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ORIGINAL ARTICLE

Neural stem cell proliferation is decreased in schizophrenia, but not in depression

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The phenomenon of adult neurogenesis (AN), that is, the generation of functional neurons from neural stem cells in the dentate gyrus of the hippocampus, has attracted remarkable attention, especially as it was shown that this process is also active in the human brain. Based on animal studies, it has been suggested that reduced AN is implicated in the etiopathology of psychiatric disorders, and that stimulation of AN contributes to the mechanism of action of antidepressant therapies. As data from human post-mortem brain are still lacking, we investigated whether the first step of AN, that is, the level of neural stem cell proliferation (NSP; as quantified by Ki-67 immunohistochemistry), is altered in tissue from the Stanley Foundation Neuropathology Consortium comprising brain specimens from patients with bipolar affective disorder, major depression, schizophrenia as well as control subjects ($n=15$ in each group). The hypothesis was that stem cell proliferation is reduced in affective disorders, and that antidepressant treatment increases NSP. Neither age, brain weight or pH, brain hemisphere investigated nor duration of storage had an effect on NSP. Only in bipolar disorder, post-mortem interval was a significant intervening variable. In disease, onset of the disorder and its duration likewise did not affect NSP. Also, cumulative lifetime dose of fluphenazine was not correlated with NSP, and presence of antidepressant treatment did not result in an increase of NSP. Concerning the different diagnostic entities, reduced amounts of newly formed cells were found in schizophrenia, but not in major depression. Our findings suggest that reduced NSP may contribute to the pathogenesis of schizophrenia, whereas the rate of NSP does not seem to be critical to the etiopathology of affective disorders, nor is it modified by antidepressant drug treatment.
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Introduction

Neural stem cells, located in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG), produce new neuronal and glial cells in the hippocampus of adult mammals (neural stem cell proliferation; NSP). Some of these neuronal cells differentiate into neurons, which eventually integrate functionally and structurally into existing neural networks (adult neurogenesis, AN;[1,2]), thereby maintaining neuronal plasticity and probably memory formation[3] or stress response.[4] As the hippocampus is thought to be involved in the etiopathology of depression, AN was suggested to be involved in the pathophysiology of mood disorders (reviewed by Duman,[5] Benninghoff *et al.*[6] and Kempermann and Kronenberg.[7]) Evidence for this is mainly derived from animal studies: factors linked to depression – like stress,[8–10] early life experience[11] and social hierarchy[11] – decrease AN, whereas antidepressive strategies were found to increase AN. An elevated number of newly formed neurons were reported following exercise,[12] biological antidepressant (AD) treatments like electroconvulsive therapy (ECT)[13] and treatment with ADs of all classes (for review, see Duman[5]). However, it remains controversial whether this is necessary for the AD action or merely an epiphenomenon.

Support for a role of AN in depression could be provided by animal models of depression in that animals with a depression-related phenotype also display reduced AN. Animals selected for high and low learned helplessness were not different with regard to their levels of AN,[14] although early survival of newly formed cells was decreased in both groups by exposure to the learned helplessness paradigm. This indicates that this stressful procedure, just like

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immobilization or restraint stress,[15] reduces AN, which however does not relate to a depressive phenotype. Corresponding to these results, we demonstrated that NOS-III knockout mice are less helpless, that is, show an antidepressive phenotype, yet have significantly lower levels of AN.[16] Furthermore, an irradiation procedure that results in reduced levels of AN does not induce depression-like behaviors in mice.[17] Together, these results argue against AN being an important factor underlying depression-related traits in mice.

It is a consistent finding that depression in humans is associated with a reduced hippocampal volume *in vivo*, as evidenced by MRI techniques. This was confirmed in two recent meta-analysis studies.[18,19] Unipolar – but not bipolar – depression led to a significant 10% reduction of the right hippocampus volume (left side, 8%), correlated to the number of depressive episodes.[19] Post-mortem studies investigating the underlying biological mechanisms however remain sparse. One study revealed increased density in the packing of neurons, thus accounting for the observed hippocampal atrophy found in imaging studies.[20] Hippocampal volume reduction therefore was attributed to a loss of neuropil, but not to neurodegeneration or to reduced cell counts. Likewise, no hippocampal cell loss and only slightly increased neuronal apoptosis was detected in post-mortem tissue of patients suffering from depression.[21,22] In contrast, in patients with bipolar affective disorder (Bip), a significant reduction of CA2 non-pyramidal cells was demonstrated.[23]

