

## Low proliferation and differentiation capacities of adult hippocampal stem cells correlate with memory dysfunction in humans

Roland Coras,<sup>1,\*</sup> Florian A. Siebzehnrubl,<sup>1,2,\*</sup> Elisabeth Pauli,<sup>3,\*</sup> Hagen B. Huttner,<sup>3</sup> Marleisje Njunting,<sup>1</sup> Katja Kobow,<sup>1</sup> Carmen Villmann,<sup>4</sup> Eric Hahnen,<sup>5</sup> Winfried Neuhuber,<sup>6</sup> Daniel Weigel,<sup>7</sup> Michael Buchfelder,<sup>7</sup> Hermann Stefan,<sup>3</sup> Heinz Beck,<sup>8</sup> Dennis A. Steindler<sup>2</sup> and Ingmar Blümcke<sup>1</sup>

1 Institute of Neuropathology, University Hospital Erlangen, 91054 Erlangen, Germany

2 Department of Neuroscience, McKnight Brain Institute, University of Florida, Gainesville, FL 32610, USA

3 Epilepsy Centre, Department of Neurology, University Hospital Erlangen, 91054 Erlangen, Germany

4 Institute for Biochemistry, Emil-Fischer-Centre, Friedrich-Alexander-University of Erlangen-Nürnberg, 91054 Erlangen, Germany

5 Institute of Human Genetics, Institute of Genetics and Centre for Molecular Medicine Cologne (CMMC), University of Cologne, 50931 Cologne, Germany

6 Department of Anatomy, Friedrich-Alexander-University of Erlangen-Nürnberg, Krankenhausstr. 9, 91054 Erlangen, Germany

7 Department of Neurosurgery, University Hospital Erlangen, 91054 Erlangen, Germany

8 Experimental Epileptology and Cognition Research, Life and Brain Centre, University of Bonn Medical Centre, 53105 Bonn, Germany

\*These authors contributed equally to this work.

Correspondence to: Ingmar Blümcke,  
University Hospital Erlangen,  
Schwabachanlage 6,  
D – 91054 Erlangen, Germany  
E-mail: bluemcke@uk-erlangen.de

The hippocampal dentate gyrus maintains its capacity to generate new neurons throughout life. In animal models, hippocampal neurogenesis is increased by cognitive tasks, and experimental ablation of neurogenesis disrupts specific modalities of learning and memory. In humans, the impact of neurogenesis on cognition remains unclear. Here, we assessed the neurogenic potential in the human hippocampal dentate gyrus by isolating adult human neural stem cells from 23 surgical *en bloc* hippocampus resections. After proliferation of the progenitor cell pool *in vitro* we identified two distinct patterns. Adult human neural stem cells with a high proliferation capacity were obtained in 11 patients. Most of the cells in the high proliferation capacity cultures were capable of neuronal differentiation ( $53 \pm 13\%$  of *in vitro* cell population). A low proliferation capacity was observed in 12 specimens, and only few cells differentiated into neurons ( $4 \pm 2\%$ ). This was reflected by reduced numbers of proliferating cells *in vivo* as well as granule cells immunoreactive for doublecortin, brain-derived neurotrophic factor and cyclin-dependent kinase 5 in the low proliferation capacity group. High and low proliferation capacity groups differed dramatically in declarative memory tasks. Patients with high proliferation capacity stem cells had a normal memory performance prior to epilepsy surgery, while patients with low proliferation capacity stem cells showed severe learning and memory impairment. Histopathological examination revealed a highly significant correlation between granule cell loss in the dentate gyrus and the same patient's regenerative capacity *in vitro* ( $r=0.813$ ;  $P<0.001$ ; linear regression:  $R^2_{\text{adjusted}}=0.635$ ), as well as the same patient's ability to store and recall new memories ( $r=0.966$ ;  $P=0.001$ ; linear regression:  $R^2_{\text{adjusted}}=0.9$ ). Our results suggest that encoding new memories is related to the regenerative capacity of the hippocampus in the human brain.

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**Keywords:** stem cells; neurogenesis; hippocampus; memory; learning; epilepsy; doublecortin

**Abbreviations:** BDNF = brain-derived neurotrophic factor; cdk5 = cyclin-dependent kinase 5; HPC = high proliferation capacity; IAT = intracarotid amobarbital testing; LPC = low proliferation capacity

## Introduction

Multipotent progenitor cells reside below the granule cell layer of the dentate gyrus in the adult mammalian hippocampus (Altman, 1962; Eriksson *et al.*, 1998; Gage, 2000) and give rise to newborn granule cells that mature and functionally integrate into neuronal microcircuits (van Praag *et al.*, 2002; Tashiro *et al.*, 2006; Zhao *et al.*, 2006; Jessberger *et al.*, 2008). The role of the specific regenerative capacity in the dentate gyrus, herein defined as capacity of adult hippocampal stem cells to proliferate and differentiate into neurons *in vitro*, has remained enigmatic until recent years, in which a number of studies have examined the role of the dentate gyrus in specific forms of memory and pattern processing. Particularly strong evidence is available for a role of both human and rodent hippocampus for pattern separation, which constitutes the distinct representation of similar inputs (Squire *et al.*, 2004; Leutgeb *et al.*, 2007; Bakker *et al.*, 2008; Nakashiba *et al.*, 2008). Pattern separation as a general principle may be applicable to different processing modalities both in human and in rodents and is probably a key process, important for spatial and episodic memory formation (Leutgeb *et al.*, 2007; Bakker *et al.*, 2008; Nakashiba *et al.*, 2008). More importantly, recent evidence indicates that pattern recognition is impaired if neurogenesis is reduced by irradiation (Clelland *et al.*, 2009). In addition to pattern separation, other forms of hippocampal-dependent memory tasks are also impaired when neurogenesis is experimentally reduced. For instance, hippocampus-dependent trace conditioning is impaired following pharmacological ablation of neurogenesis in the adult rat (Shors *et al.*, 2001). Transgenic inhibition of adult-born granule cells revealed deficient long-term spatial memory and further supports the notion that immature neurons that undergo maturation make an important contribution to learning and memory (Deng *et al.*, 2009; Jessberger *et al.*, 2009). Timing of neuronal differentiation plays a critical role in the functional integration of newly generated hippocampal neurons, and continuous production of neurons may be required not only for new memory acquisition, but also to use previously consolidated memories (Farioli-Vecchioli *et al.*, 2008). Taken together, these studies point to neurogenesis as a required determinant of dentate gyrus-dependent information processing and memory. Prospective studies that address the importance of neurogenesis in the human hippocampus are impossible to perform, for obvious reasons. We have taken advantage of the opportunity to obtain hippocampal specimens from epilepsy surgery to quantitatively assess the regenerative potential in the dentate gyrus in individual human subjects (Roy *et al.*, 2000; Walton *et al.*, 2006). We found two distinct groups of patients, one with a high potential for generation of neural precursors [high proliferation capacity (HPC)] and subsequent differentiation, and another with a severe deficiency in these processes [low proliferation capacity (LPC)]. As many patients

with epilepsy also suffer from variable degrees of declarative learning and memory impairment (Helmstaedter and Elger, 2009), we have used these patient groups to ask if these dramatic differences in neurogenesis are reflected in altered hippocampus-dependent memory processes in human subjects.

## Materials and methods

### Human hippocampal tissue

Hippocampal specimens were obtained from 23 consecutive patients who underwent epilepsy surgery at the Erlangen Epilepsy Centre during 2006–09 (Table 1), in which drug-resistant unilateral mesial temporal lobe epilepsy was diagnosed by preoperative evaluation. Pre-surgical epilepsy monitoring included interictal and ictal video EEG monitoring, using 32–64 EEG channels, as well as MRI (1.5 Tesla Sonata Siemens, Munich, Germany) and neuropsychological evaluation. Intracarotid amytal testing (see below), PET, magnetic encephalography and intraoperative electrocorticography were applied when necessary to characterize the epileptogenic zone (Engel, 1994; Stefan *et al.*, 2004, 2009). Anti-epileptic drug treatment (at the time of surgery as well as previously administered during the course of the disease) is shown in Table 2. Mean duration of epilepsy was 24.7 years, ranging from 4 to 40 years. The average age at surgery was 36.9 years, ranging from 21 to 55 years. Tailored anterior temporal resections were performed in all patients including *en bloc* resection of the hippocampus. Available clinical data are summarized in Table 1. Informed and written consent was given by all patients included in our study for additional scientific investigations approved by the local ethics committee of the University of Erlangen. All procedures were conducted in accordance with the Declaration of Helsinki (1964).

