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1 **Title page**

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

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4 **Johnson<sup>4</sup>, Elaine K. Perry<sup>4</sup>, Johannes Attems<sup>4</sup>, Alyma Somani<sup>1</sup>, Stephen Minger<sup>1c</sup>, Clive Ballard<sup>1</sup>, on**  
5 **behalf of The Medical Research Council Cognitive Function and Ageing Neuropathology Study**

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21

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28 **Abstract**

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

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

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

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

48

**49 Introduction**

50 Dementia currently affects more than 34 million people worldwide, with estimations that more than  
51 110 million people will be affected by dementia in 2050 (1). Alzheimer's disease (AD), the most  
52 common form of dementia, causes enormous personal, social and financial burdens on the patients,  
53 their caregivers and society. Current pharmacological treatments offer symptomatic benefits, thus  
54 effective disease-modifying therapies are urgently needed. As AD is a neurodegenerative disease,  
55 cell replacement strategies are a potential target for therapeutic intervention; such as promoting  
56 endogenous neurogenesis.

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

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

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

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

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

99 to identify alterations in the various phases of endogenous neurogenesis in relation to dementia and  
100 AD-associated pathology.

101

## 102 **Methods and Materials**

103 Tissue was obtained from brains donated to the UK MRC CFAS. Details of the study have been  
104 described elsewhere (33, 34) and can also be found at the website ([www.cfas.ac.uk](http://www.cfas.ac.uk)). In brief, MRC  
105 CFAS included an initial cohort of 13,004 individuals, representative of the population aged 65 and  
106 over recruited from general practice lists in five areas of England and Wales. The cohort for the  
107 current study consists of 28 brain samples selected from those participants of the MRC CFAS who  
108 agreed to donate their brain on death and among whom a successful autopsy was performed. At the  
109 time of sampling, 456 brain donations had been made to the study, 114 of which were available and  
110 had sufficient tissue for the current analysis (those from the Cambridgeshire and Newcastle centres).  
111 Those who received a neuropathological diagnosis of ‘normal brain’, ‘possible AD’, ‘probable AD’  
112 or ‘definite AD’ were considered for selection into the study. Those with any diagnosis of Lewy  
113 body disease, cerebrovascular disease or other neuropathology were excluded. Neurofibrillary tangle  
114 (NFT) pathology was assessed using Braak staging (35-37) after histochemistry, by experienced  
115 neuropathologists working in the MRC CFAS study, blinded to clinical findings (supplementary  
116 figure S1). Neurofibrillary Braak stages are stated based on the topographical distribution of  
117 neurofibrillary tangles and neuropil threads which are neuropathological hallmark lesions of AD; at  
118 Braak stages I and II, NFTs are confined mainly to the transentorhinal region of the brain; at stages  
119 III and IV they are also found in limbic regions such as the hippocampus, and at the severe stages V  
120 and VI they are extensively located in other brain areas, including the neocortex (35, 36). The  
121 neuropathological diagnosis of AD was done according to internationally accepted criteria that  
122 include the assessment of amyloid- $\beta$  pathology, which progresses spatially and temporally  
123 differently than the tau pathology. Of note, similar to many other studies, we used Braak stages to

124 indicate the overall severity of AD pathology, but not to directly compare the severity of tau  
125 pathology with neurogenesis; in this study neither tau nor amyloid- $\beta$  pathology was directly  
126 compared with neurogenesis in the same topographic locations.

### 127 *Diagnosis of dementia*

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

### 134 *Immunohistochemistry*

135 8 $\mu$ m thick, paraffin- embedded sections were obtained at the level of basal ganglia, including the  
136 anterior SVZ, and also at the level of hippocampus, including the temporal horn of the SVZ. Slides  
137 were processed for immunohistochemistry and double immunofluorescence (described in  
138 supplementary *Methods and Materials*), according to previously published procedures (22, 23, 26,  
139 27).