As investigation of AN, usually accomplished by immunohistochemical 5-bromo-2-deoxyuridine (BrdU) staining, was hitherto impractical in humans, it is still unclear whether decreased AN possibly reflected by hippocampal atrophy detected in imaging studies is a consistent feature of affective disorders. Recently, several antibodies have been developed to substitute for BrdU in the examination of neuroneogenesis in the recent 2 years: proliferating cell nuclear antigen (PCNA) staining was used to demonstrate increased AN in Huntington's disease brain,[24] but decreased precursor cell proliferation in patients suffering from Parkinson's disease.[25] Antibodies against doublecortin (DCX) were used in tissue from Alzheimer patients, showing that hippocampal AN is increased.[26] Another mitotic marker shown to be an alternative to BrdU staining is the nuclear protein Ki-67, which is expressed during all phases of cell cycle except G_0.[27] Ki-67 thus is a marker of stem cell proliferation, the first step of AN, which thereafter is followed by migration and terminal differentiation. As these techniques also allow to study AN or NSP in humans, we investigated in the present study whether NSP is altered in unipolar major depression (MD), Bip or schizophrenia (Sz) by the use of Ki-67 antibodies. The *a priori* hypothesis was that the number of Ki-67-positive cells is reduced in the hippocampus of patients with affective disorders. Furthermore, we examined whether confounding variables like age, post-mortem interval (PMI) and pH have an influence on neuroneogenesis and whether treatment, especially with ADs, increases NSP in humans.

Materials and methods

Subjects

Frozen sections of the anterior hippocampus, mounted on glass slides, were obtained from the Stanley Foundation Neuropathology Consortium.[28] The collection consisted of 15 subjects with Sz, 15 with bipolar disorder, 15 with MD without psychotic features and 15 controls. All groups were matched for age, sex, race, PMI and hemispheric side. Demographic information and medical data including lifetime use of psychotropic medications, history of drug abuse and last prescribed medication were provided to us by the Stanley Foundation Neuropathology Consortium (an overview is given in Table 1). All but three schizophrenic subjects received neuroleptic treatment at the time of death, and all but three depressive subjects received AD treatment at the time of death. Bipolar patients received a wide range of psychotropic drugs, and two of them were unmedicated at the time of death. However, medication-free subjects did not differ in the amount of Ki-67-positive cells. Basic information about the Stanley Medical Research Institute (SMRI) brain bank can be found online at http://www.stanleyresearch.org/programs/brain_collection.asp. Psychiatric diagnoses had been established by two psychiatrists using DSM-IV criteria. All experiments were performed blinded to the diagnosis of each subject.

Animals, BrdU administration and tissue processing

For double-labeling experiments with Ki67 and BrdU, C57BL/6 mice from Jackson Laboratories (Bar Harbor, MA, USA) were used. All mice were housed under identical conditions with water and food *ad libitum*. All animal protocols have been reviewed and approved by the review board of the Government of Lower Franconia and the University of Würzburg and were in accordance with international guidelines on animal testing. For analysis of stem cell proliferation by means of BrdU labeling, mice were administered BrdU (4×100 mg/kg body weight every 2 h; Roche, Mannheim, Germany) and transcardially perfused with 4% paraformaldehyde (PFA) 48 h after the last injection. Brains were fixated with 4% PFA overnight at 4°C and cryoprotected with sucrose solution. Serial cryostat sections (40 μm thick) were cut through the entire hippocampus and stored until use at −20°C.

Immunohistochemistry

For Ki-67 and BrdU co-labeling, mouse brain sections (prepared as described above) were mounted on glass slides and rinsed $3\times$ in TBS. Afterwards, the sections were incubated in 0.01 M citrate buffer heated to 95°C for 5 min and washed in TBS again. Thereafter, all slices were incubated for 1 h in 3% normal horse serum (NHS) in TBS followed by an overnight

