Molecular Psychiatry (2004) 9, 278–286

www.nature.com/mp

ORIGINAL RESEARCH ARTICLE

Blockade of CRF_1 or V_{1b} receptors reverses stress-induced suppression of neurogenesis in a mouse model of depression

R Alonso*[1], G Griebel[2], G Pavone[1], J Stemmelin[2], G Le Fur[3] and P Soubrié[1]

[1]*Sanofi-Synthelabo Recherche, Rue du Professeur J. Blayac, Montpellier, France;* [2]*Avenue P.V. Couturier, Bagneux, France;* [3]*Avenue de France, Paris, France*

Repeated exposure to stress is known to induce structural remodelling and reduction of neurogenesis in the dentate gyrus. Corticotrophin-releasing factor (CRF) and vasopressin (AVP) are key regulators of the stress response *via* activation of CRF_1 and V_{1b} receptors, respectively. The blockade of these receptors has been proposed as an innovative approach for the treatment of affective disorders. The present study aimed at determining whether the CRF_1 receptor antagonist SSR125543A, the V_{1b} receptor antagonist SSR149415, and the clinically effective antidepressant fluoxetine may influence newborn cell proliferation and differentiation in the dentate gyrus of mice subjected to the chronic mild stress (CMS) procedure, a model of depression with predictive validity. Repeated administration of SSR125543A (30 mg/kg i.p.), SSR149415 (30 mg/kg i.p.), and fluoxetine (10 mg/kg i.p.) for 28 days, starting 3 weeks after the beginning of the stress procedure, significantly reversed the reduction of cell proliferation produced by CMS, an effect which was paralleled by a marked improvement of the physical state of the coat of stressed mice. Moreover, mice subjected to stress exhibited a 53% reduction of granule cell neurogenesis 30 days after the end of the 7-week stress period, an effect which was prevented by all drug treatments. Collectively, these results point to an important role of CRF and AVP in the regulation of dentate neurogenesis, and suggest that CRF_1 and V_{1b} receptor antagonists may affect plasticity changes in the hippocampal formation, as do clinically effective antidepressants.
Molecular Psychiatry (2004) **9,** 278–286. doi:10.1038/sj.mp.4001464
Published online 23 December 2003

Keywords: depression; hippocampus; vasopressin; corticotrophin

Despite extensive investigation, the mechanisms by which antidepressants exert their therapeutic effects are far from being fully understood. The dentate gyrus of the hippocampal formation is a brain region, which has focused much attention with respect to the effects of stress and the action of antidepressants. It is one of the few brain regions where adult neurogenesis has been documented in different species, including humans.[1] Neurogenesis is defined by the proliferation of progenitor cells, giving rise to cells that migrate into the granule cell layers, and ultimately differentiate into neurons.[2,3] Among the regulatory factors of neurogenesis, stressful events have been identified as potent inhibitors of dentate cell proliferation.[4–8] Chronic antidepressant treatment was reported to increase the rate of neurogenesis in adult brain.[9,10] Together, these findings led to the proposal that suppression of hippocampal neurogenesis in response to stress could be part of the structural remodelling occurring under pathological conditions and, accordingly, that restoration of this form of neural plasticity could be involved in the therapeutic effects of antidepressant treatment.[11–13] The precise mechanisms by which stress exerts these deleterious effects on hippocampal neurogenesis are unclear. However, this reduction appears to be mediated partly via stress-induced activation of the hypothalamic–pituitary–adrenal (HPA) axis, which results in the elevation of glucocorticoids.[14,15]

Intense attention is being given to antagonists of the CRF_1 or the V_{1b} receptor, the major CRF and AVP receptor subtypes, respectively, involved in the regulation of the HPA axis.[16,17] Both CRF_1[18,19] and V_{1b}[20,21] receptor antagonists were found to inhibit acute stress-induced adrenocorticotropin hormone (ACTH) secretion and exert clear anxiolytic- or antidepressant-like effects in rodents. For example, recent findings from our laboratory have demonstrated that the CRF_1 receptor antagonist SSR125543A and the first nonpeptide V_{1b} receptor antagonist SSR149415 were able to improve the degradation of the physical state, anxiety, despair, and the loss of

*Correspondence: Dr R Alonso, Sanofi-Synthelabo Recherche, Rue du Professeur J. Blayac, Montpellier 34000, France.
E-mail: richard.alonso@sanofi-synthelabo.com
Received 04 July 2003; revised 26 September 2003; accepted 06 October 2003

coping behaviour produced by unpredictable repeated stress in mice.[21,22] Moreover, the antidepressant paroxetine was recently shown to reduce hypothalamic AVP mRNA overexpression occurring in anxious rats, suggesting that vasopressinergic systems are likely to be critically involved in the behavioural and neuroendocrine action of antidepressants.[23] Together, these findings suggest that blockade of certain CRF and AVP receptor subtypes may represent an innovative approach for treatment of depression.

The present study aimed at determining whether repeated administration of SSR125543A or SSR149415 influences newborn cell proliferation and differentiation in the dentate gyrus of adult mice exposed to chronic mild stress (CMS). The CMS procedure has been proposed as a model of depression, in that it satisfies some criteria for face, predictive, and construct validity.[24,25] Although the effects of antidepressants on neurogenesis in normal[9,10] or stressed[26,27] animals have been investigated previously, no attempt was made so far to draw a parallel between reduced neurogenesis and appearance of depressive-like symptoms in animal models. Therefore, since CMS causes the occurrence of physical abnormalities reminiscent of certain symptoms of human depression, we measured physical state over the entire stress and treatment period. For comparison purpose, the clinically effective antidepressant fluoxetine was included in this study.

Drug treatment started once animals displayed maximal degradation of the physical state, and continued during the following 4 weeks in parallel with stress exposure.[21,22] Proliferation and phenotypic development of the newborn cells were determined using 5-bromo-2′-deoxyuridine (BrdU) immunohistochemistry and specific markers of neuronal and glial cells.

