Microtubule associated proteins and plasticity in the developing and diseased brain

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Increased proliferation reflects glial and vascular-associated changes, but not neurogenesis in the presenile Alzheimer hippocampus.

Karin Boekhoorn, MSci, Marian Joels, PhD and Paul J. Lucassen, PhD
Abstract

Adult proliferation and hippocampal neurogenesis are stimulated by injury. In agreement, aberrant cell-cycle-related protein expression has been reported in senile Alzheimer’s disease (AD), where the hippocampus is particularly affected. Recently, increased expression of Doublecortin (DCX), a neurogenesis marker, was reported in senile AD. Here, we addressed whether proliferative and neurogenic responses also occur in younger, i.e. presenile AD cases, using immunohistochemistry for Ki-67, GFAP and DCX. Increased numbers of Ki-67+ cells with a healthy, non-mature appearance were found in CA1-3. These were mainly due to glial and vasculature-associated changes, while DCX immunostaining appeared sensitive to postmortem breakdown. We found no indications for altered dentate gyrus neurogenesis. Our data obtained using validated methodology in a well characterized, presenile cohort thus differ from data obtained in senile AD. They reflect clear differences in proliferative responsivity, particularly in the glia and vascular components and suggests different underlying mechanisms in these groups.

Introduction

Alzheimer’s disease (AD) is clinically characterized by progressive dementia. Neuropathologically, the hippocampus is one of the first and most severely damaged structures in AD. Extensive pathology, with large numbers of neurofibrillary tangles (NFTs) and β amyloid (Aβ) plaques, are characteristic of the AD hippocampus (Braak and Braak, 1991). Also, prominent inflammatory responses take place in AD, that involve the activation of microglia and astrocytes (Akiyama et al., 2000; Meda et al., 2001; Nagele et al., 2004). In addition, almost all AD patients (around 90%) show cerebral amyloid angiopathy (CAA), that correlates with cognitive impairment (Mandybur, 1975; Jellinger, 2002) and may play an important role in AD pathogenesis (Castellani et al., 2004; Nicollet al., 2004; Zlokovic, 2005).

In addition to these well-known processes, various cell-cycle related events have been identified in AD. For instance, an increased expression of various cyclins and cyclin-dependent kinases has been reported in the hippocampus in AD (Smith and Lippa, 1995; Arendt et al., 1996; Kondratick and Vandre, 1996; McShea et al., 1997; Vincent et al., 1997; Busser et al., 1998). Most authors suggest this ectopic expression occurs in AD-affected degenerating cells. However, one recent publication shows increased expression of doublecortin (DCX), a marker for new neurons, in a senile cohort of AD patients (Jin et al., 2004a). These studies are especially interesting since the hippocampal dentate gyrus (DG) is one of the few brain areas where adult neurogenesis occurs, albeit at very low rates in aged subjects (Heine et al., 2004). In rodents, proliferation and neurogenesis is stimulated by hippocampal damage (Parent et al., 1997; Covolan et al., 2000; Kuhn et al., 2001; Radley et al., 2003). Interestingly, Aβ can also exert neurogenic effects on stem cells in vitro (Ohsawa et al., 1999; Lopez-Toledano and Shelanski, 2004). Changes in neurogenesis have furthermore been reported in some mouse models for AD (Jin et al., 2004b; Chevallier et
al., 2005), although these results are still far from conclusive (Haughey et al., 2002; Dong et al., 2004; Wang et al., 2004; Wen et al., 2004; Boekhoorn et al., 2006).

Possible proliferative and compensatory responses are considered more likely to occur in younger presenile patients than in older AD patients, while the former condition generally also has a more aggressive nature and shorter disease duration. To address whether proliferative changes are indeed different in a younger versus older AD group, we studied a well defined cohort of presenile AD cases and performed a detailed quantitative immunohistochemical inventory on the hippocampus, using the proliferation marker Ki-67 (Gerdes et al., 1991). Subsequently, we investigated associations with gliogenesis and vasculature using GFAP and Von Willebrand Factor (VWF) immunohistochemistry, respectively.

Most reports have examined cell-cycle changes predominantly in close association with AD affected neurons (Arendt et al., 1996; Busser et al., 1998). This ectopic expression is presumed to be associated with cell death rather than cell birth. Since this was not the topic of our investigation, we here specifically focused on Ki-67 expression in apparently healthy, small, presumably precursor cells, and excluded Ki-67 expression- if any- in mature profiles. DCX immunohistochemistry was further applied as a new marker for adult generated, migrating neurons in rodents. Unlike Bromodeoxyuridine (BrdU) immunocytochemistry, DCX does not require prior injections in live subjects. As such, DCX has an interesting potential to detect neurogenesis in human brain as well (Francis et al., 1999; Brown et al., 2003; Jin et al., 2004a; Rao and Shetty, 2004; Couillard-Desprets et al., 2005).

Materials and methods

Subjects

Human hippocampal tissue was obtained through the rapid autopsy program of the Netherlands Brain Bank (Coordinator: Dr. R. Ravid). The Netherlands Brain Bank abides to all local ethical legislation. All tissue was obtained with informed consent of the donor or next of kin to perform brain autopsy and the subsequent use of brain tissue for scientific purposes. Permission to use the medical records was also requested in advance.