Table 1 Demographic data on the investigated subjects

Diagnosis (n = *15 each)*	*Age*	*pH*	*PMI (h)*	*Storage (days)*	*Onset of disease (age)*	*Disease duration (years)*	*Neuroleptic treatment (lifetime quantity of fluphenazine or equivalent; mg)*	*Never received neuroleptic treatment* (n)	*Psychosis present* (n)	*Suicide* (n)
Control	48 ± 10	6.2 ± 0.2	23 ± 9	338 ± 234	NA	NA	NA	15	NA	NA
Schizophrenia	44 ± 13	6.1 ± 0.2	33 ± 14	621 ± 233	23 ± 8	21 ± 11	52 266 ± 62 061	1	15	4
Bipolar disorder	42 ± 12	6.1 ± 0.2	32 ± 16	620 ± 172	21 ± 8	20 ± 10	20 826 ± 24 015	3	11	9
Major depression	46 ± 9	6.1 ± 0.2	24 ± 11	434 ± 290	34 ± 13	13 ± 11	NA	15	0	7

NA, not applicable; PMI, post-mortem interval.
Bold represents significant results.

incubation (4°C) with a mouse anti-Ki-67 monoclonal antibody (NCL-KI67-MM1, NovoCastra Labs Ltd) diluted 1:200 in 3% NHS/TBS. After three further rinses in TBS, the primary antibody was detected using the fluorescent secondary antibody Cy™2-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, PA, USA; 1:200 in TBS). After 2 h of incubation, the tissue was washed 3 × in TBS and post-fixed in 8% PFA for 15 min to protect Ki-67 signals from the HCl treatment (45 min, 45°C) that follows, used to denature the DNA thus exposing the antigens. The sections were washed 3 × in TBS, then incubated overnight at 4°C with the second primary antibody (monoclonal rat anti-BrdU; Diagnostic International GmbH, Schriesheim, Germany), 1:5000 in TBS containing 5% NHS, 0.25% Triton X-100 and 2% BSA). After three more rinses in TBS, the BrdU antibody was detected using the secondary antibody Cy™3-conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories, PA, USA; 1:200 in TBS containing 2% NHS, 0.25% Triton X-100 and 2% BSA). After 2 h of incubation, sections were washed again in TBS, embedded in a water-based medium and visualized with a Bio-Rad MRC 1024 laser-scanning confocal imaging system (Bio-Rad, Cambridge, MA, USA) mounted onto a DMRBE Leica microscope (Leica, Wetzlar, Germany).

For detection of newborn cells in the DG of human brain, seven sections of the anterior hippocampus of each brain were immunohistochemically labeled with a Ki-67 antibody. Therefore, slices were fixed in 4% PFA for 10 min and washed in TBS three times. To block endogenous peroxidases, the tissue was incubated for 30 min in 0.6% H_2O_2 in TBS. Afterwards, the sections were washed in TBS, treated with citrate buffer and blocked with NHS as described above. For the following overnight incubation (4°C), the mouse anti-Ki-67 monoclonal antibody (NCL-KI67-MM1, NovoCastra Labs Ltd) was diluted 1:100 in 3% NHS/TBS. After three more rinses in TBS, the primary antibody was detected with a biotinylated goat anti-mouse IgG antibody (Vector Laboratories Inc., USA; 1:200 in 3% NHS/TBS) in combination with the avidin–biotin–peroxidase complex technique (Vector Laboratories) and DAB (Roche, Mannheim, Germany). Thereafter, sections were counterstained with Nissl.

Morphometric analysis and quantification

Human brain sections (seven per brain) of corresponding levels of the anterior hippocampus, stained with an anti-Ki-67 antibody, were examined using a bright-field Leica microscope (DMRBE, Wetzlar, Germany) at × 400 magnification. Ki-67-positive cells in the granular cell layer (GCL) were counted and expressed as the average number of Ki-67-positive cells/mm of GCL. The length of the GCL was determined by scanning magnifications of each DG section using an AGFA duoscan *f*40 scanner (AGFA, Köln, Germany), followed by measuring the GCL using the ImageJ 1.33u software package (Wayne

Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). The examined region was defined as follows: the inner edge of the GCL was positioned horizontally in the center of the visible field at $\times 400$ magnification, and every Ki-67-positive neuron within the upper and lower limits of the visible field was counted. Only cells that could be sharply focused by three-dimensional scanning through the slice were regarded as Ki-67 positive. Cell counts were performed blindly with respect to diagnosis. Ki-67-positive cells in the DG were only found in the SGZ, defined as two cell diameters to either side of the inner edge of the GCL.[29]

