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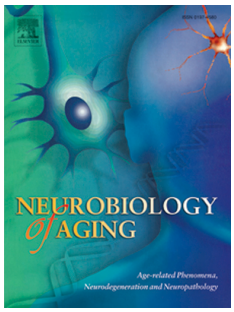

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The age-related slow increase in amyloid pathology in APP.V717I mice activates microglia, but does not alter hippocampal neurogenesis

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Abbreviations

Aβ, amyloid β; AD, Alzheimer's disease; AHN, adult hippocampal neurogenesis; CA, cornu ammonis; CR, calretinin; DCX, doublecortin; DG, dentate gyrus; GCL, granular cell layer; SGZ, sub granular zone; WT, wild-type.

Abstract

In Alzheimer's disease (AD), the hippocampus is characterized by abundant deposition of amyloid peptides (Aβ) and neuroinflammation. Adult hippocampal neurogenesis (AHN) is a form of plasticity that contributes to cognition and can be influenced by either or both pathology and neuroinflammation. Their interaction has been studied before in rapidly progressing transgenic mouse models with strong overexpression of APP and/or PS1. So far, however, changes in AHN and neuroinflammation remain poorly characterized in slower progressing models at advanced age, which approach more closely sporadic AD. Here, we analyzed 10- to 26-month-old APP.V717I mice for possible correlations between Aβ pathology, microglia and AHN.

The age-related increase in amyloid pathology was closely paralleled by microglial CD68 upregulation, which was largely absent in age-matched wildtype (WT) littermates. Notably, aging reduced the AHN marker doublecortin, but not calretinin, to a similar extent in WT and APP.V717I mice between 10 and 26 months. This demonstrates that AHN is influenced by advanced age in the APP.V717I mouse model, but not by Aβ and microglial activation.

Keywords

APP; neuroinflammation; neurogenesis; doublecortin; calretinin; Alzheimer's disease; aging.

1. Introduction

Age is the major risk factor for cognitive decline and for neurodegenerative disorders including Alzheimer's disease (AD) (Jagust, 2013; Prince et al., 2013; Small et al., 2002). The hippocampus in particular is implicated in cognition and undergoes functional and volumetric changes during aging (Bobinski et al., 2000; Hara et al., 2012; Pereira et al., 2014; Raz et al., 2004; Small et al., 2002; West et al., 1994, 2004) and is affected early in AD (Barnes et al., 1997; Bizon et al., 2009; Devanand et al., 2007; Frick et al., 1995; Small et al., 2004; Stoub et al., 2010). Indeed this brain region displays extensive neurodegeneration and functional deficits, both in AD patients and mouse models (Breyhan et al., 2009; Bobinski et al., 2000; Dodart et al., 2000; Fotenos et al., 2005; Jack et al., 1999; Schmitz et al., 2004; Stoub et al., 2010).

Different forms of neuronal as well as synaptic plasticity contribute to hippocampal functions. Adult hippocampal neurogenesis (AHN) is the generation of new neurons in the dentate gyrus (DG) and represents a unique form of structural plasticity, implicated in cognition and memory (Deng et al., 2010; Eriksson et al., 1998; Oomen et al., 2014). AHN decreases with age in both rodents (Cameron and McKay, 1999; Heine et al., 2004; Kuhn et al., 1996) and humans (Göritz and Frisén, 2012; Knoth et al., 2010; Manganas et al., 2007; Spalding et al., 2013). It furthermore responds to acute pathological insults like ischemia and epilepsy (Kuhn et al., 2001; Mattiesen et al., 2009; Parent et al., 2002; Shetty et al., 2012; Taupin, 2006), as well as to more chronic, slower developing pathologies like Parkinson's disease and AD (Curtis et al., 2003; De Lucia et al., 2016; Boekhoorn et al., 2006).

Alterations in AHN have been postulated to contribute to hippocampal dysfunction and/or disease progression (Maruszak et al., 2014; Mu & Gage, 2011; De Lucia et al., 2016; Gomez-Nicola et al., 2014; Richetin et al., 2015). However, considerable variation with respect to AHN in AD is reported in clinical studies, including increased, decreased or unchanged AHN and this variance in outcome seems to depend on the disease stage, the patient age and the examined AHN markers (Boekhoorn et al., 2006; Briley et al., 2016; Ekonomou et al., 2014; Jin et al., 2004; Li et al., 2008; Perry et al., 2012).

More controlled, pre-clinical studies have indicated that AHN diminishes when Aβ pathology becomes apparent (Demars et al., 2010; Donovan et al., 2006; Haughey et al., 2002a; 2002b; Kuhn et al., 2007; Krezymon et al., 2013; Lucassen et al., 2015; Marlatt and Lucassen, 2010; Mirochnic et al., 2009; Rodríguez et al., 2008; Verret et al., 2007), although conversely exceptions are reported as well (Donovan et al., 2006; Haughey et al., 2002b; Krezymon et al., 2013; Mirochnic et al., 2009; Unger et al., 2016; Verret et al., 2007; Yu et al., 2009). It must be noted that all these findings relied on transgenic mice that strongly overexpress (combinations of)

human amyloid precursor protein (APP) and presenilin 1 (PS1) mutant transgenes, resulting in high to very high levels of Aβ early in life. These high Aβ levels cause a rapid development of the pathology, a wanted characteristic for drug-development, which however deviates from the progression of the neuropathology in humans. Moreover, in these earlier studies, the transgenic mice were often studied at relatively young ages for practical reasons. Considering that AD is the most typical age-related neurodegenerative disorder, the pre-clinical mouse model APP.V717I that exhibits a slow progression of amyloid neuropathology might be a source of novel insights in the relation between AHN and amyloid pathology.

Related to humans, mice over 18 months of age can be considered old-age, whereas mice between 6- and 12-month-old can be considered adult (Flurkey et al., 2007). APP.V717I mice first develop amyloid plaques in the entorhinal cortex around 10 months of age. Soon after this, the plaques appear in the subiculum and other hippocampal sub-regions of APP.V717I mice, where it further develops to more extensive pathological levels by 15 to 18 months of age (Dewachter et al., 2000a; 2000b; Heneka et al., 2005; Moechars et al., 1999; Tanghe et al., 2010).

Besides amyloid-induced alterations in AHN, neuroinflammatory responses can modulate AHN as well and in particular microglia are thought to be instrumental (De Lucia et al., 2016; Ekdahl et al., 2009; Gebara et al., 2013; Sierra et al., 2010; 2014; Solano Fonseca et al., 2016; Varnum et al., 2015; Olmos-Alonso et al., 2016). Pre-clinical and clinical studies have revealed an association between brain aging and enhanced inflammatory signaling by microglia (Cribbs et al., 2012; Deng et al., 2006; Henry et al., 2009; Holtman et al., 2015; Sheng et al., 1998; Sierra et al., 2007; von Bernhardi et al., 2011). The inflammatory response of microglia to amyloid pathology (Cribbs et al., 2012; Holtman et al., 2015; Heneka et al., 2015a; 2015b; Marlatt et al., 2014) might hamper neurogenesis and hippocampal plasticity (Barrientos et al., 2006; Biscaro et al., 2012; Chapman et al., 2012; Chugh et al., 2013; von Bernhardi et al., 2015). It is therefore of interest to consider if and how age and AD-related changes in microglia in concert with emerging amyloid pathology may affect AHN.