Materials and methods

Animals

A total of 137 male BALBc mice weighing 17–32 g (Charles River Breeding Laboratories, Iffa Credo or Janvier) were used in the present study. They were housed singly, maintained under standard laboratory conditions and kept on a 12-h light/dark cycle with light onset at 0600 h. All experimental procedures described herein were approved by the Animal Care and Use Committee of Sanofi-Synthelabo Recherche, and fully comply with French legislation on research involving animal subjects.

Compounds

SSR125543A, SSR149415, and fluoxetine were synthesized by Sanofi-Synthelabo Recherche, France. Compounds were prepared as solutions or suspensions in physiological saline or distilled water containing 0.1% Tween 80 (fluoxetine) or 5% dimethyl sulphoxide (DMSO) plus 5% Cremophor EL (SSR125543A and SSR149415), and given in a constant volume of 20 ml/kg.

CMS and drug treatments

This test was performed as described previously.[21,22] Briefly, the CMS procedure consists of the sequential application of a variety of mild stressors, including restraint, forced swimming, water deprivation, and pairing with another stressed animal, in a schedule that lasts for 3 weeks, and is repeated thereafter. Parallels between human depression and chronically stressed animals have been drawn from the reduction of the efficiency with which even the smallest tasks are accomplished in depressed patients, leading to the inability to maintain minimal personal hygiene, and the decrease in grooming behaviour seen in stressed animals. In this latter case, there is a degradation of the physical state of the coat, consisting of a loss of fur and dirty fur. Thus, physical state was measured about once a week over the entire 7-week stress period using a scale from 1 to 3: a health state was noted 3 and damaged state with piloerection and/or dirty fur was noted 1. Intermediate state was noted 2. Each measure was scored by an experimenter unaware of the treatment group. In stressed mice, fluoxetine (10 mg/kg), SSR125543A (30 mg/kg), SSR149415 (30 mg/kg) or vehicle (0.1% Tween 80 and 5% DMSO-5% Cremophor EL) was administered i.p. once a day for 28 days, starting 3 weeks after the beginning of the CMS. The doses were chosen on the basis of our previous experiments in mice showing that fluoxetine and SSR125543A or SSR149415 had a significant impact on behaviour at 10 and 30 mg/kg, respectively.[21,22] Control, nonstressed animals received similar treatments. Six to eight mice from each group were randomly selected for histological analysis.

BrdU injections

For analysis of cell proliferation, mice received a single i.p. injection with 75 mg/kg of BrdU (Sigma-Aldrich) 3 weeks after the beginning of stress or on the last day of the 7-week stress period (Figure 1a). Animals were anaesthetized with sodium pentobarbital (80 mg/kg i.p.) 24 h later and perfused transcardially with saline, followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). This survival time allows for completion of at least one cell cycle by cell in S phase at the time of BrdU injection.[28] For determination of newborn cell differentiation, mice received three daily consecutive injections of BrdU (3×75 mg/kg every 2 h) at the end of the 7-week stress period, and were killed 30 days after the last BrdU injection (Figure 1b).

Histological procedures

After overnight postfixation of brains in 4% paraformaldehyde, a vibratome was used to collect 40-μm coronal sections through the rostro-caudal extent of the hippocampus. Every sixth section from each brain was processed for immunohistochemistry. Adjacent sections were stained with haematoxylin for volumetric measurements. Additional sections were collected through the striatum (0.7–0.9 mm anterior to

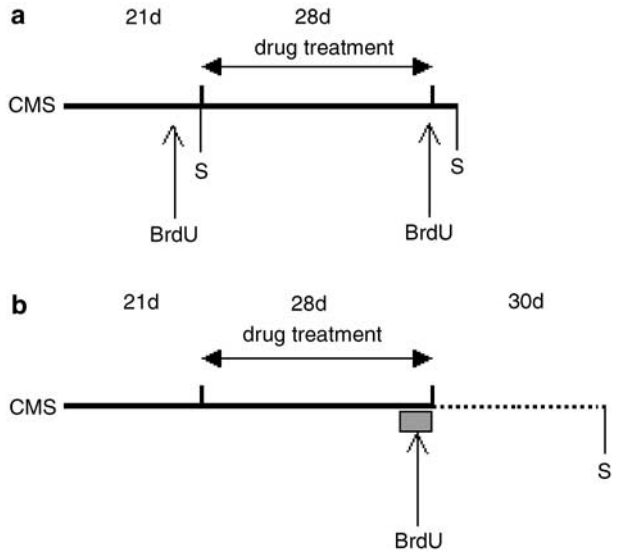

Figure 1 Schematic representation of the experimental procedure. Mice were subjected to a variety of mild stressors (CMS) during 7 weeks. Drugs were administered once a day for 28 days, starting 3 weeks after the beginning of the CMS. For analysis of cell proliferation (a), a first group of mice received a single injection of BrdU (75 mg/kg i.p.) on day 20 of the CMS. A second group was administered with BrdU on the last day of the 7-week stress period. Animals were killed (S) 24 h after BrdU administration. In experiments designed to determine newborn cell differentiation (b), mice received daily injections of BrdU (3×75 mg/kg i.p. every 2 h) the last 3 days of the 7-week stress period, and were killed 30 days after the last BrdU administration.

the bregma according to the atlas of Franklin and Paxinos[29]) to investigate BrdU labelling in the subventricular zone. BrdU immunohistochemistry was performed on free-floating sections according to Lemaire *et al*[7] with minor modifications. Briefly, sections were treated with 2 N HCL (30 min at 37°C), rinsed in borate buffer for 5 min (0.1 M, pH = 8.4), and incubated (24 h at 4°C) with a monoclonal mouse anti-BrdU antibody (1 : 200; Dako, Carpinteria, CA, USA) in 0.1 M PBS containing 0.3% Triton X-100 and 1% normal horse serum. Immunohistochemistry was completed by using a biotinylated horse anti-mouse antibody (1 : 200; Vector Laboratories, Burlingame, CA, USA) and the diaminobenzidine visualization method (Elite ABC Kit, Vector).