Statistics

The average number of Ki-67-positive cells is reported as means $\pm$ s.e.m. Differences between diagnostic groups were determined by ANOVA as well as Student's *t*-test with subsequent Bonferroni correction, with $P<0.05$ considered significant. The potential confounding effects of age, brain pH and weight, days of storage, PMI, onset and duration of disease as well as total lifetime quantity of fluphenazine with regard to Ki-67 cell number were assessed using variance analysis with covariates. To investigate the impact of severity of alcohol or substance abuse with regard to Ki-67-positive cells, Student's *t*-test with subsequent Bonferroni correction was performed. The influence of AD treatment on NSP has been calculated by forming four groups (no AD treatment, selective serotonin reuptake inhibitors (SSRI), tricyclic antidepressant (TCA) or both at the time of death) irrespective of diagnosis and subsequent ANOVA as well as individual Student's *t*-test. All calculations were performed using SPSS for Windows 9.0 (SPSS Inc., Chicago, IL, USA).

Results

Ki-67 colocalizes with BrdU staining in mice

To demonstrate that Ki-67 can be used to label proliferating cells not only in rats, as already shown,[27] but also in other mammals, brain sections of BrdU-injected mice were double labeled using antibodies against BrdU and Ki-67. Confocal microscopy revealed that in the SGZ – at the border between hilus (HI) and GCL – both markers are present in the nuclei of labeled cells (Figure 1a–c). The SVZ at the lateral wall of the lateral ventricles is another neurogenetic brain region, and we could also detect cells reliably co-labeling with Ki-67 and BrdU (Figure 1d–f, indicated by arrows), which was in part only evident when scanning along the *z* axis (40 μm) through the entire slice (compare Figure 1g–i, arrowheads). Ki-67 thus was found to colocalize with BrdU in mice, and as BrdU is a common marker for mitotic cells in studies of AN, we concluded that Ki-67 staining can be used as a reliable method for measuring cell proliferation.

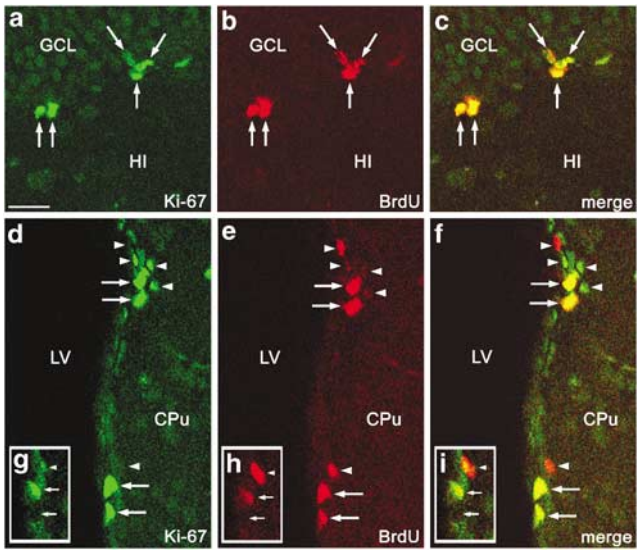

Figure 1 Double-labeling experiments demonstrate Ki-67 expression in BrdU-stained newborn cells. (**a–c**) Representative confocal laser-scanning images displaying a section of mice DG labeled for Ki-67 (**a**, green) and BrdU (**b**, red). The merged image (**c**) shows that all Ki-67-expressing cells in the SGZ of the DG are also stained for BrdU (arrows). (**d–i**) Immunohistochemical double labeling for Ki-67 (**d, g**; green) and BrdU (**e, g**; red) in the mice SVZ. Arrows in (**d–f**) point at cells that are clearly stained with both antibodies (compare the merged image f) and arrowheads indicate cells that, on first glance, do not seem to be labeled for both markers. Regarding deeper layers of the 40 μm thin slice, as demonstrated in panels g–i, we observed that even these cells are double labeled (indicated by arrowheads), whereas others cannot be detected in this layer anymore (indicated by the lowest arrow in panels g–i). Scale bar-25 μm (given in panel a but applicable to panels a–i); HI, hilus; GCL, granular cell layer; LV, lateral ventricle; CPu, corpus putamen.

Neural stem cell proliferation in humans and confounding variables

To analyze NSP in humans, anterior human hippocampus sections obtained from the SMRI were stained for Ki-67 and analyzed as described in Materials and methods. Ki-67-expressing cells were only located in the SGZ between the GCL and the polymorphic layer of the human DG (Figure 2b and d). Ki-67-expressing nuclei of stained cells in humans (Figure 2b and d) were comparable to the morphology of Ki-67-positive cells in mice (Figure 2a and c). Together, Ki-67 thus appears to be a suitable proliferation marker not only in rodents, but also in humans.