Hippocampal tissue of 9 AD patients and 10 controls was studied. Patients ranged from 63 to 70 years of age. Based on the medical and neuropathological records, careful selection and matching was performed. Subjects with Reisberg stages 3-7 (Reisberg et al., 1982) and Braak stages (Braak and Braak, 1991) 5 and 6 were assigned to the AD group whereas subjects with Reisberg stage 0 and Braak stage 0-2 were assigned to the control group. Post mortem delay (PMD) was on average 9.7 h ± 5.9 (SD) for the control subjects and 5.2 h ± 1.1 (SD) for the AD patients. The presenile AD patients had a mean disease duration of 8.6 years. Further clinicopathological details are presented in Table 1.

None of the control subjects were reported to suffer at the moment of death, or to have suffered before, from a known neurological or psychiatric disease. Also controls did not suffer from brain metastases, or from
other conditions, like ischaemia, that might have affected the integrity of the blood brain barrier or endothelial cells. Controls also did not exhibit prolonged arterial blood pressure changes, prolonged fever or the presence of multiple brain lesions, nor was brain irradiation applied. Moreover, as this may possibly influence neurogenesis, none of the selected subjects were treated with synthetic steroids, antidepressant drugs or brain irradiation, at any time during their life. For subject 95-092, particular attention was paid when analysing the results because of the cytostatic drug treatment until death. Also patients 96-129 and 97-157 suffered from cancer. But chemotherapy was not continued until death. Ki-67 countings for all 3 patients were well within the average range of the control group.

The AD group had been clinically assessed as having "probable AD" by excluding other possible causes of dementia by history, physical examination and laboratory tests (McKhann et al., 1984). For every subject, a standard set of hippocampal and neocortical brain areas has been carefully investigated (Ravid and Swaab, 1993) using Bodian Silver, H&E, Nissl, Alz-50 as well as Congo red staining for amyloid angiopathy changes (by neuropathologists Prof. Dr. F.C. Stam and Dr. W. Kamphorst, Free University, Amsterdam). The presence or absence of large numbers of amyloid plaques and amyloid angiopathy, tangles and dystrophic neurites in the above areas were noted. Further microscopical examination failed to find any obvious malformations, infiltrations or neoplasms in either group of patients. Final diagnosis was established by relating this neuropathological examination to the outcome of the clinical diagnosis.

All AD patients displayed extensive neuropathology in the neuronal fibre network, and an extensive presence of plaques and tangles, whereas tissue from the control subjects was free of any such changes. The AD patients displayed abundant amyloid angiopathy in all cases.

Brain tissue

At autopsy, the hippocampus proper was dissected at a mid-anterior-posterior level and fixed in 10% buffered formalin at room temperature for different periods of time, ranging from 30 - 646 days (see Table 1 for details). Tissue was then dehydrated in graded ethanols and embedded in paraffin and serial sections were cut on a microtome at 8 μm. Sections were then mounted on Superfrost Plus slide (Menzel-Gläser).
Lieberkuhn, where apoptotic cells predominate at the luminal side.

For 70-year-old, formalin-fixed and paraffin-embedded. The colon holds large numbers of proliferating cells in the basal parts of the crypts of Lieberkuhn, whereas apoptotic cells predominate at the luminal side.

Secondly, colon biopsies, pathologically confirmed as healthy, were obtained from a male control of 70 years of age, formalin-fixed and paraffin-embedded. The colon holds large numbers of proliferating cells in the basal parts of the crypts of Lieberkuhn, whereas apoptotic cells predominate at the luminal side.

### Table 1. Clinicopathological details of the subjects used in this study.

Patient 97-005 was included as a positive control for Ki-67 staining. Apo E; Apo E genotype, f; female, m; male, PMID: post mortem delay. NBB no.; Netherlands Brain Bank number, ND; not determined, AD: Alzheimer cases. CVA: cardiovascular accident.

**. PMD and brain weight are significantly lower in AD patients compared to controls (p= 0.038 and 0.02 resp)

**. Patient 00-018 was diagnosed with vascular type dementia, declaring the high Reisberg stage.

#### Methodological validation

Ki-67 immunohistochemistry is unaffected by PMD (Bowers et al., 2003). Masking of this epitope occurs after prolonged fixation, and microwave enhanced antigen retrieval (AR) is essential for formalin fixed tissues (Smith and Lippa, 1995; Nagy et al., 1997; Del Bigio, 1999; Blumcke et al., 2001). AR is generally done for short periods, and at neutral pH values. For long fixation times, as in our patient cohort, more vigorous AR procedures are suggested, that are proportional to an increased heating duration and/or the use of lower pH values (Taylor et al., 1994). AR for Ki-67 at pH 6.0 was e.g. already suboptimal for tissue fixed longer than 24 h (Munakata and Hendricks, 1993; Suurmeijer and Boon, 1993). For the MIB-1/Ki-67 antibody, AR at low pH is optimal (Shi et al., 1995). We compared the following pH values: pH 1.0 (0.1 M HCL), pH 3.0 (0.01 M citrate buffer), pH 6.0 (0.01 M citrate buffer) and pH 9.0 (0.01 M Tris).

To validate the Ki-67 staining protocol, we studied 4 positive control tissues known to contain large numbers of proliferating cells and fixed for prolonged periods of time. First, brain tissue of 3 human foetuses of 23 weeks of gestation was studied. Sections were cut at the level of the hypothalamus and associated cortical areas including the basal telencephalon, a foetal structure that appears as a stream of proliferating and migrating neurons at these ages (Letinic and Kostovic, 1997). Secondly, colon biopsies, pathologically confirmed as healthy, were obtained from a male control of 70 years of age, formalin-fixed and paraffin-embedded. The colon holds large numbers of proliferating cells in the basal parts of the crypts of Lieberkuhn, whereas apoptotic cells predominate at the luminal side.
Thirdly, young rat hippocampal tissue of 10 days old was included as large numbers of proliferating cells are present at this age. Finally, brain sections from a patient suffering from leukemia were included as positive control for each experiment (subject 97-005, Table 1).