### Human hippocampal cell culture

After surgical *en bloc* resection, the hippocampus was coronally sliced along the anterior–posterior axis. One naïve 5 mm thick section from the anteromedial body and adjacent to that used for histological analysis was available from each patient and processed for cell culture. The dentate gyrus was micro-dissected from adjacent structures (in particular from the ventricular wall) under a stereomicroscope (Olympus SZX9, Tokyo, Japan) and dissociated mechanically, followed by enzymatic digestion as described (Siebzehnrubl *et al.*, 2007). Isolated cells were plated in N5 medium (Walton *et al.*, 2006), supplemented with 20 ng/ml epithelial growth factor (CellSystems, St Katharinen, Germany), 20 ng/ml fibroblast growth factor 2 (R&D Systems, Minneapolis, MN, USA) and 10 ng/ml leukaemia inhibitory factor (Millipore, Billerica, MA, USA) onto poly-L-ornithine/laminin-coated (Sigma-Aldrich, Schnellendorf, Germany) culture plates. Growth factors were added every third day and medium was exchanged once a week. For differentiation, 20 000 cells were plated onto poly-L-ornithine/laminin-coated coverslips in N2 medium

**Table 1** Patients included in this study

Patient ID	Clinical history							
	Gender	First seizure (years)	Age at epilepsy onset (years)	Duration of epilepsy (years)	Age at surgery (years)	Side of resection	Seizure type	Memory (IAT)
P1	F	0	32	23	55	Right	SPS, CPS, sGTCS	-2.20
P2	F	1	26	11	37	Left	SPS, CPS, sGTCS	-2.35
P3	F	8	8	31	39	Right	SPS, CPS	0.30
P4	F	3	3	34	37	Right	SPS, CPS, sGTCS	NA
P5	F	0.5	14	23	37	Left	SPS, CPS	0.50
P6	F	1	4	28	32	Right	SPS, CPS, sGTCS	NA
P7	M	2.5	7	36	43	Left	CPS, sGTCS	NA
P8	F	1	11	31	42	Right	SPS, CPS, sGTCS	NA
P9	M	0.8	4	21	25	Right	SPS, CPS	NA
P10	F	0.1	1	40	41	Right	SPS, CPS	-1.00
P11	M	1	1	39	40	Right	SPS, CPS, sGTCS	NA
P12	M	4	4	24	28	Right	SPS, CPS, sGTCS	NA
P13	M	3	11	23	34	Left	CPS, sGTCS	-2.10
P14	M	0.5	18	25	43	Right	SPS, CPS, sGTCS	NA
P15	F	0.5	5	16	21	Right	SPS, CPS, sGTCS	-0.70
P16	M	42	42	4	46	Right	SPS, CPS, sGTCS	NA
P17	M	2	3	32	35	Left	SPS, CPS, sGTCS	NA
P18	M	2	20	7	27	Left	SPS, CPS, sGTCS	-2.10
P19	F	15	15	40	55	Right	CPS, sGTCS	NA
P20	M	1.5	2	19	21	Right	SPS, CPS, sGTCS	-1.40
P21	M	5	26	25	51	Right	CPS, sGTCS	NA
P22	M	7	7	25	32	Right	CPS, sGTCS	NA
P23	F	18	18	10	28	Right	SPS, CPS	NA

IAT data are given as z-scores with normal values between 0 and -1, and severe memory impairment <-2.

CPS = complex partial seizures; F = female; IAT = Intracarotid amobarbital testing; M = male; NA = not analysed; sGTCS = secondary generalized tonic-clonic seizures; SPS = simple partial seizures.

containing 31.25 µg/ml bovine pituitary extract (Invitrogen, Karlsruhe, Germany), 1% foetal calf serum (Biochrom, Berlin, Germany); 500 ng/ml sonic hedgehog (R&D Systems), 100 ng/ml fibroblast growth factor 8 (Peprotech, Hamburg, Germany) and 1 µM suberoylanilide hydroxamic acid (Axxora, Lörrach, Germany) for 48 h. Thereafter, medium was changed to N2 supplemented with bovine pituitary extract, 1% foetal calf serum, 10 ng/ml nerve growth factor (Sigma-Aldrich) and 5 µM forskolin (Sigma-Aldrich). Cellular differentiation was immunocytochemically analysed after 14 days (see below).

## Fluorescence-immunocytochemical analysis

Immunocytochemistry of fixed cells and quantitative microscopic evaluation were performed as described earlier (Siebzehnrubl *et al.*, 2007). Primary antibodies were used at the following dilutions: mouse-anti-Map2ab (Sigma-Aldrich) 1:250, chicken-anti-MAP2 (Abcam, Cambridge, UK) 1:5,000, rabbit-anti-nestin (Millipore) 1:200, mouse-anti-nestin (Millipore) 1:200, rabbit-anti-Musashi (Millipore) 1:200, mouse-anti-Pax6 1:50 (Millipore), mouse-anti-βIII tubulin (Promega, Mannheim, Germany) 1:500, rabbit-anti-βIII tubulin (Covance, Berkeley, USA), mouse-anti-NeuN (Millipore) 1:1000, rabbit-anti-gial fibrillary acidic protein (Dako, Glostrup, Denmark) 1:600, mouse-anti-gial fibrillary acidic protein (Millipore) 1:600, mouse-anti-2',3'-Cyclic-nucleotide 3'-phosphodiesterase (Millipore) 1:200, rabbit-anti-doublecortin (Abcam) 1:500, mouse-anti-Ki67

(Dako) 1:100, rabbit-anti-brain-derived neurotrophic factor (BDNF; Abcam) 1:100, rabbit-anti-cyclin dependent kinase 5 (cdk5; Abcam) 1:100, goat-anti-Sox2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:100. Secondary antibodies were obtained from Invitrogen and used in appropriate dilutions. Cellular nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). After differentiation with suberoylanilide hydroxamic acid, sonic hedgehog, fibroblast growth factor 8, forskolin and nerve growth factor, neuronal profiles were assessed *in vitro* by counting MAP2-immunoreactive cells.

## Histopathological examination

Each surgical hippocampus specimen was dissected into 5 mm thick slices along the anterior-posterior axis. Tissue from the mid-hippocampal body (see above) was fixed overnight in 10% formalin and routinely processed into liquid paraffin. All specimens were cut at 4 µm on a rotation microtome (Microm; Heidelberg, Germany) and stained with haematoxylin and eosin. Hippocampal pyramidal neurons and granule cells of the dentate gyrus were detected using immunohistochemistry for NeuN (Millipore, 1:1000) and an automated staining apparatus (Ventana, Strasbourg, France). Microwave pretreatment was applied for anti-doublecortin, anti-BDNF, anti-cdk5 and anti-Ki67 labelling of paraffin embedded tissue.

**Table 2** Anti-epileptic drug treatment in our series of 23 patients

Patient ID	VPA	CBZ	CLB	ESM	GBP	LTG	LEV	OXC	PB	PHT	PGB	PRM	STM	TPM	VGB	ZNS
P1	N	Y	Y	N	P	N	Y	N	N	N	N	N	N	N	N	N
P2	P	Y	N	N	N	P	Y	Y	N	N	N	P	N	N	N	N
P7	P	Y	N	N	P	Y	P	N	P	P	N	N	N	N	N	N
P9	P	N	N	N	N	N	P	Y	N	N	N	N	N	N	N	N
P13	P	P	Y	N	P	P	Y	P	N	N	N	N	N	N	N	N
P14	N	N	P	N	N	Y	N	N	N	N	N	N	N	N	N	N
P16	P	N	N	N	N	Y	Y	N	N	N	N	N	N	N	N	N
P18	N	P	N	N	N	N	Y	Y	N	N	N	N	N	N	N	N
P20	P	N	N	N	N	Y	N	Y	N	N	N	N	N	N	N	N
P21	P	P	Y	N	N	N	Y	Y	N	N	N	N	N	N	N	N
P22	P	P	Y	N	N	N	Y	Y	N	N	N	N	N	N	N	N
P23	N	P	N	N	N	Y	Y	N	N	N	P	N	N	N	N	N
P3	P	P	P	N	N	Y	P	Y	N	P	P	P	N	P	N	P
P4	N	N	P	P	N	Y	Y	P	N	N	N	P	N	P	P	N
P5	P	N	N	N	N	Y	Y	N	N	N	N	N	N	N	N	N
P6	P	N	N	N	P	Y	Y	N	N	N	P	N	N	N	N	N
P8	P	P	N	N	P	P	Y	Y	N	N	P	N	N	P	N	P
P10	P	P	N	N	P	N	P	Y	Y	P	N	N	N	P	N	N
P11	P	P	N	N	N	Y	N	Y	P	N	N	N	N	N	N	N
P12	P	P	Y	N	P	P	Y	Y	N	N	N	N	N	N	N	N
P15	P	N	N	N	N	Y	P	P	N	P	N	N	Y	P	N	P
P17	N	P	N	N	N	N	Y	Y	P	N	N	N	N	N	N	N
P19	N	N	P	N	N	Y	N	Y	N	N	N	N	N	N	N	N

The patient ID is the same as in Table 1, listed according to LPC (12 upper rows) and HPC (11 lower rows). Statistical analysis did not reveal any correlation between anti-epileptic drug treatment and each patient's regenerative capacity (Pearson Correlation, Table 4).