The neuronal or glial phenotype of newborn cells was determined using double immunofluorescence labelling. Briefly, sections were processed as described above, except that horse serum was replaced by 1% normal goat serum. Alternate sections were incubated (24 h at 4°C) with a rat anti-BrdU monoclonal antibody (1/200, Accurate, Chemical, Westbury, NY, USA) and one of the following antibodies: mouse anti-neuronal nuclei (NeuN, 1/200, Chemicon, Temecula, CA, USA) or rabbit anti-glial fibrillary acidic protein (GFAP) (1/200, Sigma-Aldrich). After they were washed in PBS (0.1 M), sections were incubated for 2 h with fluorochrome-labelled secondary antibodies (all generated in goat): Alexa 546 anti-rat, Alexa 488-anti-mouse or Alexa 488-ant-rabbit (Molecular-Probes, Eugene, OR, USA). Sections were examined under a Leica confocal scanning laser microscope (TCS-NT) using a $\times 40$ oil-immersion lens and a 3.8 numerical zoom. Images were acquired sequentially in a line-scanning mode through an optical section of 1 μm in the *z*-axis, and merged using the Leica TCS 4D software. Colocalization with NeuN or GFAP was determined in 50–80 BrdU-labelled cells per mice.

Quantitative evaluation of staining

Hippocampal BrdU labelling was quantified according to a modified unbiased stereology protocol.[10,26,30,31] Every sixth section through the rostro-caudal extent of the hippocampus was examined. All BrdU-labelled cells in the subgranular zone and the granule cell layer, defined as a two-body-wide zone along the border of the granule cell layer, were counted in each section by two experimenters unaware of the slide code. Counts were performed at $\times 400$ and $\times 1000$ magnifications using a light microscope (Leica), cells in the outermost focal plane being omitted. The total number of BrdU-labelled cells per dentate gyrus was estimated by multiplying the number of cells counted in every sixth section by 6. BrdU immunoreactive cells were also counted within a $300 \times 300\ \mu m^2$ area positioned in the dorsolateral corner of the subventricular zone, and results from 5–6 sections per animal are expressed as the mean counts.

The volume of the entire right hippocampus (hippocampus proper plus dentate gyrus) and the granule cell layer was estimated using computer-assisted image analysis (Samba Technologies, Meylan, France) on the basis of the Cavalieri principle. Every sixth section was analysed, starting at a random position. Structures were outlined, and the computed areas were summed and multiplied with the inter-section distance and with the thickness of the sections. Volumes were expressed as mm^3.

Statistical analysis

Data referring to the physical state of the coat were analysed by a two-way ANOVA (treatment $\times$ week) with repeated measures, followed by Newman–Keuls *post hoc* test. Data from histological procedures were analysed by one- or two-way ANOVA (treatment $\times$ stress), followed by Newman–Keuls *post hoc* comparison.

Results

SSR125543A, SSR149415, and fluoxetine improved stress-induced degradation of the physical state of mice

Two-way ANOVA with repeated measures showed a significant effect for physical state ($F_{24,492} = 8.81$, $P < 0.001$). Further analysis indicated a significant

degradation of the physical state of the coat of mice due to stress. The index of physical degradation was maximally decreased 3 weeks (Figure 2) after the beginning of the stress procedure ($P<0.001$). In the vehicle-treated group, the effect of stress lasted until the end of the 7-week CMS ($P<0.001$). In contrast, the degradation of the physical state of the animal's coat was significantly improved by SSR125543A, SSR149415, and fluoxetine, following 1 week of treatment, an effect that lasted until the stress period was completed (Figure 2). Moreover, 3 (SSR125543A) or 4 (SSR149415 and fluoxetine) weeks after the beginning of drug treatment, animals showed comparable physical state as nonstressed mice (Figure 2). The physical state of nonstressed control mice was also weakly diminished at the end of the 7-week period. In fact, these animals needed to be isolated during the study to prevent fighting, which was intense in pilot experiments. Although not as severe as repeated unpredictable stress, animal isolation is considered to be a stressor.

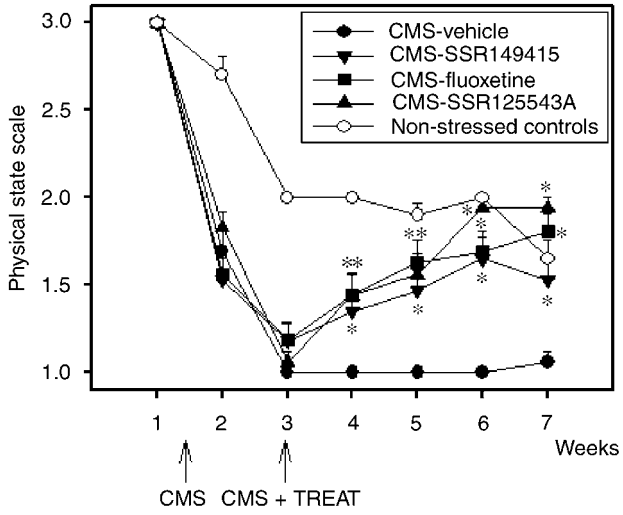

Figure 2 Effects of repeated administration of fluoxetine, SSR125543A, and SSR149415 on the degradation of the physical state of the coat of animals in chronically stressed mice. Data represent mean ± s.e.m. $^{**}P<0.01$ and $^{*}P<0.05$ (*vs* vehicle-treated stressed animals (filled circle)).

