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Decreased numbers of progenitor cells but no response to antidepressant drugs in the hippocampus of elderly depressed patients

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ABSTRACT

Imaging studies have consistently documented hippocampal volume reductions in depression. Although depressive disorders are traditionally considered to have a neurochemical basis, recent studies suggest that impairments of structural plasticity contribute to the volume reductions and the related cognitive changes. This might result from repeated periods of stress that are a wellknown risk factor for depression. Adult neurogenesis is a prominent example of neuroplasticity that in rodents, is reduced by stress but stimulated by antidepressant drugs. Although reductions in neurogenesis have been proposed to contribute to the etiology of depression, only two studies have so far examined hippocampal cytogenesis in depression, but this was in a limited number of subjects with considerable interindividual variation, and these studies came to different conclusions. We therefore collected hippocampal tissue of 10 elderly control subject and 10 well-matched depressed patients that were highly comparable in terms of age, sex, pH-CSF and postmortem delay, and tested whether the numbers of MCM2-positive progenitors and PH3-positive proliferating cells were altered by depression or antidepressant treatment. A significant reduction was found in MCM2-, but not PH3-immunopositive cells in depression. Although this result is consistent with the concept that structural plasticity is decreased in depression, we could not confirm that antidepressant drugs had a stimulatory effect on these cells. This discrepancy may relate to anatomical differences, in medication, to neurogenesis–independent mechanisms of antidepressant action, or the age of the patients that was higher than in previous studies. Whether the reduction is a cause or consequence of depression awaits to be determined.

1. Introduction

Major depression is a severe and often life threatening condition that affects millions worldwide. Among other factors, chronic stress has been proposed a risk factor for the development of depression in vulnerable individuals (De Kloet et al., 2005; Kendler et al., 1999). As glucocorticoid receptors are abundantly expressed in the hippocampal formation, this brain area is particularly sensitive to stress (De Kloet et al., 2005; Joëls et al., 2007; Wang et al., submitted for publication). Indeed, in depressed patients, an activated hypothalamo-pituitary-adrenal (HPA) axis is commonly observed (Swaab et al., 2005), while high resolution *in vivo* magnetic resonance imaging studies have consistently documented reductions in hippocampal volume (Campbell et al., 2004; Videbech and Ravnkilde, 2004), that may relate to the cognitive impairments, learning and memory deficits and diminished cognitive flexibility often seen in depression (Austin et al., 2001; Drevets, 2001).

Although depressive disorders are traditionally considered to have a neurochemical basis, recent studies suggest that impairments of structural plasticity contribute to their pathophysiology as well (Bremner, 2002; Castrén, 2005; Fuchs et al., 2004; Ressler and Mayberg, 2007). It has been hypothesized that plasticity changes may relate to the hippocampal volume reduction, which might result from repeated periods of stress exposure (Sheline, 2000; Sapolsky, 2000; MacQueen et al., 2003; Gianaros et al., 2007). Various candidate substrates such as dendritic atrophy, neuronal loss or glial changes, are known to be stress-sensitive and may contribute to these changes (Stockmeier et al., 2004; Czéh et al., 2006; Czéh and Lucassen, 2007).

A prominent example of neuroplasticity is adult neurogenesis (AN) that concerns the production of new neurons in an adult brain, a process that occurs selectively in the hippocampal dentate gyrus (DG) and subventricular zone, also of humans (Eriksson et al., 1998; Curtis et al., 2007; Sanai et al., 2007; Manganas et al., 2007). In the hippocampus, AN decreases with age (Heine et al., 2004a) and is

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regulated by stress, disturbed sleep, exercise and inflammation, all factors implicated in brain vulnerability, that may play a role in depression and anxiety (Oomen et al., 2007; Joëls et al., 2007; Czéh et al., 2001; Lucassen et al., 2009; Revest et al., 2009).

AN has attracted considerable attention regarding depression because of its hippocampal occurrence and its strong inhibition by stress, amongst others. Recently, the 'neurogenic theory' of depression has been put forward that postulates that a suppressed rate of cyto- or neurogenesis contributes to the (vulnerability for) depression (Kempermann et al., 2008; Sahay and Hen, 2007), a theory further supported by the following additional findings: 1) stress inhibits AN in animals and is a risk factor for depression; 2) depressed patients often display hippocampal volume reductions that are paralleled by cognitive deficits; 3) most antidepressant drugs do not exert their therapeutic effect until after 3–4 weeks of administration, a period that parallels the time course of maturation of new neurons. 4) Almost all antidepressants increase or normalize AN in animals, 5) disruption of AN blocks the behavioral response to antidepressant drugs (Malberg et al., 2000; Czéh et al., 2001; Oomen et al., 2007; Santarelli et al., 2003; Warner-Schmidt and Duman, 2006; Sahay and Hen, 2007).

Given its rare nature and technical limitations to visualize neurogenesis in the live elderly human brain (Manganas et al., 2007; Shapiro et al., 2006), it is still poorly understood whether AN actually contributes to hippocampal changes in depression. Only two postmortem tissue have been published on this topic so far that investigated brain tissues of a small number of depressed patients with considerable variation in age, fixation and postmortem delay (PMD). Despite the use of the same marker, i.e. Ki-67, these authors came to different conclusions, which may partly be due to the interindividual variation and the fact that Ki-67 has a short half-life and is indeed very rare in human brain (Bruno and Darzynkiewicz, 1992; Boekhoorn et al., 2006). Reif et al. (2006) failed to find changes in proliferation in depression, and antidepressant treatment had no effect. In contrast, Boldrini et al. (2009) found a reduction in proliferation in non-treated depression whereas antidepressant-treated patients had higher numbers. Given these discrepancies, further studies were warranted and we therefore studied a group of 10 control subjects that were carefully matched to 10 depressed cases in order to make these groups highly comparable in terms of age, sex, pH of the CSF and PMD (see Table 1). We then tested the hypothesis that in depression, the hippocampus holds a reduced number of proliferating or progenitor cells, for which we used 2 well validated markers with a longer half-life, i.e. PH3 and MCM2 (see below).