Here we characterized both cell-associated amyloid as well as amyloid plaque pathology in APP.V717I mice at age 10, 14, 19 and 26 months, and studied whether AHN was altered relative to the age-related changes in neuropathology and microglial activation.

2. Methods

2.1 Mice

A total of 25 male mice were analyzed in this study: 14 APP.V717I heterozygous mice (Moechars et al., 1999) and 11 wildtype mice (WT), all of the FVB/n genetic background. As described before in detail, the APP.V717I mice produce both Aβ40 and Aβ42 peptides in the brain and develop dense-cored plaques that contain primarily Aβ42 and are Congo Red and Thioflavin S-positive (Dewachter et al., 2000b; Moechars et al., 1999; Van Dorpe et al., 2000). In contrast to highly overexpressing and rapidly progressing mouse models, the APP.V717I mouse model is characterized by a long pre-plaque stage and the first plaques do not emerge until ±12 months of age (Dewachter et al., 2000b; Moechars et al., 1999; Tanghe et al., 2010). Thereby the model more closely resembles the slow, age-related development of amyloid pathology in human AD patients.

Mice were subdivided in 4 age groups with different stages of amyloid pathology: 10 ±1 months (WT n=1, APP.V717I n=4), 14 ±1 months (WT n=3, APP.V717I n=2), 19 ±1 months (WT n=1, APP.V717I n=6) and 26 ±1 months (WT n=6, APP.V717I n=2) of age. Mice of 10-14 months represented the early pathological stages with still few amyloid plaques, while mice aged 19-26 months displayed widespread amyloid pathology. All mice were housed with no more than 4 littermates per cage, and all experiments were carried out in accordance with the EU Directive 2010/63/EU on animal welfare for scientific purposes.

2.2 Tissue collection

Brains were processed as described previously (Naninck et al., 2015). Briefly, mice were anaesthetized by intraperitoneal injection of 120 mg/kg pentobarbital before transcardial perfusion with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH 7.4). The brains were removed and post-fixed in 4% PFA in 0.1M PB overnight at 4°C and stored in 0.1M PB containing 0.01% sodium azide at 4°C.

Prior to sectioning, brains were kept overnight in 30% sucrose in 0.1M PB for cryoprotection at 4°C, subsequently frozen and cut in 40 μm thick coronal sections with a sliding microtome. Sections were divided over 8 series to obtain an even representation of each brain region per series and collected in an antifreeze solution of 20% glycerol, 30% ethylene glycol and 50% 0.05M phosphate buffered saline (PBS) and stored at -20°C until further use.

2.3 Immunohistochemistry

Immunohistochemistry (IHC) was performed to determine (I) amyloid load, (II) CD68 expressing microglia, the marker present in lysosomes and endosomes of monocytes. IHC for (III)

AHN was based on the number of cells expressing doublecortin (DCX) as a marker of the differentiation of neuroprogenitor cells to post-mitotic immature neurons (Couillard-Després et al., 2005), and on the number of cells expressing calretinin (CR) as a marker for more mature post-mitotic immature neurons, in addition to DCX (Brandt et al., 2003). Parallel series of brain sections were used for all stainings. Amyloid load in APP.V717I mice was assessed by IHC using an antibody directed against the N-terminal of the amyloid peptide (rabbit polyclonal anti-Aβ[N], #18584, IBL Japan, Gumma, Japan; Marlatt et al., 2013). IHC for CD68 marked microglial lysosomal activation (rat anti-mouse CD68 clone FA-11, MCA1957, Serotec, Kidlington, UK; Hoeijmakers et al., 2016). AHN was determined by IHC for DCX (goat anti-DCX, sc-8066, SantaCruz Biotechnology, Santa Cruz, CA, USA; Naninck et al., 2015) and CR (rabbit anti-calretinin, 7697, Swant, Marly, Switzerland ; Naninck et al., 2015).

Sections for amyloid staining were pre-mounted on coated glass slides (Superfrost Plus, Menzel, Braunschweig, Germany) with antigen retrieval by sequential citrate buffer and formic acid (FA) pre-treatment as described (Christensen et al., 2009; Marlatt et al., 2013). After washing in 0.05M Tris buffered saline (TBS), and rinsing with sterile water, the slides were incubated in 0.01M citrate buffer (pH 6.0) using a standard microwave protocol at 95-99°C for 15 min. After cooling to room temperature (RT), antibody retrieval was continued by 3 min incubation in 88% FA.

Next, the pre-treated slides for amyloid IHC and the free-floating sections for CD68, DCX and CR staining were washed in 0.05M TBS. Subsequently, slides and sections were incubated in 0.3% H_2O_2 for 15 min to block endogenous peroxidase activity. Non-specific antibody binding was blocked by 30 min incubation in 1% bovine serum albumin, 0.1% Triton X-100 in 0.05M TBS (Aβ, CD68), 2% milk powder in TBS (DCX), 2% normal goat serum, 0.3% Triton X-100 in 0.05M TBS (CR). Primary antibodies were diluted in blocking mix (1:1000 Aβ, 1:400 CD68, 1:5000 CR) or in an incubation mix of 0.25% gelatin, 0.1% Triton X-100, in 0.05M TBS (1:800 DCX), and incubated for 1 hour at RT followed by overnight incubation at 4°C.

Secondary biotinylated antibodies for Aβ and CR (goat anti-rabbit IgG 1:500 Vector Laboratories, Burlingame, CA), for CD68 (donkey anti-rat IgG 1:500 Vector Laboratories, Burlingame, CA) and for DCX (donkey anti-goat IgG 1:500 Jackson Laboratories, Bar Harbor, Maine, USA) were diluted in the same buffers as the primary antibodies and added to the sections for 2 hours at RT, followed by 90 min incubation with avidin-biotin complex (Vectastain elite ABC peroxidase kit, 1:800 in 0.05M TBS, Vectastain, Brunschwig Chemie, Amsterdam, the Netherlands). DCX staining included an additional signal amplification step for 30 min with biotinylated tyramide (1:500 in 0.01% H_2O_2 in 0.01M TBS) followed by a second incubation of 90

min with avidin-biotin complex. Finally, the tissues were thoroughly washed in 0.05M Tris buffer (TB) prior to incubation in 0.5 mg/ml diaminobenzidine, 0.01% H_2O_2 in 0.05M TB for chromogen development, followed by washing in 0.05M TB. Free-floating sections were mounted on pre-coated glass slides (Superfrost Plus slides, Menzel-Glaser, Braunschweig, Germany) and cover slipped with Entellan (EMD Millipore, Billerica, MA, USA).