DCX is a relatively novel marker for human brain. In view of the well known sensitivity of particularly dendritic markers to postmortem decay (Swaab and Uylings, 1988), DCX IR was first studied in a series of postmortem rat brains (8-10 weeks of age, n=2 per group) that were left at room temperature for 1, 3, 8, 12 and 24 hours after death. Six µm thick sections were stained for DCX according to the protocol described below. Paraffin sections of perfused brain were included for comparison.

Immunohistochemistry

Table 2 lists the antibodies used for immunohistochemistry. The Ki-67 antigen is a 345 to 395 KDaltion non-histone protein complex present only in the nucleus of proliferating cells during G1, S, G2 and M, but not the G0 phase of the cell cycle (Endl and Gerdes, 2000). Ki-67 antisense treatment strongly reduces thymidine uptake in cell lines, indicating an important role in the cell-cycle (Duchrow et al., 2001). Of the various Ki-67 antibodies tested in pilot studies (NCL-Ki67p (Novocastra), Ki67 (Zymed), Ki67 Ab-3 (Immunocor) and MIB-1 (Dako)), the MIB-1 antibody yielded the best results on human brain. The MIB-1 antibody is furthermore a well-accepted tool in the detection of proliferation in tumour biology (Kee et al., 2002).

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Table 2. Detailed information on the antibodies used in this study

For all washes and antibody incubations, 0.1 M Tris buffered saline (0.15 M) pH 7.6 (TBS) was used. Mounted hippocampal sections were cleared in xylene and hydrated in graded ethanols and then placed in plastic Coplin jars for microwave pre-treatment. AR was performed for 15 min with 2x200 ml buffer in a household microwave device starting at 800 W and gradually decreasing to 100 W once boiling commenced. After 30 min of subsequent cooling at room temperature, endogenous peroxidase activity was blocked for 15 min with 0.5% peroxide treatment. Five percent normal goat serum (NGS)/ 0.5% Triton X-100 was applied for 30 min to prevent aspecific binding. Incubation of the first antibody was in 0.25 % gelatin/0.5% triton X-100 in TBS (Supermix) for 1 h. It was then incubated overnight at 4°C. The secondary
antibody was amplified with the Elite Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories, Burlingame) and biotinylated tyramide (1:500, kindly provided by Dr I. Huitinga, Neth. Inst. for Brain Research, Amsterdam) and 0.01% peroxide for 30 min, followed by a 45 min incubation with ABC. The last 2 washes were in 0.05 M tris buffer, which was also used to dissolve diaminobenzidine (DAB) as a chromogen and for Ki-67 staining with 0.04 % nickel intensification.

Often, an association of Ki-67+ cells with the vasculature was readily apparent, and only in a few cases, it appeared somewhat difficult to establish this unequivocally. In a sample of sections adjacent to Ki-67 stained ones, and taken from various patients, we subsequently confirmed an endothelial origin of the Ki-67+ cells using immunolabelings for Von Willebrand Factor (VWF). The same protocol as for Ki-67 was used except for MW pretreatment in 0.01 M citrate buffer at pH 9.0.

DCX immunohistochemistry on human hippocampal sections was performed according to the same protocol as for Ki-67, except the microwave pretreatment was at pH 6.0.

For GFAP immunohistochemistry, deparaffinized sections were pretreated in 10 mM citrate buffer at pH 6.0 in a microwave oven for 10 min. The pretreatment was followed by immersion in 0.3% H₂O₂ in methanol for 30 min. PBS containing 1% (w/v) bovine serum albumin (BSA; Boehringer Mannheim, Germany) was used in all subsequent steps. Preincubation of the sections with normal rabbit serum (DAKO, Glostrup, Denmark) for 10 min was followed by incubation with a GFAP-specific monoclonal mouse antibody at room temperature for one hour. After washing, the slides were incubated with a biotin-conjugated rabbit anti-mouse F(ab')2 secondary antibody (DakoCytomation, Glostrup, Denmark; 1:500 dilution) for 30 min, and streptavidin-biotin horseradish peroxidase complex (strepABComplex; DakoCytomation, Glostrup, Denmark; 1:200 dilution) for 60 min. Colour was developed (3 min) using 3,3'-diaminobenzidine (0.1 mg/ml, 0.02% H₂O₂) as chromogen.

**Morphometry**

Cross sectional areas of the main hippocampal subregions, i.e., DG-GCL, hilus, CA1/2 and CA3 were determined in cresyl violet stained sections adjacent to the midlevel sections stained for Ki-67. The public domain program "Object image", an extended version of "NIH Image", was used.

**Quantification**

For each patient, 3-4 sections at comparable midlevels of the hippocampus were used. Numbers of Ki-67 positive nuclear profiles were determined in the DG, hilus, CA3, CA1-2 of the hippocampus. Specific care was exerted to exclude Ki-67 staining in mature neurons and only include non-mature, healthy looking Ki-67 IR nuclear profiles, (i.e., cells without a large soma or extensive neurites visible). As double labelling for phenotypic analysis with mature neuronal markers is not possible, specific attention was paid to the location of Ki-67 IR profiles over the various subregions. Subareas
known to be largely occupied by a specific cell type were denoted as either a) neuron-rich (e.g., the granular layer of the DG and the pyramidal layer of CA areas), b) glia-rich areas (like the stratum radiatum), or c) associated with blood vessels. The number of Ki-67 positive cells was normalized to the cross sectional area of the subregion involved and expressed per mm². Subsequently, immunohistochemistry for VWF and GFAP was performed on adjacent sections.