2. Materials and methods

2.1. Patient selection

Brain material was obtained from the Netherlands brain bank (coordinator Dr. I. Huitinga) that works with a rapid autopsy program and aims to keep postmortem delay (PMD) to a minimum. The NBB abides to all local ethical legislation and permission was obtained for all brain autopsies and for the use of the tissues and clinical data for research purposes. Ten clinically well defined patients were selected that had suffered from recurrent episodes of major depression or depression with bipolar disorder, based on psychiatric reports and previous admissions to psychiatric wards in the patient's records. The clinical diagnosis of all depressed patients had been established by psychiatrist Prof.Dr. W.J.G. Hoogendijk, Amsterdam, based on DSM-IV criteria (see Table 1 for a clinicopathological summary of the patients). Moreover, from some of the depressed patients, it was known that they displayed elevated levels of corticotrophin-releasing hormone or vasopressin in the hypothalamic paraventricular nucleus, indicative of an activated HPA axis (Raadsheer et al., 1995; Lucassen et al., 2001; Müller et al., 2001; Swaab et al., 2005; Meynen et al., 2006). Specific care was further taken to exclude subjects that had suffered from other brain disorders or comorbidities affecting the hippocampus, like dementia.

To minimize methodological variation as much as possible, control subjects were carefully matched on a one-to-one basis with a depressed subject for age, sex, fixation time, pH of the cerebrospinal fluid and PMD. None of the control subjects had suffered from any primary neurological or psychiatric disease or brain metastases, nor did they have a history of steroid or antidepressant treatment (Table 1). In previous studies on some of the same patients, the hippocampus had already been studied for various structural alterations (Lucassen et al., 2001; Müller et al., 2001), and based on the thorough analysis by a team of trained neuropathologists, the absence of neuropathological changes was confirmed in the hippocampus and other brain regions in both groups, with all subjects having a Braak score for neuropathological alterations of 0 or 1.

2.2. Human brain tissue preparation

At autopsy, the hippocampus proper was dissected and fixed in 0.1 mol/L phosphate-buffered 4% formaldehyde (Sigma, St. Louis, MO) solution (pH 7.2) for a period of approximately 4–5 weeks. Tissue was then dehydrated in graded ethanol and embedded in paraffin. Eight μm thick tissue sections were cut for all patients from the mid-anteroposterior level on a Leica microtome and mounted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany).

2.3. Marker selection

For postmortem brain, various markers are available that identify different stages of the neurogenic process, or of specific phases of the cell cycle e.g. (Von Bohlen and Halbach, 2007; Curtis et al., 2007; Boekhoorn et al., 2006). Of these, the minichromosome maintenance protein 2 (MCM2) is involved in the control of DNA replication and commonly used in cancer research where it was shown to be a reliable marker for detecting dividing and slowly cycling putative stem cells in situ (Hashimoto et al., 2004; Yang et al., 2006; Fahrner et al., 2007; Reena et al., 2008). MCM2 expression starts in early G1 and is maintained throughout the cell cycle, also in cells that proliferate without actually synthesizing DNA and is thus present in higher numbers than the short-lived proliferation marker Ki-67+ (Bruno and Darzynkiewicz, 1992; Maslov et al., 2004; Reena et al., 2008; Platel et al., 2009). Moreover, 96% of the cells that express MCM2 also express the immature neuronal marker doublecortin and MCM2 has therefore also been used to study neurogenesis. As such, it is a promising neural progenitor marker (Amrein et al., 2007; Maslov et al., 2004; Fahrner et al., 2007; Sivilia et al., 2008; Liu et al., 2008; Platel et al., 2009).

Another commonly used marker for proliferating cells is phosphorylated histone H3 (PH3). Histone H3 is a part of the histone octamer making up the nucleosome; the phosphorylated form of histone H3 is present during the late G2 phase and in the mitotic phase of cell division. PH3 has been widely used to identify proliferating and mitotic cells in amongst others the hippocampus, also of humans (Saka and Smith, 2001; Dupret et al., 2008; Liu et al., 2008; Tamai et al., 2008; Goodarzi et al., 2009).

2.4. Immunocytochemistry

After deparaffination and rehydration of the human hippocampal paraffin sections, antigen retrieval was performed by placing the slides in Tris buffer (pH 8.5) and heating them in a household steamer device (Braun # 3216) for 60 min at 100 °C. Nonspecific staining was blocked with 1% non-fat dry milk (ELK, Campina, The Netherlands) in 0.05 M Tris-buffered saline (TBS) at pH 7.6. The first antibody (goat anti-MCM2 polyclonal antibody sc-9839, lot #H0508, Santa Cruz Biotechnologies) was applied to the sections at a dilution of 1:200 in supermix (0.5% Triton X-100, 0.25% gelatin in 0.05 M TBS pH 7.6). Following one hour of incubation in a humidified chamber at room temperature (RT), slides were stored overnight at 4 °C. The next day, tissue was incubated with secondary antibody (biotinylated donkey anti-goat, Jackson ImmunoResearch) 1:500 in supermix for 1 h at RT. Hereafter the incubation with first and second antibody was repeated, both for 1 h at RT. The tissue sections were then pre-incubated with 1% bovine serum albumin (BSA) in TBS for 30 min followed by incubation with avidin–biotin complex (ABC, Vectastain ABC Elite kit, Vector Laboratories) 1:800 in TBS with 1% BSA for 1 h. The signal was amplified by tyramide incubation 1:750 in TBS with 0.01% H_2O_2 for 30 min, followed by incubation with ABC for 45 min. The antibody-ABC complex was visualized with diaminobenzidine (DAB) in Tris buffer (TB, pH 7.55) and 0.01% H_2O_2 with nickel sulphate amplification. Slides were dehydrated through graded ethanol series and xylene and coverslipped with Entellan (Merck).

For PH3 immunostaining, sections were first incubated for 20 min in 0.3% H_2O_2 diluted in methanol to quench endogenous peroxidase activity. Antigen retrieval was then performed by incubation for 10 min at 121 °C in citrate buffer (0.01 M, pH 6.0). Sections were washed with phosphate-buffered saline (PBS), and incubated for 30 min in 10% normal goat serum (Harlan Sera-Lab, Loughborough, Leicestershire, UK). Incubation with primary antibody was overnight at 4 °C, with anti-phospho-Histone H3 (Upstate/06-570; rabbit polyclonal IgG) at 1:500. The next day, the sections were washed in PBS and we used the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3′-diaminobenzidine (DAB; Sigma) as chromogen. Some sections were counterstained with cresyl violet for morphometry and then dehydrated and coverslipped.

