting cells in the outermost focal plane. The total number of BrdU-labeled cells was estimated by multiplying the number of cells counted in every fifth section by 5.

Measurement of hippocampal volume

After cutting the left hemisphere, every fifth serial section was mounted and stained with cresyl violet, dehydrated and coverslipped with Eukit. Slides were coded before quantitative analysis, and the code was not broken until the analysis was completed. Hippocampal volume estimation was made using the Neurolucida system (Neurolucida 4.04, Microbrightfield), which superimposed optical image of the light microscope field on a computer monitor. The volume of the entire hippocampal formation (hippocampus proper together with the dentate gyrus) was estimated on the basis of the Cavalieri principle (Gundersen et al. 1988). Starting at a random position, every fifth section was used for the volume estimations, i.e. an average of 27 sections was analyzed. The cross-sectional hippocampal areas were measured first by tracing the borders of the hippocampus (Ammon's horn together with the dentate gyrus), then surface areas were

computed using the NeuroExplorer software (Microbrightfield). The cross-sectional surface areas were then summed, and multiplied with the thickness of the sections and with the intersection distance to estimate the entire volume of the hippocampus.

Analysis of urine samples

Norepinephrine was quantified by LC-MS (analytical column; Alure Basics, Restek, 50 mm, 2 mm ID) with electrospray ionization (5,500 V, 200°C) after liquid–liquid extraction. To correct for physiological alteration in urine dilutions, the resulting concentrations were related to creatinine concentrations, which were determined with a Roche Modular P800 clinical chemistry analyzer with creatinine reagents (Jaffe method).

Testosterone

Before perfusing the animals, blood samples were collected. For determination of testosterone, 100 µl serum was twice extracted with 1 ml of diethylether by vortexing for 10 min. After 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 enzyme immunoassay, as described in detail by Kraus et al. (1999).

Data analysis

Results are presented as the mean±SEM. Treatment effects were assessed either with a two-tailed unpaired Student's *t* test or with a one-way or two-way ANOVA, followed by the Newman–Keuls test or Dunnett's multiple comparison test as post hoc analysis for further examination of group differences.

Results

The following sections summarize the effects of chronic psychosocial stress and concomitant treatment with SLV-323 on neuroendocrine and behavioral parameters as well as on brain metabolite levels, dentate cell proliferation, and hippocampal volume.

Neuroendocrine parameters

Activation of the sympatho-adrenomedullary system is an important and reliable indicator by which tree shrews can be classified as subordinates (Fuchs and Flügge 2002). The intensity of psychosocial stress in subordinate tree shrews was demonstrated by an immediate and sustained activation of the neurosympathetic tone, as indicated by the pronounced and significant elevation of urinary norepinephrine excretion in the Stress and Stress + SLV-323 groups (Fig. 2a). From the beginning of the drug treatment, animals of the Stress+SLV-323 group always had lower levels of urinary norepinephrine compared with the Stress group, and by the end of the experiment, their norepinephrine concentrations were close to the baseline level (Fig. 2a). Comparison of urinary norepinephrine concentrations between the Stress and Stress+SLV-323 group with a two-way ANOVA (drug× time) revealed a significant main effect of drug treatment ($F_{1,5}$=6.24; P<0.01), and the effect of stress was indicated by the highly significant difference between the experimental weeks (time factor) ($F_{1,5}$=41.10; P<0.0001).

Furthermore, repeated territorial conflict significantly reduced (−40%) serum testosterone concentrations in the animals of the Stress group (Student's *t* test, P<0.05). Interestingly, treatment with SLV-323 induced a profound increase of testosterone levels (Fig. 2b). One-way ANOVA revealed a significant difference between the three groups ($F_{2,15}$=5.25; P<0.05). Animals of the Stress+SLV-323 group had sixfold higher (P<0.05) testosterone levels than the Stress group; even compared with Control animals, SLV-323 treatment resulted in a twofold increase (P<0.05) of serum testosterone concentrations.

Behavioral parameters

Chronic psychosocial stress had a significant suppressive effect on both scent-marking behavior and locomotor activity (Fig. 3a, b). During the first stress week (Week 2), animals of both stressed groups spent about 50% less time (P<0.05) displaying scent-marking behavior compared with the Control week (Week 1, baseline period). In the last stress week, after 5 weeks of stress, scent-marking behavior was almost completely abolished in the untreated animals (about −90%, P<0.001, Fig. 3a). In contrast, animals of the Stress+SLV-323 group somewhat increased their scent-marking, although their mean value of marking after 4 weeks of SLV-323 treatment was not statistically different, neither from the control week nor from the first stress week (Fig. 3a).

Locomotor activity was significantly suppressed by chronic stress, and treatment with the NK_1R antagonist did not counteract this effect (Fig. 3b).

Cerebral metabolite concentrations

The cerebral metabolite concentrations of *N*-acetyl-aspartate (NAA), total creatine (Cr), choline-containing compounds (Cho), and *myo*-inositol (Ins) were determined in all animals. As summarized in Table 1, psychosocial stress resulted in statistically significant decreased concentrations of NAA (−10%; P<0.05), Cr (−11%; P<0.05), and Cho (−13%; P<0.01), while Ins remained unaffected. Treatment with the NK_1R antagonist SLV-323 diminished stress-induced alterations, yielding metabolite concentrations between the respective means of the Control and Stress group.