SSR125543A, SSR149415, and fluoxetine reversed stress-induced reduction of cell proliferation

The majority of BrdU-labelled cells were located within the subgranular zone of the dentate gyrus where they appeared either singly or in clusters (Figure 3a). After 3 weeks of stress, the number of

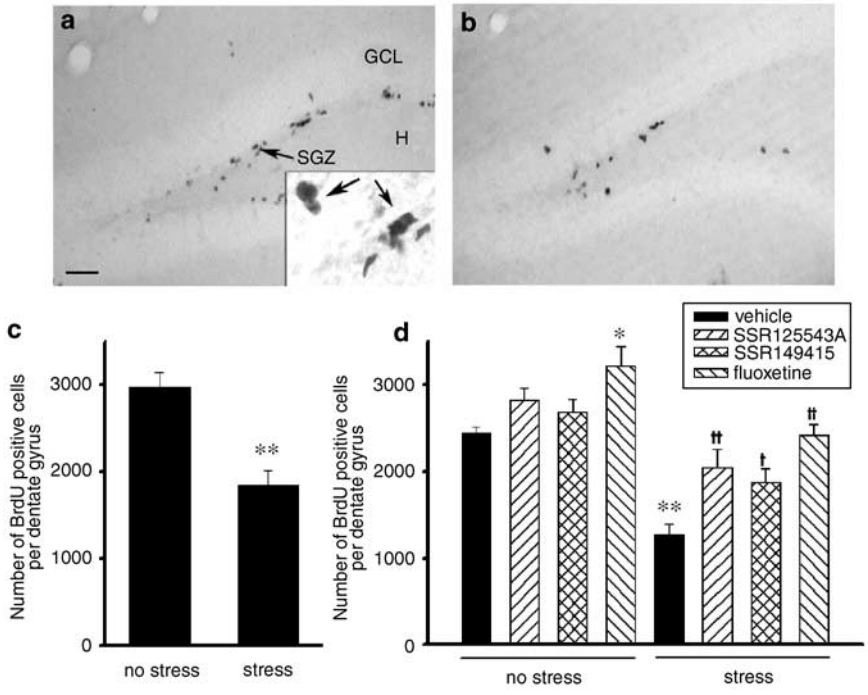

Figure 3 SSR125543A, SSR149415, and fluoxetine reversed stress-induced reduction of cell proliferation. Mice received BrdU 3 weeks after the beginning of stress or on the last day of the 7-week stress period and were killed 24 h later. (a, b) Representative photomicrographs from nonstressed (a) and stressed (b) mice after the 3-week stress period. The majority of BrdU-positive cells are located in the subgranular zone (SGZ) of the dentate gyrus, where they appeared singly or in clusters (arrows in (a)-inset). Scale bar = 100 μm. GCL: granule cell layer; H: hilus. (c, d) The number of BrdU-positive cells is reduced in mice stressed for 3 (c) or 7 weeks (d). Treatment with fluoxetine, SSR125543A or SSR149415 during the last 4 weeks reversed stress-induced reduction of cell proliferation, while fluoxetine, but not SSR125543A or SSR149415, increased cell proliferation in nonstressed mice (d). The results are mean ± s.e.m. of the number of BrdU-positive cells per dentate gyrus ($n=6$–8 per group). $^{**}P<0.01$ and $^{*}P<0.05$: significantly different from vehicle-treated, nonstressed mice; $\dagger\dagger P<0.01$ and $\dagger P<0.05$: significantly different from vehicle-treated, stressed mice.

BrdU immunoreactive cells in the dentate gyrus of stressed mice was significantly decreased by 38% ($F_{1,15}=21$, $P<0.01$) as compared to nonstressed mice (Figure 3b, c).

The proliferation of newborn cells was then evaluated at the end of an additional period of 4 weeks, during which mice were treated with SSR125543A, SSR149415 and fluoxetine (Figure 3d). Two-way ANOVA (stress × drug treatment) revealed a significant main effect for stress ($F_{1,47}=88.56$, $P<0.01$) and drug treatment ($F_{3,47}=9.98$, $P<0.01$). The CMS produced a 44% decrease ($P<0.01$) of the number of BrdU-labelled cells in vehicle-treated stressed mice relative to control nonstressed animals. Further analysis in stressed animals revealed that treatment with fluoxetine, SSR1255443A or SSR149415 produced a significant elevation of the number of BrdU immunoreactive cells as compared to control-stressed mice (SSR125543A: +60%, $P<0.01$; SSR149415: +46%, $P<0.05$; fluoxetine: +68%, $P<0.01$). Comparison of BrdU labelling in nonstressed mice showed that fluoxetine, but not SSR125543A or SSR149415, significantly increased the number of dentate BrdU-positive cells (Figure 3d).

Given that the reversion of stress-induced reduction of cell proliferation by drug treatment was paralleled by a marked improvement of the physical appearance of stressed mice, additional analysis was performed to access whether the two parameters were correlated, assuming that the correlation between the physical state and the number of BrdU-immunoreactive cells is independent of the treatment group. The BrdU labelling data from each animal killed at the end of the 7-week stress period were plotted against the corresponding physical state value, and analysis was performed on the whole set of data, regardless of the group distinction (stressed and nonstressed mice, either treated or not; $n=48$). The nonparametric Spearman correlation coefficient revealed a highly significant correlation ($r=0.76$, $P<0.01$) between the two parameters.

To determine whether stress or drug treatment effects on newborn cell proliferation were specific to the dentate gyrus, we examined BrdU labelling in another brain region known to contain progenitor cells, namely the subventricular zone of the lateral ventricle.[32] As shown in Table 1, no significant modification of the number of BrdU immunoreactive cells was observed in this brain region after the 3- or 7-week stress period, nor by drug treatment in stressed or unstressed mice.