When counting Ki-67-stained cells in the GCL of the DG, five subjects (healthy controls as well as schizophrenic and depressive subjects) were found to display dramatically increased levels of proliferation and thus were excluded from subsequent statistical analysis. These subjects on the average had 6.9 Ki-67-positive cells/mm GCL, whereas the mean value calculated without these patients was 0.13 ± 0.12 cells/mm GCL (calculated including the

excluded subjects: 0.76 ± 2.81 cells/mm CGL). There were no differences with regard to age, brain pH and weight, days of storage, PMI, onset and duration of disease as well as total lifetime fluphenazine doses. Further details on these subjects are displayed in Table 2.

We then investigated whether age, brain pH and weight, days of storage, PMI (in all subjects), onset and duration of disease as well as total lifetime quantity of fluphenazine (patients only) had an influence on the amount of Ki-67-labeled cells, using analyses of variance with covariates. In the analyses using all subjects, PMI, treated as a covariate, yielded significant effects only in the analysis involving depressive subjects ($P = 0.039$). Note that, however, tendentially significant PMI covariate effects were observed also for the analyses involving the two other patients' groups, whereas the other variables had no effect on the amount of Ki-67-labeled cells (Table 3). In the analyses using patients only, neither onset/duration of disease nor total lifetime quantity of fluphenazine had a significant effect ($P \geqslant 0.39$). Left and right hippocampus were also not significantly different ($P = 0.93$, Student's *t*-test). When all groups were compared, Ki-67-specific cell quantity was also not affected by lifetime neuroleptic treatment ($P = 0.11$, Student's *t*-test). Thus, apart from PMI, intervening variables did not seem to have an impact on neurogenesis in this sample.

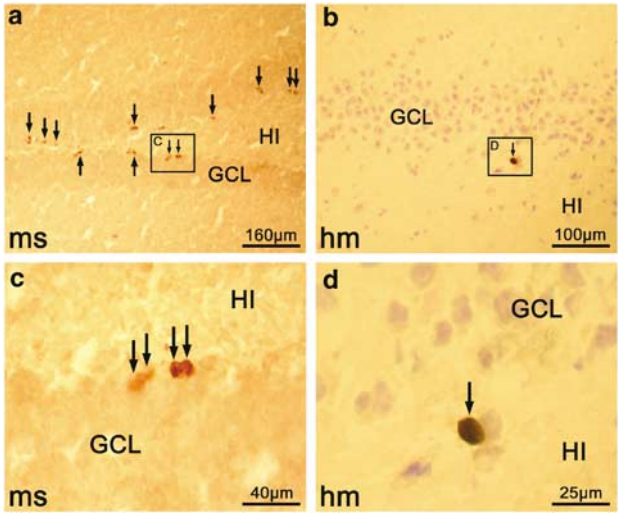

Figure 2 Ki-67 antibody labeling in the DG of mouse and man. (**a, c**) Representative photomicrographs of coronal sections through the DG of a Ki-67-stained mouse brain. Labeled cells are located in the SGZ of the DG and appear most often in clusters and are indicated by arrows. (**b, d**) Representative photomicrographs of an anterior human DG section stained for Ki-67 (Nissl counterstaining). The Ki-67-expressing cell nucleus in the SGZ is indicated by an arrow. As Ki-67 detection is accomplished with DAB, the labeled cell can be distinguished from Nissl-stained cells by its dark brown color. HI, hilus; GCL, granular cell layer; ms, mouse; hm, human.

Effects of alcohol and substance abuse

As alcohol consumption has been suggested to impair neurogenesis,[30] we were interested in whether alcohol abuse, along with substance abuse, has an impact on Ki-67 staining. Alcohol as well as substance abuse of investigated subjects was reported by the Stanley Foundation, ranging from 0 to 5, with 0 being the lowest and 5 the highest grade of abuse. Furthermore, substance abuse was classified as 'current', 'past' or 'never'. We could not detect significant differences in Ki-67 staining between any of these groups (Student's *t*-test, with severity levels 0–2 and 3–5 grouped together owing to the low number of subjects with high abuse).

