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Title page

Stage-specific changes in neurogenic and glial markers in Alzheimer's disease

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Abstract

Background: Reports of altered endogenous neurogenesis in people with Alzheimer's disease (AD) and transgenic AD models have suggested that endogenous neurogenesis may be an important treatment target, but there is considerable discrepancy between studies. We examined endogenous neurogenesis and glia changes across the range of pathological severity of AD in people with/without dementia to address this key question.

Methods: Endogenous neurogenesis and glia in the subventricular zone and dentate gyrus neurogenic niches were evaluated using single and double immunohistochemistry and a validated antibody selection for stage-and-type-specific markers in autopsy tissue from a representative cohort of 28 participants in the MRC Cognitive Function and Ageing study (MRC CFAS). Immunopositive cells were measured blinded to diagnosis, using brightfield and fluorescent microscopy.

Results: The number of newly-generated neurons significantly declined only in the dentate gyrus of patients with severe tau pathology. No other changes in other neurogenic markers were observed in either of the neurogenic niches. Interestingly, alterations in astrocytes and microglia were also observed in the dentate gyrus across the different stages of tau pathology. No change in any of the markers was observed in individuals who died with dementia compared to those who did not.

Conclusions: Alterations in endogenous neurogenesis appear to be confined to a reduction in the generation of new neurons in the dentate gyrus of AD patients with severe neurofibrillary tangle pathology and were accompanied by changes in the glia load. These data suggest that intervention enhancing endogenous neurogenesis may be a potential therapeutic target in AD.

Introduction

Dementia currently affects more than 34 million people worldwide, with estimations that more than 110 million people will be affected by dementia in 2050 (1). Alzheimer's disease (AD), the most common form of dementia, causes enormous personal, social and financial burdens on the patients, their caregivers and society. Current pharmacological treatments offer symptomatic benefits, thus effective disease-modifying therapies are urgently needed. As AD is a neurodegenerative disease, cell replacement strategies are a potential target for therapeutic intervention; such as promoting endogenous neurogenesis.

Endogenous neurogenesis is evident in two areas of the brain: the hippocampal dentate gyrus (DG) and the wall of the lateral ventricles (subventricular zone–SVZ) (2-5). In mammals, neural progenitors at the base of the DG granular layer (subgranular layer, SGL) give rise to neurons that can be functionally integrated in the granular cell layer, whereas the SVZ neural progenitors follow a distinct pathway, the Rostral Migratory Stream (RMS), to the olfactory bulb (OB) where they create interneurons. In the healthy adult brain, SVZ neurogenesis maintains cellular turnover in the OB, contributing to olfactory adaptation and learning (6-8), whereas in DG endogenous neurogenesis is crucial for the hippocampal-dependent spatial learning and memory throughout life (8-11).

Groundbreaking work over the last two decades has demonstrated the presence of the same neurogenic niches in the adult human brain, including the temporal horn of the lateral ventricles, located adjacent to the hippocampal formation (12-15). Consequently, there has been evolving interest in the therapeutic potential of strategies that aim to enhance endogenous neurogenesis. Many available compounds, of which some are already in clinical use, such as retinoid agonists, cannabinoids, Selective Serotonin Reuptake Inhibitors (SSRIs), cholinesterase inhibitors and certain hormones have a substantial positive impact on neurogenesis in animals, by either stimulating proliferation of endogenous neural stem cells and/or increasing their differentiation into neurons (for reviews see 16 and 17).

However, the potential clinical relevance for AD patients is less clear, with contradictory results from the small number of human autopsy studies that have been undertaken. Ziabreva et al. (2006) and Perry et al. (2012) identified an increase at the proliferation stages of neurogenesis in the anterior SVZ (18) and the temporal horn SVZ and DG (19) respectively, but a reduction in the early stage neural progenitors in the SVZ of AD patients compared to age-matched controls (18). In a previous study from our group foucssing on a different cohort of AD patients, including people with concurrent cerebrovascular disease, no statistically significant difference was observed in early neuronal marker immunoreactivity between AD and controls (19). In contrast, in another study increased numbers of neural progenitors were detected in the DG of AD patients, which resulted in an unsuccessful maturation to newly-generated neurons (20). In a report focusing on younger patients with AD, increased glial proliferation was reported in the SGL, but no alteration in neurogenesis was identified (21). Other studies suggested that both concurrent cerebrovascular pathology (22-26) and the severity of cortical cholinergic system deficits (18, 19, 27) are likely to represent key mediating factors in either increasing or decreasing endogenous neurogenesis, respectively. Hence, the influence of age-associated neuropathological changes on neurogenesis is not fully elucidated, in particular with respect to the early stages of the AD process.

Similar to the often contradictory data from studies on human tissue, studies investigating neurogenesis in transgenic animal models carrying the human mutations for APP and/or PS1 or PS2 and/or tau proteins reported increased, decreased or unchanged progenitor activity (28-32).

To further elucidate the role of neurogenesis in AD, we examined *post mortem* brain tissue from a subset of participants of the Medical Research Council Cognitive Function and Ageing Study (MRC CFAS), including individuals who died with and without dementia, and who showed all neuropathological stages of AD-associated tau pathology (i.e., Braak stages 0 to VI), without any other neuropathology such as cerebrovascular disease. For the first time, the levels of astrocytic and microglia cell numbers were also identified in the different Braak stages. Our study primarily aimed to identify alterations in the various phases of endogenous neurogenesis in relation to dementia and AD-associated pathology.

Methods and Materials

Tissue was obtained from brains donated to the UK MRC CFAS. Details of the study have been described elsewhere (33, 34) and can also be found at the website (www.cfas.ac.uk). In brief, MRC CFAS included an initial cohort of 13,004 individuals, representative of the population aged 65 and over recruited from general practice lists in five areas of England and Wales. The cohort for the current study consists of 28 brain samples selected from those participants of the MRC CFAS who agreed to donate their brain on death and among whom a successful autopsy was performed. At the time of sampling, 456 brain donations had been made to the study, 114 of which were available and had sufficient tissue for the current analysis (those from the Cambridgeshire and Newcastle centres). Those who received a neuropathological diagnosis of ‘normal brain’, ‘possible AD’, ‘probable AD’ or ‘definite AD’ were considered for selection into the study. Those with any diagnosis of Lewy body disease, cerebrovascular disease or other neuropathology were excluded. Neurofibrillary tangle (NFT) pathology was assessed using Braak staging (35-37) after histochemistry, by experienced neuropathologists working in the MRC CFAS study, blinded to clinical findings (supplementary figure S1). Neurofibrillary Braak stages are stated based on the topographical distribution of neurofibrillary tangles and neuropil threads which are neuropathological hallmark lesions of AD; at Braak stages I and II, NFTs are confined mainly to the transentorhinal region of the brain; at stages III and IV they are also found in limbic regions such as the hippocampus, and at the severe stages V and VI they are extensively located in other brain areas, including the neocortex (35, 36). The neuropathological diagnosis of AD was done according to internationally accepted criteria that include the assessment of amyloid-β pathology, which progresses spatially and temporally differently than the tau pathology. Of note, similar to many other studies, we used Braak stages to indicate the overall severity of AD pathology, but not to directly compare the severity of tau pathology with neurogenesis; in this study neither tau nor amyloid-β pathology was directly compared with neurogenesis in the same topographic locations.