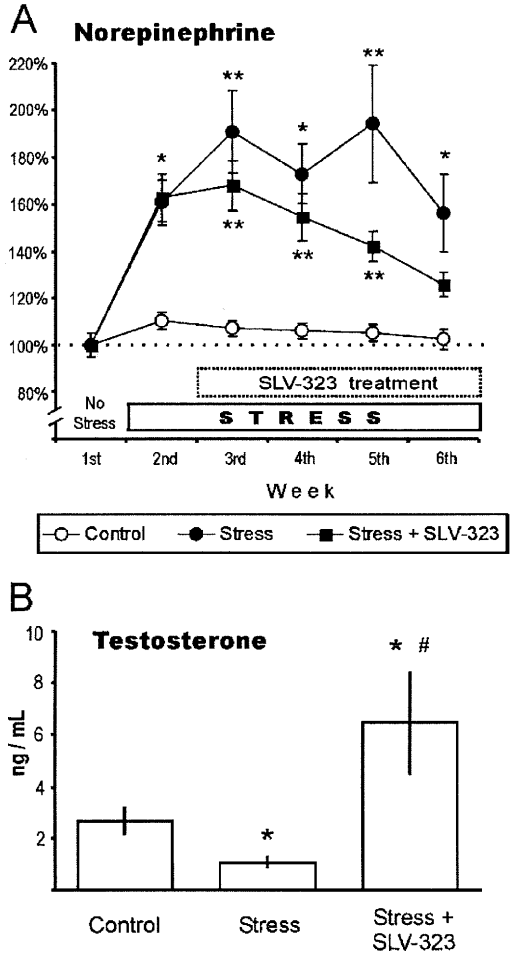

Fig. 2 Effects of chronic psychosocial stress and concomitant SLV-323 treatment on urinary norepinephrine (**a**), and serum testosterone (**b**) concentrations. **a** Stress produced a sustained hyperactivation of the neurosympathetic tone, as evidenced by the elevated urinary norepinephrine levels in both the Stress and Stress+SLV-323 treated animals; however, in the last 2 weeks this effect was significantly attenuated by the drug treatment. Data represent the mean±SEM, normalized to the average urinary norepinephrine during the No Stress period. One-way ANOVA with repeated measurements followed by Dunnett's multiple comparison test: $^{**}P<0.01$, $^{*}P<0.05$ compared with baseline (No Stress). Furthermore, comparison of the Stress and Stress + SLV-323 groups with a two-way ANOVA (drug × time) revealed a highly significant main effect of drug ($P<0.01$), and time ($P<0.0001$). **b** Serum testosterone levels were measured from blood samples collected before perfusing the animals. Serum testosterone concentrations were significantly decreased (−40%; Student's t test, $P<0.05$) in Stress animals, whereas treatment with the NK_1R antagonist SLV-323 induced a profound increase of testosterone levels, resulting in a significant difference compared with both the Control and Stress groups. One-way ANOVA followed by Newman–Keuls test as post-hoc analysis with $^{*}P<0.05$ versus Control, # $P<0.05$ versus Stress

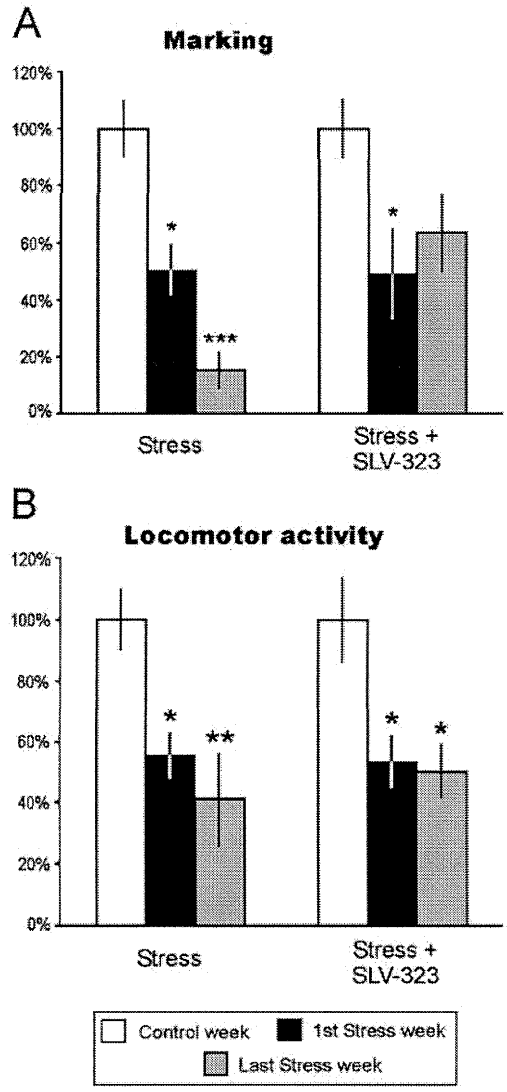

Fig. 3 Effect of chronic psychosocial stress and concomitant SLV-323 treatment on scent-marking behavior (**a**) and locomotor activity (**b**). **a** Stress had a dramatic suppressive effect on marking behavior, whereas treatment with SLV-323 could at least partially counteract the effect of stress. **b** Stress significantly suppressed the duration of locomotor activity in the Stress group, but treatment with the NK_1R did not block the reduction in locomotor activity. One-way ANOVA followed by Newman–Keuls test as post hoc analysis with $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus No Stress (week 1)

Dentate cell proliferation

Generation of new neurons is the end product of a series of steps consisting of proliferation, survival, migration, differentiation, and establishment of functional connections with other neurons. BrdU injection is the most common way to label newly generated cells. When the animals were killed shortly (2–24 h) after BrdU injection, the amount of BrdU-positive cells in the dentate gyrus represent the number of newly generated cells (cell proliferation rate, or cytogenesis).