Table 1
Clinicopathological data of the 10 depressed (D) patients matched to the 10 control (C) subjects in a paired, one-by-one selection. Abbreviations used; ND; not known/not determined; COPD: chronic obstructive pulmonary disease; CVA: cerebrovascular accident; AD; antidepressant drug; SSRI: selective serotonin reuptake inhibitor; TCA: tricyclic antidepressant; MAO: monoamine oxidase inhibitor, ATAD; atypical and/or other types of antidepressant (AD) medication.

Group	Age	Sex	PMD	pH	BW	Cause of death	Diseases (depression and other)	Age at diagnosis	AD drug class	Length of time on drugs	Details on medication last month before death
C1	73	F	04:00	6.47	1360	Respiratory insufficiency	Lung fibrosis, rhabdomyolysis, renal insufficiency, respiratory insufficiency				Antihypertensive drugs, azathioprine, prednison
D1	75	F	04:00	ND	1123	Intoxication, CVA	Depression	40	SSRI	35 years	Seroxat, Rastinon (intoxication), Imovane
C2	48	M	05:30	6.88	1500	Legal euthanasia	Diabetes type I, nephrotic syndrome, hypertension, decompensatio cordis, CVAs				Antihypertensive drugs, anticoagulant, insulin, dormicum
D2	45	M	07:00	6.55	1427	Brainstem haemorrhage	Major Depression	32	SSRI	13 years	Fluvoxamine
C3	79	M	07:40	6.02	1334	Bronchopneumonia and sepsis	Bronchopneumonia, sepsis, cardiac arrhythmia				Radiotherapy 8 months before death
D3	78	M	07:35	6.27	1227	Metastized intestinal cancer	Depression, hypertension, coronary sclerosis	69	ATAD	9 years	Aspirin, insulin, carbamazepine, periciazine, morphin, temazepam, bisoprolol
C4	56	M	14:00	7.03	1323	cachexia	ND				ND
D4	55	M	>10.00	ND	ND	Suicide (cranial trauma after gun shot)	Major depression, bipolar disorder	49	ATAD/SSRI	4 years	bupropion, risperidon, trazodon, temazepam, Erythromycin, ECT treatment 2 years before death
C5	58	F	06:15	6.31	991	Circulatory failure, cardiac pathology	Multiple organ failure, COPD, angina pectoris				Antihypertensive drugs, oxazepam, ipratropium
D5	57	F	05:30	6.28	1345	Legal euthanasia	Depression, fobia, multiple sclerosis	43	TCA, ATAD	14 years	lormetazepam, trazodon
C6	80	M	07:15	5.80	1345	Cachexia and dehydration	Diabetes type II, hypertension, CVA				haloperidol
D6	81	M	06:00	6.50	1345	Renal insufficiency, liver cirrhosis	Depression, COPD	53	–	>1 year	Renitec, furosemide, allopurinol, ranitidine, salmeterol, amoxicilline, loperamide, enalapril
C7	68	M	10:10	7.08	1547	Heart infarction	Massive heart failure, cardial hypertension, obesity				Last 24 h: atropine, adrenalin, lidocain
D7	68	M	16:46	6.64	1424	Subdural haematoma (caused by fall)	Depression, bipolar disorder, coronary insufficiency	42	MAO	24 years	Moclobemide, famotidine, verapamil
C8	69	M	19:15	6.40	1325	Pneumonia, sudden death (infarction in distal brainstem)	Pneumonia, infarction in brainstem, multiple TIAs, hypertension, familial hyper-cholesterolemia, alcohol excess				Haloperidol, temazepam
D8	68	M	05:55	6.82	1204	Cardiac ischemia	Bipolar disorder, mania, TIA, CVAs	37	–	nd	Aspirin
C9	71	M	07:40	6.20	1190	Sepsis, respiratory insufficiency	Sepsis, respiratory insufficiency, renal insufficiency, diabetes type II, hypertensive cardiomyopathy, diverticulosis				Antihypertensive drugs, anticoagulants, haloperidol
D9	70	M	04:50	6.26	1490	Cardiac arrest (after haemorrhage)	Bipolar disorder type 1	35	ATAD	17–28 years	ECT, zuclopentixol, risperidon, valproic acid, lithium, temazepam, lorazepam, clorazepine acid
C10	85	F	15:15	6.15	1196	CVA	Massive CVA, obesity, pulmonary hypertension				Carbaspirin calcium, enalapril, hydroxyzine, piracetam, last hours: diazepam, morphine
D10	84	F	<28:00	ND	1250	Thromboembolism	Depression, hypertension	78	–	nd	Motilum, Digoxin, Capoten

2.5. Validation of MCM2 and PH3 in human brain tissue

Although specificity tests of the MCM2 and PH3 antibodies have been shown before (Platel et al., 2009; Maslov et al., 2004; Reena et al., 2008; Saka and Smith, 2001; Goodarzi et al., 2009; Dupret et al., 2008), we additionally tested our immunocytochemical protocols on sections from formalin-fixed, paraffin-embedded young rat brain and human colon samples that were processed and embedded in the exact same way as the human brain tissue (see Boekhoorn et al., 2006). We further tested MCM2 and PH3 on cortical tissue of a 2 year old female with a PMD of 18 h that had died from pneumonia and sepsis, and in which large numbers of proliferating new cells were expected. This was compared to cortical tissue of a 69 year old control subject (patient C8, see Table 1) in which proliferation and neurogenesis were assumed to be rare events.

In addition, in every experiment, we included as positive control, brain tissue from a patient that had suffered from leukemia and where many proliferating and neoplastic cells were present next to the blood cells in the vasculature, as we had shown before for Ki-67 (Boekhoorn et al., 2006). Furthermore, particular attention was paid to the morphology of the individual MCM2+ and PH3+ cells as to whether they were dividing and/or had an immature, elongated or migratory appearance, and to one depressed patient that had recently received electroconvulsive therapy, a treatment known to potently stimulate neurogenesis in rodent brain.