Diagnosis of dementia

Dementia status at death was determined based on interviews during the last years of life, including the full GMS-AGECAT diagnostic algorithm that was equivalent to that in the *Diagnostic and Statistical Manual of Mental Disorders,* third edition, revised (DSM-III-R), interviews with informants after the respondent's death when this was possible and death certification (37). Of the 28 individuals included in the present study, 13 received a study diagnosis of dementia at death. Demographic data are shown in table 1.

Immunohistochemistry

8μm thick, paraffin- embedded sections were obtained at the level of basal ganglia, including the anterior SVZ, and also at the level of hippocampus, including the temporal horn of the SVZ. Slides were processed for immunohistochemistry and double immunofluorescence (described in supplementary *Methods and Materials*), according to previously published procedures (22, 23, 26, 27).

Cell counts

Cell counting was performed twice, blind to the clinical and neuropathological diagnosis, using a Nikon Eclipse E800 microscope and the NIS elements software for bright field microscopy (version. 2.3, both from Nikon Europe, Nederlands) and a Carl Zeiss Apotome Axioplan 2 microscope and the AxioVision software for the immunofluorescence (version 4.7.2, all from Carl Zeiss, UK). The length of the hippocampus and that of three areas of the SVZ and the neighbouring ependymal cell layer in either the anterior ventricle horn or the temporal ventricle horn were measured on each slide section under a very low magnification and using the relevant microscope software tools. Results were expressed as the number of immunopositive cells for each antibody/mm of length for all the markers, in order to adjust for variances due to different lengths of the areas measured.

Statistical analysis

For all markers except doublecortin, negative binomial regression analysis was used to model the difference in the number of immunopositive cells per mm length across groups defined by Braak stage. Regression models were adjusted for age at death and for gender. Differences in cell counts per mm between those with and without dementia at autopsy were assessed by negative binomial regression adjusting for age at autopsy, gender and Braak stage. Negative binomial regression is a count based regression model, and allows a number of events (immunopositive cells) to be modelled in terms of covariates of interest (Braak stage and dementia diagnosis), as well as potentially confounding covariates (age and sex), while also taking into account differences in an ‘exposure’ variable (length of tissue sample being examined). Negative binomial regression is a generalisation of Poisson regression in that it allows for heterogeneity in the number of cells per mm across individuals within a group. Poisson regression assumes an even distribution of immunopositive cells across samples within groups and would lead to Type 1 errors since this assumption is highly unlikely to be met. Negative binomial regression results in an estimate of ‘rate’ (cells per mm) in each group standardised to remove the effect of any differences in age and sex across groups and an estimate of difference in terms of an ‘incident rate ratio’ corresponding to the ratio of the rates across groups, assuming all other covariates held constant. It should be noted that the rate estimate is very similar to the raw count of cells/mm in each case, suggesting that the effect of any difference in age and sex across groups in this analysis is minimal.

As a secondary albeit more conventional analysis, we conducted an analysis of variance (ANOVA) across groups treating total cells per mm as a continuous outcome. This led to substantively similar results, however it is likely that the outcome measure of cells per mm violates the assumptions of an

ANOVA (normally distribution with equal variances across groups) and so we prefer the negative binomial regression as the primary analysis.

The numbers of cells positive for doublecortin were too small for meaningful multivariate analysis or analysis using a count-based regression model, and so for each brain area the proportion of cases with any doublecortin positive cells was compared across groups using Fisher's exact tests.

An initial p value of $p<0.05$ was set for statistical significance, however owing to the large number of hypotheses considered, we subsequently corrected for multiple testing using the method of Benjamini and Hochberg (38) by setting a false discovery rate of $q=0.05$, leading to a revised critical value of $p<0.0016$ for each individual hypothesis. Finally, Spearman's correlation coefficients were estimated to identify any association between the detected changes. For the data analysis, IBM SPSS (version 19) and STATA (version 12.1) statistical software were used.

Results

Demographic data for the cohort are presented in Table 1. Across the entire study cohort, the mean age at death was 84.8 years (+/- 8.6, range 71-103) and 50% of the participants were female (for details see table 1). Statistical analysis showed that the post mortem delay was not related either to Braak Stage and dementia diagnosis ($p>0.05$ for both) or to the cell counts of any of the markers examined (data not shown).

The pattern of immmunoreactivity of the neural stem/progenitor cells and their progeny were consistent with our previous descriptions (22, 23). Nestin and doublecortin immunoreactivity was observed in cellular somata and processes, although in the case of doublecortin, immunoreactivity in the processes was rarely observed, possibly due to the post mortem delay, as described previously (21). As doublecortin is also expressed in astrocytes (39), any doublecortin-positive cells with astrocytic appearance (multiple processes) were excluded from counting. PCNA and HuC/D immunoreactivity were detected in the cell nucleus. Figure 1 (A and B) shows the different cell types, as detected by DAB immunohistochemistry and double immunofluorescence in the hippocampal DG and the subventricular zone.

Dentate gyrus

The estimated number of immunopositive cells per mm length in the DG and the estimated frequency of cells across Braak stages, standardised to the average age at the time of autopsy and for gender is shown in figure 2 (A and B) for both neurogenic markers and glia. There was a lower number of HuC/D-positive post mitotic early neurons in the DG in individuals with Braak staging V-VI (severe neuropathology) compared to those with Braak staging 0-II ($p=0.032$, Figures 1B and 2A).

A significantly higher number of microglia cells, as identified by Iba1 immunohistochemistry, were detected in individuals with Braak stage III-IV compared to the other two groups ($p=0.033$, Figures 2B and 3). However, the differences in early neurons and microglia cells detected were not statistically significant after adjusting for multiple testing.

There was a significantly lower number of GFAP-positive cells (identifying both neural stem cells and astrocytes) among those with Braak stage III-IV (IRR=0.6; 95% CI=0.4-0.9) compared to those with Braak stage 0-II, but a higher number among those in those in Braak stage V-VI (IRR=1.9; 95% CI=1.3-2.8), and this difference across Braak groups remained statistically significant after correction for multiple testing ($p<0.0001$, Figures 2B and 3).