Influence of antidepressant treatment on Ki-67 staining

As it has been suggested that an increase in AN is intrinsic to the mechanism of action of all ADs, we were interested in whether this notion holds true in humans. To do so, patients were grouped according to their last prescribed medication irrespective of their diagnosis and allocated to four different groups: no AD treatment, treatment with TCA, treatment with SSRI or both (Table 4). Significant differences between groups could not be shown by ANOVA ($P = 0.21$). Also, comparing 'no treatment' to 'any AD treatment' did not result in a significant difference (Student's *t*-test, $P = 0.22$). A subgroup analysis in MD only was not feasible, as only three MD patients did not receive ADs at the time of death.

Table 2 Excluded subjects because of excessive amounts of neurogenesis

Subject no.	*Ki-67-labeled cells/mm GCL*	*Diagnosis*	*Substance abuse*	*Medication at the time of death*
1	0.8	MD	Current severe alcohol and substance abuse	Fluoxetine, trimipramine
2	1.4	CTRL	None	None
3	5.3	MD	Very low alcohol abuse	Trazodone
4	8.3	Sz	Very low alcohol abuse	Clozapine
5	18.5	Sz	None	Thioridazine, clozapine, amitryptiline

Table 3 Differences in neural stem cell proliferation, as evidenced by Ki-67 staining, in psychiatric disorders

Source of variation	*Bip vs controls*			*MD vs controls*			*Sz vs controls*		
	$F_{1,21}$	P	η^2	$F_{1,16}$	P	η^2	$F_{1,19}$	P	η^2
Factor									
Diagnosis	1.06	0.317	0.05	0.06	0.811	<0.01	**5.84**	**0.027**	**0.26**
Covariates									
Age	0.19	0.665	0.01	0.42	0.526	0.03	0.59	0.452	0.03
pH	0.55	0.466	0.03	1.20	0.293	0.08	0.27	0.610	0.03
Weight	<0.01	0.984	<0.01	2.85	0.113	0.17	0.02	0.903	<0.01
PMI	3.62	0.072	0.16	5.20	0.039	0.27	3.60	0.075	0.18
Days of storage	0.30	0.588	0.02	0.19	0.668	0.01	3.86	0.066	0.19

Bip, bipolar affective disorder; MD, major depression; Sz, schizophrenia; PMI, post-mortem interval.
Bold represents significant results.

Table 4 Influence of antidepressant treatment on counts of Ki-67-expressing cells

Treatment	*None*	*TCA*	*SSRI*	*Both*
	n = *33*	n = *10*	n = *7*	n = *5*
Ki-67-positive cells/mm granule cell layer (means ± s.d.)	0.14 ± 0.12	0.12 ± 0.12	0.12 ± 0.16	0.08 ± 0.03

SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant.

Adult neurogenesis and psychiatric disorder

Following the analysis of potential confounding factors and medication effects, we investigated whether NSP, as evidenced by Ki-67 staining, in the DG is altered in psychiatric disorder (Figure 3). All three diagnostic groups (Bip, MD and Sz) were compared to controls by means of variance analysis (Table 3). NSP was significantly reduced in schizophrenic patients only, even when considering potential covariate effects of intervening variables (0.07 ± 0.02 vs 0.19 ± 0.04 cells/mm in the controls; $P = 0.027$). Both MD and Bip feature phasic course, and it remains unknown whether patients were actually ailing at the time of death. Although suicide may be considered as an indicator of present illness, patients suffering from affective disorders who died from suicide did not display altered levels of NSP compared to those who died for other reasons (suicide group, 0.12 Ki-67-positive cells/mm GCL; $n = 16$; non-suicide group, 0.13 cells/mm GCL; $n = 12$; $P = 0.77$, Student's *t*-test).