### 140 *Cell counts*

141 Cell counting was performed twice, blind to the clinical and neuropathological diagnosis, using a  
142 Nikon Eclipse E800 microscope and the NIS elements software for bright field microscopy (version.  
143 2.3, both from Nikon Europe, Netherlands) and a Carl Zeiss Apotome Axioplan 2 microscope and the  
144 AxioVision software for the immunofluorescence (version 4.7.2, all from Carl Zeiss, UK). The  
145 length of the hippocampus and that of three areas of the SVZ and the neighbouring ependymal cell  
146 layer in either the anterior ventricle horn or the temporal ventricle horn were measured on each slide  
147 section under a very low magnification and using the relevant microscope software tools. Results

148 were expressed as the number of immunopositive cells for each antibody/mm of length for all the  
149 markers, in order to adjust for variances due to different lengths of the areas measured.

150 *Statistical analysis*

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

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



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

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

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

183

## 184 **Results**

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

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

197 types, as detected by DAB immunohistochemistry and double immunofluorescence in the  
198 hippocampal DG and the subventricular zone.

199

#### 200 *Dentate gyrus*

201 The estimated number of immunopositive cells per mm length in the DG and the estimated  
202 frequency of cells across Braak stages, standardised to the average age at the time of autopsy and for  
203 gender is shown in figure 2 (A and B) for both neurogenic markers and glia. There was a lower  
204 number of HuC/D-positive post mitotic early neurons in the DG in individuals with Braak staging V-  
205 VI (severe neuropathology) compared to those with Braak staging 0-II ( $p=0.032$ , Figures 1B and  
206 2A).

207 A significantly higher number of microglia cells, as identified by Iba1 immunohistochemistry, were  
208 detected in individuals with Braak stage III-IV compared to the other two groups ( $p=0.033$ , Figures  
209 2B and 3). However, the differences in early neurons and microglia cells detected were not  
210 statistically significant after adjusting for multiple testing.

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

216 Table 2 shows the difference in cell numbers per mm between those with and without dementia, and  
217 the rate ratio standardised for age, gender and Braak stage. There is some evidence of an increase in  
218 HuC/D-positive cell numbers in those with dementia (adjusted IRR=2.1; 95% CI=1.0-4.3;  $p=0.05$ ).  
219 As the number of doublecortin-positive cells was very low in the DG, a different statistical analysis  
220 was performed, as described in *Methods and Materials* section. Table 3 shows the proportion of  
221 cases with doublecortin-immunopositive cells in each brain area examined, with some evidence that

222 these are more commonly found in the DG of those with higher Braak stages (Fisher's exact test  
223  $p=0.05$ ). All of the samples with doublecortin immunopositive cells in the DG were from  
224 participants who died with dementia (table 3, Fisher's exact test  $p=0.04$ ), however owing to small  
225 numbers of samples and the large number of hypotheses considered these findings should be treated  
226 with caution.

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

231

232 *SVZ and ependymal cell layer (anterior and temporal horn)*

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

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

240

## 241 **Discussion**

242 A detailed analysis of endogenous neurogenesis in both neurogenic niches at various stages of tau  
243 pathology in 28 people with and without AD was undertaken from a representative community  
244 cohort of patients followed to autopsy (MRC CFA Study). This is a population-based representative  
245 pilot study of endogenous neurogenesis in AD using human autopsy tissue, and focuses on the full  
246 range of pathological disease severity, the cognitive status and both neurogenic niches from each  
247 individual, compared to our previous studies (18, 19). Furthermore, the study has the methodological

248 advantage of excluding patients with concurrent cerebrovascular pathology, Lewy body disease and  
249 any other pathology that have been found to affect adult endogenous neurogenesis (22-27, 40), as  
250 opposed to previous studies of endogenous neurogenesis in AD (20, 21), including our previous  
251 studies focussing on different patient cohorts (18, 19) where the percentage area of immunoreactivity  
252 was measured rather than number of immunopositive cells.

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

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

268 Earlier studies examining endogenous neurogenesis in the course of AD have produced conflicting  
269 results, possibly because of the predominant focus on more severe AD, variable concurrent vascular  
270 changes and the limited focus on the later stages of neurogenesis, i.e. the newly-generated neurons.

271 The only other study to examine early neurons, as well as progenitors, reported a significant

272 reduction on their maturation as marked by the the decreased levels of MAP2a and MAP2b isoforms  
273 in the DG of AD patients (20), consistent with our current findings.