Histological analysis of the left hemisphere revealed that psychosocial stress resulted in a substantial decrease (−31%) in the number of BrdU-positive cells relative to unstressed Controls (Fig. 4a). One-way ANOVA revealed a significant difference between the groups ($F_{2,15}=11.51$; $P<0.001$) and post hoc comparisons showed a significant difference between the Control and the Stress group ($q=4.09$; $P=0.01$). Treatment of stressed animals with SLV-323

Table 1 Cerebral concentrations of *N*-acetyl-aspartate (NAA), total creatine (Cr), choline-containing compounds (Cho), and *myo*-inositol (Ins)

	Control	Stress	Stress+SLV-323
NAA	10.0±0.3	9.0±0.3*	9.5±0.6
Cr	7.7±0.2	6.9±0.3*	7.4±0.6
Cho	2.5±0.1	2.2±0.1**	2.3±0.2
Ins	6.5±0.2	6.3±0.2	6.2±0.4

Metabolite concentrations (mM/VOI) were obtained in vivo and are shown as mean±SEM. Control (n=15), Stress (n=17) and Stress+SLV-323 (n=6). Additional MRS data for animals of the Control and the Stress group were available from a cohort of age-matched males acquired in multiple studies. Student's t test revealed significant differences between the Stress and Control groups: *P<0.05, **P<0.01

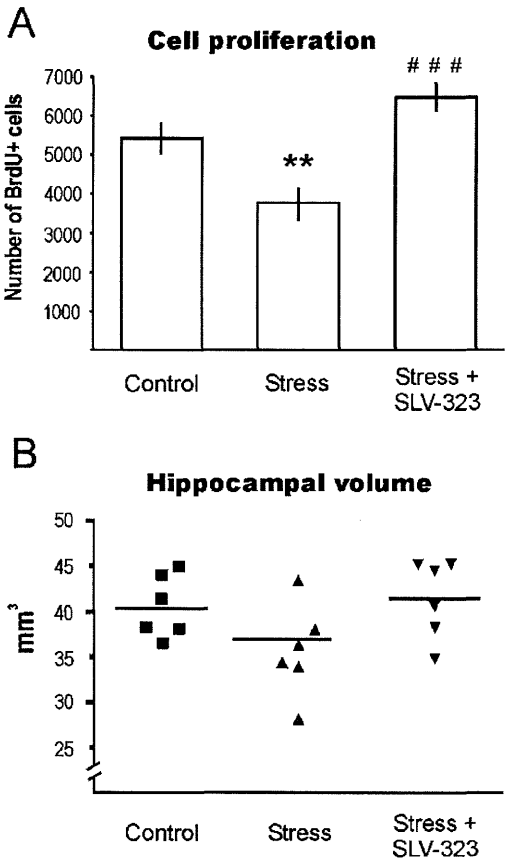

Fig. 4 Effects of chronic psychosocial stress and concomitant SLV-323 treatment on dentate cytogenesis (**a**) and hippocampal volume (**b**) in the left hemisphere. **a** Stress significantly suppressed cell proliferation in the hippocampal dentate gyrus, whereas SLV-323 treatment prevented the stress-induced effect. Results are given as the mean±SEM number of BrdU-positive cells in the hippocampal dentate gyrus. **P<0.01 significantly different from untreated Controls, ###P<0.001 compared with Stress. **b** There was a minor decrease of hippocampal volume (−11%) in Stress animals, which was counteracted by NK_1R antagonist treatment (one-way ANOVA: P=0.07). Note the substantial individual differences in reaction to stress and drug treatment

resulted in a significant increase in the number of BrdU-labeled cells in the dentate gyrus (q=6.73; P<0.001 vs Stress). This demonstrates that chronic treatment with the NK_1R antagonist could overcome the stress-induced reduction of cell proliferation in the dentate gyrus.

Hippocampal volume

Chronic exposure to psychosocial stress resulted in a mild decrease (−11%) of the hippocampal volume in subordinate tree shrews (Stress) compared with non-stressed controls (Fig. 4b). Statistical analysis with one-way ANOVA revealed a between-groups difference approaching the level of significance ($F_{2,15}$=3.04; P=0.07). In stressed animals, treatment with SLV-323 resulted in a tendency of volume normalization (Fig. 4b).

Discussion

In the present study we investigated the effectiveness of SLV-323, a novel member of the NK_1R antagonist family, in the chronic psychosocial stress model for depression. Using this new NK_1R antagonist, we repeated the same experimental paradigm that we have previously adapted for evaluating the efficacy of another NK_1R antagonist, L-760,735 (van der Hart et al. 2002). This allowed us to compare the efficiency of these two compounds.