Table 2 shows the difference in cell numbers per mm between those with and without dementia, and the rate ratio standardised for age, gender and Braak stage. There is some evidence of an increase in HuC/D-positive cell numbers in those with dementia (adjusted IRR=2.1; 95% CI=1.0-4.3; $p=0.05$).

As the number of doublecortin-positive cells was very low in the DG, a different statistical analysis was performed, as described in *Methods and Materials* section. Table 3 shows the proportion of cases with doublecortin-immunopositive cells in each brain area examined, with some evidence that these are more commonly found in the DG of those with higher Braak stages (Fisher's exact test p=0.05). All of the samples with doublecortin immunopositive cells in the DG were from participants who died with dementia (table 3, Fisher's exact test p=0.04), however owing to small numbers of samples and the large number of hypotheses considered these findings should be treated with caution.

In order to examine potential correlations between the neurogenic and glial markers we performed Spearman's correlation (rho) analysis. There was a significant positive association between newly-generated neurons and the activated microglia (R=0.52, p=0.005), but negatively associated with the changes of astrocytic cell numbers (R=-.396, p=0.045) in the DG (Table 4).

SVZ and ependymal cell layer (anterior and temporal horn)

The neurogenic markers remained unchanged at the anterior (at the level of basal ganglia) and temporal (at the level of hippocampus) horns of the SVZ and the ependymal cell layer adjacent to those when analysed by Braak stage and by the presence of dementia (supplemental table S1 and table 3). PCNA-HuC/D double and HuC/D single immunolabelled cells were not detected in these areas.

The astrocytic and microglia markers also remained unchanged in the SVZ neurogenic niche and neighbouring ependymal cell layer in Braak stages 0 to VI (supplemental table S1).

Discussion

A detailed analysis of endogenous neurogenesis in both neurogenic niches at various stages of tau pathology in 28 people with and without AD was undertaken from a representative community cohort of patients followed to autopsy (MRC CFA Study). This is a population-based representative pilot study of endogenous neurogenesis in AD using human autopsy tissue, and focuses on the full range of pathological disease severity, the cognitive status and both neurogenic niches from each individual, compared to our previous studies (18, 19). Furthermore, the study has the methodological advantage of excluding patients with concurrent cerebrovascular pathology, Lewy body disease and any other pathology that have been found to affect adult endogenous neurogenesis (22-27, 40), as opposed to previous studies of endogenous neurogenesis in AD (20, 21), including our previous studies focussing on different patient cohorts (18, 19) where the percentage area of immunoreactivity was measured rather than number of immunopositive cells.

There were limited but statistically significant changes in one of the markers used in this study in individuals with dementia or at the different Braak stages. Specifically, we observed a significant reduction in newly generated neurons, as determined by single HuC/D immunohistochemistry, but not neural progenitors as determined by nestin, doublecortin and double PCNA-HuC/D immunolabelling in people with severe tau pathology (Braak stage V and VI), compared to those with no significant tau changes (Braak stage 0-II). Interestingly and consistent with other recent studies about the role of microglia in modulating endogenous neurogenesis, there was a significant relationship with activated microglia.

There were no changes in neural stem cells or progenitors in the anterior and temporal horn of the SVZ, which is in contrast with the increased numbers of neural stem cells and progenitors observed in the SVZ of patients suffering from stroke, vascular dementia, dementia with Lewy bodies and small vessel disease, as our previous studies have shown (22, 23, 26, 27, 40). Markers of neurogenesis did not vary between those with and without dementia after adjusting for Braak stage, thus the reduced numbers of new neurons were specifically associated wih the severe Alzheimer pathology.

Earlier studies examining endogenous neurogenesis in the course of AD have produced conflicting results, possibly because of the predominant focus on more severe AD, variable concurrent vascular changes and the limited focus on the later stages of neurogenesis, i.e. the newly-generated neurons. The only other study to examine early neurons, as well as progenitors, reported a significant reduction on their maturation as marked by the the decreased levels of MAP2a and MAP2b isoforms in the DG of AD patients (20), consistent with our current findings.

The present study exhibits some limitations, mainly due to methodology. As there are no reliable and/or applicable markers to “visualise” and follow the fate of neural stem cells in the adult brain, it is impossible to draw any conclusions about adult endogenous neurogenesis during the lifespan of the participants in the study, so all our results represent adult neural stem cells and their progeny at a single time point, that of autopsy. Optimistically, the development of new technologies will facilitate this and clarify more the role of adult neurogenesis and its incolvement in cognitive decline in ageing and AD.

As exclusion criteria were applied to the cases in reference to the presence of other neurodegenerative diseases, our sample was modest (n=28), but it was a population-representative it is pilot study of endogenous neurogenesis focussing upon “pure” AD in human autopsy tissue, including individuals with different stages of AD pathology.

The use of antibodies as markers of endogenous neurogenesis on human autopsy tissue represents a challenge, but we employed a validated battery of antibodies for the identification of progenitors and newly-developed neurons at the various stages of neurogenesis in human autopsy tissue. Long post-mortem delay, quite common factor for obtaining human tissue, has been shown to alter but not eliminate the immunostaining pattern for doublecortin, with similar overall levels of staining but reduced staining within the soma. For example, Boekhoorn et al. (2006) have shown that post mortem delay reduced immunoreactivity within the dendrites of doublecortin-positive neuroblasts (21). A similar pattern was seen for doublecortin in our study, but importanly the changes in the overall pattern of staining by dementia stage were similar for other markers of neuroblasts/immature neurons (HuC/D with PCNA). As it has been suggested that DCX can also be expressed in astrocytes (39) and in dormant cells in non neurogenic areas (41, 42), the use of HuC/D as an additional marker for neuronal progenitors/early neurons is important and limits the possibility of overinterpreting results obtained from DCX immunohistochemistry. In addition, post-mortem delay was not significantly correlated with the overall level of staining. Many hypotheses were explored and when a correction was applied to ensure a false discovery rate of less than 0.05 only the changes detected in the numbers of GFAP-immunopositive cells in the DG remained still statistically significant across groups.

As there is an age-related decline in neurogenesis observed also in humans (43), our estimates have also been adjusted for age, which further confirms the validity of our results. Physical activity and certain pharmacological treatments such as SSRIs (for a review see 16, 17 and references therein) can have an impact on neurogenesis in rodent models (44-51 and for a review 16, 17). These were not specifically examined in the current study, but it is unlikely that the magnitude of these effects would be sufficient to confound the analysis.

The PCNA immunohistochemistry (Figure 1B) also revealed a number of cells that are not co-expressing the neuronal fate marker HuC/D (green only cells in figure 1B). Although these cells were not counted for the study, we can speculate that they could represent astrocytic, microglial, or endothelial cell proliferating progenitors or cells re-entering the cell cycle according to Yang et al. (2003) (52), although that was not observed in the DG.