2.6. Quantification

For standardization purposes, we collected hippocampal sections around the anterior-to-midlevel of every subject only when prominent DG and CA subregions

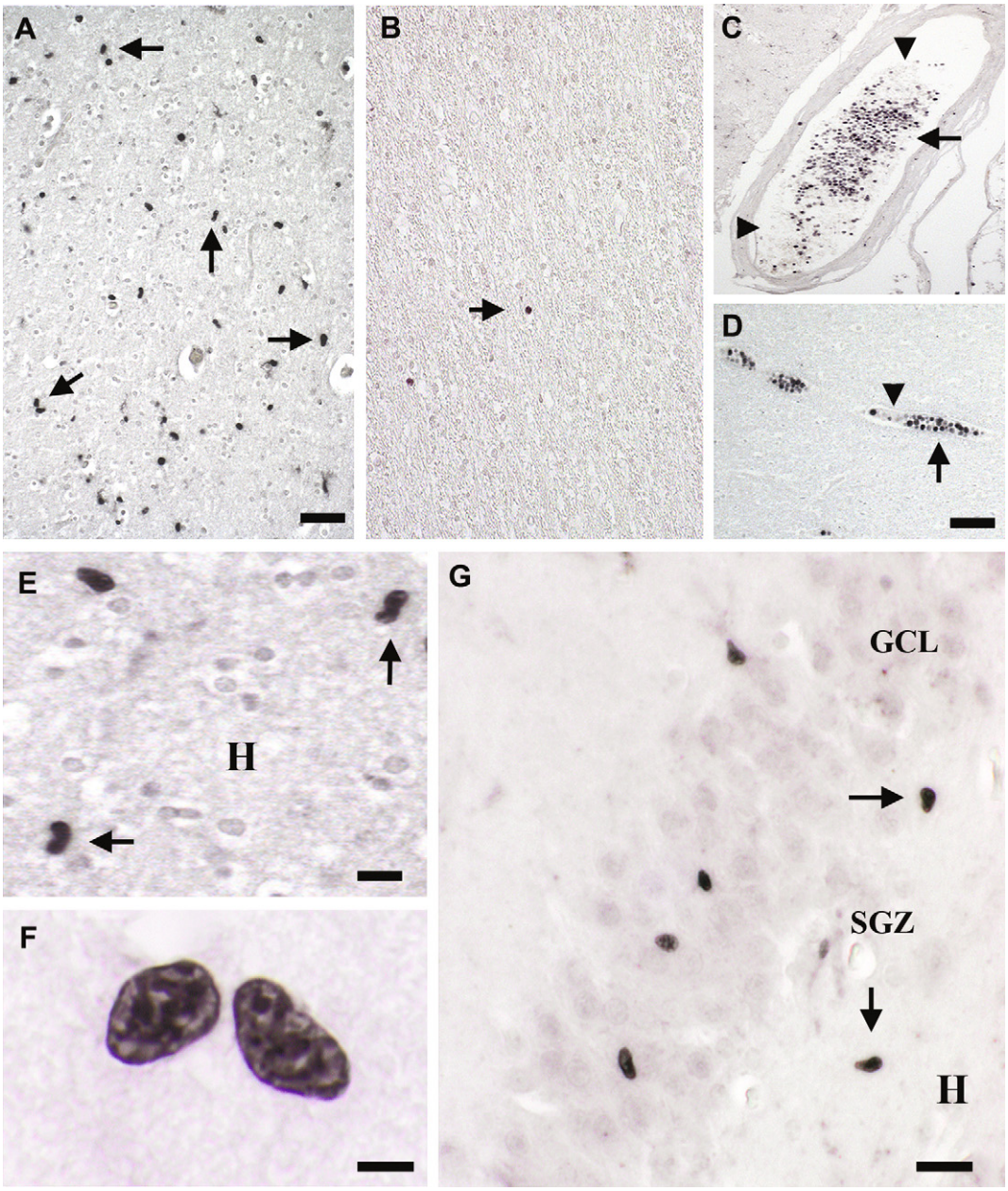


Fig. 1. Photomicrographs illustrating immunoreactivity for the cell cycle marker MCM2 in human brain tissue. A) Many MCM2 immunopositive cells, recently divided cells and doublets (arrows) are observed in cortical tissue of a 2 year old subject that served as positive control. B) MCM2+ cell numbers are dramatically reduced to very low numbers (arrow) in cortical tissue of a 69 year old control male subject. C) Numerous MCM + cells (arrow) are found in between many non-stained blood cells (2 arrowheads) within a large blood vessel located on top of the cortex of a patient that was known to have suffered from leukemia and also served as positive control. D) A large number of MCM2+ cells (arrow) was observed also inside smaller blood vessels inside brain tissue from the same leukemia patient (arrowhead again points to non-stained blood cells). E) Higher magnification of proliferating cells and doublets (arrows) in the hippocampal hilus of 48 year old control subject C2. F) High magnification of a single doublet showing 2 MCM2+ cells that have recently separated. G) Considerable numbers of MCM2 cells are found in the hippocampal hilus (H), subgranular zone (SGZ) (arrows) and granular cell layer (GCL) of a patient that received electroconvulsive therapy, a treatment known to strongly increase hippocampal proliferation in rodent models. These sections were not-counterstained. H. Low power overview of a cresyl violet counterstained section of the human hippocampus illustrating a representative midlevel as was studied in the present cohort. DG: dentate gyrus; H: hilus, CA1-3: cornu ammonis 1 to 3 subregion.

were present. To further standardize differences in anatomical level or hippocampal size, we determined the surface area of the granular cell layer (GCL) in cresyl violet counterstained sections as well as the mean cellular density in 3 reference areas chosen in the outer tips and middle part of the granular cell layer averaged for every patient. Cellular density was expressed as cell number per square micrometer. Surface area of the GCL was determined using manual outlining in combination with the appropriate tools on the StereoInvestigator setup (MicroBrightField, MBF Biosciences, Williston, VT, USA) based on Cavalieri's principle. Furthermore, in 5–6 coded sections, MCM2- and PH3-immunopositive cells were quantified in the DG by 2 independent observers (PJL, MS) unaware of the history of the subject. Only those cells displaying a clearly immature, isolated, mitotic or migratory morphology (see examples in Figs. 1 and 2) were included and categorized according to their location in the various subregions of the hippocampal DG; i.e. hilus (H), molecular layer (mol) subgranular zone (SGZ) or granular cell layer (GCL), and then averaged per subregion per patient. Subregions were identified according to Duvernoy (1988). Total numbers were calculated for GCL and SGZ together (SGZ + GCL) and for all subregions (i.e. h + mol + SGZ + GCL) together (Total) as listed in (Table 2). Finally, the average number of cells in the SGZ and GCL were taken together and normalized to the GCL surface area (Graph 1).