In view of our PMD test series, DCX-labeled human hippocampal sections were scored by distinguishing DCX IR cellular profiles from the small granular elements frequently present in the DG. Cellular profiles were scored separately, while semiquantitative scores were assigned for the granular pattern, ranging from 0 for no IR present, to 1) weak DCX signal in small parts of the region of study, to 2) weak DCX IR detected in considerable parts of the region, to 3) moderate levels of DCX IR throughout the region, to 4) abundant DCX-IR throughout the region.

GFAP staining was assessed semiquantitatively for the main hippocampal subregions, including the granular cell layer (GCL), subgranular zone (SGZ), molecular layer (Mol), hilus (hil) and CA1 region, as well as associated cortical white (Cx WM) and grey matter (Cx GM). A scoring range from - (no GFAP immunoreactivity apparent) to +/- (modest), + (moderate), ++ (strong) and +++ (extensive GFAP immunoreactivity and activated astroglia present) was used.

Statistical analysis

Having established that the data were normally distributed, Ki-67 results were analyzed using a parametric two-tailed student's t-test. DCX qualitative numerical scores of the extent of the granular staining in the SGZ were analyzed non-parametrically using a Mann Whitney U test.

Results

Morphometry

Measurement of the cross-sectional area of the various hippocampal subareas revealed a significant decrease (p = 0.034) of the CA1/2 region in AD compared to controls (Fig. 1). Other subareas did not differ significantly between the two groups.
Ki-67 Antigen retrieval (AR) optimization

The AR test series revealed a clear improvement at pH 1.0 over other pH values. Using pH 1.0, large numbers of Ki-67 positive, proliferating cells were found in the ganglionic eminence and telencephalon, while clear and isolated Ki-67 positive cells were detected outside these regions. In contrast, treatment at pH 3.0 displayed no signal at all, while at pH 6.0 and pH 9.0, staining was only present in locations with high proliferation, but with high background levels and a poor signal to noise ratio. Notably, the isolated Ki-67 positive cells located outside the zone of massive proliferation were only observed with AR at pH 1.0. Application of our protocol on intestinal tissue (Fig. 2A), yielded many Ki-67 positive cells at the basal (arrows, Fig. 2A), but no apical/lumenal side of the villi (arrowheads), thus excluding possible crossreactivity with non-proliferative or apoptotic cells that predominate the latter region. Secondly, the hippocampus of a 10-day-old rat (Fig. 2B) revealed large amounts of Ki-67 positive cells, often found in pairs or clusters (arrows), in hilus and granule cell layer (GCL). Finally, a control subject (patient # 97-005, see Table 1) that suffered from leukaemia displayed extensive Ki-67 staining in blood cells throughout the hippocampus and cortex (Fig. 2C). In conclusion, retrieval at pH 1.0 was found to be optimal (Shi et al., 1995) for tissue with prolonged fixation times, and this condition was subsequently applied to our experimental tissues.
Figure 2. Positive controls showing Ki-67 signal in proliferating cells

Low power photomicrographs are shown on the left, details on the right. Figure A shows Ki-67 positive, proliferating cells at the basal side of the crypts of Lieberkühn in a non-counterstained section of human colon, an area where abundant proliferation occurs (arrows). Positive cells are absent from the apical (lumenal) side of the crypts (arrowheads), a region where apoptosis is known to prevail. In 10-day-old rat hippocampus (B), Ki-67 identified large amounts of dividing and proliferating cells in the hilus and DG (arrows) and in smaller amounts also in other areas of the hippocampus of animals this age (arrowhead). In the entorhinal cortex of a patient who suffered from leukaemia (C), many Ki-67 positive cells were found inside blood vessels (arrows). Scale-bars on the overviews indicate 100 μm, whereas scale-bars on the details (right side) indicate 20 μm.
Ki-67 quantification in AD brains

Ki-67 positive cells were found at very low frequencies in the hippocampus of AD patients and controls (Fig. 3A-C). Nuclear Ki-67 staining was present often in doublets or pairs close to each other (Fig. 3D-G), or in cells in close contact with the vasculature (Fig. 3B). Never was Ki-67 staining observed in tangle-like structures, nor in mature cellular or neuronal profiles, or with characteristics suggestive of apoptosis. This was expected based on the absence of Ki-positive cells from the lumenal side of the intestinal villi (see above). Specific attention was further paid to one patient that received chemotherapy until death and one suffering from leukemia. The Ki-67 counts of the former patient were within the range of the control group, suggesting the drugs had not passed the blood-brain barrier. The patient suffering from leukemia (Fig 2C) consistently showed strong staining of many proliferating cells within the vasculature.

Quantification of the mean number of Ki-67 positive cells in all hippocampal subregions together yielded a significant increase in the AD hippocampus (p = 0.018). This was mainly due to an increased proliferation in CA1-3 (p = 0.015)(see Fig. 4). Further subdivision in 3 phenotypic groups (Fig. 5) revealed proliferation in neuron-rich areas to be almost exclusively present in the DG of both groups (Fig. 5D). Virtually no Ki-67 positive cells were found within the pyramidal neuron layers of CA1-3. The increased proliferation in CA1-3 in AD was largely attributable to cells located in glia-rich areas, such as the stratum radiatum, and to blood vessel associated cells. Additional stainings for VWF in a series of adjacent sections confirmed in all cases that the Ki-67+ cells reside in VWF+ blood vessels (Fig. 6).
Figure 3. Ki-67 immunohistochemistry identifies adult proliferation in sub-regions of the adult human hippocampus.