2.4 Image analysis and quantification

Quantification of immunoreactive staining was performed by an observer unaware of the experimental conditions. The hippocampus was subdivided over the rostral-caudal axis based on the pre-determined bregma points, with all sections from bregma -1.22 mm to bregma -2.30 mm representing the rostral/dorsal hippocampus, and all sections from bregma -2.70 mm to -3.64 mm representing the caudal/ventral hippocampus. 6 bilateral sections with an approximate intersection distance of 480 µm per animal were chosen for analysis for all four quantifications (amyloid, CD68, DCX, CR), thereby including 3 sections representing the rostral hippocampus and 3 sections representing the caudal hippocampus.

2.4.1 Amyloid and CD68 immunoreactivity

Amyloid plaque load and CD68 immunoreactivity (referred to as CD68 coverage) were quantified in the hippocampal sub-regions subiculum, DG, cornu ammonix (CA) by image analysis based on standard thresholding method (Hoeijmakers et al., 2016; Marlatt et al., 2013). The respective regions were viewed and recorded with a Leica CTR5500 microscope (10x objective for Aβ and 20x objective for CD68) using dedicated software (Leica MetaMorph AF, version 1.6.0; Molecular Devices Sunnyvale, CA, USA). Images were processed using publicly available software (Image J; NIH, Bethesda, Maryland, USA). The respective regions were delineated in all images and converted to 8-bit grayscale pictures. A fixed threshold was set to include all IHC positive signal in the delineated regions, allowing us to determine the relative areas that define amyloid plaque load and CD68 coverage.

Next, we manually counted the number of CD68+ cells and the number of Aβ+ cells. CD68+ cells were sampled in the subiculum (250 µm x 250 µm area), CA1 (250 µm x 250 µm area) and molecular layer of the DG (150 µm x 150 µm area) of the aforementioned selected brain sections. The CD68+ individual cell surface (µm2) was further measured by dividing the CD68+ surface coverage (µm2) in the individual squares by the number of counted cells in this area, as a proxy for the changes in CD68 expression at the individual cell level. The number of Aβ+ cells is referred to as cell-associated and intraneuronal amyloid (Christensen et al., 2009; Jeong et al.,

2006; LaFerla et al., 2007). In each image, we quantified the cells in a 200 μm x 200 μm area in the center of the subiculum, and in a 100 μm x 400 μm area covering part of the pyramidal cell layers of the CA1 and CA3. Quantification of cell-associated amyloid in CA1 and CA3 pyramidal cell layers were combined to represent the cell-associated amyloid in the CA.

2.4.2 DCX and CR immunoreactive cell numbers

DCX immunoreactive cells (DCX+) and CR immunoreactive cells (CR+) were manually quantified at 20x magnification (Zeiss Axiophot microscope, Microfire camera; Optronics, Goleta, CA, USA) using dedicated software (Stereo Investigator software; MicroBrightField, Magdeburg, Germany). DCX+ and CR+ cells were counted in the DG granular cell layer (GCL) and subgranular zone (SGZ). DCX+ cells were further classified in three different developmental stages based on their morphological appearance (Oomen et al., 2010). The proliferative stage represents cells with no or very short, plump processes; the intermediate stage refers to cells with one process approaching or reaching the molecular layer; the post-mitotic/immature neuron stage includes cells with dendritic branching into the GCL and/or molecular layer (Oomen et al., 2010).

2.4.3 Volume estimation

Estimations of the volumes of GCL, DG and total hippocampus were based on the Cavalieri principle (Gundersen and Jensen, 1987, Naninck et al., 2016). A surface estimation of the regions was obtained based on the 6 bilateral hippocampal sections by outlining the contour of the specific region of interest. The total estimated surface was then multiplied by the section thickness (40 μm), by the number of series (8) and by the ratio of bilateral hippocampal sections sampled out of the total number of hippocampal sections within a series (6 out of 9).

2.5 Statistical analysis

Data graphs present means ± standard error of the mean. Statistical outliers were not present in the data, as test by the freely available online Grubb's test (Graphpad software, San Diego, CA, USA). Further statistical analysis was performed with SPSS 22.0 software. Significance was accepted for $p<0.05$. Amyloid pathology in APP.V717I mice was analyzed by one-way ANOVA and post-hoc analyses were performed using Bonferroni multiple comparison tests. Data of CD68, DCX+ cell numbers and CR+ cell numbers in WT and APP.V717I mice were analyzed using the two-way ANOVA model with genotype and age as independent factors. For these analyses, the two youngest (10 and 14 months) and two oldest (19 and 26 months) age groups were combined in order to compare an early pathological stage (10 and 14 months) with more

abundant pathology (19 and 26 months). The relatively low power prevented the assessment of age-specific effects within all 4 age groups. Overall, inter-parameter relationships were tested using Pearson's bivariate correlation analysis.

3. Results

3.1 APP.V717I expression, but not age, induced hippocampal atrophy

Hippocampal atrophy is one of the hallmarks of AD and we therefore estimated the volume of the hippocampus with specific attention for DG and GCL. Hippocampal, DG and GCL volumes were smaller in APP.V717I compared to age- and gender-matched WT mice, independent of age (fig 1A-C: Hippocampus: *age F(1,20)=0.282, ns, genotype F(1,20)=7.773, p=0.011, interaction F(1,20)= 1.886, ns*; DG: *age F(1,20)=0.020, ns, genotype F(1,20)=6.278, p=0.021, interaction F(1,20)= 1.522, ns*; GCL: *age F(1,21)=0.105, ns, genotype F(1,21)=13.708, p<0.001, interaction F(1,21)=0.487, ns*).

3.2 Amyloid pathology increased with age in APP.V717I mice

Quantification confirmed the age-related progression of amyloid pathology between age 10- and 26-month-old APP.V717I mice, which was brain region specific (fig 2A*).* Amyloid load was low 10-14 months of age in all brain regions and progressed at older age, primarily in the subiculum (fig 2B, *F(3,10)=17.704, p<0.001; Bonferroni post-hoc test 10 months vs. 19 months p<0.001, 10 months vs. 26 months p=0.011, 14 months vs. 19 months p=0.005, 14 months vs. 26 months p=0.047*). Amyloid plaque load was further elevated in the DG and CA at 26 months of age (fig 2C,D; DG: *F(3,10)=7.166, p=0.007; Bonferroni post-hoc test 10 months vs. 26 months p=0.009, 14 months vs. 26 months p=0.023, 19 months vs. 26 months p=0.018, all other comparisons ns;* CA: *F(3,10)=7.166, p=0.007; Bonferroni post-hoc test 10 months vs. 26 months p<0.001, 14 months vs. 26 months p<0.001 and 19 months vs. 26 months p<0.001*).