To address whether antidepressant drugs had affected structural plasticity in these patients, we compared the contribution of the different types of medication to the number of MCM2+ cells in the SGZ + GCL normalized to the GCL surface area.

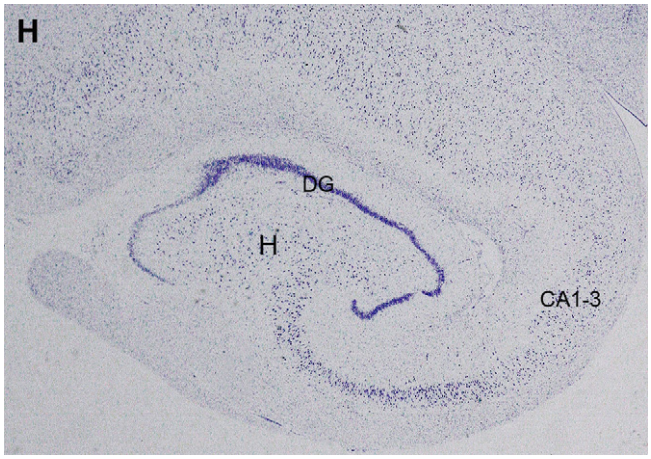


Fig. 1. (*Contiuned*).

In addition to selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAO) and tricyclic antidepressants, some of the depressed patients were prescribed atypical medication (D3, D4, D5, D9), like anti-psychotic drugs, anti-epileptic drugs or combinations thereof, or had taken no antidepressant medication (D6, D8, D10) until their death. We therefore categorized patients in three classes of medication (see Table 1); classical antidepressants (SSRI, TCA or MAO), atypical antidepressants and no medication. Differences in GCL surface area and cellular density, in Total, in SGZ + GCL and in the normalized SGZ + GCL number of MCM2 and PH3+ cells were compared between the groups and on basis of the antidepressant medication, by means of a two sample *t*-test assuming unequal variances (two-tailed, $P < 0.05$ was considered significant).

3. Results

3.1. Validation of MCM2 and PH3 specificity

In the young rat brain and human colon sections, numerous proliferating cells were observed in the dentate gyrus and crypts of Lieberkuhn, respectively (results not shown) in a pattern very similar to the Ki-67 tests we reported before (Boekhoorn et al., 2006). Notably, positive cells were absent from the tips of the villi thereby excluding possible crossreactivity with apoptotic cells. In the positive control samples, numerous MCM2- and PH3-immunopositive cells were found in cortical tissue of a 2 year old subject. Allmost all immunopositive cells had the characteristic morphology of isolated, dividing or migratory cells, or appeared as doublets (Figs. 1E, F and 2A, B, F). However, in the cortex of a 69 year old control subject, the number of cells positive for both markers was strongly reduced and occasionally a positive cell was seen (arrow in Fig. 1B).

In the other positive control, large numbers of MCM + cells were found in blood vessels situated on top of, or inside brain tissue of a leukemia patient (Fig. 1C and D). Also, considerable numbers of doublets (arrows) were found in the hippocampal hilus (H) (Fig. 1E) and granular cell layer (GCL) of depressed patient D9 (Fig. 1G) that had received electroconvulsive therapy, a treatment known to increase hippocampal proliferation in rodents.

Higher magnifications of low numbers of MCM2+ and PH3+ cells in the hippocampus of control and depressed cases revealed the characteristic morphology of proliferating cells; rare, isolated, small and often clearly dividing MCM2+ and PH3+ cells were found among larger and non-stained mature neurons, that were generally higher in number at the subgranular zone (SGZ) and in the granular cell layer (GCL)(Fig. 2A, C, E) than in the hilus and molecular layer (Fig. 2A). Examples of positive cells (Fig. 2C, E), doublets (Fig. 2B and F), or elongated cells with a migratory appearance (arrow in D) are shown in Fig. 2.

3.2. Morphometry and quantification

When comparing control subjects with depressed cases, no differences were present in average age ((68.7 (Controls) vs 68.1 (Depressed) years of age; $P < 0.9$), in pH of the cerebrospinal fluid (6.43 (Control) vs 6.47 (Depressed); $P < 0.8$)) or postmortem delay ((9 h 45 min (Control) vs 9 h 30 min (Depressed); $P < 0.9$)) of the groups. No difference was present between the average surface areas of the granular cell layer ((controls: 1.26 ± 0.25 versus 1.37 ± 0.68 mm^2 in depression; $P < 0.6$))(two sample *t*-test assuming unequal variances, two-tailed) and neither were significant differences found in cellular density of the granular cell layer (Cont vs Dep: $5{,}1 \times 10^{-3} \pm 1.42 \times 10^{-3}$ vs $4.51 \times 10^{-3} \pm 8.21 \times 10^{-4}$ cells/square micrometer of GCL; $P < 0.12$).

Average numbers of MCM2- and PH3-immunopositive cells are depicted in Table 2, where they are expressed per hippocampal subregion (i.e. SGZ, GCL, H or Mol), per total DG (i.e. SGZ + GCL combined) and per average hippocampal section (MCM2 Total, PH3 Total). For the total numbers of MCM2+ cells in all subregions, i.e. in the hilus, molecular layer, GCL and SGZ combined, a significant reduction was found in the depressed group ($P < 0.001$). When the GCL and SGZ are taken together, the number of immunopositive cells were significantly decreased in depression ($P < 0.013$). When numbers of MCM2+ cells in SGZ + GCL were normalized to the respective GCL surface areas and expressed per square micrometer, a significant reduction in depressed patients was found ($P < 0.011$) (Graph 1). No sex differences were found ($P < 0.44$), nor was a reduction present with increasing age in the number of MCM+ cells ($P < 0.1$).

For PH3, no significant changes were found in all subregions combined (Total, $P < 0.32$), for SGZ + GCL combined ($P = 0.29$) or in the SGZ + GCL normalized to GCL surface area ($P < 0.5$) (Table 2).