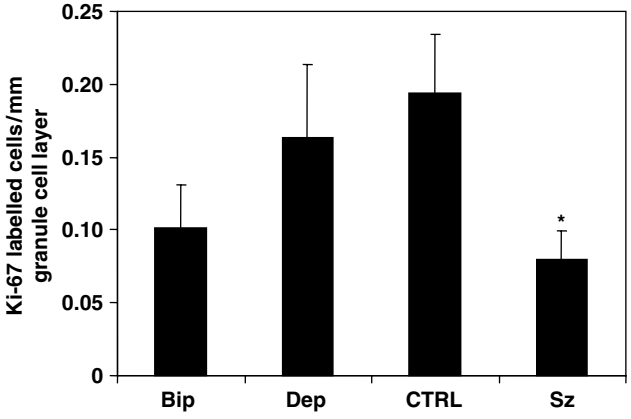

Figure 3 Neurogenesis in the DG is reduced in Sz, but not in MD. Neuroneogenesis in humans has been measured by use of Ki-67 staining, as stated in Materials and methods. Each bar represents 13–15 subjects; in each subject, seven sections of the anterior hippocampal formation have been stained and counted. Although there was a trend toward lower numbers of AN in Bip, there was no difference in the number of stained cells between MD and controls. Most remarkably, however, was a significant reduction in newborn cells in the DG of schizophrenic subjects. $^*P < 0.05$ (Student's *t*-test followed by Bonferroni correction).

Discussion

The main findings of the present study are as follows: (a) the first step of AN, NSP as evidenced by Ki-67 staining, is not reduced in patients with unipolar depression; (b) proliferation of hippocampal neural stem cells is significantly reduced in schizophrenic patients and (c) AD medication does not have an influence on NSP. Before discussing these findings, two methodological issues have to be addressed.

First, we excluded five subjects from further statistical analysis, as they featured markedly elevated levels of NSP (Table 2) hampering proper statistical evaluation. Especially, subjects 3–5 had remarkably increased NSP levels not explained by treatment or any other obvious reason. Although no information was given as to whether these patients underwent ECT, this would be a possible, although speculative explanation, as ECT was shown to stimulate AN in the animal model.[13,31] As MD and Sz have both been suggested to be a heterogeneous group of disorder, the possibility exists that some subforms of psychoses go along with markedly increased NSP. Finally, the chance that these measures represent artefacts cannot be ruled out. As cell distribution was not different to other sections and there were no morphological differences in staining in these subjects, we consider this however unlikely; furthermore, there were no other apparent differences with regard to intervening variables compared to the remaining subjects.

The second concern to be discussed is whether Ki-67 staining actually reflects AN in the human brain. In previous studies investigating AN in human tissue, antibodies against PCNA[24,25] or DCX[26] have been used. Although initial tests with PCNA in the present SMRI tissue have been promising, labeling was not reliable in subsequent experiments in contrast to Ki-67 staining. In contrast to Ki-67, PCNA also labels resting cells[32] as it is also involved in DNA repair, and the correlation between Ki-67 and BrdU is far better than between PCNA and BrdU.[33] For these reasons, we chose to use the Ki-67 antibody in subsequent experiments. In contrast to BrdU, which is incorporated in the S-phase of dividing cells, Ki-67 is expressed during the whole mitotic phase of the cell except G_0.[34] Thus, the amount of Ki-67-expressing cells in the DG of rats is about twice the amount of BrdU-stained cells. However, there remains the possibility that also some dying neurons were labeled, as both Ki-67 and BrdU are expressed following ischemia/hypoxia-induced DNA syntheses during apoptosis.[35] Furthermore, Ki-67 labeling does not discriminate between neural and glial cells, as the marker is expressed before cells undergo terminal differentiation and thus Ki-67 labeling can only provide a means of NSP, but not quantify the generation of functional neurons, that is, AN in a strict sense. Double labeling of Ki-67 with neural (NeuN) or glial (GFAP) markers therefore cannot be accomplished, and it has already been shown that there is no coexpression of Ki-67 and NeuN.[27] Despite these limitations, Ki-67 staining appears to be the best-estimate marker for AN in the human DG; especially, as Ki-67 colocalizes with BrdU and as it has been shown that 75–90% of BrdU-positive cells express NeuN,[13,36–38] Ki-67 can be considered to approximate the level of AN. Presuming that Ki-67 actually reflects at least the first step of neurogenesis, that is, stem cell proliferation, and mirrors AN, what do our findings mean?