Next to amyloid plaque pathology, we quantified cell-associated amyloid, which was not present in the DG, and at only low and stable levels in the CA pyramidal cell layers (fig 2E; *F(3,10)=2.146, p=0.158*). In contrast and remarkably, cell-associated amyloid was high in the subiculum 10- and 14-month-old mice, but became reduced upon further aging (fig 2F; *F(3,10)=6.432, p=0.011; Bonferroni post-hoc test 10 months vs. 26 months p=0.021*). Furthermore, in the subiculum, cell-associated amyloid was inversely correlated with amyloid load (fig 2G; *r=-0.667 p=0.011*).

3.3 Microglial phagocytic CD68 increased in relation to Aβ pathology

The microglial response in WT and APP.V717I mice during aging was analyzed by IHC for the phagocytic marker CD68 (fig 3A). This staining showed CD68 immunoreactive cells (fig 3A') with a small CD68+ soma and a more punctate CD68+ pattern in their processes in 10-month-old WT and APP.V717I mice (arrows), cells with a larger CD68+ soma in 26-month-old WT mice

(white arrowheads), and cells with thick CD68+ processes that cluster together in the subiculum of 26-month-old APP.V717I mice (black arrowheads).

CD68 coverage increased overall in the subiculum of APP.V717I mice as well as WT mice with advancing age, although this increase tended to be primarily observed in 19- to 26-month-old APP.V717I mice (fig 3B; *age F(1,20)=5.453, p=0.030, genotype F(1,20)=13.501, p=0.002, interaction F(1,20)=3.907, p=0.062*). In CA and DG, CD68 coverage was upregulated in APP.V717I mice, irrespective of age (fig 3C,D; CA: *age F(1,18)=0.138, ns, genotype F(1,18)=7.695, p=0.013, interaction F(1,18)=1.728, ns*; DG: *age F(1,18)=0.247, ns, genotype F(1,18)=10.614, p=0.004, interaction F(1,18)=1.603, ns*).

In addition, we distinguished whether changes in CD68 coverage resulted from a higher number of CD68+ cells, or from changes in CD68 expression at the individual cell level. The number of CD68+ cells in the subiculum was increased in APP.V717I mice in an age-independent manner (fig 3E; *age F(1,20)=0.000, ns, genotype F(1,20)=19.874, p<0.001, interaction F(1,20)=0.403, ns*). CD68 expression at the individual cell level was significantly increased in the 19- to 26-month-old APP.V717I mice, compared to all other groups (fig 3F; *age F(1,20)=5.5336, p=0.029, genotype F(1,20)=17.152, p<0.001, interaction F(1,20)=4.920, p=0.038*; Bonferroni post-hoc test: *WT 10-14 months vs. APP.V717I 19-26 months p<0.001, WT 19-26 months vs. APP.V717I 19-26 months p<0.001, APP.V717I 10-14 months vs. APP.V717I 19-26 months p<0.001*). The number of CD68+ cells was increased in the CA1 and DG of APP.V717I, but not WT mice (data not shown; CA1: *age F(1,18)=0.003, ns, genotype F(1,18)=16.980, p<0.001, interaction F(1,18)=0.002, ns*; DG: *age F(1,18)=0.138, ns, genotype F(1,18)=7.034, p<0.016, interaction F(1,18)=0.002, ns*). CD68 expression at the individual cell level in these regions was not significantly altered by either age or genotype (fig 3G, CA1: *age F(1,18)=2.002, ns, genotype F(1,18)=1.672, ns, interaction F(1,18)=1.509, ns*; DG, data not shown: *age F(1,18)=1.453, ns, genotype F(1,18)=0.864, ns, interaction F(1,18)=0.761, ns*).

We next questioned whether the observed elevation in microglial CD68 in APP.V717I mice was associated with either or both types of amyloid pathology studied in these regions. We observed that CD68 coverage overall is positively correlated with amyloid plaque load in subiculum, in the DG and in the CA (data not shown; Subiculum: *r=0.580, p=0.030*; DG: *r=0.744, p=0.004*; CA: *r=0.639, p=0.019*). Interestingly, CD68 expression at the individual cell level was correlated with plaque load in both the subiculum and CA1, but not with plaque load in the DG (fig 3H: subiculum: *r=0.597, p=0.004*; CA, data not shown: *r=0.647, p=0.017*; DG, data not shown: *r=0.089, ns*). CD68 cell numbers did not correlate with the plaque load in these regions (data not shown; subiculum*: r=0.180, ns*; CA: *r=-0.096, ns*; DG: *r=0.079, ns*). Interestingly, cell-associated

amyloid was inversely correlated with both CD68 individual cell expression and CD68 coverage in the subiculum (fig 3I; subiculum CD68 coverage: *r=-0.718, p=0.004*; subiculum CD68 individual cell expression, data not shown: *r=-0.721, p=0.004*), but these measures did not correlate in the CA1 (fig 3J; CA CD68 coverage: *r=0.038, ns*; CA CD68 individual cell expression, data not shown: *r=-0.045, ns*).

3.4 Amyloid pathology in APP.V717I mice does not correlate with neurogenesis

DCX+ cell numbers (fig 4A,B) and CR+ cell numbers (fig 5A,B) were quantified in the hippocampus of WT and APP.V717I mice as a representative measure of AHN. The DCX+ cells were classified based on their developmental stage (fig 4B). The number of DCX+ cells decreased with age in both WT and APP.V717l mice (fig 4C, *age F(1,20)=5.776, p=0.026, genotype F(1,20)=0.107, ns, interaction F(1,20)=0.027, ns*). The decrease was statistically significant in the rostral part of the hippocampus, but not in the caudal part (rostral: *age F(1,20)=8.372, p=0.009, genotype F(1,20)=0.068, ns, interaction F(1,20)=0.546, ns*; caudal: *F(1,20)*=3.592, *p=0.073, genotype F(1,20)=0.607, ns, interaction F(1,20)=0.790, ns*).

Further classification of DCX+ cells based on their developmental stages, revealed that age specifically reduced the number of DCX+ cells in the intermediate and immature stages, but not the DCX+ cells in the proliferative stage (fig 4C; proliferative: *age F(1,20)=1.965, ns, genotype F(1,20)=0.637, ns, interaction F(1,20)=0.116, ns*; intermediate: *age F(1,20)=4.444, p=0.048, genotype F(1,20)=0.031, ns, interaction F(1,20)=0.036, ns*; immature neuron: *age F(1,20)=5.744, p=0.026, genotype F(1,20)=0.090, ns, interaction F(1,20)=0.045, ns*). Numbers of DCX+ cells in the DG of APP.V717I mice were neither associated with amyloid plaque load, nor with microglial CD68 coverage (fig 4D; DCX and plaque load: *r=-0.319 ns*; fig 4E DCX and CD68: *r=-0.314, ns*).