Our results still showed some evidence that although the early stages of endogenous neurogenesis remained unchanged throughout the different Braak stages, severe AD pathology had a detrimental effect on the numbers of newly-generated neurons in the DG of the affected individuals. In contrast, endogenous neurogenesis at all stages and areas remained unchanged in individuals with dementia compared to people without, suggesting that severe AD pathology impaired only the production of new neurons.

We used Braak staging, a neurofibrillary tangle-based staging system, to describe the overall severity of AD. Amyloid and tau pathology both increase with increasing disease severity, and many other concurrent pathways related to a broad range of changes including inflammation and mitochondrial function are activated. The specific mechanisms associated with the reduced production of new neurones in people with severe AD are therefore difficult to unravel from the results of the current study. Previous work has however suggested that tau transgenic mice (29, 53) do have reduced neurogenesis, supporting the potential role of tau pathology as a contributor to this effect.

There was a significant positive correlation between the cell numbers of activated microglia and those of the newly-generated neurons in the DG, suggesting that the reduction in activated microglia in people with Braak stage V- VI tangle pathology may be a key driver for the decline in the newly-produced neurons in these individuals, along with the presence of tangles. Microglia have an important role in adult neurogenesis in the healthy brain, as it has been demonstrated to control the numbers of newly-produced neuron in the hippocampus through apoptosis (54) and can have both pro- and anti- neurogenic effects, finely “tuning” adult neurogenesis (for a review see 55).

For the first time, changes in GFAP-positive astrocytes have been examined at the various Braak stages. Though we have no knowledge of the causal factors of these changes, one can hypothesize that as astrocytes have a significant role in the support and protection of neurons in the healthy brain, the decrease identified at Braak stage III-IV, below the levels seen in individuals with Braak stage 0-II, could be another contributing factor to the disease progression and pathology. The two-fold increase above the levels observed in the healthy brain in astrocytes numbers in the Braak stage V-VI could have a detrimental effect on the diseased brain, as it has been described for certain neurodegenerative diseases, including dementia (for a review see 56). A separate study further investigating this hypothesis could clarify that observation and the underlying mechanism(s).

Our study examined the fate of neuronal progenitors and their progeny at the DG and the anterior and temporal horn SVZ and adjacent ependymal layers in various stages of AD, without any effect from concurrent cerebrovascular or other neuropathology, and showed that specific and significant reductions in newly-generated neurons were detected only in the DG of those with severe AD pathology, and were associated to the microglial load of the area. Previous studies (57-59) identifying that abnormal endogenous neurogenesis relates to age-related learning impairment have indicated that the manipulation of endogenous neurogenesis may be a potential treatment target in people with AD. As a cross-sectional autopsy study, our results have to be interpreted cautiously, but our findings do support the concept for an enhancement of aspects of endogenous neurogenesis, as a possible treatment target in AD.

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Within the last three years, Prof. Clive Ballard has received research grants from Lundbeck pharmaceutical company and fees for consultancy or speaking from Lundbeck, Acadia, Bristol-Myer Squibb, Bial and Novartis pharmaceutical companies. None of this work is however directly relevant to the submitted manuscript. Within the last three years, Prof. Paul Francis has received research grants from Lundbeck pharmaceutical company and fees for consultancy or speaking from Lundbeck and Novartis pharmaceutical companies. He has also received payment related to expert witness testimony related to cases involving Novartis and Janssen Alzheimer Immunotherapy. None of this work is however directly relevant to the submitted manuscript.

All other authors report no biomedical financial interests or potential conflicts of interest.

Table 1: Demographic data for the cases used in the present study

	Braak stage		
	0-II	**III-IV**	**V-VI**
	n=12	n=11	n=5
Age (years) ± SD	80.3±8.4	88.9±8.2	86.8±5.3
Gender	Female: 5; Male: 7	Female: 8; Male: 3	Female: 1; Male: 4
Dementia	n=3	n=5	n=5
Gender	Female: 2; Male: 1	Female: 4; Male: 1	Female: 1; Male: 4
PM delay in hours median (IQR)	17.5 (12-28)	25 (7-27)	17.5 (9.5-33)

Data represent the mean or median in each group. SD: standard deviation, PM delay: Post-mortem delay, IQR: InterQuartile Range.

Table 2: Cell numbers and area lengths (mm) for the neurogenic markers in the human dentate gyrus by study diagnosis of dementia at death.

Antibody	**No dementia** (n=15)			**Dementia** (n=13)		
	Cells/mm (raw data)	Adjusted Cells/mm	IRR	Cells/mm (raw data)	Adjusted Cells/mm	IRR
Nestin	0.094	0.13 (0.08)	Ref.	0.10	0.09 (0.05)	0.7 (0.1-4.0)
HuC/D-PCNA	0.88	1.0 (0.3)	Ref.	0.61	0.6 (0.2)	0.6 (0.2-1.6)
HuC/D	0.74	0.6 (0.1)	Ref.	0.73	1.3 (0.4) *	2.1 (1.0-4.3)
GFAP	4.79	5.2 (0.7)	Ref.	2.46	4.9 (0.5)	0.9 (0.7-1.4)
Iba1	7.82	7.3 (1.9)	Ref.	5.57	7.0 (2.2)	1.0 (0.4-2.3)

Rates and incident rate ratios for the difference in cell density in the dementia group compared to the no dementia group are estimated by negative binomial regression, adjusted for Braak stage. Cells per mm are standardised to the sample age and gender and numbers in parentheses represent standard error; IRR = Incidence rate ratio adjusted for age and gender, numbers in parentheses represent 95% confidence interval.*: p=0.045.

Table 3: Occurrence of Doublecortin immunoreactivity in the adult human brain neurogenic niches

Antibody	**Braak 0-II** (n=12)	**Braak III-IV** (n=11)	**Braak V-VI** (n=5)	**Fisher's exact test p-value**	**No dementia** (n=15)	**Dementia** (n=13)	**Fisher's exact test p-value**
DG	0 (0)	2 (20)	2 (40)	0.05	0 (0)	4 (31)	0.04
SVZ	1 (8.3)	2 (20)	0 (0)	0.57	1 (7)	2 (15)	0.58
EP	1 (8.3)	1 (10)	0 (0)	1.00	1 (7)	1 (8)	1.00
SVZ BG	4 (33)	1 (9)	0 (0)	0.22	2 (13)	3 (23)	0.64
EP BG	7 (58)	6 (54)	1 (20)	0.47	9 (60)	5 (38)	0.45

The numbers represent number of samples with doublecortin immunopositive cells in each brain area by Braak stage and dementia status where number in parentheses show the % of the same samples in each subgroup by Braak stage and dementia status. SVZ BG: Subventricular Zone at the level of basal ganglia (anterior horn). EP BG: Ependymal cell layer at the level of basal ganglia, adjacent to SVZ BG.