Examples of Ki-67 positive cells present in neuron-rich areas (A), blood vessels (B) or glia rich areas (C, D). Note that the frequency of the Ki-67 positive cells is very low. Figure A shows the subgranular zone (SGZ) and granular cell layer (GCL) of the DG with a Ki-76 positive cell at the border of the SGZ. Figure B shows a blood vessel (lumen is depicted by the asterisk) associated cell (arrow). Figure C shows positive cells (arrow) in the polymorphous layer (PML) of the DG, a relatively glia-rich area. Figure D shows the positive cells in C in detail, illustrating a recently separated doublet. Figure E-G are representative examples of Ki-67 positive cells often present as doublets. Arrows indicate pairs of proliferating and/or clearly dividing cells that are about to separate. Proliferation was observed in control subjects (E, G) and AD patients (A-D, F). Scale-bar in C: 50 μm. Scale-bars in the rest of the figure are 20 μm.
In the entire hippocampus proliferation was significantly increased in AD patients compared to control subjects (second right pair of bars, p = 0.019). This was mainly due to increased proliferation in the CA1-3 area (most right pair of bars, p = 0.015). Numbers are normalized for the total hippocampal surface for each individual patient. Similarly, the average of CA1 to 3 has been determined. Error bars indicate SEM.

Figure 4. Quantification of Ki-67 labelled cells in the hippocampus of AD patients and controls.

Figure 5. Quantification of subtypes of Ki-67 positive cells in the AD hippocampus.

A: CA1/2, B: CA3, C: hilus, D: DG, E: total.

Proliferation in neuron rich areas (neuron) is almost completely limited to the DG (Figure D), but not different between groups. Proliferation in glia rich areas (gla) and bloodvessels (vascular) occurs in all areas of the hippocampus. Within the different categories, proliferation did not differ significantly between AD patients and controls. The increase in proliferative cell number is mainly due to increases in proliferation in the glia-rich areas and bloodvessel-associated cells and not from neuron-rich areas, that is only present in the DG-SG2 and not different between groups.
Figure 6. Proliferation in endothelial cells.

(A) Ki-67 positive cells (arrows) are located inside a Von Willebrandt Factor (VWF) positive vessel (*), indicating their endothelial origin. VWF immunohistochemistry was performed on adjacent sections. GCL: granular cell layer. Scale-bar in overview indicates 100 μm, in the detail 10 μm.
GFAP immunohistochemistry

GFAP immunostaining revealed extensive astrogliosis in the cortical white and grey matter in AD as compared to controls, with local differences in the hippocampus (Fig. 7, color figure). In control subjects, faint GFAP IR was apparent in the hilus and SGZ, while the CA1 region was generally devoid of GFAP IR. In AD, abundant astrogliosis was found in these regions. Within the DG, in almost all AD patients (8/9) and in only 1 control subject, an extensive GFAP IR and large numbers of GFAP positive reactive astrocytes were apparent in the SGZ (Fig. 7E, Table 3) and hilus (Fig. 7F+G). Also, these astrocytes often appeared in clusters (Fig. 7F). Many of their protrusions frequently extended into the GCL (Fig. 7H) that was otherwise devoid of GFAP staining. In 2 AD cases, reactive astrocytes were found in the molecular layer, and in 4 cases, astrocytes were also present in the GCL (Fig. 7H+I, Table 3).

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Table 3. Semiquantitative scoring of GFAP immunohistochemistry in the hippocampus and associated cortex.

Expression is scored as: -; no. +/-; weak. +/--; modest; +; moderate and ++; strong GFAP expression, and +++; extensive GFAP expression with astrogliosis. NBB: Netherlands brain bank number, pmd: post mortem delay [h], SGZ: subgranular zone, GCL: granular cell layer, mol: molecular layer, CA1 pyr: pyramidal layer of CA1, CA1 rad: stratum radiatum of CA1, CX GM: associated cortex gray matter, CX WM: associated cortex white matter.
Proliferation in Alzheimer hippocampus

Figure 7. Gliarial Fibrillary Acidic Protein (GFAP) immunohistochemistry

A. Very little signal is present in the CA1 area of a control subject immunostained for GFAP.
B. Conversely, abundant gliosis and many GFAP positive reactive astrocytes (arrow) are present in CA1 and cortical grey matter of an AD patient. C. Abundant glia staining is seen in the hilus and SGZ of most of the AD patients with only occasionally glial activation apparent in the molecular layer. D. In most controls subjects, very little glial activation was found in the SGZ, GCL or molecular layer.
E. In AD patients, abundant glial activation in the SGZ (arrows) and hilus was often observed.
F. Detail of E showing clusters of GFAP positive astrocytes (arrow) present in the SGZ.
(G) and hilus (H). Often GFAP positive protrusions were extended and traversed through the GCL into the molecular layer. I. Only in a few cases, abundant activation of GFAP positive astrocytes was seen within the GCL and the molecular layer.
SGZ: Subgranular zone, mol: molecular layer, H: hilus, GCL: granular cell layer.