CR+ cells were not altered by the age or genotype of the mice, although the numbers of CR+ cells tended to increase in APP.V717I mice (fig 5C; *age F(1,20)=0.23, ns, genotype F(1,20)=4.31, p=0.051, interaction F(1,20)=1.73, ns*). Numbers of CR+ cells in the DG were neither associated with amyloid plaque load in APP.V717I mice, nor with microglial CD68 coverage in WT and APP.V717I mice (fig 5D,E; CR and plaque load: *r=-0.168, ns*; CR and CD68: *r=0.359, ns*).

4. Discussion

The current study demonstrates that AHN is reduced by age, but that this decline is neither affected by the progressive accumulation of amyloid pathology, nor by the paralleled microglial activation in middle-aged and old APP.V717I mice. We confirmed and extended on the characterization of age-related accumulation of amyloid pathology in APP.V717I mice. Amyloid pathology progressed slowly, with plaques appearing most abundantly in the subiculum and a plaque coverage of approximately 40% in this brain region in mice over 19 months of age. Lower levels are present in the DG and CA. Particularly in the subiculum was an inverse correlation between plaque- and cell-associated amyloid accumulation, with cell-associated amyloid diminishing from age 10-14 months onwards, which was paralleled by the increase in amyloid plaque load in this region. The age-related increase in amyloid plaque load in old APP.V717I mice was further paralleled by increased microglial CD68 expression. The elevated level of microglial CD68 coverage in APP.V717I was accounted for by both an increase in the number of CD68 expressing microglia, as well as by an upregulation of CD68 expression at the individual cell level, which correlated with the plaque pathology. Except for an age-related increase in CD68+ cell numbers in the subiculum, microglial changes were absent in age-matched WT mice. Interestingly, DCX+ new born cells in the DG decreased with advancing age in both WT and APP.V717I mice, whereas the more matured CR+ immature neurons were not significantly affected by age or genotype. These results indicate that the reduction in AHN with aging, measured at different stages, is neither modified by the increased amyloid neuropathology nor by the microglial CD68 changes in APP.V717I mice.

4.1 Amyloid pathology correlates with microglial lysosomal activity during aging in APP.V717I mice

Amyloid pathology was primarily present in the form of cell-associated amyloid at 10 months, which diminished with increasing age to give rise to increased extra-cellular amyloid plaque deposition. Such a pattern and progression of amyloid pathology confirms and extends the earlier, detailed descriptions of these mice using Aβ antibodies, Congo Red or Thioflavin S staining (Dewachter et al., 2000a; 2000b; Heneka et al., 2005; Moechars et al., 1999; Tanghe et al., 2010; Van Dorpe et al., 2000). Cell-associated or intraneuronal Aβ has been observed in AD patients and in several other mouse models and is generally accepted to precede amyloid plaque pathology (Bayer & Wirths 2011; Christensen et al., 2009; Christensen et al., 2010; Giménez-Llort et al., 2007; Oddo et al., 2006; Youmans et al., 2012; Wirths et al., 2002). In our study, the subiculum in particular displayed early and abundant cell-associated amyloid at middle age,

converting to amyloid plaques in old APP.V717I mice, similar to the inverse relation of intracellular and extracellular Aβ deposition observed in other AD-related mouse models, as well as in human AD brain tissue (Oddo et al., 2006).

The shift in the pathological amyloid pattern appeared most specific for the subiculum, while the DG and CA were less affected in APP.V717I mice. This dynamic shift in pathology is consistent with the concept that amyloid accumulates mainly in the cell-associated, internal pool at early stages, until amyloid is "trapped" in the extracellular plaque deposits at later stages, preventing further intracellular accumulation and detection (Oddo et al., 2006). However, the still open question remains why the subiculum is subject to the most early and abundant amyloid-related pathological changes: what factor(s) determine(s) this regional selectivity? A logical explanation would be a region-specific difference in promotor-driven transgene expression. Conversely, vascular amyloid deposition is not more abundant in the subiculum than in other brain regions in the APP.V717I model (Van Dorpe et al., 2000), implying that amyloid deposition in the subiculum is perhaps regulated by other factors than simply the level of APP transgene expression. Aside from this, the observed regional specificity in deposition might be modulated by altered APP and Aβ-peptide intracellular trafficking and/or processing in neurons projecting to the subiculum, a major output region of the hippocampal circuit (Lazarov et al., 2002; Thinakaran & Koo, 2008; Wirths et al., 2002).

We went on to investigate whether changes in amyloid pathology throughout life were associated with alterations in microglia and their activation. Although aging slightly increased the number of CD68+ cells in the subiculum of WT mice, the progression of the plaque pathology in APP.V717I mice was paralleled by a strong upregulation in microglial CD68 expression in all hippocampal sub-regions. This upregulation was primarily accounted for by elevated CD68 expression at the individual cell level, and to a lesser extent by an increased number of CD68+ cells. This observation is consistent with reports indicating that the gradual buildup of amyloid pathology triggers a neuroinflammatory response, and with changes in microglia indicative of a response to amyloid peptides (Jung et al., 2015; Nagele et al., 2004; Serrano-Pozo et al., 2013, Zhu et al., 2014). Previous studies have demonstrated that the age-related progression of amyloid pathology in APP.V717I mice is largely driven by an impaired clearance of Aβ peptides, rather than increased production (Dewachter et al., 2000a). The observed shift from cell-associated amyloid to extracellular plaques paralleled by increase in microglial CD68 suggests that microglia might be involved in this process. Indeed microglia can become dysfunctional with increasing age and/or change their response to amyloid peptides, thereby affecting amyloid pathology and its

progression (Daria et al., 2017; Zhao et al., 2014; Hoeijmakers et al., 2016; Bates et al., 2009; Deane et al., 2009; Heneka et al., 2015b).

Activation of microglia in response to accumulating Aβ alters the release of inflammatory factors as well as their support for neuronal functioning, and probably for AHN (Béchade et al., 2013; Biscaro et al., 2012; De Lucia et al., 2016; Ekdahl et al., 2009; 2012; Fuster-Matanzo et al., 2013). This raised the question as to whether the alterations in Aβ pathology and the concomitant responses may have also altered AHN in APP.V717I mice.