CBZ = carbamazepine medication in medical history; CLB = clobazam; ESM = ethosuximide; GBP = gabapentin; LEV = levetiracetam; LTG = lamotrigine; N = drug not obtained; OXC = oxcarbazepine; P = previously administered anti-epileptic drug medication during course of disease; PB = phenobarbital; PGB = pregabalin; PHT = phenytoin; PRM = primidon; STM = Sultiam; TPM = topiramate; VGB = vigabatrin; VPA = valproate (note that any valproate medication was stopped 3 months before surgery); Y = prescribed drug medication at time of surgery; ZNS = zonisamide.

## Neuronal cell counts

Semi-quantitative cell density measurements were obtained from all patients using 4 µm thin paraffin sections and NeuN immunohistochemistry (Wolf *et al.*, 1996). Hippocampal sectors CA1, CA2, CA3 and CA4 and the dentate gyrus were examined at ×40 objective magnification. Ten randomly placed microscopic fields were examined for each anatomical subregion. Measurements were performed with a microcomputer imaging system (ColorView II CCD camera, Soft imaging system SIS, Stuttgart, Germany) attached to a BX51 microscope (Olympus). Immunohistochemically stained neuronal cell bodies were tagged on the computer screen, counted within the region of interest and expressed as the mean number of neurons/mm<sup>2</sup> using AnalySIS imaging software (SIS) and Excel software (Microsoft, Redmond, Washington, USA). Histopathological data are summarized in Table 2. The same methodology was applied for assessing the proliferation activity *in vivo* using Ki67 immunoreactivity. The subgranular and granule cell layers were analysed in 18 patients from which sufficient material was available for preparing 10 serial 4 µm sections. We could not perform these experiments in patients P1, P2, P3, P5 and P7 (Table 1).

Nestin-, Sox2-, PAX6-, doublecortin-, BDNF- and cdk5-immunoreactive cells were semi-quantitatively estimated by the same method using fluorescence labelling. Four adjacent microscopic fields were placed into the dentate gyrus granule cell layer at ×20 objective magnification and immunoreactive cell bodies were identified using appropriate filter combinations.

## Electrophysiological recordings from human hippocampal progenitor cells

Membrane currents were measured by applying the patch-clamp technique in a whole-cell recording configuration. Current signals were amplified with an EPC-9 amplifier (HEKA, Lambrecht, Germany). Whole-cell recordings were performed after three expansion periods and induced differentiation (see above). After 14 days *in vitro*, cells with a neuronal morphology by phase contrast imaging were chosen (Fig. 2C). All cells were held at −70 mV. Following a 40 ms prepulse to −120 mV, voltage steps incremented by 10 mV were applied from −80 to +10 mV every 2 s. The external buffer consisted of 145 mM NaCl, 5 mM KCl, 2.4 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1.8 mM glucose, 10 mM HEPES, pH adjusted to 7.4 with NaOH; the internal buffer contained 150 mM CsCl, 5 mM EGTA, 10 mM HEPES, and the pH adjusted to 7.2 using CsOH. All experiments were carried out at room temperature (~22°C). Recording pipettes were fabricated from borosilicate capillaries with open resistances of 5–6 MΩ.

## Neuropsychological examination

Intracarotid amobarbital testing (IAT; WADA) was carried out separately in both hemispheres as part of the presurgical evaluation in nine patients. The test is employed in patients in whom the risk for post-operative memory loss has to be clarified preoperatively. The greatest potential risk in surgical treatment is verbal memory loss in patients

suffering from left-sided temporal lobe epilepsy (Chelune, 1995), but suspicion of atypical memory dominance in right-sided temporal lobe epilepsy should in some cases also require examination. Thus 5 out of 6 patients with left-sided temporal lobe epilepsy and 4 out of 17 patients with right-sided temporal lobe epilepsy underwent IAT following the Erlangen Wada Test protocol. This protocol is described in detail elsewhere (Pauli *et al.*, 2006). In brief, double encodeable memory items are tested under recall and recognition conditions and the results are transformed into z-scores according to normative values specific for speech dominant and for non-dominant hemispheres, respectively. Since healthy control data are not available for IAT, standardization was based on values from the contralateral, non-affected left or right temporal lobes, including only those patients from our database ( $n > 200$ ) presenting with: (i) unilateral mesial temporal lobe epilepsy; (ii) unilateral left-sided speech dominance; (iii) complete postoperative seizure freedom; and (iv) normal range IQ. IAT memory scores were transformed into z-values following the calculation rule: (i) if the resected hippocampus was taken from the speech dominant hemisphere:  $z(\text{IAT memory}) = [(\text{Total memory score} - \text{Mean}_{\text{left}}) / \text{SD}_{\text{left}}]$ ; and (ii) if the resected hippocampus was taken from the non-dominant hemisphere:  $z(\text{IAT memory}) = [(\text{Total memory score} - \text{Mean}_{\text{right}}) / \text{SD}_{\text{right}}]$ . Thus, a z-score of 0 indicates full functional integrity of the investigated left or right hippocampal structure; i.e. indicates equality with the average score of non-affected left or right hippocampi in unilateral temporal lobe epilepsy.

## Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 16) was used for statistical evaluation. The threshold for significance was set to 0.05. Correlation analysis, linear multiple regression and partial correlations were calculated to evaluate the relationship between clinical histories, histopathological data, cell culture and memory performance. Cluster analysis and discriminant analysis were used to explore neurogenesis *in vitro*. Correlation analysis (Pearson) was performed to relate (i) neurogenesis *in vitro* with clinical data (age, onset and duration of epilepsy); (ii) neurogenesis *in vitro* with cell densities in hippocampal subfields and the dentate gyrus; and (iii) neurogenesis *in vitro* with IAT memory capacity. Only variables that significantly correlated with the dependent variable were included in linear regression and partial correlation analysis. Since the multiple correlation coefficient  $R$  tends to overestimate the correlation between observed and predicted values of dependent variables, adjusted  $R^2$ , which is thought to more closely reflect the goodness of fit of the model in a population, was calculated.  $R^2_{\text{adjusted}}$  describes the proportion of variance of the dependent variable that is explained by the independent variables. To assess the relative importance of each independent variable, we applied the  $t$ -statistic for the linear regression coefficient and only values  $> 2$  were considered significant. To check for multicollinearity, tolerance statistics were used. In addition, partial correlation analysis was calculated to assess the correlation of the independent variable with the dependent variable after removing the linear effect of the other variables in the model. Neuropsychological parameters as well as cell densities in the hippocampal subfields were transformed into z-scores, representing, in standard deviation units, the amount score deviates from the mean of the population from which the score is drawn (null hypothesis). For histopathological data (cell densities in the hippocampal subfields and the dentate gyrus), standardization was based on age-matched non-epileptic autopsy controls (Blumcke *et al.*, 2007). For IAT, standardization was based on normal values from contralateral, non-affected left or right temporal lobes in unilateral temporal lobe epilepsy.