According to findings reported by Hesselink et al. (2003), SLV323 is a potent, centrally available NK_1R antagonist. It shows high affinity for (pK_i=8.8±0.2), as well as potent antagonism (inhibition of NK_1R-mediated IP3 production in CHO cells, pA_2=8.9±0.2) of the human NK_1R. It is 1,000-fold selective for NK_1R compared to other neurokinin receptors. Its affinity for the rat NK_1R was tenfold less than for the human NK_1R. Plasma concentrations exceeding 10–20 ng ml^{-1} were effective in antagonizing both peripherally and centrally mediated effects of NK_1R agonists (antagonism of substance P-induced hypotension in guinea pigs and GR73632-induced foot-tapping in gerbils, respectively), with the brain concentration being approximately twofold higher than the plasma concentration.

Stress-induced alterations of endocrine levels are attenuated by SLV-323 treatment

Chronic psychosocial stress activates the HPA axis, increases the neurosympathetic tone, and suppresses gonadal activity. The increased concentration of urinary norepinephrine demonstrates that the animals in the Stress and the Stress+SLV-323 groups were stressed throughout the entire experimental stress period. SLV-323 treatment had a significant attenuating effect on the elevated urinary norepinephrine levels, as displayed by the steadily decreasing amount of norepinephrine concentrations throughout the drug treatment period. In contrast, animals of the Stress

group displayed sustained activation of the neurosympathetic tone throughout the entire experiment.

Serum testosterone levels were significantly suppressed in animals of the Stress group, confirming earlier findings on gonadal hypofunction and hypotrophy in animals submitted to chronic stress (Fischer et al. 1985; Flügge et al. 1998). Interestingly, SLV-323 treatment not only blocked the stress-induced decrease of testosterone concentrations, but even stimulated testosterone production. Similarly robust increases of testis weight have been reported for the same compound (Czéh et al. 2005). This finding clearly supports recent theories suggesting that tachykinins may have a physiological modulatory role on testicular function (Debeljuk et al. 2003). The observation that SLV-323 substantially stimulated testosterone production above the normal level suggests that SLV-323 treatment may avoid the common and unwanted side effect of many antidepressant drugs: sexual dysfunction (Gregorian et al. 2002). However, high testosterone concentration may lead to other side effects (e.g., aggression). Results of clinical studies also indicate that NK_1R antagonist treatment has a reduced side effect profile (such as lesser sexual dysfunction), when compared with SSRIs such as paroxetine (Kramer et al. 1998, 2004).

L-760,735, another NK_1R antagonist that we tested earlier under the same experimental paradigm, had no effect at all on the stress-induced elevation of urinary norepinephrine (testosterone was not measured, see van der Hart et al. 2005).

Stress-induced alterations of behavior are not affected by SLV-323 treatment

As in our earlier reports, chronic psychosocial stress significantly suppressed scent-marking behavior and locomotor activity (Fuchs et al. 1996; Kramer et al. 1999). Treatment with SLV-323 had no effect on locomotor activity, but had a tendency to improve scent-marking behavior. L-760,735, the other NK_1 receptor antagonist that we had tested earlier, had similarly no effect on locomotor activity, but had more pronounced restoring effect on scent-marking (van der Hart et al. 2005). This is in contrast to our previous findings that the antidepressant clomipramine was able to prevent the negative effects of stress on various behavioral parameters (Fuchs et al. 1996). One possible explanation for the absence of a behavioral effect due to SLV-323 might be that our treatment period was too short to observe a putative normalization of behavior for this particular drug. It should be noted, however, that a similar treatment schedule was used in our earlier, above-mentioned studies as well (Fuchs et al. 1996; van der Hart et al. 2005). We treated the animals with SLV-323 for 4 weeks, because this mimics the realistic situation of antidepressant medication in patients, where a similar time period is necessary to observe the appearance of clear treatment effects. Thus, for the chosen experimental conditions and despite a treatment-related prevention of stress-induced neurobiological alterations in the hippocampus, the expected drug-induced recovery of behavior does not inevitably occur (see also Wood et al. 2004).

Stress-induced reductions of brain metabolites are diminished by SLV-323 treatment

In line with earlier proton MRS studies of other antidepressants (Czéh et al. 2001; van der Hart et al. 2002), psychosocial stress-induced decreases of NAA, Cr, and Cho were similarly diminished by treatment with SLV-323. A detailed comparison with clinical proton MRS studies of depressed subjects is hampered by the common use of metabolite ratios rather than metabolite concentrations, which led to conflicting results in the respective literature (Renshaw et al. 1997; Frey et al. 1998; Winsberg et al. 2000; Steingard et al. 2000; Auer et al. 2000).

A decrease of the cerebral NAA level is commonly understood as a reduction of neural cell density and/or dysfunction. Interestingly, recent clinical studies reported increased NAA levels after lithium treatment or electroconvulsive therapy (Moore et al. 2000; Michael et al. 2003). Similar increases of NAA were observed in epileptic rats after creatine administration, and were histologically demonstrated to reflect alterations of metabolism rather than neural cell density (Vielhaber et al. 2003). In accordance with these findings, our results suggest that treatment with antidepressants may reestablish neuronal resilience.