Table 1 Lack of effect of stress and drug treatments on cell proliferation in the subventricular zone

	Number of BrdU-positive cells	
	3-week stress period	*7-week stress period*
Control		
Vehicle	107±14	115±8
SSR125543A	—	108±8
SSR149415	—	93±17
Fluoxetine	—	134±19
Stress		
Vehicle	135±13	93±7
SSR125543A	—	139±22
SSR149415	—	118±14
Fluoxetine	—	89±10

Fluoxetine (10 mg/kg), SSR125543A (30 mg/kg), and SSR149415 (30 mg/kg) were administered i.p. once a day for 28 days, starting 3 weeks after the beginning of the CMS. Mice were injected with BrdU on the last day of the 3-week or the 7-week stress period and were killed 24 h later. The results are the mean±s.e.m. of the number of BrdU-positive cells ($n=8$ and 6 mice per group for the 3- and 7-week stress period, respectively). There was no significant effect for the number of BrdU-positive cells after the 3- ($F_{1,15}=2.09$, $P>0.05$) or 7-week stress period (two-way ANOVA; stress: $F_{1,47}=3.31$, $P>0.05$; drug treatment: $F_{3,47}=0.77$, $P>0.05$).

SSR125543A, SSR149415, and fluoxetine prevented stress-induced reduction of neurogenesis

The number and phenotype of dentate BrdU-labelled cells was determined 30 days after the last BrdU administration, a time interval sufficient to allow newly generated cells to migrate and differentiate.

At this time, BrdU-positive cells were almost concentrated within the granule cell layer and their absolute number in nonstressed mice was lower than that evaluated 24 h after BrdU injection (number of BrdU-positive cells 24 h after BrdU: 2449±62, $n=6$; 30 days after BrdU: 1219±120, $n=4$). Analysis of BrdU labelling revealed a significant main effect for the number of BrdU-positive cells in the dentate gyrus ($F_{4,19}=3.64$, $P<0.05$). CMS produced a 57% decrease of the number of BrdU immunoreactive cells ($P<0.05$) in vehicle-treated mice relative to unstressed animals (Figure 4a), while in fluoxetine-, SSR125543A- or SSR149415-treated animals, the number of BrdU immunoreactive cells was significantly increased when compared to vehicle-treated mice (fluoxetine: +155%, $P<0.05$; SSR149415: +143%, $P<0.05$; SSR125543A: +96%, $P<0.05$).

The phenotype of BrdU-immunoreactive cells that survived 30 days after the end of the 7-week stress period was determined using specific markers for mature neurons (NeuN) and astrocytes (GFAP). Confocal microscopy analysis was carried out to verify that colocalization of fluorescent signals originated from the same cells. In nonstressed mice, 73% of BrdU-labelled cells expressed NeuN, and 9% displayed GFAP staining (Figure 4b, c), indicating that the population of surviving BrdU-positive cells essentially matures into neurons. There was no major difference in phenotypic expression patterns between

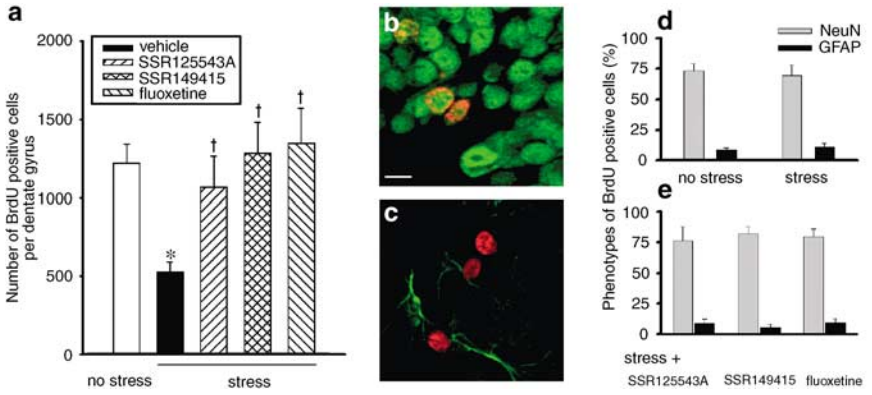

Figure 4 SSR125543A, SSR149415, and fluoxetine prevented stress-induced suppression of neurogenesis. Mice were subjected to a 7-weeks stress period and were treated with fluoxetine, SSR125543A or SSR149415 during the last 4 weeks. They were left undisturbed for an additional 30-days period. BrdU was pulsed during the 3 last days of the stress period. The number of surviving BrdU-positive cells in the dentate gyrus was severely decreased in stressed as compared to nonstressed controls (a). This stress-induced alteration was prevented by fluoxetine, SSR125543A, and SSR149415. Data are mean $\pm$ s.e.m. of the number of BrdU-positive cells per dentate gyrus ($n=4$ per group). *$P<0.05$: significantly different from nonstressed controls; †$P<0.05$: significantly different from vehicle-treated, stressed mice. (b, c) Representative confocal laser-scanning microscopic images in the dentate gyrus of a nonstressed mice showing red staining for BrdU and green staining for NeuN (b) or GFAP (c). Images, sequentially acquired using appropriated filters and merged, showed that BrdU immunolabelling is colocalized with NeuN, but not GFAP. Scale bar $=10\,\mu$m. In nonstressed and stressed mice, the majority (73%) of surviving BrdU immunoreactive cells matured into neurons (d). The phenotypic expression patterns remained unchanged in drug-treated mice (e).

stressed (vehicle or drug-treated) and control nonstressed animals (Figure 4d).

SSR125543A, SSR149415, and fluoxetine prevented stress-induced reduction in hippocampal volume

At the end of the 7-week stress period, there was a significant main effect for the entire right hippocampus volume ($F_{4,28}=4.42$, $P<0.05$). CMS produced a 11% decrease of the hippocampal volume ($P<0.05$) in stressed, vehicle-treated mice relative to unstressed animals, while in fluoxetine-, SSR125543A- or SSR149415-treated animals, the hippocampal volume was significantly increased ($P<0.05$) when compared to vehicle-treated mice (in mm^3; no stress: 20.44 ± 0.44; stress: 18.10 ± 0.27; stress + SSR125543A: 20.95 ± 0.92; stress + SSR149415: 20.35 ± 0.67; stress + fluoxetine: 21.36 ± 0.47). No changes in the volume of the granule cell layer were observed in any of the groups (in mm^3; no stress: 0.98 ± 0.03; stress: 0.93 ± 0.08; stress + SSR125543A: 0.92 ± 0.07; stress + SSR149415: 1.00 ± 0.05; stress + fluoxetine: 0.94 ± 0.05; $F_{4,28}=0.33$, $P>0.05$).