Since only MCM2 yielded significant differences, we next compared the contribution of different types of medication to the number of MCM2+ cells in the SGZ + GCL normalized to the GCL surface area in three classes (see Table 1); classical antidepressants (SSRI, TCA or MAO), atypical antidepressants and no medication. When comparing the controls to the non-medicated cases, a significant reduction was found in the non-medicated depressed patients ($P < 0.035$). When controls were compared to all medicated depressed patients together, a significant reduction was found in depression ($P < 0.02$). Comparing control subjects to patients that were prescribed atypical medication yielded no significant difference ($P < 0.27$). Furthermore, no significant differences were found when comparing non-medicated patients with patients on atypical medication ($P < 0.32$), or comparing patients treated with atypically medication with the combined patients receiving either SSRI, TCA or MAO type of medication.

4. Discussion

We found a significant reduction in the number of MCM2+, but not of PH3+, immunopositive cells in the hippocampus of a well-matched group of depressed patients, from which important methodological parameters like age, fixation time and PMD were not different from the controls. Also the GCL surface area and cellular density in the GCL were not different between groups, indicating we had selected a comparable anatomical plane of the hippocampus for our measurements. A significant reduction in MCM2+ numbers was found in non-medicated, depressed patients ($P < 0.035$), and also when all medicated, depressed patients, irrespective of the type of medication, were taken together, a significant reduction in depression persisted ($P < 0.02$). Only when controls were compared to depressed patients that were prescribed atypical medication, the significant difference disappeared. Although

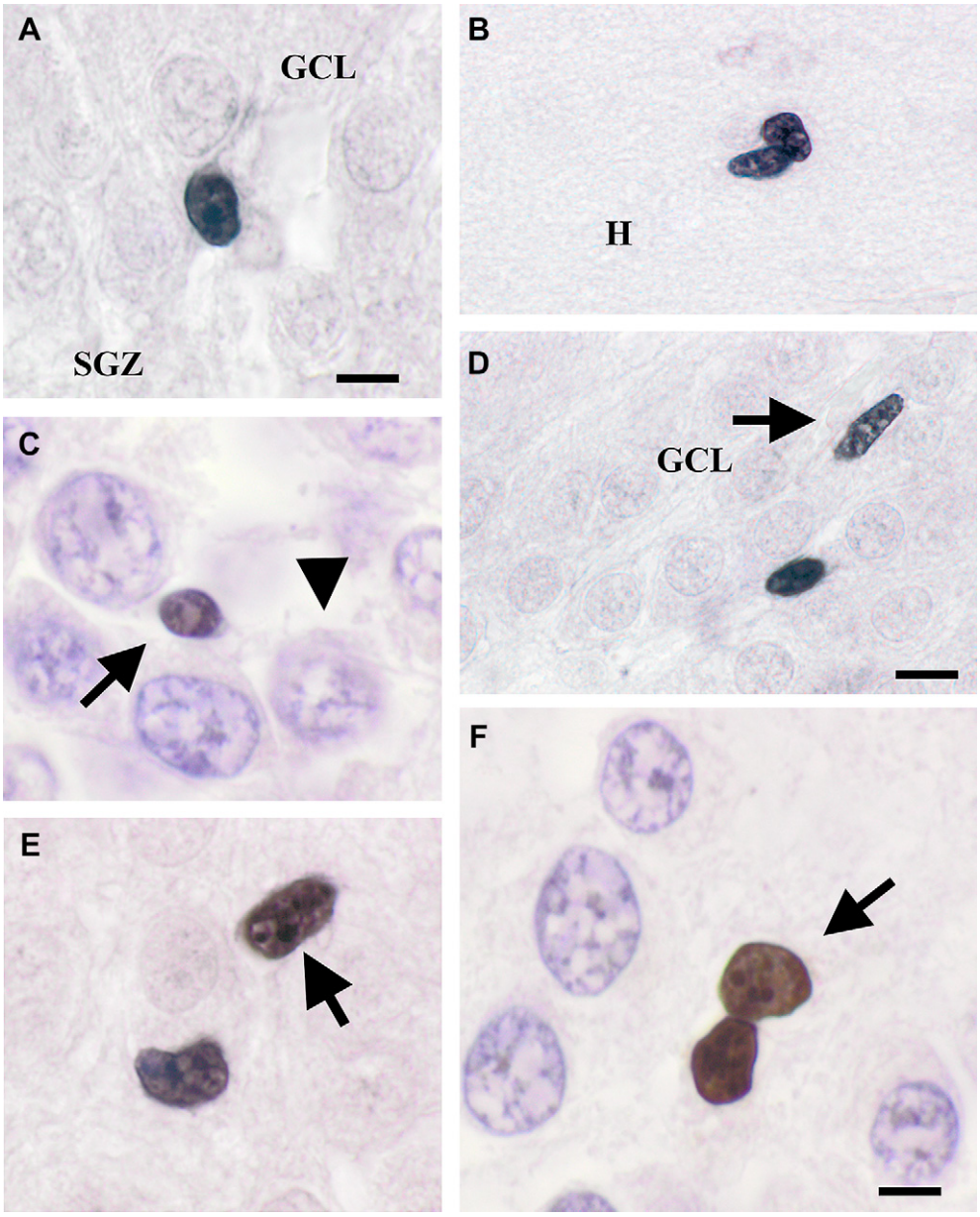
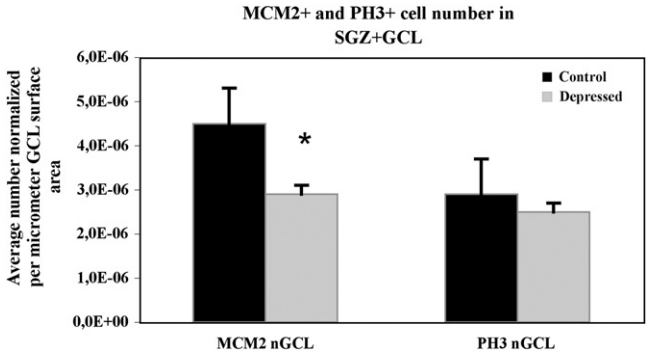


Fig. 2. High power magnifications of MCM2- and PH3-immunopositive cells in the hippocampus of control and depressed cases illustrating the different typical morphologies of proliferating cells. A) Isolated, oval shaped MCM2+ cell located at the border of the subgranular zone (SGZ) and granular cell layer (GCL). B) MCM2+ doublet of 2 still closely attached cells in the hilus (H) that appear about to separate. C) PH3+ cell with a condensed and small appearance (arrow) located in between larger, mature granular neurons in the GCL (arrowhead), cresyl violet counterstain. D) Two MCM2+ cells with an elongated, migratory appearance (arrow) within the granular cell layer (GCL). E + F) Examples of 2 closely associated MCM2+ (E) and PH3+ (F) cells within the GCL (E), and hilus (F) that are about to divide (arrow in F) or seem to have recently divided (E).