Alterations of the Cho level were reported to result from changes of cytosolic choline compounds due to disturbances in the formation and degradation of cell membranes. Because Cho is highly concentrated in oligodendrocytes (Urenjak et al. 1993), alterations may also reflect a respective reduction of cell number, size, and density. Interestingly, a reduction of oligodendrocytes was reported postmortem in the amygdala of patients with major depressive disorder (Hamidi et al. 2004). This finding is not paralleled in astrocytes, as evidenced by the observation of a normal concentration of the respective marker compound Ins after chronic stress, but is in line with a similar decrease of Cr as a major constituent of all cells (Table 1). Based on these results, one may speculate that, in stressed tree shrews, SLV-323 administration may preserve or even stimulate oligodendritic activity, and consequently counteract neural resilience.

L-760,735, the other NK_1R antagonist that we tested earlier, had a much more pronounced enhancing effect on brain metabolites (van der Hart et al. 2002). In particular, it significantly elevated the Ins concentration, which was not at all affected by SLV-323.

Stress-induced reduction in dentate cytogenesis is prevented by SLV-323 treatment

Recently, various groups suggested that disturbances of adult hippocampal neurogenesis play an important role in the etiology of major depressive disorder (Jacobs et al. 2000; Duman 2004). Although this theory has been seriously questioned (Henn and Vollmayr 2004), growing

experimental evidence indicates that different classes of antidepressant treatments are indeed able to stimulate dentate cyto- or neurogenesis (Malberg et al. 2000; Czéh et al. 2001; van der Hart et al. 2002). Furthermore, it has been suggested that the behavioral effects of chronic antidepressants may be mediated by the stimulation of neurogenesis in the hippocampus (Santarelli et al. 2003). Certainly, without clinical data on altered hippocampal neurogenesis in depressed patients and its normalization after antidepressant treatment, the involvement of adult neurogenesis in mood disorders remains to be disputed.

Regulation of neurogenesis is a complex process, and several hypotheses can be considered regarding the mechanisms by which new neurons are produced in the adult dentate gyrus. We already demonstrated that, among others, NK_1R antagonist treatment may affect dentate cytogenesis (van der Hart et al. 2002) and, in line with this, a recent study reported enhanced hippocampal neurogenesis in NK_1R knockout mice (Morcuende et al. 2003). It is not yet clear how exactly substance P affects dentate neurogenesis, but what is currently known is that NK_1R knockout mice exhibit a twofold increase in hippocampal levels of brain-derived neurotrophic factor (BDNF) (Morcuende et al. 2003), and that exogenous BDNF can trigger an immense proliferation and appearance of new neurons in the parenchyma of the forebrain (Pencea et al. 2001).

In comparison to L-760,735 (van der Hart et al. 2002), SLV-323 was more effective in stimulating dentate cell proliferation.

Stress-induced decrease of hippocampal volume is counteracted by SLV-323 treatment

Both SLV-323 and L-760,735 (van der Hart et al. 2002) had a modest counter-acting effect on the stress-induced shrinkage of hippocampal volume.

Recent imaging studies in humans revealed that the hippocampus undergoes a selective volume reduction in several stress-related neuropsychiatric illnesses such as recurrent depressive illness or post-traumatic stress disorder (Bremner et al. 2000; Sheline 2000). Besides reduced neurogenesis, the hippocampal formation was also shown to undergo another morphological change in response to stress, namely the retraction of apical dendrites of CA3 pyramidal neurons (Magarinos et al. 1996; Kole et al. 2004). Based on these findings in animals, the clinically observed hippocampal volume loss may—at least partially—be explained by dendritic retraction and reduced cell proliferation.

Among the mechanistic explanations for the remodeling, one may speculate that the reversibility is due to alterations in the dendritic, axonal and synaptic components, as well in the glia cells of the hippocampal neural network. To determine the specific cellular bases of these observations and to identify the cellular components involved, detailed immunohistochemical and molecular studies are needed.

Methodical considerations

In this study we examined a novel putative antidepressant compound in the chronic psychosocial stress paradigm in male tree shrews, which is regarded as a valid animal model for studying the pathophysiology of major depression (Fuchs and Flügge 2002; van Kampen et al. 2002; Fuchs et al. 2004). In several earlier reports, we demonstrated that this paradigm fulfils the three major criteria for modeling mental illness (Willner 1991): face, predictive and construct validity (for reviews, see Fuchs and Flügge 2002; van Kampen et al. 2002; Fuchs et al. 2004). Importantly, whereas different classes of antidepressant compounds were able to block, or even reverse several stress induced alterations (Fuchs et al. 1996; Czéh et al. 2001; van der Hart et al. 2002, Simon et al. 2004), this did not hold true when animals were treated with the prototypic anxiolytic diazepam (van Kampen et al. 2000).

Conclusions

This work demonstrates that SLV-323, a novel NK_1R antagonist, partially prevents several chronic stress-induced alterations in the central nervous system. In contrast, no attenuation of behavioral abnormalities was observed for a treatment protocol of 4 weeks. Interestingly, SLV-323 had a robust effect on testicular function, profoundly stimulating testosterone production. Altogether, SLV-323 showed a different efficacy profile compared to the other NK_1R antagonist, L-760,735, tested earlier under the same experimental paradigm.