4.2 Amyloid pathology does not modulate AHN in old APP.V717I mice

In the DG, DCX+ cells were similarly reduced with advancing age in both WT and APP.V717I mice, with low numbers of immature cells present at age 19-26 months. In the DG, DCX+ cells were similarly reduced with advancing age in both WT and APP.V717I mice, with low numbers of immature cells present at age 19-26 months. In addition to the DCX+ cells, the CR+ cells in DG were not reduced with age, and even tended to be increased, in APP.V717I mice. Very few cells proliferate in the brain of rodents older than 10 months (Ben Abdallah et al., 2010; Ihunwo and Schliebs, 2010; Heine et al., 2004). We consequentially used DCX as the marker of choice to study AHN in older mice, because new-born neurons express DCX from 3 to 14 days after their birth, a relatively long time window that allows labeling of a relatively large number of neurogenic cells (Couillard-Després et al., 2005; Kempermann et al., 2003). In addition, we assessed the CR+ cell numbers to also quantify a later stage of neurogenesis, since CR expression partly overlaps with DCX expression, but is still present in 4-week-old cells (Brandt et al., 2003, Kempermann et al., 2004). Interestingly, both DCX+ and CR+ cell numbers failed to correlate with the amyloid or microglial changes in APP.V717I mice. This indicates that neither the young immature stage nor a later maturational stage of the young neurons are influenced by the (slow) emergence of pathology. It is further important to note that both DCX+ and CR+ cell numbers reflect subsets of the newborn cell pool. The fact that we did not find changes in CR+ numbers, therefore does not fully exclude the possibility that newborn cell survival per se is altered in these mice. This question should be answered by future studies using timed injections with cell birth-date markers such as BrdU and subsequent co-labeling for NeuN.

The continued reduction in DCX+ cells from middle age up to 19-26 months, notably at ages when amyloid pathology and microgliosis began to increase, further indicates that a ‘floor effect’ does not relate to the DCX reduction in the younger age groups. The low power in some of the aged groups may possibly have prevented the detection of more subtle effects on AHN in APP.V717I mice. As such, caution is required when considering such (floor) effects. For the same

reason, the association between pathology and AHN could not be addressed within the different age groups. Despite this limitation, the lack of an impact of Aβ pathology on AHN indicates that, in contrast to the changes seen in stronger overexpressing APP and PS1 models (Cotel et al., 2012; Demars et al., 2010; Hamilton and Holscher, 2012; Taniuchi et al., 2007), AHN does neither respond to a slower and more gradually progressing development of amyloid pathology in ageing APP.V717I mice, nor to the concomitant changes in microglia.

To our knowledge, only few other studies have described effects of APP mutations on neurogenesis in old age. Tg2576 mice were reported to (visually) have more proliferating cells than non-transgenic mice at 16 months of age, and a general absence of proliferating or DCX+ cells by 18 months of age (Ihunwo & Schliebs, 2010). 18-month-old APP23 mice showed a reduction in DCX+ and CR+ cell numbers, but no difference in the survival of newborn (BrdU+/NeuN+) neurons compared to WT mice (Mirochnic et al., 2009). Interestingly, PS1 knock-in mice showed a reduction in DCX+ neurogenic cells at both 6 and 18 months of age, which aggravated in APP/PS1 double knock-in mice that develop Aβ neuropathology (Zhang et al., 2007). Mutant APP knock-in alone did not lead to alterations in two different plasticity markers or in amyloid deposition, indicating that PS1 mutations on their own affect neurogenesis, and that APP mutant knock-in requires a secondary modulating factor like a mutated PS1 knock-in to induce Aβ neuropathology and affect neurogenesis. The overexpression of mutant PS1 thus complicates the interpretation of Aβ effects on AHN, because of its intrinsic role in neuronal fate and neurogenesis (Veeraraghavalu et al., 2013). AHN is indeed affected in bigenic mouse lines; DCX+ cells were reduced in 2- to 10-month-old APP751SL/PS1 and APPswe/PS1dE9 mice (Cotel et al., 2012; Demars et al., 2010; Hamilton and Holscher, 2012; Taniuchi et al., 2007), whereas increased DCX+ cell numbers were reported in APPswe/PS1dE9 mice at 10 months of age (Yu et al., 2009). The differences in our findings and what was reported so far in the literature are possibly due to the fact that these AD mouse models were mostly studied at considerably younger ages, contained strong neuronal promotors to reach high overexpression of APP as well as co-expression of mutant PS1 which more than doubles the resulting Aβ levels (Borchelt et al., 1997; Götz et al., 2004). As a result, the rapid progressing of amyloid and associated PS1-mediated pathology is often already present around a such very young age (4 to 6 months). Consequently, these aggressive models differ considerably from the APP.V717I mice in which amyloid plaque pathology is not observed until 10-12 months of age. We propose a possible explanation for the currently observed lack of impact of amyloid pathology on AHN. In mouse models with early and rapid development of amyloid pathology, the high Aβ levels will impact all cellular processes already at considerably younger ages than in our current model. At such young age, the level of neurogenesis is higher and might be

more responsive. The neurogenic pool in the more rapidly progressing AD-models will therefore be more vulnerable to the pathological changes. These differences make neurogenic progression, in particular in single-APP-mutant models, an interesting topic for future studies. Furthermore, such studies should also consider the inclusion of (aging) female mice, given the changing levels of sex-hormone levels over the lifespan that might impact neurogenesis differentially with age (Duarte-Guterman et al., 2015; Pawluski et al., 2009). Such differential, sex-specific effects are indeed reported for hippocampal plasticity and pathological progression in AD mouse models (Rodriguez et al., 2008; Richetin et al., 2017).

The lack of reduction in DCX+ and CR+ cells in the current APP.V717I model suggest that AHN is not affected by the progression of amyloid pathology at these older ages. Modeling of amyloid pathology based on multiple clinical imaging studies in AD patients suggests it to follow a sigmoidal build-up over time, starting in a slowly progressive manner, evolving into the extensive pathological hallmarks commonly present in the elderly (Jack et al., 2013). The slow, age-related accumulation of amyloid in APP.V717I mice therefore better resembles the gradual build up in humans than the more aggressive models, that display rapidly developing amyloid pathology already at younger age. This important age-related component of amyloid pathology in humans, and the observed lack of impact on AHN in old age, highlights the necessity to study AD-models, and the consequences of amyloid pathology, in a proper and moderate age-related framework and context.

In AD-patients, the question remains to what extent AHN contributes to their clinical phenotype: is it causally involved in the cognitive deficits, or is it a secondary phenomenon or consequence? The accumulation of Aβ peptides in the human brain is accepted to start several decades before the onset of any cognitive impairments, when both the level and the potential involvement of AHN is still substantial (Spalding et al., 2013; Weissleder et al., 2016). AHN might therefore still be vulnerable in such earlier pathological stage, although this remains unresolved to date.

One more interesting option is whether 'boosting' AHN at an earlier age will be beneficial, to e.g. build a cognitive 'reserve' and/or to prevent, or at least provide some protection, against neurodegeneration in the elderly (Stern, 2002; 2012). Of note, physical activity in adult and aged rodents has potent neurogenic effects and benefits cognitive performance (Marlatt et al., 2013; Ryan & Nolan, 2016; Van Praag et al., 2005). In addition to physical activity, enrichment and diet are part of the life style factors that were shown to be important in determining the development and progression of AD (Jack et al., 2013 Rolandi et al., 2016; Scheltens et al., 2016). These factors all benefit AHN and cognition in rodents (Maruszak et al., 2014; Mirochnic et al., 2009; Scarmeas

et al., 2009; Van Praag et al., 2005). Life-style factors that impact AHN may thus be influential in AD patients (Grande et al., 2014; Kandola et al., 2016; Singh et al., 2014; Sofi et al., 2011; Vivar et al., 2013), making AHN an interesting substrate to study in relation to cognitive reserve and its possible role in providing protection against age-related cognitive decline and AD.