Discussion

The main findings of this study show that decreased neurogenesis induced by chronic stress (CMS) in mice is reversed by repeated treatment with the reference antidepressant fluoxetine, the CRF_1 receptor antagonist SSR125543A, and by the nonpeptide V_{1b} receptor antagonist SSR149415. Moreover, we show that dentate neurogenesis strongly correlates with the physical appearance of mice, likely to reflect depressive states in the CMS model.

The CMS paradigm in mice is a valid model of depression, as it satisfies the criteria of correlation, isomorphism, and homology.[24,25] Here, we confirm that chronically stressed mice exhibit a marked degradation of the physical state of the coat, an effect which lasted until the end of the stress period. This effect can be tentatively explained by a decrease in the animal's grooming in favour of coping behaviours, which are vital in a particularly stressful situation. This measure, which is easy to score, rapidly observed and reproducible, has been shown to be a good index of the response of mice to CMS. In line with the idea that stress-induced physical degradation may represent a valid measure of depression in mice are the present findings that repeated treatment with the antidepressant fluoxetine significantly improved the degradation of the physical state of the coat of stressed animals. Similar effects were obtained with SSR125543A and SSR149415. Previous experiments performed at the end of the CMS period have indicated that these compounds produce behavioural changes indicative of antidepressant-like activity.[21,22] For example, we have shown that repeated administration of SSR149415 improved despair and the loss of coping behaviour produced by stress.[22] The present data, which are in line with our previous findings on the antidepressant-like properties of the CRF_1 and V_{1b} receptors antagonists,[21,22] provide further evidence for the efficacy of these drugs in models of depression, notably in the CMS procedure.

Our results show that repeated stress severely reduced the rate of newborn cell proliferation, as evidenced by the marked decrease in the number of BrdU-labelled cells in the dentate gyrus. The stress-induced alteration was observed after the initial 3-week stress period, and was maintained throughout the entire CMS procedure. These results are in line with recent evidence highlighting the deleterious effect of stressful events on hippocampal newborn cell proliferation in various animal species. Profound alteration of cell proliferation was reported following predator odour exposure in rats,[6] social stress in marmosets[5] and tree shrews,[4] and prenatal or repeated restraint stress in rats.[7,8] The stress-induced reduction of BrdU labelling was not seen in animals treated by fluoxetine and the CRF_1 and V_{1b} receptor antagonists. The finding that cell proliferation rate was maximally affected by stress before drug treatment started suggests that SSR125543A, SSR149415, and fluoxetine were able to reverse the suppression of cell proliferation. Interestingly, this effect parallels the time course of physical state modifications. Since chronic, but not short-term, treatment is required for antidepressants to stimulate cell proliferation,[9,10] further characterization of the time course of newborn cell proliferation under blockade of CRF_1 or V_{1b} receptors is the focus of current investigation. So far, antidepressant drugs such as fluoxetine were shown to stimulate newborn cell proliferation in unstressed rat,[9,10] a finding confirmed here in mice. However, it is noteworthy that, unlike fluoxetine, SSR125543A and SSR149415 did not significantly stimulate cell proliferation in unstressed animals, thus indicating a specific action of these drugs under stressful conditions.

Our data show that modifications of BrdU immunolabelling following stress or drug treatment occurred in the dentate gyrus but not in the subventricular zone, another brain region known to contain progenitor cells in adulthood.[32] In addition to further highlighting the critical impact of stress and antidepressants on hippocampal functioning,[33,34] these results suggest that changes of BrdU labelling were unlikely to be caused by a modification in the amount of BrdU entering in the brain or into the DNA of dividing cells.

Over time, progenitor cells in the dentate gyrus give rise to cells that migrate into the granule cell layers and ultimately differentiate into mature neurons or astroglia.[35–38] Since many newborn cells do not survive, the total number of BrdU immunoreactive cells found here 30 days after BrdU administration was lower than that evaluated after a 24 h time interval, as reported previously.[9,37] We show that, at this later time, the chronic stress still markedly reduced the number of BrdU-labelled cells, an effect prevented by all drug treatments. In agreement with previous data,[7–9,35,37,38] the population of surviving BrdU-positive cells essentially matured into neurons, as evidenced by the great majority of BrdU immunoreactive cells that expressed the neuronal (NeuN), as compared to the glial (GFAP) marker. Moreover, this phenotypic expression pattern remained unchanged between groups. Together, these findings indicate that the incorporation of new neurons into the dentate gyrus was severely impaired by chronic stress and, importantly, that the CRF_1 or V_{1b} receptor antagonists not only reversed stress-induced reduction of cell proliferation, but they also normalized hippocampal neurogenesis. These results point for the first time to a critical role of CRF and AVP in stress-induced alteration of hippocampal neurogenesis, a finding consistent with the involvement of these neuropeptides on behavioural responses to stress.

Studies in animals have demonstrated that, in addition to their stimulatory effect on dentate neurogenesis, antidepressants attenuate morphological and structural changes in the hippocampus, such as dendritic remodelling in CA3 pyramidal neurons and volume reduction that occurred following stress exposure.[26,27,39,40] Moreover, a decrease of the hippocampal volume has been documented in patients suffering from recurrent major depression and post-traumatic stress disorders.[41,42] The present finding that SSR125543A and SSR149415 prevented the reduction of hippocampal volume produced by repeated stress suggests that these compounds may affect neural plasticity in the hippocampus, as do clinically effective antidepressants. Moreover, the fact that the volume of the granule cell layer remained unchanged following stress and drug treatment indicates that the decrease of dentate cell proliferation does not contribute to the observed reduction of hippocampal volume.