Acknowledgements This study was partially supported by Solvay Pharmaceuticals, Weesp, the Netherlands.

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CHAPTER 6

General discussion

and

summary of the main findings

Summary of the main findings

The exact neurobiological processes leading to depression and the mechanisms responsible for the effects of antidepressant drugs are not yet completely understood. This dissertation based on an animal model of depression aims to elucidate some aspects of cellular plasticity in the hippocampal formation.

The results show:

1. In agreement with previous studies, chronic psychosocial stress significantly suppressed dentate cell proliferation. Older animals were significantly more vulnerable to the adverse effect of chronic stress on dentate cytogenesis. This observation was surprising, since the animals used in this study were all relatively young (5-30 months old), compared with the average life span of 5-7 years for tree shrews living under laboratory conditions. One may assume that this difference may be even more pronounced in older animals.
2. Long-term psychosocial stress resulted in a significantly fewer parvalbumin-immunoreactive cells in the dentate gyrus and in the CA2-CA3 regions of the Ammon's horn, whereas the CA1 subfield was not affected. Additionally, we found that this effect of long-term stress was prevented by concomitant treatment with the NK_1 receptor antagonist SLV-323. Treatment with fluoxetine partially counteracted the stress-induced changes: it normalized the number of parvalbumin-immunoreactive interneurons in the dentate gyrus, but had no effect in the CA2 and CA3 areas. This suggests that chronic stress and antidepressant drugs directly or indirectly modulate the hippocampal GABAergic network.
3. Chronic psychosocial conflict can result in significantly fewer astroglia in the hippocampus while concomitant treatment with fluoxetine can block this effect of long-term stress.
4. Testing a novel NK_1R antagonist in the chronic psychosocial stress paradigm, we revealed that SLV-323 partially prevents several chronic

stress-induced alterations in the central nervous system. In contrast, no attenuation of behavioral abnormalities was observed for a treatment protocol of 4 weeks.

Human implications and clinical relevance of the findings

Morphological alterations of human hippocampal formation in patients with MDD

A growing body of *in vivo* volumetric studies indicates the selective functional and structural changes of limbic structures such as the prefrontal cortex, anterior cingulate, and hippocampus in patients with MDD (Manji and Duman, 2001; Sheline, 2003; Drevets, 2004). The most frequently replicated finding in recurrent major depression is a small, but consistent reduction in hippocampal volume as documented by the *in vivo* magnetic resonance imaging) studies (Campbell et al., 2004; Videbech and Ravnkilde, 2004). The degree of volumetric decrease seems to be closely associated with the duration of the depressive episodes (Sheline et al., 1996, 1999; MacQueen et al., 2003). Even though it can not be excluded that smaller hippocampal volume might be trait characteristic for MDD (Neumeister et al., 2005), the reversibility of volume loss during recoveries in MDD has been described (Sheline et al., 1996), hence hippocampal volume is not necessarily stable throughout the lifespan.

However, hippocampal volume loss is not specific for depression: it has been observed in other stress-related psychiatric disorders, including posttraumatic stress disorder (PTSD) (Smith, 2005), borderline personality disorder with early abuse (Driessen et al., 2000) and probably with dissociative identity disorder (Vermetten et al., 2006) as well. Additionally, hippocampal shrinkage has been reported in various neuropsychiatric and neurological disorders, such as schizophrenia (Heckers, 2001), dementia, Alzheimer's, Huntington's disease, epilepsy, chronic alcohol dependence, herpes simplex encephalitis and traumatic brain injury (Geuze et al., 2005).

Various classical and recent clinical findings described the hyperactivity of the HPA axis in MDD (Swaab et al., 2005), especially in that with psychotic feature (Rihmer et al., 1984). Considering the findings

that depressed patients are frequently hypercortisolemic and that the cortisol level during human aging can predict hippocampal atrophy and memory loss (Lupien et al., 1998), it has been proposed that the neuroendocrine changes in depression may account for the reduction of hippocampal size. Because of its more or less tight connection with the high levels of GCs, hippocampal shrinkage was often explained with the cortisol neurotoxicity hypothesis. According to this theory, dendritic atrophy and even neuronal death, due to elevated level of GCs, may form the cellular basis for hippocampal volume loss (Sapolsky, 2000). However, hippocampal volume loss can also be seen in schizophrenia, which is a disease not associated with enduring hypercortisolemia. Moreover, hippocampal volume reduction in schizophrenia seems to arise at least in part from neurodevelopmental abnormalities (Weinberger, 1999), and it is not necessarily related to neuronal death (Nelson et al., 1998). Furthermore, in patients with PTSD, decreased hippocampal volume is linked with normal or reduced plasma or urine glucocorticoid levels (Yehuda, 2001). Thus, it is likely that diverse cellular mechanisms underlie hippocampal shrinkage in the different psychiatric disorders. In MDD, stress and GCs may have the most decisive impact on cellular changes, although their direct cytotoxic effect has not been unambiguously proved so far.

