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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.

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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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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

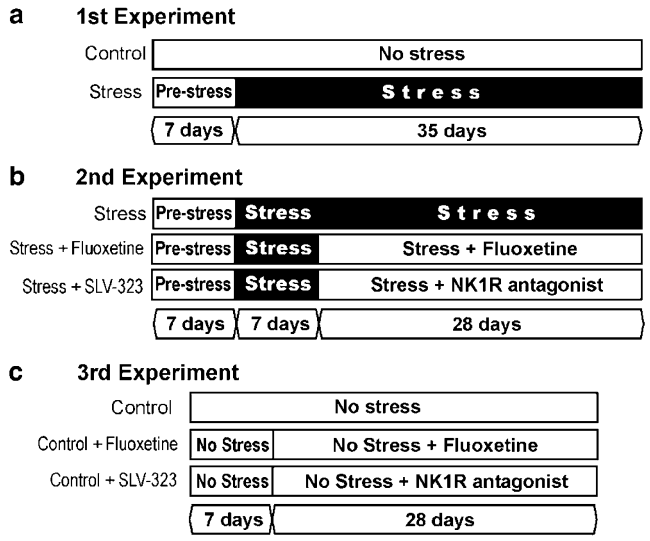

Figure 1 Experimental design. (a) In the first experiment a *Control* ($n=6$) and a *Stress* ($n=6$) group were compared, where animals of the *Stress* group were submitted to daily psychosocial conflict for 5 weeks and the *Control* group remained undisturbed. (b) In the second experiment a *Stress* 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

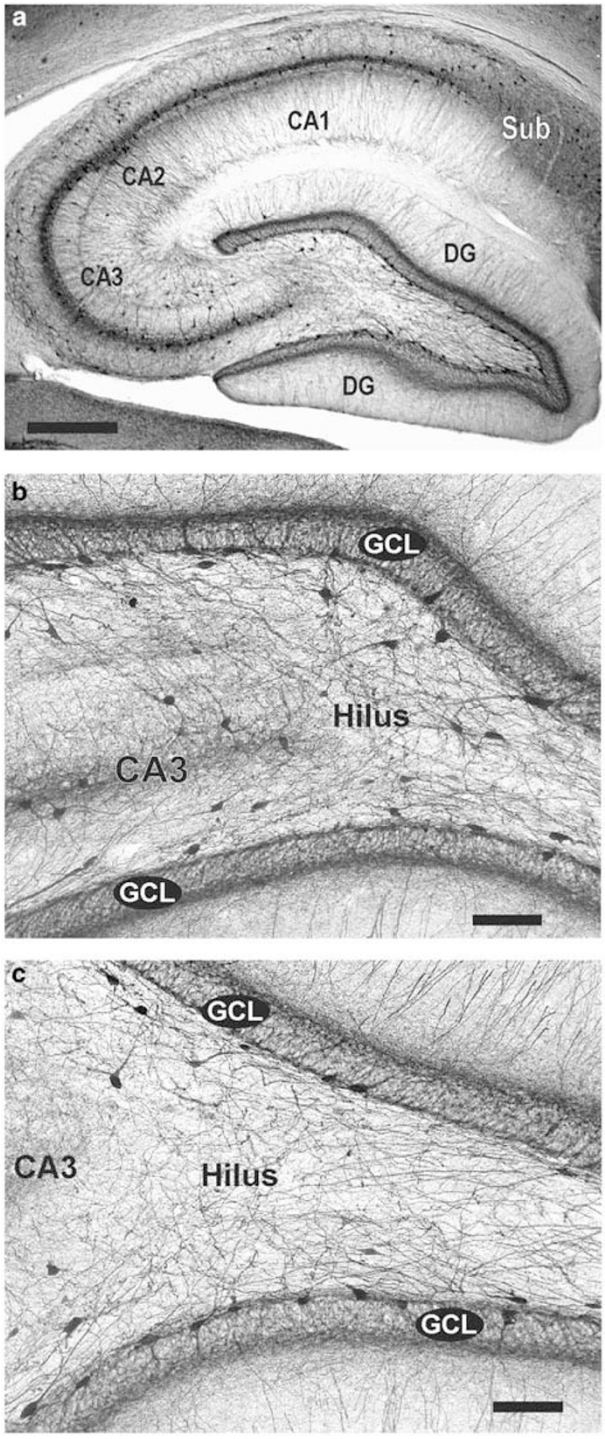

Figure 2 Representative example of a parvalbumin stained horizontal section of the tree shrew hippocampal formation (a). Parvalbumin-immunoreactive neurons were present exclusively as nonprincipal cells. Note that all subregions and layers are clearly distinguishable. Detailed images of the dentate gyrus 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