Table 4: Correlation analysis among the observed changes in the dentate gyrus

			GFAP DG	HuC/D DG	Iba1 DG
Spearman's rho	GFAP DG	*R*	1.000	-.396*	-.476*
	HuC/D DG	*R*	-.396*	1.000	.524**
	Iba1 DG	*R*	-.476*	.524**	1.000

Statistical analysis was performed using a two-tailed Spearman's rho correlation analysis. *R*: correlation coefficient. Statistical significance: *: $p < 0.05$ and **: $p < 0.01$.

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Figure legends

Figur*e* 1: Immunohistochemistry showing neurogenesis in the dentate gurus (DG) and the subventricular zone (SVZ) of the adult human brain. **A**: DAB immunohistochemistry for nestin (top left, arrows) and doublecortin (top right, arrow) depicting neural stem cell/progenitors and late neural progenitors, respectively in both neurogenic niches. Scale bar: 5um. **B**: Double immunofluorescence for HuC/D (red) and PCNA (green) in severe AD patients (Braak stage V-VI) and their age-matched controls (Braak stage 0-II). There are significantly less postmitotic immature neurons (arrows, HuC/D-immunopositive cells) in the DG of severe AD patients compared to their age-matched controls. Yellow arrowheads indicate proliferating neuronal progenitors/neuroblasts immunopositive for both PCNA (a proliferating marker) and the HuC/D antigen. Images **i** and **ii** show higher magnification of double labelled cells at Braak stage 0-II and V-VI, respectively. Scale bar: 20um. **C**: Schematic representation of the markers used for the characterization of the different stages of neurogenesis in the present study. **Gr**: dentate gyrus granular layer, **h**: hilus, **LV**: lateral ventricle, **Ep**: Ependymal cell layer.

Figure 2: Graph bars represent the adjusted mean number (±standard error) of immunopositive cells per mm of length of dentate gyrus for neurogenic (A) and glial (B) markers in AD patients at different Braak stages, adjusted for age and dementia status. *: $p<0.05$, **: $p<0.001$.

Figure 3: Immunofluorescence for astrocytes (GFAP) and microglia (Iba1) in age-matched individuals at Braak stage 0-II and patients with moderate (Braak stage III-IV) and severe (Braak stage V-VI) tangle pathology. **DG**: dentate gyrus, **h**: hilus. Scale bar: 50μm.

Figure 1

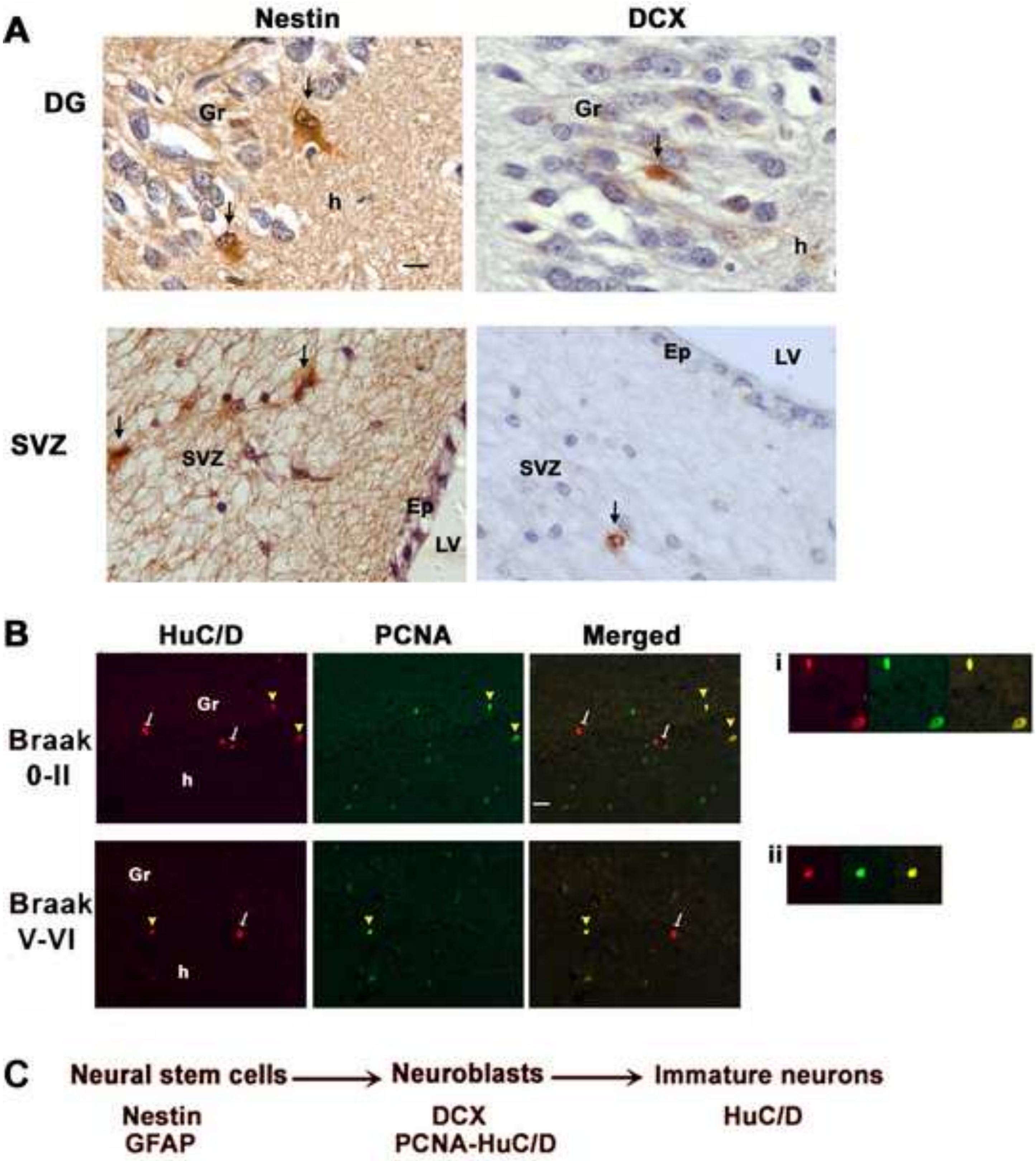

A
Nestin
DCX
DG
Gr
h
Gr
h
SVZ
SVZ
Ep
LV
Ep
LV
SVZ
B
HuC/D
PCNA
Merged
Braak 0-II
Gr
h
i
Braak V-VI
Gr
h
ii
C
Neural stem cells
Neuroblasts
Immature neurons
Nestin
GFAP
DCX
PCNA-HuC/D
HuC/D