Magnification in A, B, D, E, M, H and I: 250x, C: 100x, F and G: 400x
Doublecortin (DCX) immunohistochemistry

For DCX, PMD effects were studied first on the rat PMD series. In tissue obtained from perfused brains, DCX IR was strongly expressed throughout the SGZ, where it extensively labeled neuronal cell bodies as well as their dendrites (Fig. 8B, color figure), traversing the GCL and reaching into the molecular layer (Fig. 8A). After 1 h of PMD, a clear reduction was already apparent in DCX IR. The reduction was particularly apparent in the dendritic component (Fig. 8C, D and F), showing further progression with increased PMD. Also, weaker soma staining was detectable (Fig. 8D and G) at 8 and 12h of PMD (Fig. 8D, E and G). At 12 h of PMD, most DCX positive cells in the SGZ had further shrunken in size, whereas a smaller amount of cells was detectable at a PMD of 44 h. The number of DCX IR cellular elements per hemisphere was not strongly reduced by PMDs up to 12 h, as the average number of DCX IR cells per 4 hippocampal levels was 46, 35, 47, 47 and 18 for PMDs of 1, 3, 8, 12 and 44 hrs, respectively. In addition, at 8 and 12 hrs of PMD, a granular staining pattern became apparent mainly in the hilus and SGZ (Fig. 8E+I), likely representing DCX breakdown during PMD.

In human brain, very few examples (4 cells in a total of 19 patients) of isolated, DCX positive, cellular profiles were seen in the hilus and SGZ (Fig. 8J, K and M). DCX positive cellular profiles were never seen in the CA pyramidal layer or stratum radiatum. Also, many small granular elements, similar to those appearing in the postmortem rat series, were frequently observed in hilus, GCL and molecular layer of both groups (Table 4). In outer borders of the tissue, DCX positive corpora amyelacea were often seen. DCX IR dendrites were not observed in the human hippocampus. Between subjects, considerable variation in the number of DCX positive granular elements was present, that could not be directly related to fixation time or postmortem delay (Table 4). Quantification of the number of granular elements in the SGZ and hilus yielded no significant differences between the groups (Mann-Whitney U rank sum test; p = 0.38).

In view of the increase in granular elements in our PMD series, we conclude that only the cellular elements inside the SGZ, reflect DCX levels present antemortem. Their numbers were very low and did not change significantly with PMDs up to 8 hrs. Therefore, the presence of a discernable nuclear membrane and a nucleolus should be a morphological criterium when addressing DCX IR somata in tissue matched for preferably short PMDs.

When comparing identified cells in the 2 groups, the numbers of DCX IR cellular profiles were too low to draw any conclusions. Given the effect of PMD which will continue to contribute to the variability of the data, and the very low frequency of DCX positive cellular elements, e.g. increasing the number of sections would still not be sufficient to draw any reliable and meaningful conclusions.
Proliferation in Alzheimer hippocampus

A

B

C

D

E

F

G

H

I

J

K

L

M

N
Fig 8. DCX Immunohistochemistry

A. Representative illustration of the arrangement of newly generated neurons in the two blades of the adult mouse dentate gyrus as shown by doublecortin immunohistochemistry (perfused brain tissue). Prominent staining of branching apical dendrites is visible that traverse the granular cell layer and cross the inner and middle molecular layers to often reach into the outer molecular layer (OML, arrows).

B. High magnification showing the typical distribution pattern of the microtubule associated protein doublecortin in rodent brain that is prominent in the soma as well as in the dendrites (arrowheads).

C. Representative illustration of a DCX positive neuron in the rat dentate gyrus (immersion fixed) after a 1 hour post mortem delay. A prominent reduction in immunoreactivity is apparent, not only in the soma, but even more so in the dendrites.

D. DCX immunoreactivity of a rat brain with a PMD of 8 hours, showing only some DCX staining of the soma (arrows) remaining in the GCL, yet very little, if any, dendritic staining remains present. *: hilus.

E. At 12 h PMD, DCX soma staining is very weak in rat brain, while an additional, granular staining pattern is apparent in the SGZ and hilus (arrows).

F. High magnification of newly born neurons in the SGZ/GCL after 3 h PMD, illustrating poor dendritic staining (arrowhead).

G. High magnification of a DCX positive, newly born neuron in the rat DG after a PMD of 8 hours. Note the presence of a nucleolus (arrow) and the complete absence of dendritic staining, as compared to figs A and B.

H. Illustration of probably the remnant of a DCX positive cell, in the SGZ after a PMD of 12 h.

I. The hilar region of a rat at a PMD of 8 h, illustrating the occurrence of an abundant granular staining DCX-IR pattern (arrows).

J. DCX positive cell clearly positioned in the SGZ of the human DG of an Alzheimer patient.

K. Higher magnification of J.

L. Illustration of the granular pattern of DCX immunostaining in the human hippocampus, that was mainly found in the SGZ (arrows)/hilus area.

M. DCX positive soma in the hilar/SGZ border of an AD patient. The arrow indicates the presence of a nucleolus. The inset shows a similar example in the hilus.

N. DCX staining in the hippocampal SGZ of an AD patient, showing both granular staining (arrows) and an occasional cellular profile.

Magnifications in A: 100x; B, I, K and L: 400x; C, F, G, H, K, M and N: 1000x; D and E: J: 250X

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2.57 AVG
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3.71 AVG
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Table 4. Semi quantitative scoring of doublecortin (DXC) IR in AD hippocampus.

The table only shows the scoring of DCX IR small granular elements. The following scoring was used: 0: no immunoreactivity (IR), 1: weak IR in small parts of the region of study, 2: weak DCX IR detected in considerable parts of the region, 3: moderate levels of DCX IR throughout the region, 4: abundant IR throughout the region. NBB no, netherlands brain bank number; pmd, post mortem delay; DG, dentate gyrus; CA, total cornu ammonis; Total, sum of the scores in the separate regions for one patient.
Discussion

Using a maximized immunohistochemical protocol for Ki-67, we presented data supporting the hypothesis that adult hippocampal proliferation is increased also in presenile AD. In the GCL of the DG, the only neuron rich area where Ki-67 was found, no differences were present between control subjects and presenile AD patients. This suggests that no differences in neurogenesis existed between groups. The increased amount of Ki-67 positive cells in the entire AD hippocampus was attributable to glia-rich areas and cells associated with the vasculature. Based on these results, and on our additional DCX, GFAP and VWF stainings, we conclude that increased proliferation in the CA areas of presenile AD brains does not reflect neurogenesis but rather represents glial proliferation and vasculature associated changes.