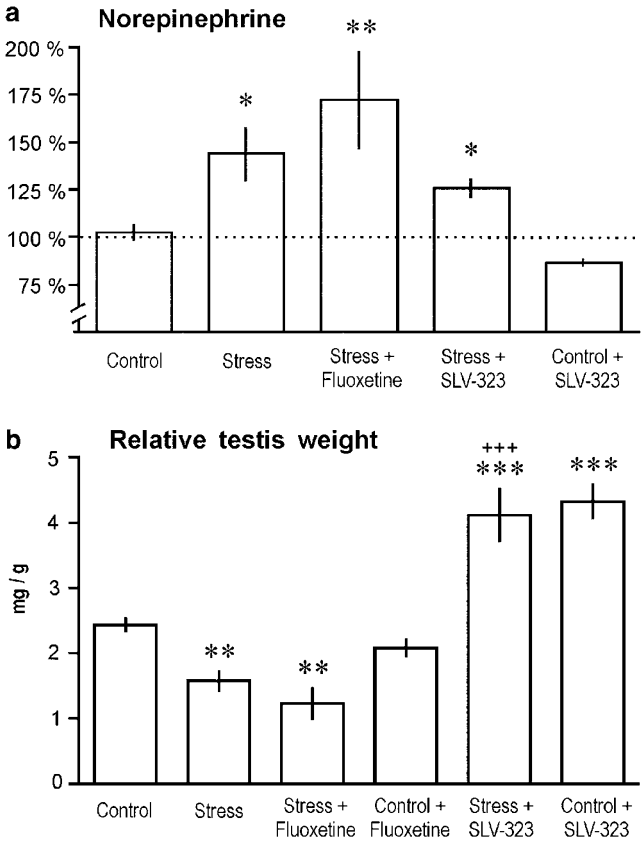

Figure 3 (a) Concentration of urinary norepinephrine. Long-term stress resulted in the sustained activation of the sympatho-adrenomedullary system, as indicated by the significant elevation of urinary norepinephrine levels in all stressed groups after the 5 weeks of social encounters. Horizontal dashed 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.

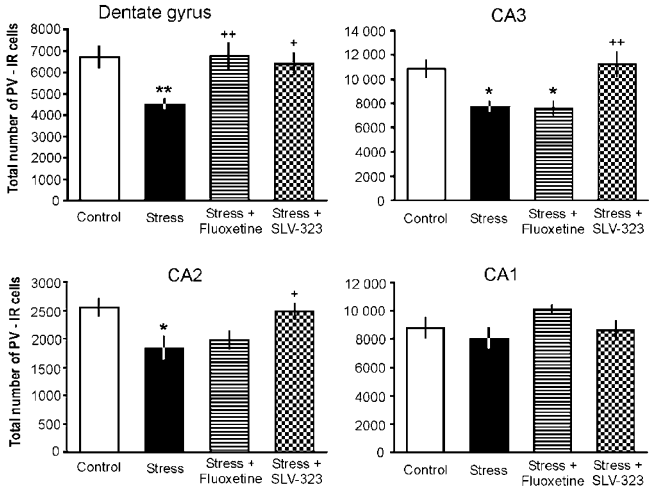

Figure 4 Effects of chronic psychosocial stress and concomitant antidepressant drug treatment on the total number of parvalbumin-immunoreactive (PV-IR) cells in the different hippocampal subregions. Stress significantly decreased the number of PV-IR cells in the dentate gyrus, CA2, and CA3, whereas the 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*	10 848	7696*	7575*	11 248##
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.

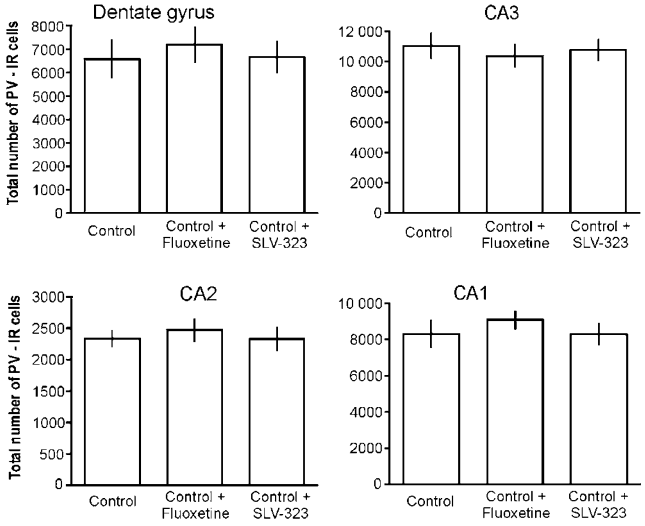

Figure 5 Chronic treatment with fluoxetine or the NK_1R antagonist SLV-323 did not affect the total number of parvalbumin-IR neurons in the hippocampi of control animals. Results are presented as mean ± SEM.

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 ($\sim$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.

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