The molecular and cellular mechanisms underlying the actions of SSR125543A and SSR149415 on hippocampal neurogenesis remain to be fully elucidated. Antidepressants have been suggested to influence neural remodelling via upregulation of the cAMP response element-binding protein (CREB) cascades and expression of brain-derived neurotrophic factor (BDNF) in the hippocampus.[43] It can be speculated that CRF_1 or V_{1b} receptor antagonists produce their antidepressant effects through a similar action. Our preliminary findings in normal rats indicate that 3 weeks of administration of SSR149415 or SSR125543A increase the expression of CREB mRNA in the dentate gyrus (manuscript in preparation).

SSR125543A and SSR149415 are devoid of affinity for *N*-methyl-D-aspartate and 5-HT_{1A} receptors, that are known to be implicated in the modulation of hippocampal neurogenesis.[4,44,45,46] Both drugs have been shown to decrease CRF and AVP functions at the level of the HPA axis. Following exposure to psychological stress, AVP is secreted into the median eminence and is transported to the pituitary portal circulation, where it strongly potentiates the effects of CRF on ACTH release.[17] Both SSR125543A and SSR149415 were found to inhibit ACTH secretion induced by acute restraint stress.[20,47] Moreover, elevated levels of adrenal steroids have been shown

to profoundly reduce dentate cell proliferation.[14,15] Based on these findings, the action of SSR125543A and SSR149415 on hippocampal neurogenesis may be partly explained by a reduction of stress-induced hyperactivity of the HPA axis. However, the possibility that extrahypothalamic sites may be involved cannot be totally discarded since anatomical and biochemical data have characterized V_{1b} and CRF_1 receptors in brain structures associated with the integration and transduction of stressful stimuli, such as the amygdala, the lateral septum, and the hippocampus itself.[48,49] In support of this idea is our recent finding showing that V_{1b} receptor blockade produces antidepressant-like activity in hypophysectomized animals, although the magnitude of these effects was weaker than that of intact subjects.[21]

Collectively, the present series of experiments demonstrate that antagonists of CRF_1 or V_{1b} receptors, which exert clear antidepressant-like effects, selectively reversed stress-induced alteration of hippocampal neurogenesis in mice. This suggests that stress peptides such as CRF and AVP play a central role in stress-induced alteration of hippocampal neurogenesis, and that pharmacological blockade of CRF_1 and V_{1b} receptors may provide an innovative approach for reversing altered brain plasticity.

Acknowledgements

We thank M Fournier, C Aliaga, H Lacroix, and Y Biron for technical assistance, and J Alexander for critical reading of the manuscript.

References

1 Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA *et al.* Neurogenesis in the adult human hippocampus. *Nat Med* 1998; **4**: 1313–1317.
2 Gross CG. Neurogenesis in the adult brain: death of a dogma. *Nat Rev Neurosci* 2000; **1**: 67–73.
3 Gould E, Gross CG. Neurogenesis in adult mammals: some progress and problems. *J Neurosci* 2002; **22**: 619–623.
4 Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci* 1997; **17**: 2492–2498.
5 Gould E, Tanapat P, McEwen BS, Flügge G, Fuchs E. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc Natl Acad Sci USA* 1998; **95**: 3168–3171.
6 Tanapat T, Galea LA, Gould E. Stress inhibits the proliferation of granule cell precursors in the developing dentate gyrus. *Int J Dev Neurosci* 1998; **16**: 235–239.
7 Lemaire V, Koehl M, Le Moal M, Abrous DN. Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. *Proc Natl Acad Sci USA* 2000; **97**: 11032–11037.
8 Pham K, Nacher J, Hof PR, McEwen BS. Repeated restaint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. *Eur J Neurosci* 2003; **17**: 879–886.
9 Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 2000; **20**: 9104–9110.
10 Manev H, Uz T, Smalheiser NR, Manev R. Antidepressants alter cell proliferation in the adult brain *in vivo* and in neuronal cultures *in vitro*. *Eur J Pharmacol* 2001; **411**: 67–70.
11 Duman RS, Malberg J, Thome J. Neural plasticity to stress and antidepressant treatment. *Biol Psychiatry* 1999; **46**: 1181–1191.
12 Jacobs BL, Praag H, Gage FH. Adult brain neurogenesis and psychiatry: a novel theory of depression. *Mol Psychiatry* 2000; **5**: 262–269.
13 Kempermann G. Regulation of adult hippocampal neurogenesis—implications for novel theories of major depression. *Bipolar Disord* 2002; **4**: 17–33.
14 Cameron HA, Gould E. Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* 1994; **61**: 203–209.
15 Rodriguez JJ, Montaron MF, Petry KG, Aurousseau C, Marinelli M, Premier S *et al.* Complex regulation of the expression of the polysialylated form of the neuronal cell adhesion molecule by glucocorticoids in the rat hippocampus. *Eur J Neurosci* 1998; **10**: 2994–3006.
16 Holsboer F. The rationale for corticotrophin-releasing hormone receptor (CRH-R) antagonists to treat depression and anxiety. *J Psychiatr Res* 1999; **33**: 181–214.
17 Aguilera G, Rabadan-Diehl C. Vasopressinergic regulation of the hypothalamic–pituitary–adrenal axis: implications for stress adaptation. *Regul Pept* 2000; **96**: 23–29.
18 Reul JM, Holsboer F. Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr Opin Pharmacol* 2002; **2**: 23–33.
19 Griebel G. Is there a future for neuropeptide receptor ligands in the treatment of anxiety disorders. *Pharmacol Ther* 1999; **82**: 1–61.
20 Serradeil-Le Gal C, Wagnon J, Simiand J, Griebel G, Lacour C, Guillon G *et al.* Characterization of (2S,4R)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidine carboxamide (SSR149415), a selective and orally active vasopressin V1b receptor antagonist. *J Pharmacol Exp Ther* 2002; **300**: 1122–1130.
21 Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, Scatton B *et al.* Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V_{1b} receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. *Proc Natl Acad Sci USA* 2002; **99**: 6370–6375.
22 Griebel G, Simiand J, Steinberg R, Jung M, Gully D, Roger P *et al.* 4-(2-Chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1, 3-thiazol-2-amine hydrochloride (SSR125543A), a potent and selective corticotrophin-releasing factor(1) receptor antagonist. II. Characterization in rodent models of stress-related disorders. *J Pharmacol Exp Ther* 2002; **301**: 333–345.
23 Keck ME, Welt T, Muller MB, Uhr M, Ohl F, Wigger A *et al.* Reduction of hypothalamic vasopressinergic hyperdrive contributes to clinically relevant behavioral and neuroendocrine effects of chronic paroxetine treatment in a psychopathological rat model. *Neuropsychopharmacology* 2003; **28**: 235–243.
24 Wilner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology* 1997; **134**: 319–329.
25 Kopp C, Vogel E, Rettori MC, Delagrange P, Misslin R. The effects of melatonin on the behavioural disturbances induced by chronic mild stress in C3H/He mice. *Behav Pharmacol* 1999; **10**: 73–83.
26 Czéh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, Van Kampen M *et al.* Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. *Proc Natl Acad Sci USA* 2001; **98**: 12796–12801.
27 Van De Hart MG, Czeh B, De Biurrun G, Michaelis T, Watanabe T, Natt O *et al.* Substance P receptor antagonist and clomipramine prevent stress-induced alteration in cerebral metabolites, cytogenesis in the dentate gyrus and hippocampal volume. *Mol Psychiatry* 2002; **7**: 933–941.
28 Takahashi T, Nowakowski RS, Caviness Jr VS. BUdR as an S-phase marker for quantitative studies of cytokinetic behaviour in the murine cerebral ventricular zone. *J Neurocytol* 1992; **21**: 185–197.
29 Franklin KBJ, Paxinos G. *The Mouse Brain in Stereotaxic Coordinates*. Academic Press: San Diego, 1997.