Medication effects

As discussed by Duman[5] and Henn and Vollmayr,[39] a considerable number of studies demonstrated that all modes of AD drug treatment consistently resulted in an increase of BrdU-labeled cells. These studies were primarily performed in rats and, with similar results, in mice. Based on findings showing that inhibition of neuroneogenesis by irradiation blocks AD effects, it has been suggested that efficiency of AD treatment depends on stimulation of AN,[17] although several points of criticism have been raised.[39] The finding that ADs of any class do not alter the rate of NSP in humans, raised in this study, argues against the notion that stimulation of neurogenesis is required for the effectiveness of AD treatments. In fact, when all ADs were grouped together, patients receiving antidepressive treatment had (insignificantly) *lower* rates of NSP irrespective of their diagnosis. Total lifetime dosage of neuroleptics also did not impact on the level of NSP. A limitation, however, may be that ADs were not taken as prescribed; yet, this may account only for some and not all patients. Another caveat to be considered is the use of polypharmacy as well as substance heterogeneity, so that the final class numbers (diagnosis $\times$ medication) are too small to allow definite statistics, a problem however inherent to every human post-mortem study published to date.

Affective disorders and AN

Based on findings in animal models, it has been suggested that depression may be caused in part by decreased AN. Studies on AN in patients suffering from affective disorders have thus been eagerly awaited. Although subjects with Bip had a trend toward lower levels of AN, our finding that NSP is unchanged in MD is unexpected and argues against the notion that AN plays a central role in the etiopathogenesis of affective disorders, at least not with respect to that total net number of newborn cells. It has however to be considered that the power of the present study to detect mild to moderate effects was low (33%), and that the rate of differentiation or the structural integration of new cells in pre-existing neural networks may however still be relevant to the pathophysiology of affective disorders. The latter has to be clarified in further studies incorporating assessment of neuronal fine structure. Furthermore, despite the above considerations, there still remains the possibility that the number of Ki-67 cells – indicating NSP – is unchanged in depression; however, their survival rate is reduced resulting in decreased net neurogenesis. Before conclusions are drawn regarding the role of AN in affective disorders, attempts thus should be made to replicate our findings by the use of alternative proliferation markers.

Adult neurogenesis and schizophrenia

Surprisingly, the only variable that was significantly associated with lower levels of NSP was a diagnosis of Sz. Until now, this disease has not been associated with reduced rates of neurogenesis. In an animal

model of Sz (ketamine administration), neurogenesis was stimulated twofold following ketamine injection.[40] On the other hand, atypical neuroleptics such as olanzapine,[38] but not the prototypic antipsychotic haloperidol, increase AN in the SVZ, suggesting that these compounds stimulate cell proliferation in the rostral migratory stream to replenish cells in the olfactory bulb, but not in the hippocampus.[41] A post-mortem study accordingly found neuronal abnormalities in the olfactory bulb of schizophrenic patients, indicating disturbance of cell proliferation as well.[42] While this does not indicate changes in the DG *per se*, the contribution of atypical neuroleptics to hippocampal neurogenesis is controversial.[38,41] A thoroughly conducted study therefore aimed to further elucidate the connection between neuroleptics and neurogenesis:[43] low-dose clozapine, but not high-dose clozapine or haloperidol, stimulated stem cell proliferation in the DG of rats. Finally, olanzapine but not haloperidol stimulated AN in the prefrontal cortex,[44] another region implicated in the pathophysiology of Sz. Atypical neuroleptics might therefore exert at least some of their effects by stimulating the proliferation of neural stem cells, thus compensating decreased AN found in Sz.

Does the finding of decreased AN in Sz make sense? Sz is known to go along with several cognitive deficits, which is stable over time,[45] that is, trait rather than state dependent. The prime role of hippocampus is rather memory formation than affect regulation. Thus, diminished AN, which has been suggested to result in impaired memory formation,[3] might well contribute to the cognitive impairment seen in Sz; improvement of cognitive functioning in Sz by clozapine might be due to the increase of AN seen in animal studies. As learning,[46] exercise[12] and enriched environment[47] all increase AN as well, this directly points toward non-pharmacological treatment of schizophrenics. The preliminary finding of reduced AN in Sz provided in the present study is thus worth being pursued further. The trend toward lower levels of AN in bipolar disorder might indicate that there is some overlap in the pathophysiology of both disorders, as also indicated by genetic[48] and morphological[49] data.

Duman,[5] in his critical appraisal of the neurogenesis theory of depression, called for a test of this hypothesis by examining AN in patients suffering from depression by means of immunohistochemical studies using antibodies against cell cycle markers. Although our study, being the first investigation of AN in humans suffering from psychiatric disorders, does not support this hypothesis, is suggests that AN may have a role in Sz, probably explaining cognitive deficits encountered in this disorder. Thus, research on AN in humans has just begun.

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