Since cortisol level rises throughout the life, the age-related susceptibility to stress observed in the tree shrew model might have clinical relevance. However, one should be very careful when extending data from animal models to humans, particularly because the distribution of corticosteroid receptors in primates highly differs from those in rodents. Sanchez et al. (2000) demonstrated very low levels of glucocorticoid receptor (GR) in the primate hippocampal formation, particularly in the Ammon's horn subfields (CA1/2 and CA3), where high levels of mineralocorticoid receptor (MR) were detected. The hypothesis that the GR:MR ratio reflects regional susceptibility to stress or GCs (de Kloet et al., 1993) and that MRs may be neuroprotective in response to excitotoxic challenge (McCullers and Hermann, 1998) can serve as a possible explanation to the negative findings of postmortem histopathological studies in humans and non-human primates chronically exposed to elevated levels

of cortisol. A postmortem study of chronically cortisol treated aged nonhuman primates could not detect any hippocampal neuronal loss (Leverenz et al, 1999). The detection of hippocampal apoptosis in the brains of depressed, as well as GC-treated patients has found only minimal cellular changes in the hippocampus overall, with no changes in CA3 region (Lucassen et al., 2001a). This latter finding is in sharp contrast to preclinical data, which suggest that the CA3 pyramidal neurons are the most vulnerable to the deleterious effects of stress (Lucassen et al., 2006). Tree shrews, however, can be placed between primates and rodents (Martin, 1990). According to an *in situ* hybridization study, where the semiquantitative evaluation of the expression of MR and GR receptors were carried out by Meyer et al. (2001) in tree shrews, the GR:MR ratio was found to be far below 0.5 in almost all subregions of the hippocampal formation, indicating that the receptor distribution in tree shrews is more similar to that in primates. Indeed, similarly to findings in humans and nonhuman primates, elevated cortisol levels due to chronic psychosocial stress did not increased the incidence of apoptotic cell death or resulted in the loss of CA3 pyramidal neurons in tree shrew hippocampus (Vollmann-Honsdorf et al., 1997; Lucassen et al., 2001b).

In summary, excitotoxic neuronal cell death does not provide definite explanation to the hippocampal volume loss observed in depressed patients. The findings, presented in this thesis, rather support the involvement of astroglial cells in hippocampal shrinkage, beside the contribution of the well-documented dendritic atrophy of neurons.

Clinical interpretation of biological data related to neuroplasticity

The neuroplasticity hypothesis is a new approach to the neurobiology of depression, integrating both traditional data (e.g. changes in monoamines or cortisol) and more recently acquired results, such as subcellular and structural changes in certain brain regions. The neuroplasticity hypothesis may provide a better understanding of the pathophysiology of depression and an explanation of the mode of action of antidepressant treatment. Furthermore, it offers a plausible explanation, to well-known clinical observations, like the increased risk of further

depressive episodes as the illness progresses, the easy relapse in patient with partial remission, or the occurrence of depressive episode during certain somatic illnesses or psychiatric conditions, e.g. depressive symptoms in fetal alcoholism syndrome, Parkinsons's disease, Alzheimer's disease, vascular infarction, diabetes, Cushing's syndrome, etc.

Hippocampal neurogenesis and depression

Adult hippocampal neurogenesis persists throughout the entire lifespan of mammals, including humans (Gross, 2000; Eriksson et al., 1998). Recently, various research groups suggested that disturbances of adult hippocampal neurogenesis play an important role in the etiology of major depressive disorder (Duman, 2004; Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006). Although this theory has been seriously questioned (Henn and Vollmayr, 2004), growing experimental evidence indicates that various classes of antidepressant treatments are able to stimulate dentate neurogenesis (Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006). Furthermore, a recent preclinical study suggested that the behavioral effects of chronic antidepressant treatment may be mediated by the stimulation of neurogenesis in the hippocampus (Santarelli et al., 2003).

However, the direct proof of this hypothesis remains to be established. The most convincing evidence for a central role of reduced neurogenesis would come from examination of the brains of depressed patients. However, no clinical data are available to directly prove the involvement of dentate neurogenesis in the development of depression. In a recent study, Reif et al. (2006) compared the number of newly generated cells in human postmortem hippocampal tissues: no significant decrease in the number of newborn neuronal cells could be detected in the dentate gyrus of patients with depression. In contrast, in patients with schizophrenia, a significant reduction in the incidence of neuronal renewal was found compared to control individuals. The limitation of this study is, nevertheless, that the majority of patients with MDD were treated with antidepressant drugs up to their death.

Translating the novel hypothesis of depression into a new approach to drug discovery

The neurotrophic hypothesis of depression is based on observations in animal models that chronic stress decreases expression of BDNF in the hippocampus, and antidepressant treatment can reverse it (Duman and Monteggia, 2006). These findings also suggest that changes in BDNF could mediate the hippocampal structural alterations in depression and the effects of antidepressant treatment. Chen et al. (2001) found reduced hippocampal BDNF immunreactivity in autopsy of patients with depression, whereas antidepressant treatment seemed to compensate this. Preclinical studies support the possibility that enhancement of BDNF signaling might have antidepressant effect (Russo-Neustadt and Chen, 2005), but BDNF has proved itself to be a difficult drug target. Targeting proteins in BDNF signaling cascades seems to be more promising, particularly if their action could be localized to brain circuits involved in depression (Berton and Nestler, 2006).