Enhanced proliferation in presenile AD

Hippocampal proliferation in the adult and elderly human brain is a rare phenomenon (Eriksson et al., 1998; Del Bigio, 1999). As only neutral AR conditions have been used for Ki-67 in earlier studies, the extent of proliferation in AD, though, may have even been underestimated (Smith and Lippa, 1995; Del Bigio, 1999). AR of particularly nuclear antigens was shown to heavily depend on heat as well as pH (Lucassen et al., 1995; Shi et al., 1995) and clear improvements of immunoreactivity have been reported after AR at acidic pH values, particularly when fixation duration was prolonged (Taylor et al., 1994; Shi et al., 1995). The present pH 1.0 incubation is simple and provides an optimal Ki-67 retrieval without the need for combined treatments or ultra rapid microwave devices. So far, only one study has demonstrated the actual occurrence of dividing cells in humans using BrdU double labeling (Eriksson et al., 1998). The BrdU numbers as reported by Eriksson et al. are in the same order of magnitude as the Ki-67 numbers presently found in patients of comparable ages. We therefore conclude, together with other studies (Blumcke et al., 2001; Duchrow et al., 2001; Kee et al., 2002), that Ki-67 is a reliable and effective proliferation marker for human brain. Based on both the BrdU and Ki-67 protocols it can furthermore be concluded that the number of proliferating cells in the adult human brain is indeed very low, both in healthy and diseased brains.

Although the number of Ki-67 positive cells was low, we still observed a considerable increase in the hippocampus of presenile AD compared to control subjects. Previous studies have reported increased expression of various cell cycle related proteins in AD (Nagy et al., 1997; Vincent et al., 1997; Busser et al., 1998; Jin et al., 2004a). The occurrence of proliferative and cell cycle-related changes in close association with AD pathology has been interpreted as attempts of endangered neurons to start a new cell cycle, that may in some conditions, lead to cell death (Smith and Lippa, 1995; Kondratick and Vandre, 1996; Busser et al., 1998; Herrup et al., 2004). A major difference with the present study is that, even though we focused on non-mature, healthy appearing Ki-67 IR nuclear profiles, mature cells with ectopic Ki-67 immunoreactivity were not observed at all in presenile AD. This suggests
that the ectopic cell cycle protein expression is characteristic for senile rather than presenile AD. Even though the age of the present cohort is relatively young, AD pathology is extensive and disease duration generally short. Hence, this subgroup represents a pathophysiology quite different from senile AD.

The presence of “cycling” neurons in senile AD, that is, ectopic expression of proliferation markers in mature neurons, may indicate that these cells are “stuck” in a cycle that can not be completed (Yang et al., 2003). or, alternatively, may reflect an early stage of cell death (Raina et al., 2001; Herrup et al., 2004). The former is consistent with the fact that most “cycling” neurons were seen in older AD cohorts (> 70 years of age), and in a separate group of mildly cognitively impaired patients (Yang et al., 2003), which is often considered a prodromal state for AD. This would indicate that cell cycle changes in adult neurons may require more time to develop, and may only appear in elderly individuals or senile AD patients with a long disease duration. Alternatively, the attempts of the mature neuronal population to re-enter the cell cycle could have been unsuccessful in presenile AD patients and may have caused an early abortive exit through apoptosis. Although this latter theory does not support the concept of “cycling” neurons with a long lasting presence (Yang et al., 2003), it is consistent with the present reduction in CA1-2 cross sectional area in presenile AD, and with various papers indicating that (forced) re-entrance into the cell cycle induces cell death in mature neurons under in vitro conditions (Busser et al., 1998; Raina et al., 2001; Herrup et al., 2004). One other possibility might be that in presenile AD more plasticity remains as compared to senile cases, which would allow the brain to more efficiently respond to the occurrence of deleterious events, by preferentially inducing an abortive exit from the cell cycle.

**Neurogenesis, astrogliosis and angiogenesis**

As to the future phenotype of Ki-67 positive cells, technical limitations need to be considered. Ki-67 positive cells cannot be double labeled with glia or neuronal markers, since Ki-67 is rapidly degraded once a cell starts to differentiate (Gerdes et al., 1991; Scholzen and Gerdes, 2000). To our knowledge, only two papers have reported Ki-67 double-labelling in human brain, but this was with immature markers, like nestin, characteristic of undifferentiated cell types, and only in very young (i.e. 2-19 months of age) brains (Blumcke et al., 2001; Abraham et al., 2004). We, therefore, were confined to inferring the differentiation i) from the location of Ki-67 positive cells and ii) through staining with more specific markers in adjacent sections. Since staining with the marker of young migrating cells DCX turned out to be sensitive to PMD, data regarding neurogenesis in human brain obtained with this marker need to be considered with some care. However, as nearly all cells in the DG granule cell layer are of neuronal origin, typefication based on location is in this case unlikely to be incorrect.