Figure 2

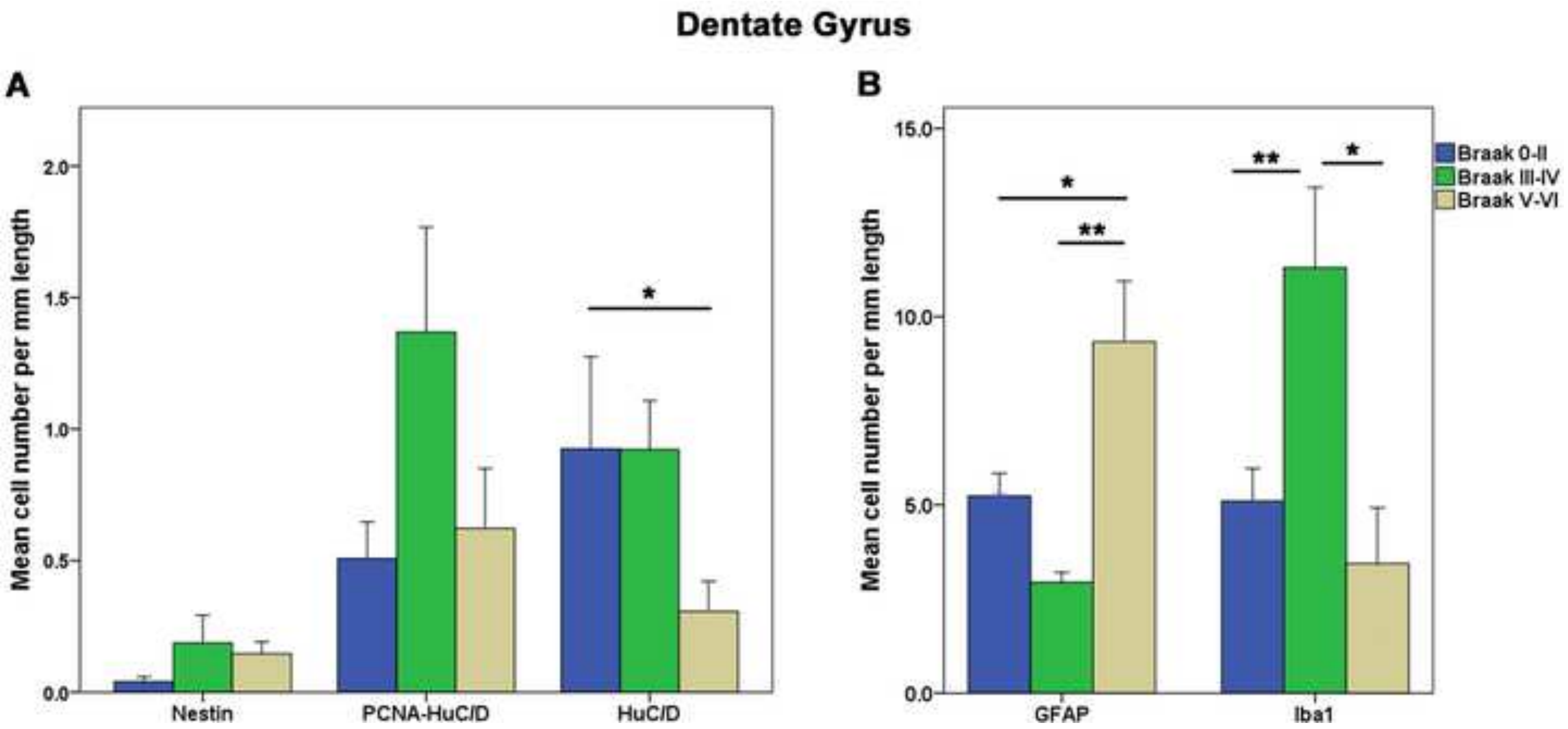
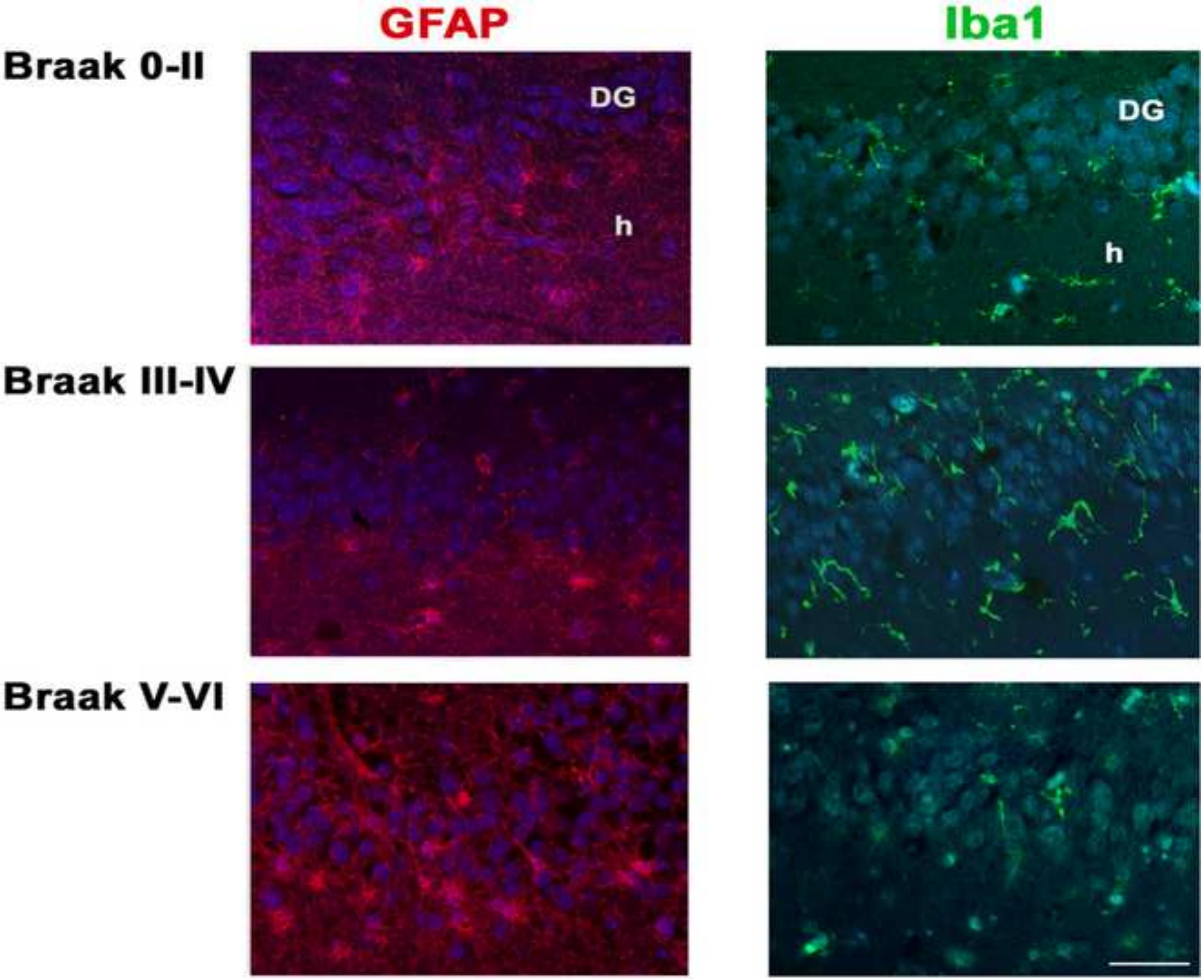