## Results

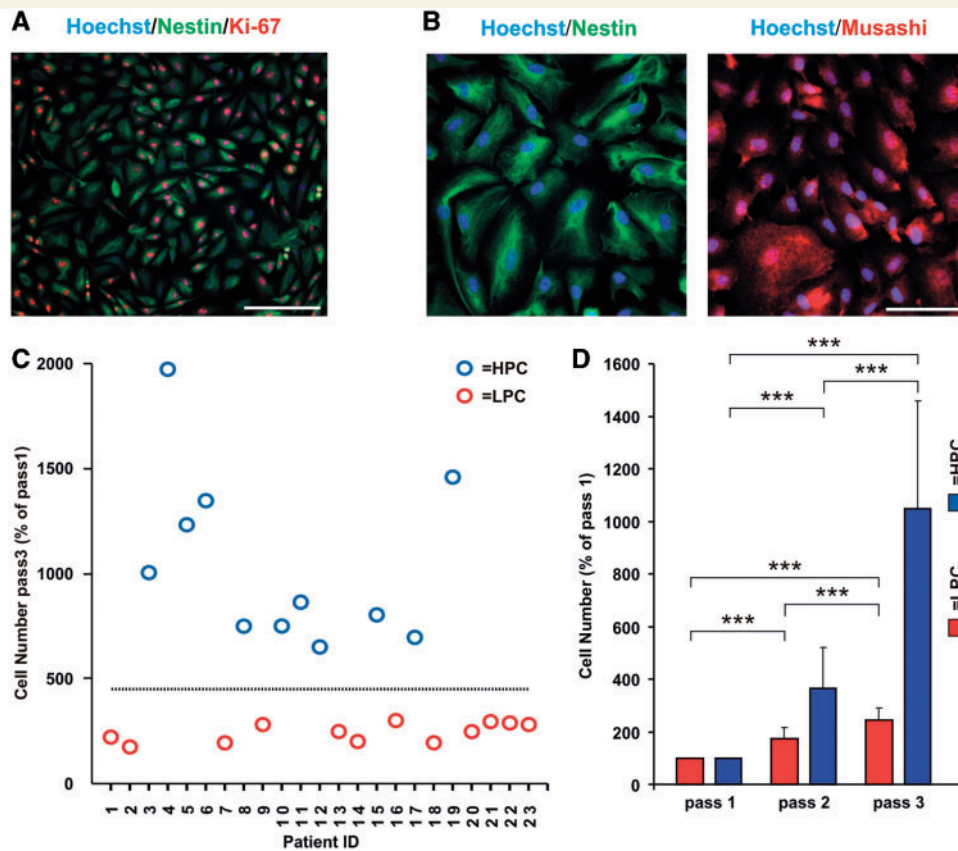
### HPC and LPC in adult human hippocampal stem cells *in vitro*

Adult hippocampal progenitor cells have the capacity for self-renewal, to generate new cells by asymmetric division and further differentiate into various cell lineages of the brain (Gage, 2000). We obtained surgical hippocampal tissue from 23 patients with drug-resistant epilepsy (Table 1). Following microdissection of the dentate gyrus, cells were dissociated and proliferated as a monolayer culture (Fig. 1A). Three cell culture passages were applied to expand the progenitor cell population ('Materials and methods' section), which were characterized by abundant expression of nestin and Musashi1 (Fig. 1B). Cluster analysis of data obtained after three cell culture passages *in vitro* from all subjects revealed two significant patterns (Fig. 1C;  $F = 45.1$ ;  $df = 1/22$ ;  $P < 0.001$ ). An HPC was observed in 11 specimens, showing doubling of primarily seeded cells after 2–3 weeks and continued proliferation through further passages *in vitro* (Fig. 1D). An LPC was evident in the remaining 12 specimens. Although cell numbers increased during each passage in LPC cultures, their amount remained significantly lower after three passages *in vitro* compared with HPC specimens.

### HPC versus LPC cultures showed different propensities for neuronal differentiation

After completing our proliferation protocols, growth factor withdrawal induced spontaneous differentiation into a neuronal phenotype in most cell cultures, ranging from 0 to 6% (mean  $2 \pm 2\%$ ) of the total cell population. Differentiation into a neuronal phenotype was significantly enhanced using suberoylanilide hydroxamic acid, an inhibitor of histone deacetylases (Hsieh *et al.*, 2004; Siebzehnrubl *et al.*, 2007), as well as sonic hedgehog and fibroblast growth factor 8 for 48 h, followed by treatment with the adenylylase activator forskolin and nerve growth factor for 12 days (mean  $28 \pm 27\%$ ). Cellular phenotype was microscopically analysed after 14 days in culture and staining with antibodies directed against antigens specific for neurons, i.e. MAP2 and NeuN (Fig. 2A and B), astrocytes (glial fibrillary acidic protein, not shown) and oligodendrocytes (2',3'-cyclic nucleotide 3'-phosphodiesterase, not shown), suggesting the maintenance of multipotent adult hippocampal stem cells in our cultures. In addition, patch-clamp recordings detected sodium inward currents of up to 300 pA (Fig. 2C) in cells with dendrite-like neuronal arborizations. These findings demonstrated that adult hippocampal stem cells can be isolated from human surgical tissue and differentiated into neurons.

There was a highly significant correlation between proliferation and the same patient's capacity to generate neurons *in vitro* ( $r = 0.834$ ,  $R^2 = 0.696$ ,  $P < 0.001$ ). Linear regression analysis revealed proliferation as the only variable meeting the demands of the predictors criterion  $|t| > 2$  ( $T = 6.94$ ,  $R^2_{\text{adjusted}} = 0.696$ ,



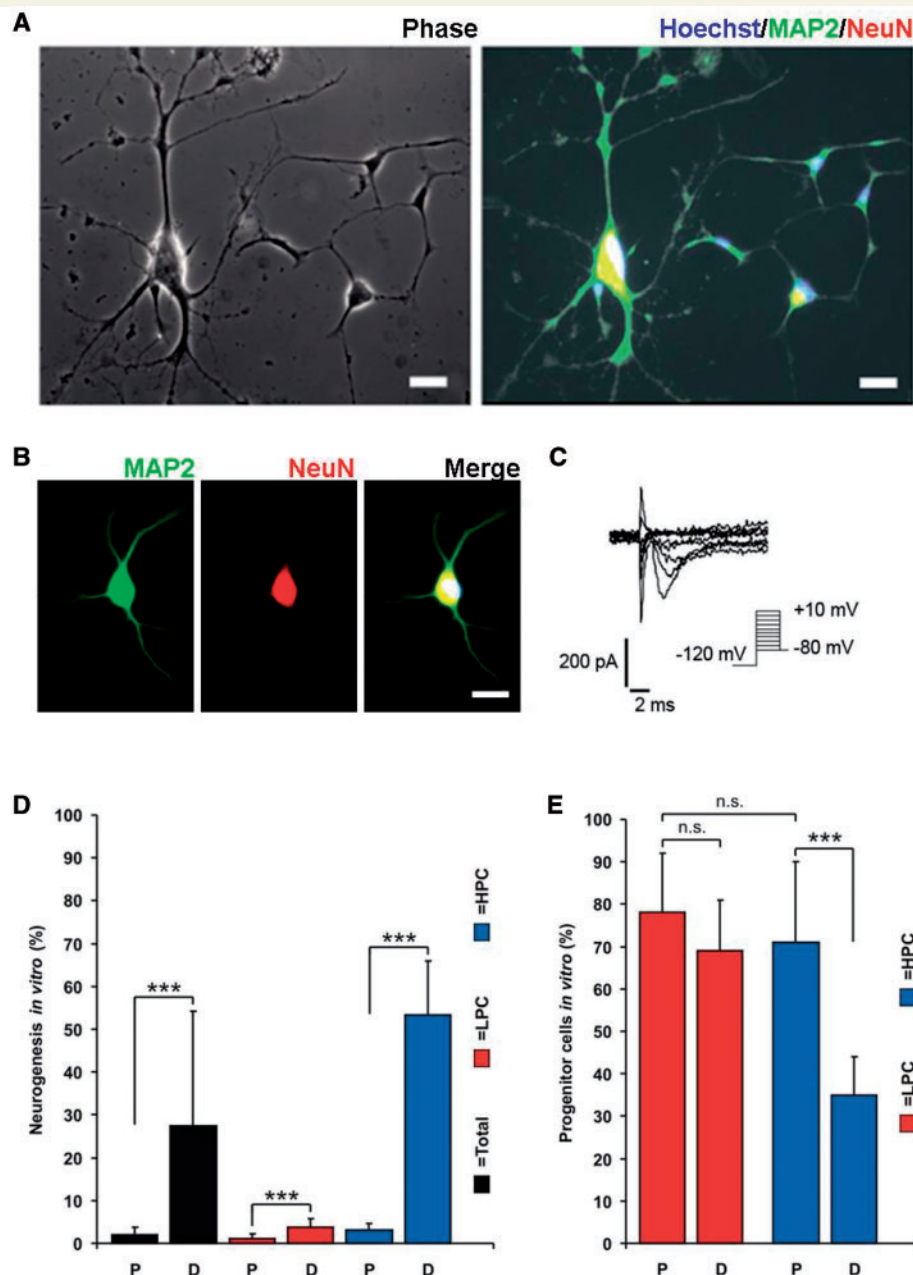
**Figure 1** Different proliferation capacities of human adult hippocampal stem cells. (A) Double-fluorescence immunocytochemistry of nestin (green) and Ki67 (red) epitopes confirmed proliferation activity of adult hippocampal stem cells. Hoechst nuclear staining shown in blue (also in B). (B) Fluorescence immunocytochemistry of nestin (green) and Musashi (red) confirmed stemness of the progenitor cell population. Scale bars in A=50  $\mu$ m and B=100  $\mu$ m. (C) Discriminance analysis of proliferation capacities obtained from all cell cultures revealed two significant growth patterns ( $P<0.001$ ). HPCs are indicated by blue dots, LPC by red dots. (D) Red bars refer to LPC culture specimens. Blue bars refer to HPC cultures, which showed a significant higher increase in cell numbers after each of the three passages (pass) *in vitro* ( $P<0.001$ ). \*\*\* $P<0.001$  (*t* test).