Table 2
Average number of MCM2 or PH3-immunopositive cells expressed per hippocampal subregion (i.e. SGZ, GCL, H or Mol), per DG (SGZ + GCL) and per hippocampal section (Total) in 10 control and 10 depressed patients. Abbreviations used: SD: standard deviation, SGZ: subgranular zone, GCL: granular cell layer, H: hilus, MOL: molecular layer., Total: SGZ + GCL + H + MOL taken together. *: $p = 0.013$, **: $p < 0.001$, see text for details.

MCM2		SGZ	GCL	H	MOL	SGZ+GCL	MCM2 total
Controls	mean	3.1	2.3	2.6	2	5.4	10
Depressed	mean	2	1.4	1.5	1.4	3.4*	6.3**
Controls	SD	0.7	0.8	0.8	0.7	1.2	1.8
Depressed	SD	0.7	0.7	0.7	0.7	0.8	1.4
PH3		SGZ	GCL	H	MOL	SGZ+GCL	PH3 total
Controls	mean	2	1.5	1.8	1.2	3.5	6.5
Depressed	mean	2	0.9	1.6	1	2.9	5.5
Controls	SD	0.8	0.7	0.6	0.8	1.4	2.1
Depressed	SD	0.8	0.6	0.5	1.1	1.1	2.3



Graph 1. Number of MCM2 and PH3 immunoreactive cells in the subgranular zone and granular cell layer of the dentate gyrus, normalized to surface area of the GCL and expressed per square micrometer. *indicates $P < 0.011$.

carefully matched to the depressed cases on a one-to-one basis, in the present control subjects, cause of death was variable and often extended as judged from the high agonal indexes. Although in particular RNA is very sensitive to agonal state and postmortem delay, and protein often remarkably stable (Ravid et al., 1992; Lucassen et al., 1995), the present controls obviously represent a selection and the variation in agonal state could, in theory, have influenced the present results. Whether the present difference is also found in a larger cohort of depressed patients and subjects, thus awaits future studies.

This significant reduction in MCM2+ cell number is consistent with a recent study (Boldrini et al., 2009) in which similar reductions were reported in non-medicated depressed cases, albeit studied with different markers, i.e. nestin and Ki-67. Ki-67 is a rare marker in human brain, and also with PH3, we failed to find changes in PH3+ number, suggesting that although the number of progenitors and/or putative stem cells may be reduced in depression, proliferation of these cells is not altered.

One other difference is that although in our cohort, most patients (7 out of 10) were on antidepressant medication, we could not find indications for a normalization in the number of progenitor or proliferating cells following antidepressant treatments. Thus, although consistent with the concept that structural plasticity is decreased in depression, the present data from our elderly cohort do not support the idea that increases in proliferation are involved in, or required for, antidepressant action, a concept based on various studies showing that antidepressants increase neurogenesis in rodent and also primate hippocampus (Sahay and Hen, 2007; Santarelli et al., 2003; Perera et al., 2007; Kempermann et al., 2008).

Several possibilities may explain these results. First, not all antidepressants stimulate neurogenesis, and both neurogenesis-dependent and neurogenesis-independent mechanisms of antidepressant action have been proposed (David et al., 2009; Sahay and Hen, 2007; Lucassen et al., 2010). Second, recent rodent studies suggested that the stimulatory action of the common SSRI fluoxetine on neurogenesis occurs only in young but not older animals (Navailles et al., 2008; Couillard-Despres et al., 2009). The average age of the current patients was 68 years, which is considerably older than the patients in a recent paper that ranged from 17 to 61 years of age (Boldrini et al., 2009). Therefore, antidepressant medication in elderly patients could have been very mild or even ineffective in terms of proliferative or neurogenic responses. Recent studies on primates have shown increases in hippocampal neurogenesis after repeated electroconvulsive shocks, an effective antidepressant therapy in humans. However, the nature of this therapy is different from the present antidepressant drugs prescribed, while also the age of the bonnet monkeys used in this study was low, i.e. 3–6 years, and not comparable to the present human cohort (Perera et al., 2007). Similarly, status epilepticus, a strong stimulus for neurogenesis in young rodents, is not associated with enhancements of neurogenesis in older animals (Rao et al., 2006).

Related to this, the duration of the depressive illness might have been important as it was shown to correlate with the reduction in hippocampal volume, and possibly also with changes in cytogenesis (Sapolsky, 2000; Czéh and Lucassen, 2007; Sheline, 2000). Given the suppressive effects of chronic stress or stress hormone exposure on neurogenesis in rodents (Mayer et al., 2006; Oomen et al., 2007), adult proliferation may have been reduced to a very low level in the current cohort, where most depressed patients had a long disease duration (see Table 1). It is of interest that elevated plasma glucocorticoid levels in older humans correlated negatively with hippocampal volume (see Lupien et al., 2009 and references therein) whereas application of exogenous, synthetic corticosteroids did not affect hippocampal number or volume of the primate or human hippocampus (Leverenz et al., 1999; Lucassen et al., 2001; Müller et al., 2001). Other studies indicate that these drugs do change apoptosis and proliferation in the rodent (Haynes et al., 2001) and human hippocampus, respectively (Lucassen et al., 2002). Since no change in GCL surface area or cellular density was found in our depressed group, this may imply that DG turnover is altered, e.g. by slowing of the cell cycle, as found before after chronic stress in rats (Heine et al., 2004b,c).

Furthermore, depression in the present patient cohort was severe and of long duration, and as a result, the brain could have become less sensitive to medication. Together with the low levels due to their age, this may have caused a failure to relieve not only the depressive symptoms, but also to normalize proliferation. The fact that no age-related reduction was found in the number of MCM2+ cells in both groups, supports the idea that only very low levels of plasticity remain, that may be difficult to further stimulate in patients this age.