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

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

285 The use of antibodies as markers of endogenous neurogenesis on human autopsy tissue represents a  
286 challenge, but we employed a validated battery of antibodies for the identification of progenitors and  
287 newly-developed neurons at the various stages of neurogenesis in human autopsy tissue. Long post-  
288 mortem delay, quite common factor for obtaining human tissue, has been shown to alter but not  
289 eliminate the immunostaining pattern for doublecortin, with similar overall levels of staining but  
290 reduced staining within the soma. For example, Boekhoorn et al. (2006) have shown that post  
291 mortem delay reduced immunoreactivity within the dendrites of doublecortin-positive neuroblasts  
292 (21). A similar pattern was seen for doublecortin in our study, but importantly the changes in the  
293 overall pattern of staining by dementia stage were similar for other markers of neuroblasts/immature  
294 neurons (HuC/D with PCNA). As it has been suggested that DCX can also be expressed in astrocytes  
295 (39) and in dormant cells in non neurogenic areas (41, 42), the use of HuC/D as an additional  
296 marker for neuronal progenitors/early neurons is important and limits the possibility of over-

297 interpreting results obtained from DCX immunohistochemistry. In addition, post-mortem delay was  
298 not significantly correlated with the overall level of staining. Many hypotheses were explored and  
299 when a correction was applied to ensure a false discovery rate of less than 0.05 only the changes  
300 detected in the numbers of GFAP-immunopositive cells in the DG remained still statistically  
301 significant across groups.

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

308 The PCNA immunohistochemistry (Figure 1B) also revealed a number of cells that are not co-  
309 expressing the neuronal fate marker HuC/D (green only cells in figure 1B). Although these cells  
310 were not counted for the study, we can speculate that they could represent astrocytic, microglial, or  
311 endothelial cell proliferating progenitors or cells re-entering the cell cycle according to Yang et al.  
312 (2003) (52), although that was not observed in the DG.

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

319 We used Braak staging, a neurofibrillary tangle-based staging system, to describe the overall severity  
320 of AD. Amyloid and tau pathology both increase with increasing disease severity, and many other  
321 concurrent pathways related to a broad range of changes including inflammation and mitochondrial

322 function are activated. The specific mechanisms associated with the reduced production of new  
323 neurones in people with severe AD are therefore difficult to unravel from the results of the current  
324 study. Previous work has however suggested that tau transgenic mice (29, 53) do have reduced  
325 neurogenesis, supporting the potential role of tau pathology as a contributor to this effect.

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

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

342 Our study examined the fate of neuronal progenitors and their progeny at the DG and the anterior  
343 and temporal horn SVZ and adjacent ependymal layers in various stages of AD, without any effect  
344 from concurrent cerebrovascular or other neuropathology, and showed that specific and significant  
345 reductions in newly-generated neurons were detected only in the DG of those with severe AD  
346 pathology, and were associated to the microglial load of the area. Previous studies (57-59)

347 identifying that abnormal endogenous neurogenesis relates to age-related learning impairment have  
348 indicated that the manipulation of endogenous neurogenesis may be a potential treatment target in  
349 people with AD. As a cross-sectional autopsy study, our results have to be interpreted cautiously,  
350 but our findings do support the concept for an enhancement of aspects of endogenous neurogenesis,  
351 as a possible treatment target in AD.

352

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372

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379

380

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391

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

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

393

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

395 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	<i>R</i>	1.000	-.396*	-.476*
	HuC/D DG	<i>R</i>	-.396*	1.000	.524**
	Iba1 DG	<i>R</i>	-.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

**Figure 1:** Immunohistochemistry showing neurogenesis in the dentate gyrus (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 ( $\pm$ 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: 50 $\mu$ m.