$P<0.001$ ), indicating that nearly 70% of the variance of the capacity to generate new neurons *in vitro* is explained by proliferation. HPC cell cultures showed a mean of  $53 \pm 13\%$  MAP2-immunoreactive neuronal cells (compared with spontaneous neuronal differentiation of  $3 \pm 2\%$  following growth factor withdrawal; Fig. 2D). In contrast, cell cultures obtained from LPC specimens failed to generate more cells with a neuronal phenotype (mean percentage of MAP2-immunoreactive cells =  $4 \pm 2\%$  after targeted differentiation; Fig. 2D). In the latter cultures, expression of the neural precursor filament nestin persisted in  $69 \pm 12\%$  of cells (compared with  $78 \pm 14\%$  after spontaneous differentiation; Fig. 2E) and glial markers were also not significantly increased ( $5 \pm 4\%$  glial fibrillary acidic protein- and  $2',3'$ -cyclic nucleotide 3'-phosphodiesterase-immunoreactive cells after targeted differentiation compared with  $3 \pm 2\%$  after spontaneous differentiation). Proliferation within the granule cell layer was studied using the Ki67-epitope in 18 patients from whom serial sections were available (Fig. 3A). These data showed a significant difference between the LPC and HPC groups ( $P=0.002$ ; Fig. 3B). Statistical analysis confirmed a significant correlation between the numbers of proliferating

cells *in vivo* and the proliferation capacities detected *in vitro* ( $P=0.007$ ; Fig. 3C). Although sufficient tissue was not available for a systematic serial section analysis of additional neural stem cell marker proteins (i.e. nestin, Pax6 or Sox2), double immunofluorescence was performed in each patient and confirmed an immature phenotype of Ki67-immunoreactive, proliferating cells in the dentate gyrus (Fig. 4). These data demonstrate that patients with temporal lobe epilepsy fall into two distinct groups, with high versus low capacity for proliferation *in vivo* and *in vitro* as well as a commensurately differing capacity for differentiation.

### Patients with HPC versus LPC cultures showed different granule cell densities *in vivo*

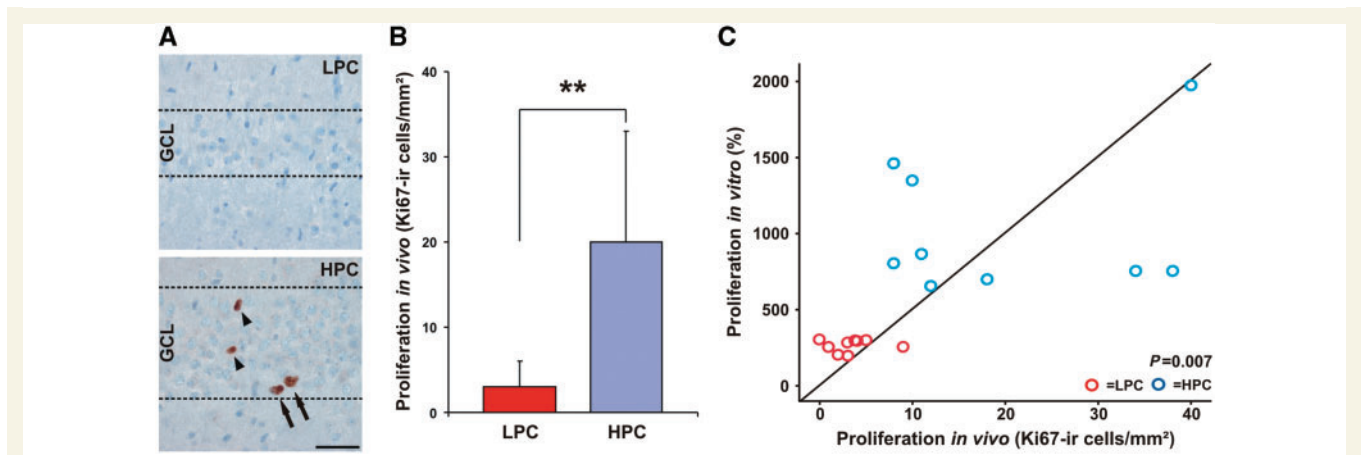
These dramatic differences in the HPC versus LPC patient groups led us to examine whether granule cell densities were higher in the HPC group. Formalin-fixed and paraffin-embedded tissue sections from the anteromedial part of the hippocampal body were