4.3 Implications of this study

The current study highlights that, in contrast to previous studies using rapidly progressing mouse models, the slower accumulation of amyloid pathology and the parallel microglial responses in APP.V717I mice did not affect AHN during middle age and advanced aging. This data-set highlights that AHN is vulnerable to the more early, fast accumulating excessive levels of Aβ peptides present in young adulthood rather than in aged individuals.

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6. Disclosure statement

The authors declare no competing interests.

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Figures and figure legends

Figure 1: Hippocampal volume is reduced in APP.V717I males

The volume of (A) the entire hippocampus, (B) the DG and (C) the GCL is reduced in APP.V717I mice, irrespective of age. *Annotations: #, genotype effect.*

Figure 2: Characterization of amyloid pathology in APP.V717I males

(A) Representative image of Aβ immunoreactive staining in the hippocampus of a 10-month-old APP.V717I mouse and 26-month-old APP.V717I mouse. (A') Enlarged images of the subiculum, revealing ample cell-associated amyloid staining in the subiculum at 10 months, and abundant plaque pathology at 26 months. Amyloid plaque load increases with age in the (B) subiculum, (C) DG and (D) CA. (E) The pyramidal cell layer of the CA shows relatively little cell-associated amyloid staining, which remains stable with aging. (F) Cell-associated amyloid is abundant in the subiculum at 10 months, but decreases significantly with aging. (G) In the subiculum, cell-associated amyloid correlates with plaque pathology, indicating that cell-associated amyloid reduces when plaque pathology increases in this region. *Scale bars: (A) 1000 μm, (A') 100 μm. Abbreviations: DG, dentate gyrus; CA, cornu ammonis; Sub, subiculum. Annotations: #, sig. from 10 months; $, sig. from 14 months; %, sig. from 19 months.*

Figure 3: Microglial CD68 coverage is elevated in APP.V717I males in association with amyloid pathology

(A) Representative images of CD68 immunoreactive staining in the hippocampus of WT and APP.V717I mice at 10 and 26 months of age. (A') Enlarged images of cells in the subiculum. The arrows point to CD68 immunoreactive cells with a small soma and dotted CD68 pattern in the processes, that are particularly present in the 10- to 14-month-old mice. The white arrowheads point to CD68+ cells observed in the subiculum of a 26-month-old WT mouse, with large soma's and little visible CD68 immunoreactive processes. The black arrowheads point the typical clustered CD68 immunoreactive cells with thick processes in the subiculum of a 26-month-old APP.V717I mouse. (B) CD68 coverage in the subiculum is increased in APP.V717I mice as well as with aging, although the age-related increase tends to be more prominent in APP.V717I mice. (C) In the CA, (D) as well as the DG, CD68 coverage is increased by APP.V717I alone. (E) The number of CD68+ cells is increased in the subiculum of APP.V717I mice. (F) The expression of CD68 in individual cells in the subiculum is increased in 19- to 26-month-old APP.V717I mice in comparison to all three other groups. (G) There is no significant difference in the CD68 expression

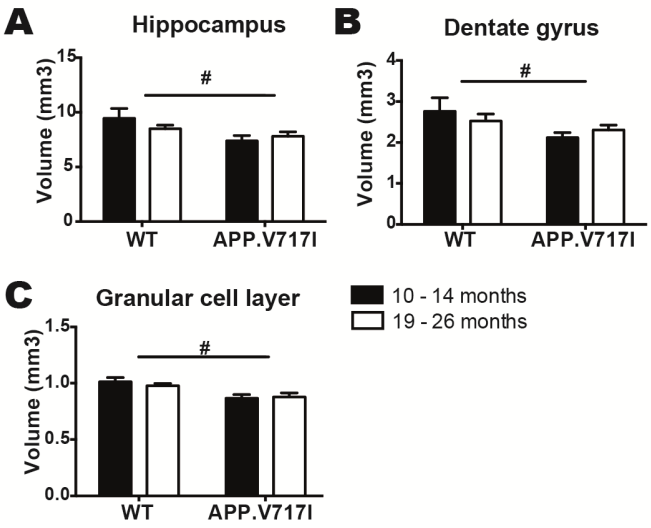
at the individual cell level in the CA1. (H) The CD68 expression at the individual cell level in the subiculum is positively correlated with the amount of plaque load in this region. (I) Cell-associated amyloid is negatively correlated with CD68 coverage in the subiculum, but (J) cell-associated amyloid in the pyramidal cell layers of the CA is not associated with CD68 coverage in this region. *Scale bars: (A, A') 100 μm. Abbreviations: DG, dentate gyrus; CA, cornu ammonis; Sub, subiculum. Annotations: *, age effect; #, genotype effect; &, interaction effect. Post-hoc annotations: %, sig from WT 10-14 months; @, sig from WT 19-26 months; $, sig from APP.V717I 10-14 months.*