Figure 1  
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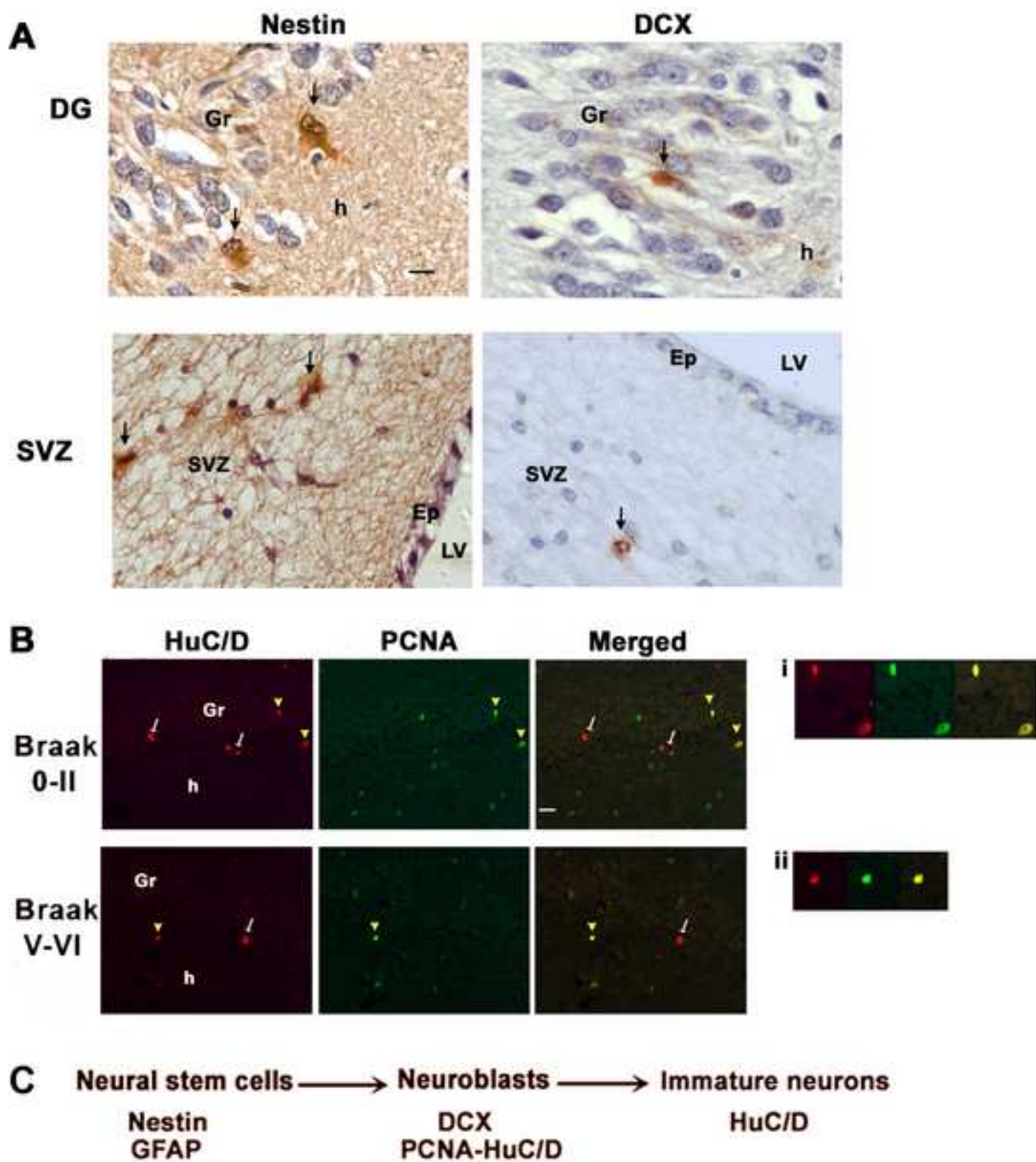


Figure 2  
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### Dentate Gyrus