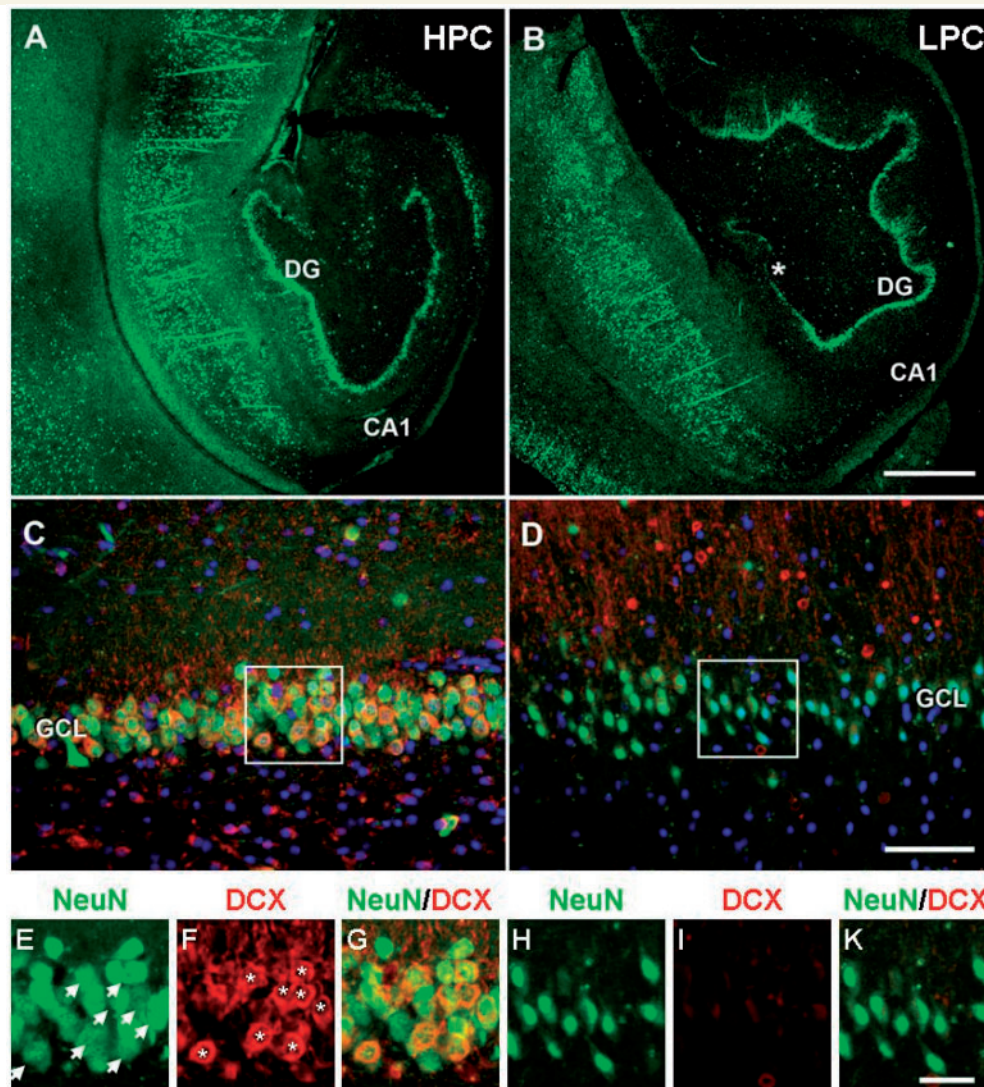


**Figure 2** Targeted neuronal differentiation of human adult hippocampal stem cells. (A) Following our differentiation protocol, cells with a neuronal phenotype showed elaborated process outgrowth (phase contrast) and expression of neuron-specific epitopes MAP2 (green) and NeuN (red). Coexpression of neuronal marker proteins in yellow, Hoechst nuclear staining in blue. (B) Single channel immunofluorescence detection from a neuron *in vitro* co-expressing MAP2 (green) and NeuN (red). (C) Voltage-clamp profile of adult hippocampal progenitor cells after 14 days of differentiation. This representative trace showed an inward  $\text{Na}^+$  current generated following a voltage step protocol from  $-80$  to  $+10$  mV incremented by  $10$  mV every  $2$  s following a  $40$  ms prepulse to  $-120$  mV (see inset). (D) After the proliferation period shown in Fig. 1D, only a few cells differentiated spontaneously into a neuronal phenotype after growth factor withdrawal (quantified by MAP2 immunoreactivity). Induction of neuronal differentiation could be achieved, however, only in HPC cultures ( $P < 0.001$ ; n.s. = not significant). (E) There was a significant decrease of the nestin-immunoreactive progenitor cell population after successful induction of a neuronal phenotype ( $P < 0.001$ ). Blue bars in D and E refer to HPC, red bars to LPC. Scale bars in A and B =  $20 \mu\text{m}$ . \*\*\* $P < 0.001$  (*t* test).

available from all surgical specimens and were adjacent to those used for cell culture experiments (Fig. 5). Neuronal cell numbers and densities were quantitatively determined in all hippocampal subfields, including the pyramidal cell layer as well as the granule

cell layer of the dentate gyrus (Table 3). There was a highly significant correlation between granule cell densities and the same patient's proliferation capacity *in vitro* ( $r = 0.813$ ,  $R^2 = 0.661$ ,  $P < 0.001$ ). Neuronal cell numbers obtained from hippocampal





**Figure 5** Histopathological examination of surgical human hippocampus. All surgical hippocampus specimens in this study were anatomically well preserved. (A) All segments of the dentate gyrus (DG) showed a compacted granule cell layer in this surgical specimen (NeuN fluorescence immunohistochemistry). In contrast, CA4, CA3 and CA1 pyramidal neurons are significantly reduced, i.e. mesial temporal sclerosis type 1a (Blümcke *et al.*, 2007). (B) Granule cell loss is visible in various dentate gyrus segments, most prominently within the internal limb (asterisk). Pyramidal cell loss is detected in all CA segments, i.e. mesial temporal sclerosis type 1b. (C) Example from a surgical specimen with a densely populated granule cell layer (GCL) showing abundant doublecortin (red) and NeuN (green) co-expressing granule cells (insert refers to magnification in E–G). (D) In this severely depleted granule cell layer we observed virtually no doublecortin-immunoreactive cells (insert refers to magnification in H–K). (E–G) Arrows in E (granule cells with predominantly nuclear NeuN-immunoreactivity) and asterisks in F (granule cells with perinuclear doublecortin-staining) indicated NeuN and doublecortin double-labelled neurons. (H–K) NeuN-immunopositive granule cells showed no co-expression of doublecortin. A, C, E–G correspond to a patient from the HPC group; B, D, H, I and K to a patient in the LPC group. Scale bar in A and B = 500  $\mu$ m; C and D = 100  $\mu$ m; and E–K = 50  $\mu$ m. Nuclear Hoechst staining in blue in C and D.

sectors CA4 ( $r=0.652$ ,  $P=0.001$ ) and CA3 ( $r=0.487$ ,  $P=0.040$ ) correlated significantly with the patient's stem cell proliferation capacity *in vitro*, although to a lower extent. Even so, there was a high intercorrelation of cell densities in the different hippocampal subfields and the dentate gyrus. Therefore, multiple linear regressions were applied to further examine these correlations and to determine the most important predictor for stem cell proliferation *in vitro*. When cell densities in CA3 and CA4 as well

as granule cell density were entered stepwise into the equation,  $R^2_{\text{adjusted}}$  reached 0.64, but only the granule cell density of the dentate gyrus fulfilled the predictor's criteria for relative importance ( $\|t\|_{\text{absolut}}=5.586>2$ ). Removing the effect of cell densities in CA3 and CA4 by partial correlation analysis, a significant partial correlation coefficient resulted [ $R(\text{partial})=0.81$ ,  $P<0.001$ ], not different from the basic correlation coefficient ( $r=0.813$ ).

**Table 3** Quantitative histopathological analysis of human hippocampal tissue *in vivo* and neurogenesis *in vitro*

Patient ID	Histopathological analysis ( <i>in vivo</i> )					Neurogenesis ( <i>in vitro</i> )		
	Neuronal densities					Diagnosis	Spontaneous (%)	Induced (%)
	CA1	CA2	CA3	CA4	DG			
P1	NA	NA	100	56	1410	MTS	3	4
P2	96	316	248	120	1330	MTS	0	2
P7	68	NA	NA	128	370	MTS	1	4
P9	80	NA	NA	112	1050	MTS	2	8
P13	52	NA	128	140	1520	MTS	1	5
P14	204	208	260	136	1200	MTS	0	1
P16	NA	NA	NA	NA	1390	no MTS	2	3
P18	68	NA	88	32	1590	MTS	0	2
P20	60	188	100	72	1170	MTS	1	4
P21	56	196	276	28	1490	MTS	2	6
P22	80	256	96	56	1030	MTS	1	4
P23	356	NA	NA	120	640	MTS	1	3
P3	368	508	420	292	2930	no MTS	3	71
P5	44	212	NA	100	2530	MTS	1	63
P4	440	404	280	292	3380	no MTS	0	66
P6	64	NA	240	184	2060	MTS	4	46
P8	144	NA	164	228	1300	MTS	5	66
P10	52	212	180	48	1800	MTS	2	51
P11	52	216	316	180	2490	MTS	2	40
P12	220	156	208	56	1810	MTS	3	31
P15	80	NA	NA	304	1910	MTS	3	58
P17	64	332	236	256	1810	MTS	4	51
P19	364	320	268	124	1860	no MTS	6	44

CA1–CA4 = anatomical segments of the human hippocampus; DG = dentate gyrus; NA = area not available for cell counting; *n* (*in vitro*) = percentage of MAP2-immunoreactive cells from the entire cell population after spontaneous (withdrawal of growth factors) or induced differentiation (supplement of sonic hedgehog, suberoylanilide hydroxamic acid, forskolin, nerve growth factor and fibroblast growth factor 8); neuronal densities = NeuN-immunoreactive neurons/mm<sup>2</sup>; MTS = mesial temporal sclerosis (Blümcke *et al.*, 2007).

Diagnostic evaluation of segmental neuronal cell loss patterns revealed mesial temporal sclerosis in 19 out of 23 patients, eight in the group with abundant neurogenesis *in vitro* and 11 in the group with a reduced regenerative capacity.