Based on the location of Ki-67 positive cells and data from our additional stainings, we presently found no indication for enhanced
neurogenesis in the brains of presenile AD patients. This is at variance with a recent study in which proliferation markers and DCX expression were found to be increased in the hippocampus of an older AD cohort (Jin et al., 2004a). While carefully validating the use of DCX immunohistochemistry for postmortem brain, we found DCX, like many other microtubule associated proteins and dendritic elements (Swaab and Uylings, 1988), to be prone to degradation during PMD. In fact, DCX IR dendrites had already disappeared after 1 h PMD, while DCX IR somata became smaller and more difficult to detect. Their numbers appeared to be stable for up to 8-12 hours PMD, but the appearance of granular elements at these PMDs likely reflect artefacts that hamper the correct interpretation of DCX in human brain. We conclude that care should be taken, particularly in matching for PMD, when interpreting DCX IR in PMD brain.

Our semiquantitative comparisons failed to detect any difference in DCX IR between control and AD cases. Clearly, we do not reproduce the robust and even increased expression of DCX in AD as reported recently (Jin et al., 2004a). This discrepancy may relate to differences in immunohistochemical protocols, antibodies or differences in AR. Also, inclusion criteria for DCX positive neurons may have been different between the two studies and it may be inferred that DCX IR granular elements, which are abundantly present in the SGZ particularly at longer PMDs (as used in the AD group by Jin et al.), were included in their study, but not in the present analysis. Moreover, we have spend great effort to quantify our immunocytochemical data in a region-specific manner. By contrast, the conclusion by Jin et al. that neurogenesis is increased in AD is primarily based on western blots for DCX and PSA-NCAM done on whole hippocampal homogenates of a few patients (n=3). This approach not only lacks morphological detail on e.g the DG, but is also sensitive to brain atrophy, which occurs in AD, and may cause a bias in relative protein concentrations. As the cohort in the paper by Jin et al. was furthermore on average 10 years older (mean age: 77.6) than the cohort in our study, and PMD was longer in their AD than in their control group, an additional possibility is that age and PMD have contributed to the present discrepancy.

Following our extensive and validated protocol, the number of Ki-67 positive cells was nevertheless found to be enhanced in presenile AD, while no indication for enhanced dentate neurogenesis could be obtained. We thus considered the possibility of enhanced gliogenesis and angiogenesis. Astrocyte activation is characterized by hypertrophy of cellular processes, upregulation of GFAP and reexpression of nestin (Wilhelmsson et al., 2004). Classic astrogliosis is known to occur extensively in the senile AD cortex and CA1 region. However, we here found a robust astrogliosis in the SGZ, an area generally not characterized by a heavy plaque load. Yet, in the molecular layer of the entorhinal cortex too, activated astrocytes loaded with Aβ are present in the absence of amyloid plaques (Nagele et al., 2004), indicating that other factors than amyloid plaques may trigger astrogliosis. The present robust GFAP immuno staining in the hilus and SGZ, but not in control subjects
was paralleled by an upregulation of Ki-67+ cells, which suggests that also glia proliferation may take place.

Our data on proliferative responses in glia-rich regions are consistent with recent reports on gliogenetic changes and an increased cell cycle protein expression in glia (Bondolfi et al., 2002; Gartner et al., 2003; Hoozemans et al., 2005; Wharton et al., 2005) in the AD hippocampus, and increased expression of cyclins in astrocytes in APP23 transgenic mice (Bondolfi et al., 2002; Gartner et al., 2003). As to the functional importance of this phenomenon, we conclude that in addition to intracellular upregulation of GFAP, this may also represent division and production of more astrocytes following pathological activation. Alternatively, as astrocytes have recently been identified as neuronal precursors in development (Noctor et al., 2001; Gotz et al., 2002), their proliferation could be an attempt to compensate for the loss of normal glial or neuronal functions.

Our current data further show that Ki-67 IR is increased in endothelial structures of presenile AD brains, as confirmed by VWF expression. Cerebral amyloid angiopathy CAA is currently being studied intensively as it may play an important role in AD etiology (Vagnucci et al., 2003; Castellani et al., 2004; Nicoll et al., 2004; Zlokovic, 2005). Various causal mechanisms for CAA have been proposed in association with AD, including hypoperfusion, altered blood brain barrier function, vascular remodeling, aberrant angiogenesis and reduced Aβ clearance. Regarding the angiogenic changes, total cortical capillary length is known to be reduced in AD (Wu et al., 2005). Also many pro-angiogenic changes are seen in AD, including increased expression of vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) (Kalaria et al., 1998; Tarkowski et al., 2002), changes that can be mediated by reactive astrocytes (Salhia et al., 2000). Also Aβ itself can induce fibroblast growth factor (FGF) expression (Cantara et al., 2004), cyclin D1 expression in brain pericytes (Rensink et al., 2004) and in vitro angiogenesis (Zand et al., 2005).

As vascular structure appears impaired in AD, the increased Ki-67 IR in vascular cells may reflect an aberrant response that could contribute to vascular dysfunction. This could be due to the lack of essential factors for angiogenesis, such as the mesenchyme homeobox 2 gene (Wu et al., 2005) which is absent in AD. We propose that vascular-associated Ki-67 expression in the presenile AD hippocampus is either non-functional or even dysfunctional, as vascular function is compromised in AD.

In conclusion, significantly increased numbers of Ki-67 IR cells were found in the presenile AD hippocampus, and were mostly accounted for by glia cells and cells associated with the vasculature. No indications for increased neurogenesis were apparent. We presently observed a significant volume loss in the CA1 area. This is well documented in AD, but with considerable variation to its extent (ranges of 12% - 86% reported) (Bobinski et al., 1997; Simic et al., 1997; Rossler et al., 2002). Clearly, if replacement of diseased hippocampal neurons is attempted, it has been unsuccessful. In contrast to senile AD, a prominent activation of glia cells and the vasculature occurs in presenile AD.