Dentate Gyrus
A
Mean cell number per mm length
2.0
1.5
1.0
0.5
0.0
Nestin
PCNA-HuC/D
HuC/D
*
B
Mean cell number per mm length
15.0
10.0
5.0
0.0
GFAP
Iba1
*
**
**
*
Braak 0-II
Braak III-IV
Braak V-VI

Figure 3



Stage-Specific Changes in Neurogenic and Glial Markers in Alzheimer's Disease

Supplemental Information

Supplementary Materials and Methods

Immunohistochemistry

After deparaffinization, rehydration and microwave pressure cooking antigen retrieval using 10 mM citrate buffer, pH 6, slides were processed for immunohistochemistry using DAB staining and double immunofluorescence. A validated battery of antibodies was used in order to identify the type of neural stem cells and progenitors as marked by nestin (1/200, Chemicon) (1) and doublecortin (1/200, Santa Cruz) (2) and immature neurons (HuC/D, 1/1000, Invitrogen) (3). In order to identify proliferating neuronal progenitors, double immunofluorescence was performed with HuC/D and an antibody against the proliferating cell nuclear antigen (1/1000, DAKO). Astrocytes were quantified using an antibody against glial fibrillary acidic protein (GFAP, 1/6000, Dako), which also marks adult neural stem cells. Although all GFAP-positive cells are astrocytes, only some of them are the adult neural stem cells. Microglial cells were labelled using the Iba1 antibody (1/1500, Wako, Japan). Counterstaining was performed using either haematoxylin (DAB staining) or Hoescht33258 (immunofluorescence). The endogenous lipofuscin autofluorescence was removed by a 10-minute incubation of the sections in a 0.5% Sudan Black B (SIGMA, UK) in 70% methanol solution, as previously described (4). Adjacent sections were incubated in the absence of the primary or secondary antibodies in order to determine non-specific antibody binding, and they were devoid of immunoreactivity (Figure S2B and S2C).

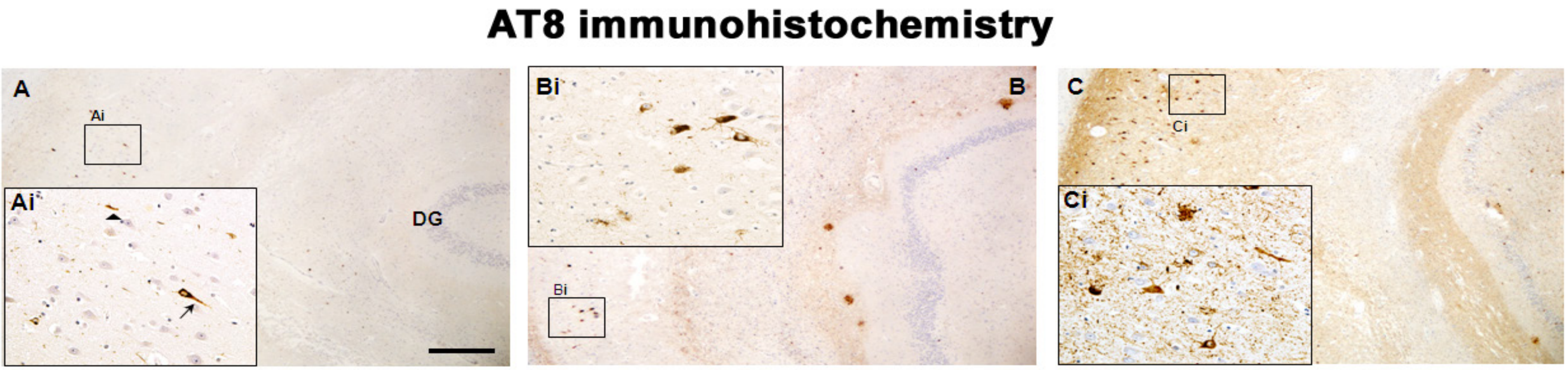


Figure S1. Examples for semi-quantitative scoring using immunohistochemistry for the AT8 antibody against hyperphosphorylated tau in hippocampal sections. (**A**) Low (arrow: neurofibrillary tangle; arrowhead: neuropil thread), (**B**) moderate and (**C**) high immunopositivity in the CA1 region of the human hippocampus. A denotes sparse pathology. Scale bar: 500 um, valid for all photomicrographs. DG, dentate gyrus.