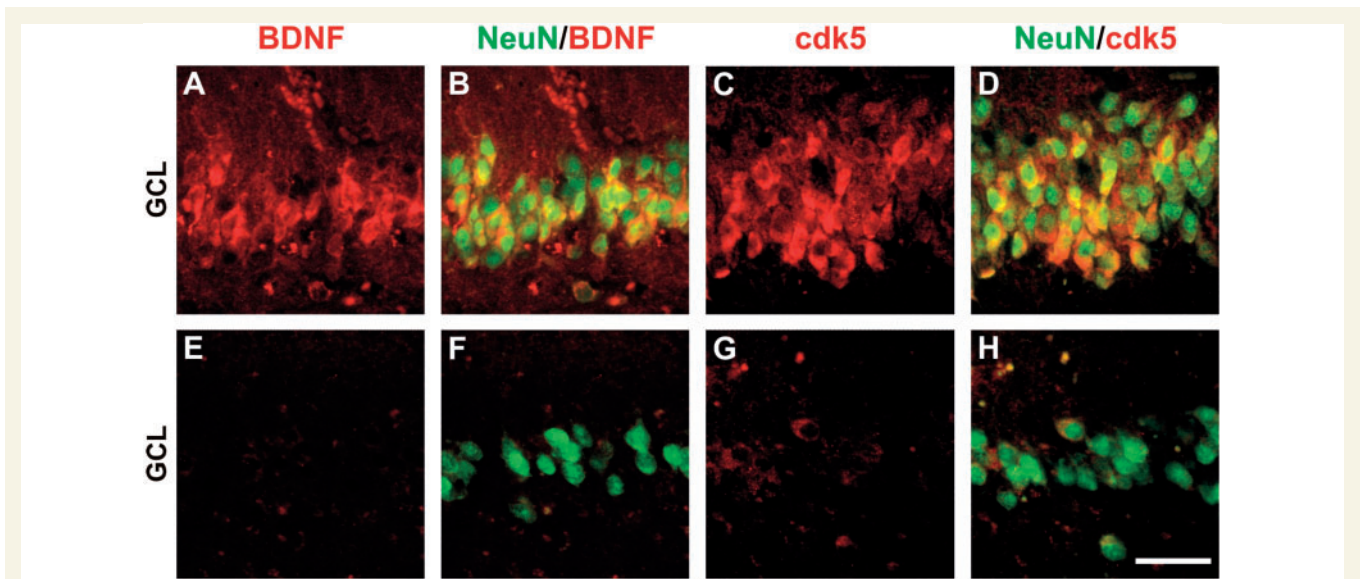
### Patients with HPC versus LPC cultures revealed different immunoreactivity patterns for doublecortin, brain-derived neurotrophic factor and *cdk5* *in vivo*

The increased neurogenesis in the HPC patient group should be reflected in an increased proportion of neurons expressing surface markers characteristic of newly generated and integrated neurons. We carried out double-immunofluorescence labelling for doublecortin and NeuN in an additional set of experiments to address this issue (Liu *et al.*, 2008). In 12 patients with LPC, doublecortin expression was observed in  $20.3 \pm 8.5\%$  of the total granule cell population. The amount of doublecortin-positive granule cells was significantly higher in the group of 11 patients with HPC *in vitro* ( $53.4 \pm 13.6\%$ ), and correlated significantly with the proliferation rate observed *in vitro* ( $r=0.790$ ,  $P<0.001$ ; Fig. 7). These cell numbers were significantly higher compared with recently published data by Knoth *et al.* (2010) in autopsy controls and

Gerber *et al.* (2009) in meningitis specimens, whereas staining patterns were similar to that reported by Jin *et al.* (2004) in the dentate gyrus of patients with Alzheimer's disease. Different antibody origin and antigen retrieval systems, as well as fixation intervals, are likely to account for these differences. Furthermore, we studied the expression of two molecules involved in the molecular signalling machinery of hippocampal neurogenesis, i.e. BDNF and *cdk5* (Fig. 6). BDNF- and *cdk5*-expression was significantly reduced in 12 specimens with LPC *in vitro* when compared with the 11 patients with HPC *in vitro*, and the numbers correlated significantly with each patient's proliferation rate observed *in vitro* (BDNF:  $r=0.596$ ,  $P=0.003$ ; *cdk5*:  $r=0.596$ ,  $P=0.003$ ).

### Significant correlation between the regenerative capacity *in vitro* and the same patient's memory

We observed a striking and highly significant correlation between each patient's regenerative capacity deduced either from our cell culture assay (*in vitro*) or from histopathological characterization (*in vivo*) and the same patient's ability to acquire and recall new memories during preoperative examination. IAT memory correlated significantly with the regenerative capacity *in vitro*



**Figure 6** Immunohistochemical characterization of BDNF and cdk5 in the human hippocampus *in vivo*. Immunohistochemical examination of the dentate gyrus in surgical hippocampus specimens showed two different patterns of BDNF and cdk5 immunoreactivities. (A–D) were obtained from a patient with HPC *in vitro*. (E–H) were obtained from a patient with LPC *in vitro*. Scale bar = 50  $\mu$ m. GCL = granule cell layer.

( $r=0.966$ ,  $R^2=0.933$ ,  $P<0.001$ ; Fig. 7B) and with granule cell density of the dentate gyrus *in vivo* ( $r=0.888$ ,  $R^2=0.789$ ,  $P=0.001$ ; Fig. 7C). Regression analysis showed a significant linear regression when entering both variables ( $R=0.966$ ,  $R^2_{\text{adjusted}}=0.923$ ,  $F=96.976$ ,  $df=1/7$ ,  $P<0.001$ ). Using the  $t$ -statistic for the linear regression coefficient to assess the relative importance of each of the independent variables, the proliferation *in vitro* ( $t=9.848>2$ ) turned out to be the crucial predictor for memory capacity (Fig. 7A and B). Partial correlation analysis, to assess the correlation of both independent variables with memory and when removing the linear effect of the other variable in the model, respectively, resulted in  $R(\text{partial})=0.966$  for regenerative capacity and  $R(\text{partial})=0.682$  for granule cell density in the dentate gyrus.

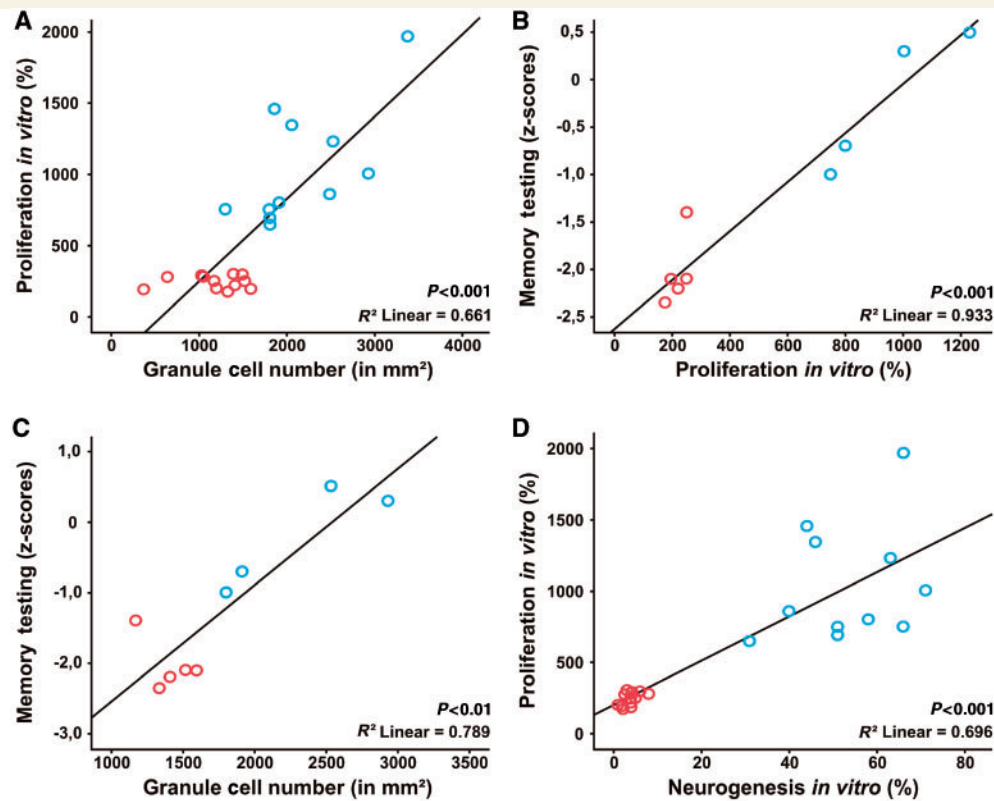
Further analysis of the divergent regenerative capacity in the human hippocampus with clinical histories from LPC versus HPC groups of patients showed fewer females in the LPC group (Table 1; three female versus nine male patients). In contrast, female gender prevailed in the HPC group (eight female and three male patients). LPC *in vitro* was only by trend correlated to a later age at epilepsy onset ( $r=-3.53$ ,  $P=0.098$ ) and positively correlated to a shorter duration of epilepsy ( $r=0.426$ ,  $P=0.042$ ), indicating that a longer seizure history is not predictive for reduced regenerative capacities *in vitro*. There was no significant correlation between age at first seizure or age at surgery. In addition, there was no significant correlation between anti-epileptic drug treatment and the regenerative capacity of the human hippocampus (Table 4).