In addition to BDNF, other neurotrophic factors can also be a possible goal for the development of new antidepressant compounds. For example, several genes of the fibroblast growth factor (FGF) family have been shown to be down-regulated in the human hippocampus of patients with depression (Turner et al., 2006). FGF is an important regulator of hippocampal neurogenesis in rodents, but it is difficult to translate these findings into new treatment discoveries, mainly because of the complexity of their receptors and signaling pathways.

Finally, there are enthusiastic attempts to develop specific compounds which could directly stimulate dentate neurogenesis, however these efforts are still in their infancy.

Beside the trials involving the neurotrophic hypothesis of depression, there are a number of other directions in the pharmacological industry to develop novel antidepressant drugs. Among others the NK_1R antagonists should be mentioned here, which were expected to become the first completely new class of antidepressants. Despite the positive preclinical findings and initially encouraging clinical results, disappointment and doubt currently surrounds the idea that substance P receptor antagonists may

become effective antidepressants. Recently, two major drug companies terminated their leading substance P programs for treatment of affective disorders. A number of other drug companies continue, however, with the development of their own NK_1R antagonists. These compounds may show better pharmacokinetic and pharmacodynamic properties than those trialed to date and may therefore achieve success or, might become useful as adjunct therapy accelerating and / or potentiating other antidepressant drugs.

In summary, neuroplastic changes of the hippocampal formation are likely to contribute to the pathophysiology of mood disorders, in particular of MDD. This assumption is based on three main facts: 1) numerous MRI studies have detected hippocampal volume loss in MDD; 2) depression can be linked to stressful life events; 3) in animals exposed to chronic stress various cellular alterations occur in the hippocampus. One may speculate that similar cellular changes that are found in laboratory animals may underlie the hippocampal volume loss detected in MDD. Clearly, more histopathological studies examining the hippocampi of depressed patients are needed.

Needless to say, the exact mechanism of the therapeutic effect of antidepressant drugs currently available on the market is not yet fully understood. Preclinical investigations can be of particular interest, as they bring into light the cellular changes after antidepressant treatment. Findings of this thesis—in line with the results of other groups— prove that these agents have a clear effect on the structural and cellular plasticity in brain structures involved in affect regulation. Thus, animal studies may help us to understand the exact way of action of these drugs, and may help us to develop novel compounds, effective in the treatment of depression.

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AKNOWLEDGEMENTS

I am particularly indebted to my mentor, Mátyás Trixler, who always supported my work. Having been the previous head of the department, he actively shaped and guided my carrier, and added orientation points to my knowledge. He always provided a critical but judicious feedback about our work, which positively influenced our professional development.

I wish to thank János Szolcsányi, the head of the PhD school 'Theoretical Sciences', and Imre Szabó, the leader of the PhD program 'Behavioral Sciences'. They generate an intellectual atmosphere, which allows many candidates to be innovative and creative at our university. Many thanks to László Lénárd, who was my first mentor during my university years, made me familiar with experimental work, and founded my lifelong interest in and enthusiasm for neuroscience.

I also thank all my colleagues, past and present, at the Department of Psychiatry and Psychotherapy, who undertook effort and overwork during my long stay in Göttingen. I am particularly thankful to Tamás Tényi for shaping my professional thinking throughout years and showing me that the integration of several theoretical domains (including neuroscience) into the everyday clinical work is one of the most exciting challenges of the psychiatry. He actively supported this dissertation with his integrative view on the psychological and biological etiology of major psychiatric disorders. Special thanks to Sándor Fekete, the current head of our department, who encouraged me–when I was hesitating–to 'hit the road' and to go to Göttingen.

Many thanks to all students, postdoctoral fellows and technical assistants of the 'Arbeitsgruppe Fuchs' of the Clinical Neurobiology Laboratory at the German Primate Center, Göttingen, particularly to the veterinary doctor, Barthel Schmelting, with whom I worked many hours in the animal facility, and who taught me useful tricks for dealing with and stressing the animals. Olga Pudovkina and Marieke van der Hart provided me important practical guidance for the behavioral analysis, and sufficient

emotional support for my futile war with electric equipments and computer software.

I am most grateful to Eberhard Fuchs, the head of the Clinical Neurobiology Laboratory in Göttingen, and Boldizsár Czéh, who most directly contributed to this dissertation. Besides allowing me to join his study group, Eberhard Fuchs provided a valuable practical and theoretical background inevitable to my work. He was always ready to share with me his comprehensive experience, and he taught me how to be keen on neuoroendocrinology. He presented that it is not impossible to be a pragmatist and a theorist at once. At last, but by no means at least, I am appreciative to Boldizsár Czéh. He introduced me in neuroanatomy and stereology; guided my practical work in the laboratory, and never gave up answering my endless questions on the relation between neuroanatomy and psychiatry. He made me understood that one can be disciplined and exact without necessarily becoming narrow-minded or boring.

LIST OF PUBLICATIONS

I. Publications related to the thesis

Scientific papers:

1. Czéh B, **Simon M** (2005) Neuroplaszticitás és depresszió. *Psychiatr Hung* 20: 4-17
2. Czéh B, **Simon M**, van der Hart MGC, Schmelting B, Hesselink MB, Fuchs E (2005) Chronic stress decreases the number of parvalbumin-immunreactive interneurons in the hippocampus: prevention by treatment with a substance P receptor (NK_1) antagonist. *Neuropsychopharmacology* 30: 67-79

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