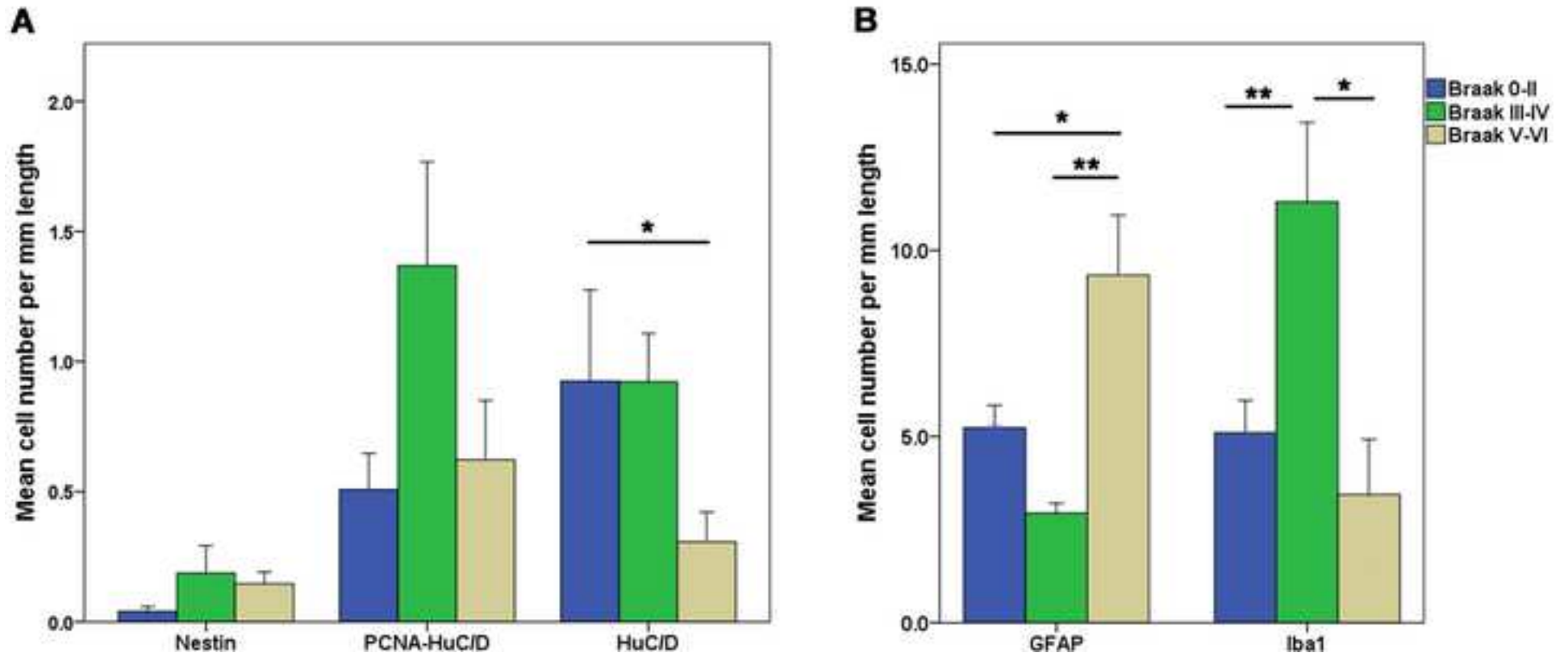
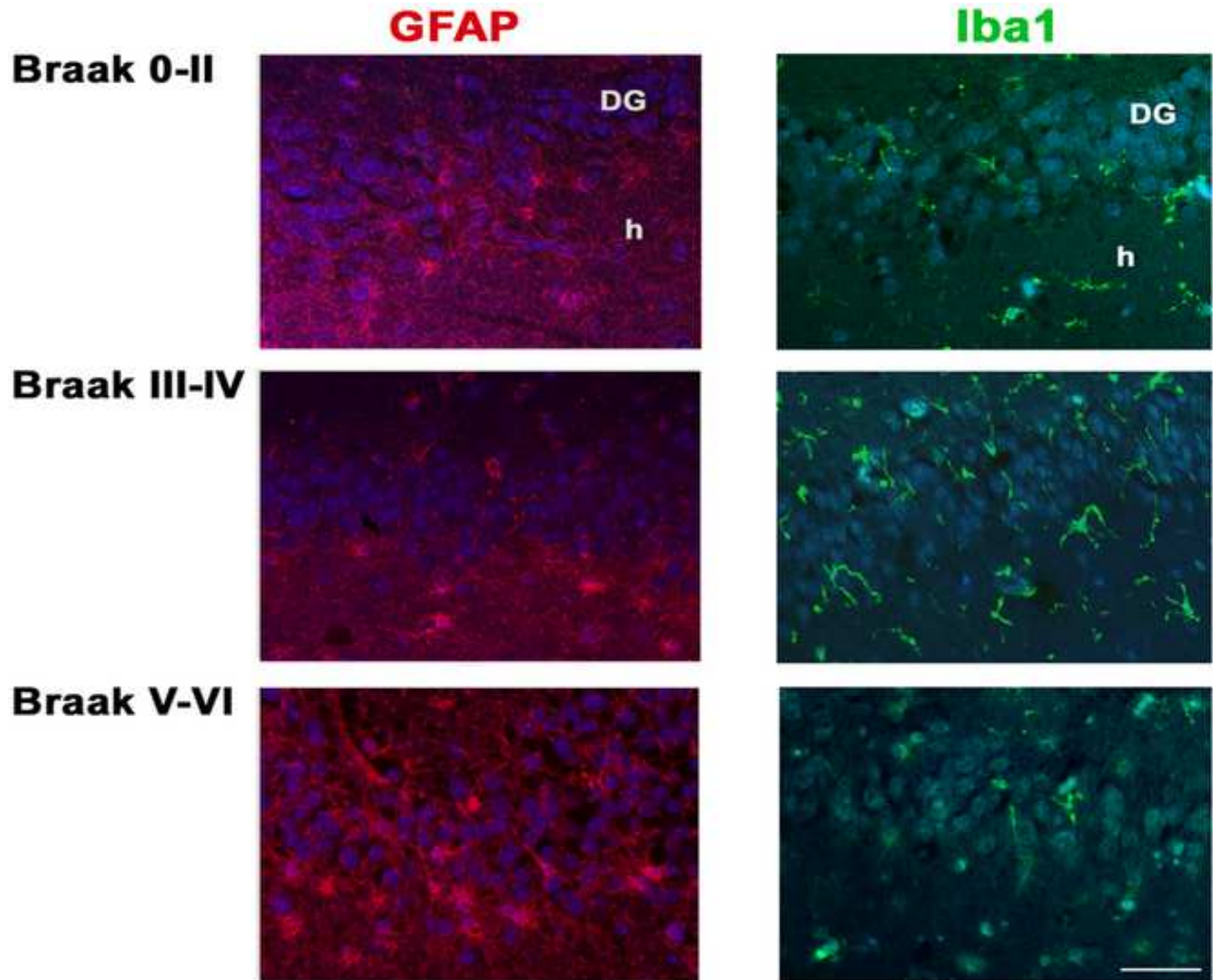