30 Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ. Opiates inhinbit neurogenesis in the adult rat hippocampus. *Proc Natl Acad Sci USA* 2000; **97**: 7579–7584.
31 West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in the subdivision of the rat hippocampus using the optical fractionator. *Anat Rec* 1991; **231**: 482–497.
32 Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. *J Neurosi* 2002; **22**: 629–634.
33 McEwen BS. Effects of adverse experiences for brain structure and function. *Biol Psychiatry* 2000; **15**: 721–731.
34 Kim JJ, Diamond DM. The stressed hippocampus, synaptic plasticity and lost of memories. *Nat Rev Neurosci* 2002; **3**: 453–462.
35 Cameron HA, Woolley CS, McEwen BS, Gould E. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience* 1993; **56**: 337–344.
36 Seki T, Arai Y. Highly polysialylated neural cell adhesion molecule (NCAM-H) is expressed by newly generated granule cells in the dentate gyrus of the adult rat. *J Neurosci* 1993; **13**: 2351–2358.
37 Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ. Learning enhances adult neurogenesis in the hippocampal formation. *Nat Neurosci* 1999; **2**: 260–265.
38 Cooper-Kuhn C, Kuhn HG. Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. *Brain Res Dev Brain Res* 2002; **134**: 13–21.
39 Watanabe Y, Gould E, Daniels DC, Cameron H, McEwen B. Tianeptine attenuates stress-induced morphological changes in the hippocampus. *Eur J Pharmacol* 1992; **222**: 157–162.
40 Magarinos AM, Deslandes A, McEwen BS. Effects of antidepressants and benzodiazepine treatments on the dendritic structure of CA3 pyramidal neurons after chronic stress. *Eur J Pharmacol* 1999; **371**: 113–122.
41 Sheline YI, Wany P, Gado MH, Csernansky JG, Vannier MW. Hippocampal atrophy in recurrent major depression. *Proc Natl Acad Sci USA* 1996; **93**: 3908–3913.
42 Bremmer JD, Randall P, Scott TM, Bronen RA, Seibyl JP, Southwick SM *et al*. MRI-based measurement of hippocampal volume in patients with combat-related post-traumatic stress disorder. *Am J Psychiatry* 1995; **152**: 973–981.
43 Duman RS, Nakagawa S, Malberg J. Regulation of adult neurogenesis by antidepressant treatment. *Neuropsychopharmacology* 2001; **25**: 836–844.
44 Cameron HA, McEwen BS, Gould E. Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. *J Neurosci* 1995; **15**: 4687–4692.
45 Brezun JM, Daszuta A. Depletion of serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. *Neuroscience* 1999; **89**: 999–1002.
46 Radley JJ, Jacobs BL. 5-HT1A receptor antagonist administration decreases cell proliferation in the dentate gyrus. *Brain Res* 2002; **955**: 264–267.
47 Gully D, Geslin M, Serva L, Fontaine E, Roger P, Lair C *et al*. 4-(2-Chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine hydrochloride (SSR125543A): a potent and selective corticotrophin-releasing factor(1) receptor antagonist. I. Biochemical and pharmacological characterization. *J Pharmacol Exp Ther* 2002; **301**: 322–332.
48 Hernando F, Schoots O, Lolait SJ, Burbach JP. Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: anatomical support for its involvement in the central effects of vasopressin. *Endocrinology* 2001; **142**: 1659–1668.
49 Chalmers DT, Lovenberg TW, Grigoriadis DE, Behan DP, De Souza EB. Corticotrophin-releasing factor receptors: from molecular biology to drug design. *Trends Pharmacol Sci* 1996; **17**: 166–172.