Aside from age and disease duration, an other factor that may explain the discrepancy with previous papers is the anatomical level of the hippocampus. Clear septo-temporal gradients have been described in neurogenesis, that have been attributed to specific functionalities, also in middle-aged rodent (Snyder et al., 2009a,b; Bannerman et al., 2004). Changes in neurogenesis after stress and antidepressant treatment are generally more pronounced in the ventral hippocampus (Banasr et al., 2006; Sahay and Hen, 2007; Snyder et al., 2009a,b). The anterior-midlevels of the body of the hippocampus that were studied here in formalin-fixed, paraffin-embedded material, show a prominent DG and CA subregion, as shown in Fig. 1H. Obviously, this level represents a selection, that is notably different from Reif et al., (2006), who analyzed selectively anterior levels of the hippocampus, and in frozen sections and failed to find changes in proliferation in depression. Similarly, Boldrini et al. (2009) studied frozen sections of the entire hippocampus and found antidepressants to increase precursor cell number, but in the anterior DG only. Clearly, these differences have to be taken into account when comparing studies.

When classifying the present antidepressant treatments in patients without medication, atypical and classic (SSRI, TCA, MAO) antidepressants, cell numbers only in the atypical antidepressant group were not different from control, suggesting this type of medication seems to be more effective than the other drugs in modulating progenitors in old individuals. However, the group of patients with atypical antidepressant treatment is small and whether the same holds for their effects on depressive symptoms, is unknown. Also, as far as we know, no effects of these atypical treatments on neurogenesis have been described so far. Hence, the therapeutic effects of antidepressants in these elderly patients may not be mediated by neurogenesis modulation alone and also neurogenesis-independent effects of antidepressants could have been involved (David et al., 2009).

In the absence of a strong effect of medication, the present reduction of progenitor number may be a typical characteristic of depression but we do not known whether it is a cause, correlate or consequence of the disorder. A recent paper showing that reductions in neurogenesis can cause increases in stress hormone levels and HPA axis activity rather than the other way around, is of interest in this respect (Schloesser et al., 2009). Also, in monkeys, hippocampal volume appears predictive for stress responsivity (Lyons et al., 2007). Although it is not known whether similar changes occur in human, various studies have shown that exposure to stressful events particular during early life poses a significant risk factor for the development of depression in vulnerable individuals (Heim et al., 2008; Kendler et al., 1999; Gianaros et al., 2007). Perinatal stress in rodents affects critical periods of brain development and alters structural, emotional and neuroendocrine

parameters in adult offspring in a persisting manner. This agrees with the longlasting reductions in neurogenesis found in adult rodents following exposure to early life stress (Lemaire et al., 2000; Oomen et al., 2009) and suggests that the setpoint for neurogenesis is determined during perinatal life. As such, a reduced rate in neurogenesis may be a vulnerability factor for adult onset depression, and thus a "state" rather than a "trait", and may have been present already from the onset of the disorder onwards. In the current "end-stage" postmortem tissue, we cannot establish whether the reduction in progenitor cell number has occurred during the course of depression, or whether it was already present from the onset of the disorder onwards. Also, whether stress-induced reductions in adult neurogenesis occur in humans, awaits further investigation. As abundant glucocorticoid receptor expression is found in the human DG, this region is at least expected to be sensitive to glucocorticoid exposure (Wang, Swaab, Lucassen, unpublished observations), consistent with known steroid induced effects on hippocampal memory and proliferation (Coluccia et al., 2008; Lucassen et al., 2002).

Our results indicate that reduced numbers of progenitor and proliferating cells are present in depression. Yet, it is important to note that the present markers only provide information on the precursor population and not on the phase of adult neurogenesis (Kempermann et al., 2008; Lucassen et al., 2009, 2010). Presently, only few markers can identify neurogenesis in formalin-fixed, paraffin-embedded archival human brain tissue samples, but they have their limitations. The microtubule associated protein doublecortin identifies neurogenesis very well in perfused rodent brain (Oomen et al., 2007; Lucassen et al., 2009), but is sensitive to postmortem delay (Boekhoorn et al., 2006; Monje et al., 2007) and may, antibody dependent, also detect a subpopulation of astrocytes in human brain (Verwer et al., 2007). Furthermore, it is important to realize that in rodents, many of the newborn cells die within a few days (Dayer et al., 2003) and of the remaining ones, only a subset differentiates into new neurons. This is consistent with the low frequency of cytogenesis in the hippocampus of middle-aged and old rodents, monkeys (Heine et al., 2004a; Rao et al., 2006; Kornack and Rakic, 1999) and humans, as recently observed in live subjects using magnetic resonance spectroscopy (Manganas et al., 2007). Clearly, the field needs better markers or techniques to identify neuronal precursors, their differentiation and fate in human brain.

The functional impact and contribution of adult neurogenesis to the hippocampal changes and clinical symptoms of depression remain unclear. Although known to be involved in spatial learning (Dupret et al., 2008; Garthe et al., 2009) and aspects of anxiety (Revest et al., 2009), so far, there is no clinical evidence that adult dentate neurogenesis can be causal to depression etiology, nor do we understand how neurogenesis can contribute to mood or other symptoms of depression, besides the cognitive and memory deficits. Interestingly, the cognitive deficits are not unique to mood disorders as alterations in hippocampal function, volume and neurogenesis have been implicated in a variety of neurodegenerative and affective disorders, including stroke and dementia (Czéh and Lucassen, 2007; Thompson et al., 2008; Marlatt and Lucassen, in press). Also, depletion of neurogenesis by other means than stress, e.g. by irradiation, fails to produce a "depressive-like" state in animals. Furthermore, also neurogenesis-independent mechanisms of antidepressant action have been demonstrated (Sahay and Hen, 2007; David et al., 2009) that may involve growth factors and/or inflammatory mediators (Greene et al., 2009; Koo and Duman, 2008). Thus, although indicative of impaired hippocampal plasticity in depression, reduced neurogenesis by itself is unlikely to produce the disorder (Henn and Volmayr, 2004) and may rather reflect the cognitive symptomatology accompanying these disorders (Kempermann et al., 2008).

In conclusion, we reproduce a reduction in proliferation and progenitor number in the hippocampus of depressed patients, but do not confirm that antidepressant medication has a stimulatory effect. This may be related to the type of antidepressant medication in combination with the age of these patients. Hence, further studies in preferably larger patient groups are warranted.

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