Figure 3  
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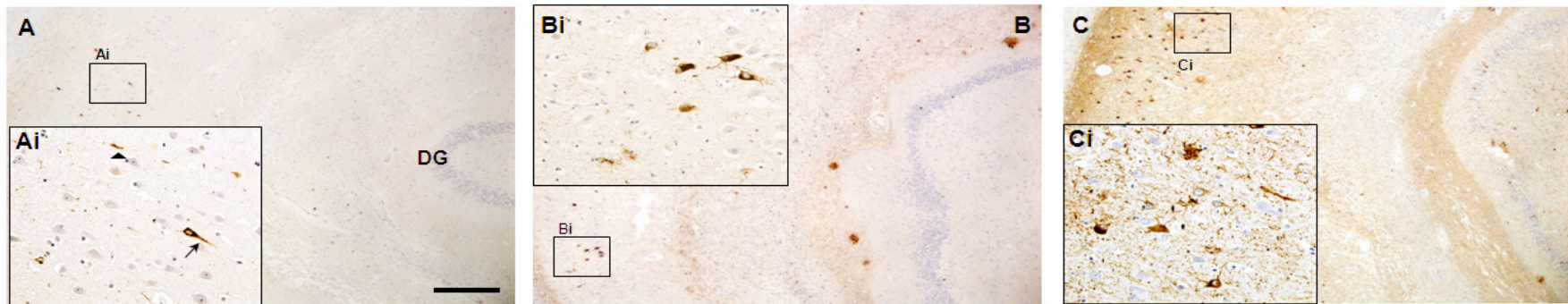


**Stage-Specific Changes in Neurogenic and Glial Markers in Alzheimer's Disease*****Supplemental Information*****Supplementary Materials and Methods****Immunohistochemistry**