## Discussion

Our study has demonstrated that patients with chronic, drug-resistant temporal lobe epilepsy fall into two clearly separable

categories with respect to their regenerative capacity within the hippocampus. We first tested the hypothesis that the proliferation and neuronal differentiation capacity *in vitro* was correlated with each patient's memory performance prior to surgery. Animal studies have demonstrated a constitutive supply and replacement of newborn neurons as a critical mechanism for hippocampal memory consolidation (Gould *et al.*, 1999; van Praag *et al.*, 1999; Shors *et al.*, 2001; Clelland *et al.*, 2009; Deng *et al.*, 2009; Jessberger *et al.*, 2009; Kitamura *et al.*, 2009), and cognitive dysfunction is a frequent finding in subgroups of patients with temporal lobe epilepsy (Pauli *et al.*, 2006; Helmstaedter and Elger, 2009). However, this has never been experimentally addressed in humans. Our data showed a highly significant and specific correlation between the regenerative capacity *in vitro* and the same patient's ability to store and recall memories, suggesting that a similar mechanism also operates in the human hippocampus.

Is the proliferative and differentiation capacity assessed *in vitro* a good measure of the neurogenic potential *in vivo*? Our data suggest that this may be the case. We were able to show a significant correlation between the proliferation capacity *in vivo* and the regenerative capacity *in vitro*. We also studied the expression of proteins involved in the molecular signalling machinery of hippocampal neurogenesis, i.e. doublecortin, BDNF and cdk5. Doublecortin and BDNF, as well as cdk5, revealed a highly significant reduction in specimens with granule cell loss and failure of neuronal differentiation *in vitro*. BDNF is required for neurogenesis in the hippocampus (Rossi *et al.*, 2006) and long-term survival of newborn granule cells (Sairanen *et al.*, 2005). In contrast, region- or cell-specific knockdown identified cdk5 to be critically involved in the maturation process of newborn neurons during adult neurogenesis (Jessberger *et al.*, 2008, 2009; Lagace *et al.*, 2008). The observed downregulation of both important molecular factors regulating adult neurogenesis in the human dentate gyrus *in vivo*



**Figure 7** Reduced proliferation capacities *in vitro* correlated with memory impairment and lower granule cell numbers *in vivo*. (A) Hippocampal granule cell densities *in vivo* (given as NeuN-immunoreactive granule cells per  $\text{mm}^2$ ) correlate significantly with the same patient's regenerative capacity as determined by proliferation capacities *in vitro* ( $r=0.813$ ,  $R^2=0.661$ ,  $P<0.001$ ,  $n=23$ ). (B) Proliferation capacities *in vitro* correlated significantly with neurogenesis *in vitro* ( $r=0.834$ ,  $R^2=0.933$ ,  $P<0.001$ ,  $n=23$ ). (C) Memory scores were significantly related to granule cell densities *in vivo* ( $r=0.888$ ,  $R^2=0.789$ ,  $P=0.01$ ,  $n=9$ ). Z-scores were calculated from intracarotid amobarbital memory testing (IAT) with normal values between 0 and  $-1$  ('Materials and methods' section). (D) Correlation analysis revealed a highly significant association between the capacity to generate new neurons *in vitro* and the proliferation capacities *in vitro* ( $r=0.834$ ,  $R^2=0.696$ ,  $P<0.001$ ,  $n=23$ ). Red dots indicate LPC patients, blue dots indicate HPC patients.

confirmed a compromised molecular machinery for hippocampal neurogenesis in the patient group with an LPC *in vitro*. This suggests that neurogenesis in the human hippocampus, first detected in five autaptic brain samples obtained from cancer patients treated with the thymidine analog bromodeoxyuridine (Eriksson *et al.*, 1998), is closely linked to the neurogenic potential *in vivo*. Finally, the close correlation of the neurogenic potential with the density of granule cells suggests that neurogenesis also regulates the granule cell numbers in human subjects.

Increased hippocampal neurogenesis has been observed in different epilepsy models (Parent *et al.*, 1997, 2006; Siebzehnrub and Blumcke, 2008), and increased numbers of nestin-immunoreactive neural precursor cells were detected in the dentate gyrus of patients with temporal lobe epilepsy younger than 4 years at time of operation (Blumcke *et al.*, 2001). In contrast, the regenerative capacity of the hippocampus declined in chronic seizure models (Hattiangady *et al.*, 2004; Hattiangady and Shetty, 2009) and is also likely to decrease with age (Fahrner *et al.*, 2007; Ahlenius *et al.*, 2009). However, our data suggest no clear effects of early epilepsy onset, longer duration of seizures or higher age at surgery on adult neurogenesis. This may be due to the very long

period of chronic seizures in all patients, far exceeding that studied in any animal model. Rather, compromised neurogenesis was observed in patients with late onset of first seizures. This observation will need further clarification. It is in line with the notion, however, that less vulnerable neuronal and stem cell populations can be detected in younger compared with older animals (Haas *et al.*, 2001; Liu *et al.*, 2003). Furthermore, nestin-expressing precursor cells persisted in cultures obtained from patients with temporal lobe epilepsy with compromised neurogenesis *in vitro* (Fig. 2E). Similar data are obtained from an animal model of temporal lobe epilepsy, showing no change in the neural precursor cell population but a dramatic decline in neuronal fate-choice decision of newly generated cells (Hattiangady and Shetty, 2009). Prolonged nestin expression at the expense of newly generated neurons was also observed in aged rats, particularly in those animals with severe spatial learning deficits during Morris Water Maze testing (Nyffeler *et al.*, 2008). Indeed, causal events in the disruption of the neurogenic fate-choice of progenitor cells may be involved and will need clarification.

There is much debate in regenerative medicine about the functional and clinical impact of hippocampal progenitor cells and

**Table 4** Statistical analysis between anti-epileptic drug treatment and neuropathological *in vivo* and *in vitro* analysis

	VPA	CLB	LTG	LEV	OXC
Proliferation <i>in vitro</i>	0.550	0.221	0.118	0.697	0.489
Proliferation <i>in vivo</i>	0.850	0.487	0.859	0.616	0.746
Neurogenesis <i>in vitro</i>	0.692	0.160	0.123	0.760	0.924
Memory (IAT)	0.136	0.376	0.149	0.408	0.993
Granule cell number	0.678	0.536	0.133	0.688	0.671
Doublecortin	0.959	0.109	0.349	0.602	0.232
BDNF	0.810	0.633	0.310	0.760	0.768
Cdk5	0.827	0.133	0.205	0.385	0.582

Statistical analysis did not reveal any impact of drug treatment on the proliferative capacity of adult human hippocampal stem cells or other parameters studied (as listed in left-hand column). CLB, LTG, LEV and OXC were the most commonly used anti-epileptic drugs continuously administered during 6 months prior to surgery. All numbers refer to *P*-values (Pearson correlation). CLB = clobazam medication in clinical history; LEV = levetiracetam; LTG = lamotrigine; OXC = oxcarbazepine; VPA = valproate.

neurogenesis (Steindler and Pincus, 2002). Adult stem cells and the endogenous regenerative capacity of the human brain are promising therapeutic targets for many neurodegenerative disorders, such as Alzheimer's or Parkinson's disease (Rodriguez *et al.*, 2008; Winner *et al.*, 2009), even though the regenerative capacity may be severely compromised during a given patient's long-term disease history. Our data show evidence that neurogenesis is not necessarily affected by chronic neurological disease, as the majority of our patients suffered from a very long period of chronic seizures originating in the hippocampus. Drugs targeting the molecular machinery towards a neuronal fate-choice of hippocampal precursor cells may thus be promising therapeutic options to ameliorate learning and memory deficits associated with a variety of neurological disorders.

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