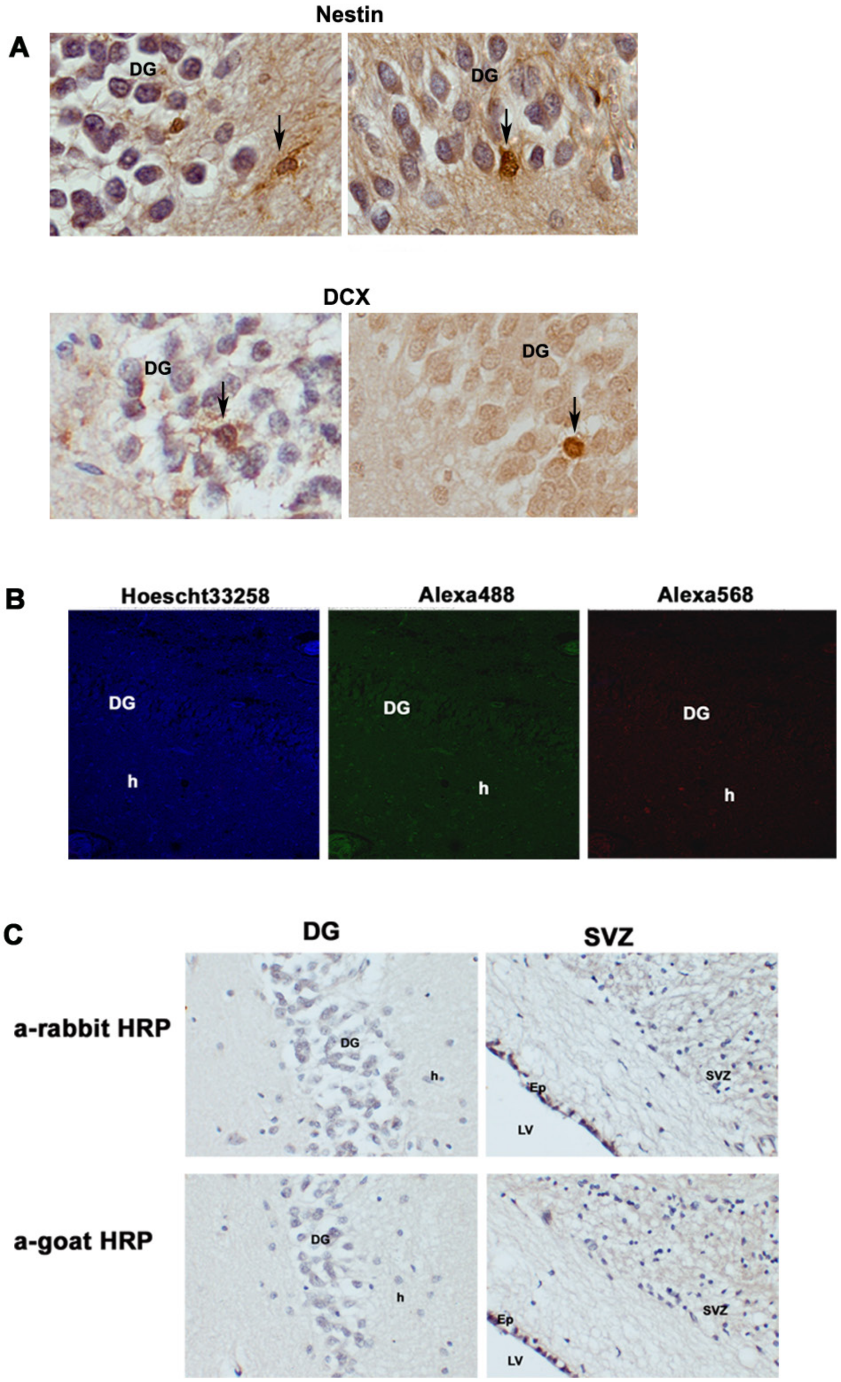


Figure S2. (**A**) DAB immunohistochemistry for nestin and doublecortin (DCX), arrows, in the hippocampal dentate gyrus (DG) under X63 magnification. (**B**) Fluorescence immunohistochemistry in the hippocampal DG after omission of the primary antibodies HuC/D and proliferating cell nuclear antibody (PCNA) and in the presence of fluorescence-conjugated donkey anti-mouse secondary antibodies Alexa®Fluor488 and AlexaFluor®568 antibodies under 20X magnification. (**C**) DAB immunohistochemistry after omission of the primary antibodies nestin and doublecortin in the presence of HRP-conjugated anti-rabbit and anti-goat secondary antibodies. h, hilus; LV, lateral ventricle; Ep, ependymal cell layer; SVZ, subventricular zone.

Table S1. Neurogenic and glia markers at the anterior and temporal horn of the subventricular zone

			Stage 0-2 (*n* = 12)			**Stage 3-4 (*n* = 11)**			**Stage 5-6 (*n* = 5)**		
			Cells/mm (raw data)	**Adjusted Cells/mm**	**IRR**	**Cells/mm (raw data)**	**Adjusted Cells/mm**	**IRR**	**Cells/mm (raw data)**	**Adjusted Cells/mm**	**IRR**
Iba1	SVZ	BG	8.28	7.5 (2.3)	Ref.	8.82	9.6 (3.3)	1.3 (0.5-3.4)	3.64	5.0 (2.9)	0.7 (0.2-2.4)
Iba1	EP	BG	1.83	2.6 (1.3)	Ref.	2.24	1.6 (0.7)	0.6 (0.1-2.4)	00.30	0.5 (0.6)	0.2 (0.02-2.3)
GFAP	SVZ	BG	4.58	5.6 (2.9)	Ref.	4.27	4.4 (2.1)	0.8 (0.2-2.7)	5.28	5.1 (3.6)	0.9 (0.2-5.0)
GFAP	EP	BG	23.3	38.8 (20.1)	Ref.	5.73	4.5 (2.6)	0.1 (0.02-0.8)	24.17	20.6 (16.5)	0.5 (0.05-5.3)
Nestin	SVZ	BG	22.12	20.9 (7.0)	Ref.	12.59	14.3 (5.9)	0.7 (0.2-2.1)	13.94	11.9 (5.7)	0.6 (0.2-1.7)
Nestin	EP	BG	93.77	86.3 (26.3)	Ref.	51.29	49.1 (15.8)	0.6 (0.2-1.5)	95.46	120.6 (54.4)	1.4 (0.5-3.8)
Iba1	SVZ		11.63	11.4 (2.2)	Ref.	21.49	21.4 (3.9)	1.9 (1.1-3.3)	16.88	19.3 (5.3)	1.7 (0.9-0.6)
Iba1	EP		1.83	2.1 (0.6)	Ref.	4.37	4.0 (0.9)	1.9 (0.9-4.1)	2.19	2.1 (0.9)	1.0 (0.4-2.7)
GFAP	SVZ		15.12	14.8 (2.7)	Ref.	19.10	19.7 (3.8)	1.3 (0.8-2.3)	17.5	17.3 (4.6)	1.2 (0.6-2.2)
GFAP	EP		10	11.3 (3.4)	Ref.	11.67	10.4 (3.1)	0.9 (0.3-2.2)	12.19	14.8 (7.0)	1.3 (0.5-3.8)
Nestin	SVZ		15.93	16.9 (2.8)	Ref.	20.67	22.1 (3.6)	1.3 (0.8-2.1)	17.81	14.2 (3.3)	0.8 (0.5-1.5)
Nestin	EP		13.58	14.6 (4.5)	Ref.	9.2	9.3 (3.1)	0.6 (0.3-1.6)	13.13	11.5 (5.0)	0.8 (0.3-2.2)

Cells/mm and incident rate ratios (IRR) (compared to the group with Braak stage 0-2 as a reference) are estimated by negative binomial regression. Adjusted cells per mm are standardized to the sample age and gender and numbers in parentheses represent standard error. None of the differences across groups are statistically significant. GFAP, glial fibrillary acidic protein; SVZ BG, subventricular zone at the level of basal ganglia (anterior horn); EP BG, ependymal cell layer at the level of basal ganglia, adjacent to SVZ BG.

Supplemental References

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