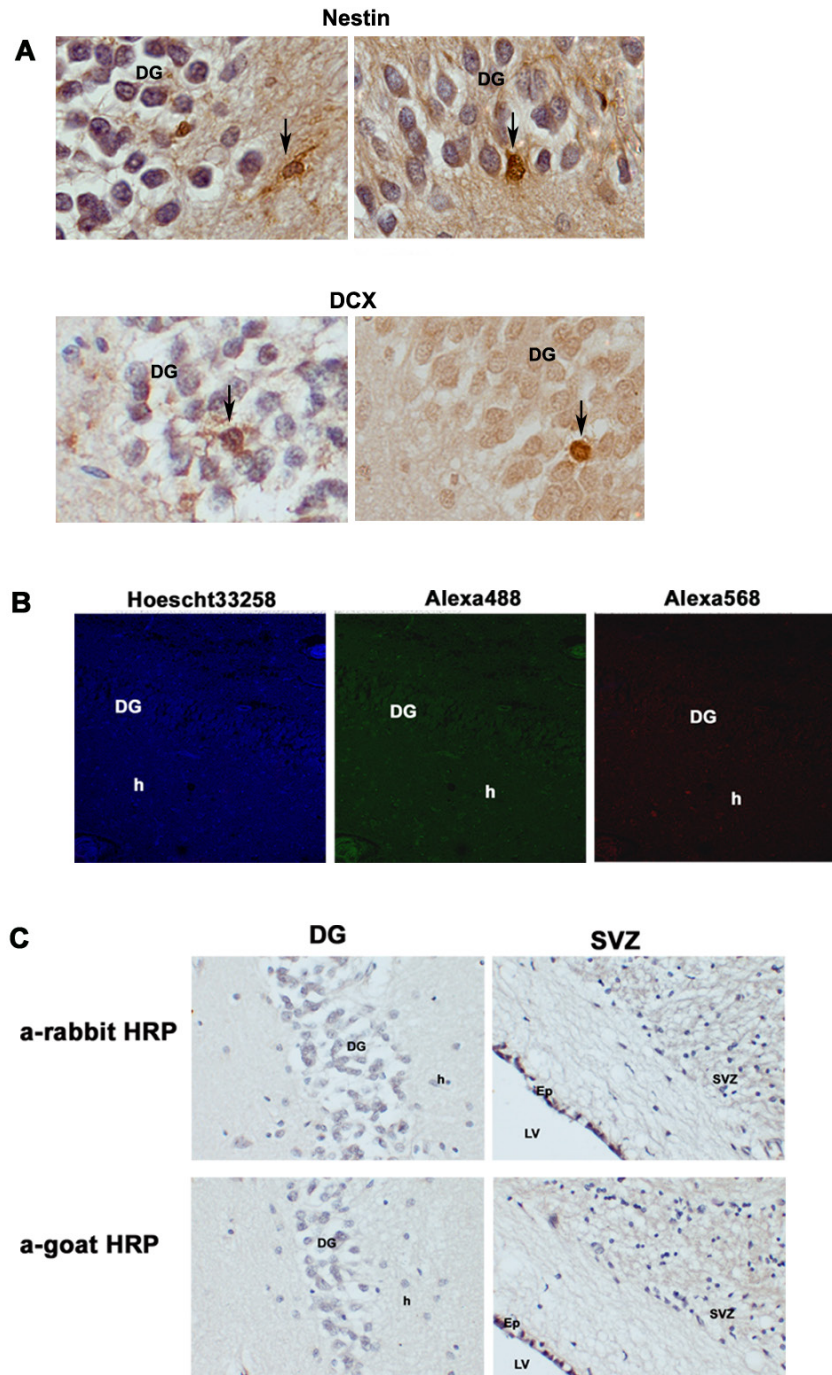
After deparaffinization, rehydration and microwave pressure cooking antigen retrieval using 10 mM citrate buffer, pH 6, slides were processed for immunohistochemistry using DAB staining and double immunofluorescence. A validated battery of antibodies was used in order to identify the type of neural stem 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).



## AT8 immunohistochemistry



**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 μm, 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<sup>®</sup>Fluor488 and AlexaFluor<sup>®</sup>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 ( <i>n</i> = 12)			Stage 3-4 ( <i>n</i> = 11)			Stage 5-6 ( <i>n</i> = 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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