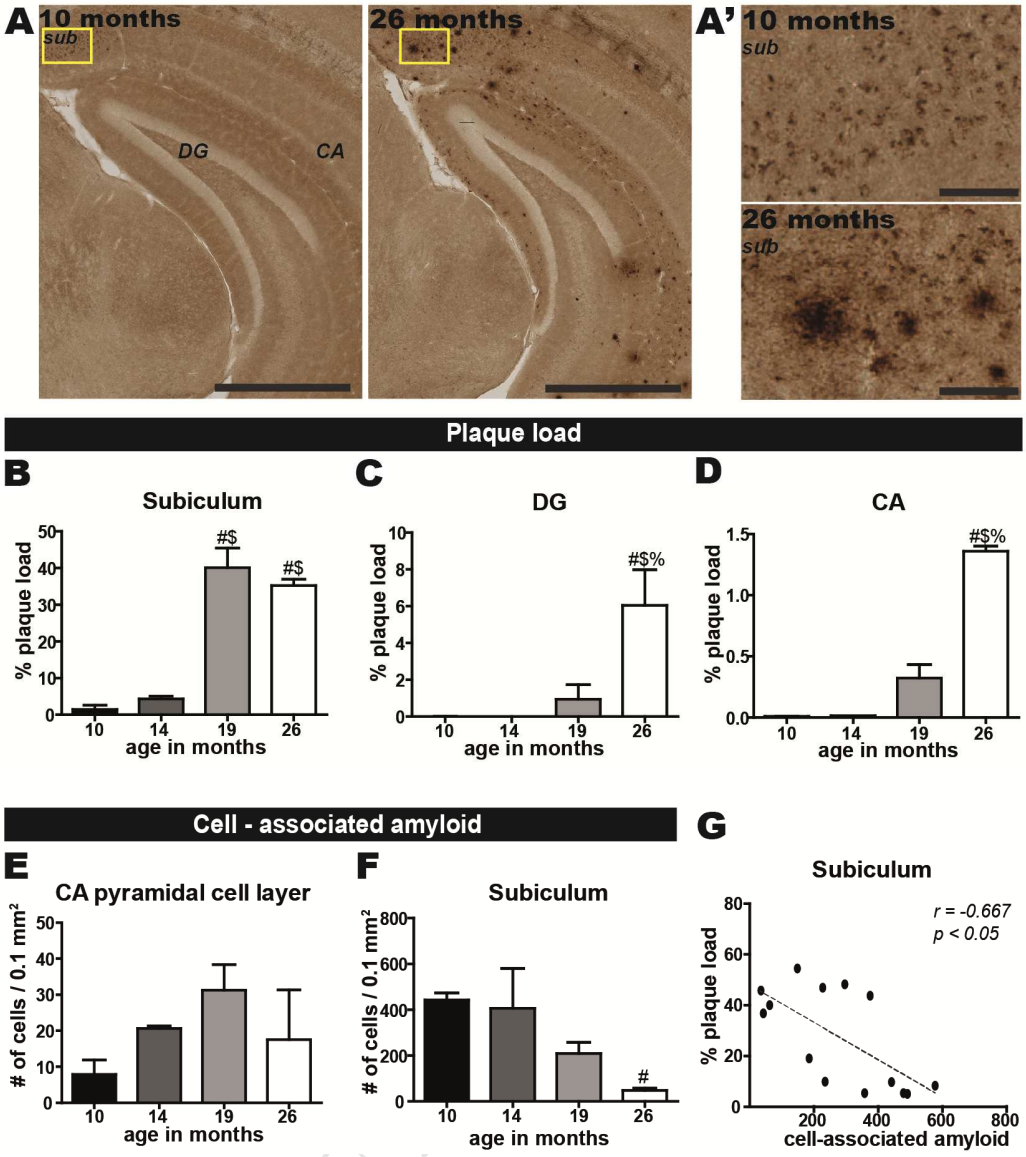
Figure 4: Hippocampal DCX+ cells are reduced in aged WT and APP.V717I mice

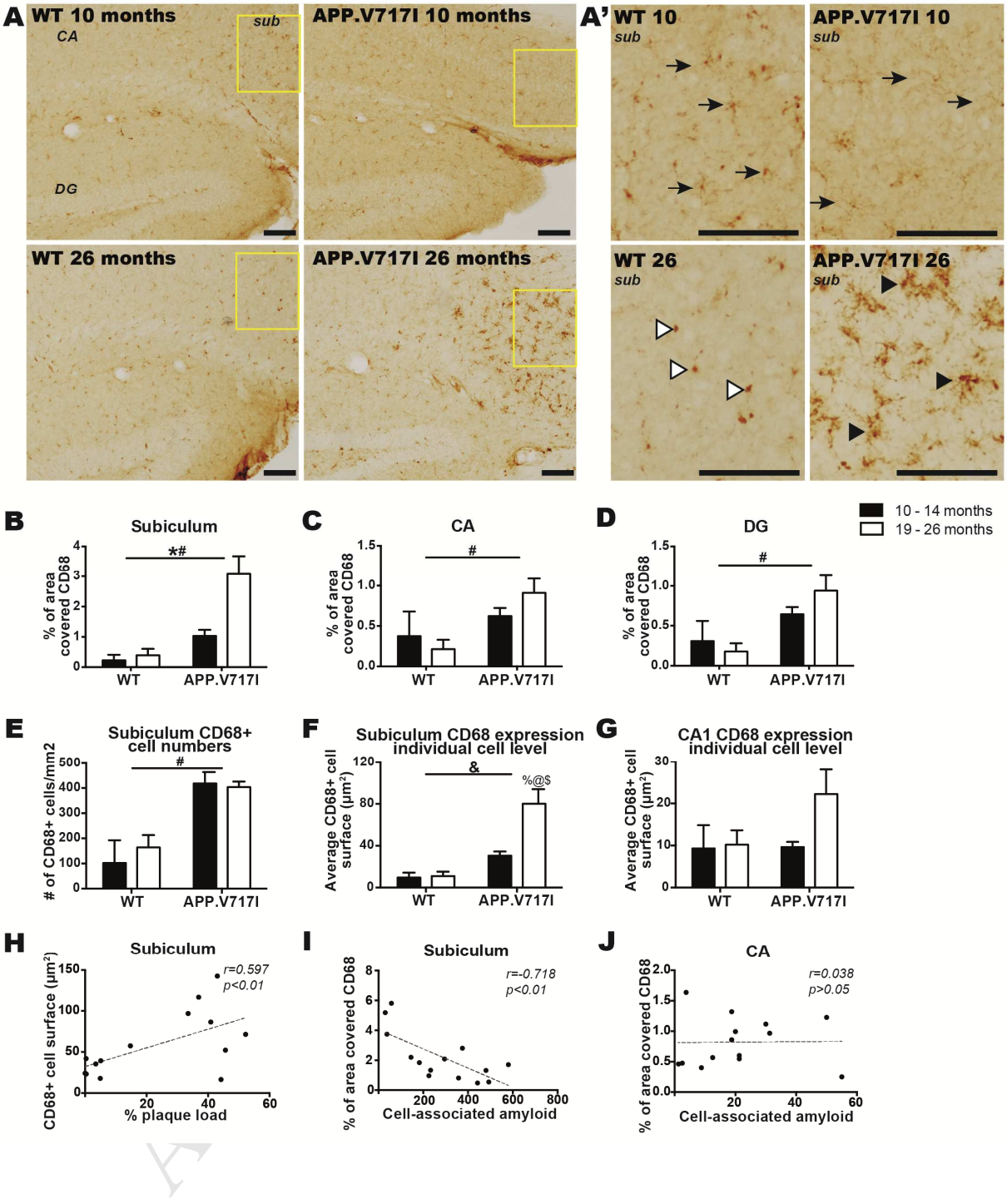
(A) Representative images of DCX immunoreactivity in 10-month-old WT and APP.V717I, as well as 19-month-old WT and APP.V717I mice. (B) DCX+ cells in the SGZ and GCL can be discriminated in 3 developmental stages as depicted; proliferative, intermediate and immature neuron. (C) The absolute DCX+ cell numbers are reduced at 19-26 months. Classification of DCX+ cells based on the developmental stages shows this reduction to primarily present in cells during the intermediate and immature neuron stage. (D) DCX+ cell numbers are not associated with Aβ plaque pathology in the DG. *Scale bars: (A) 100 μm, (B) 10 μm. Abbreviations: ML, molecular layer; GCL, granular cell layer; SGZ sub-granular zone; DCX, doublecortin. Annotations: *, age effect; %, intermediate stage significantly different from 10–14 months; &, immature neuron stage significantly different from 10–14 months.*

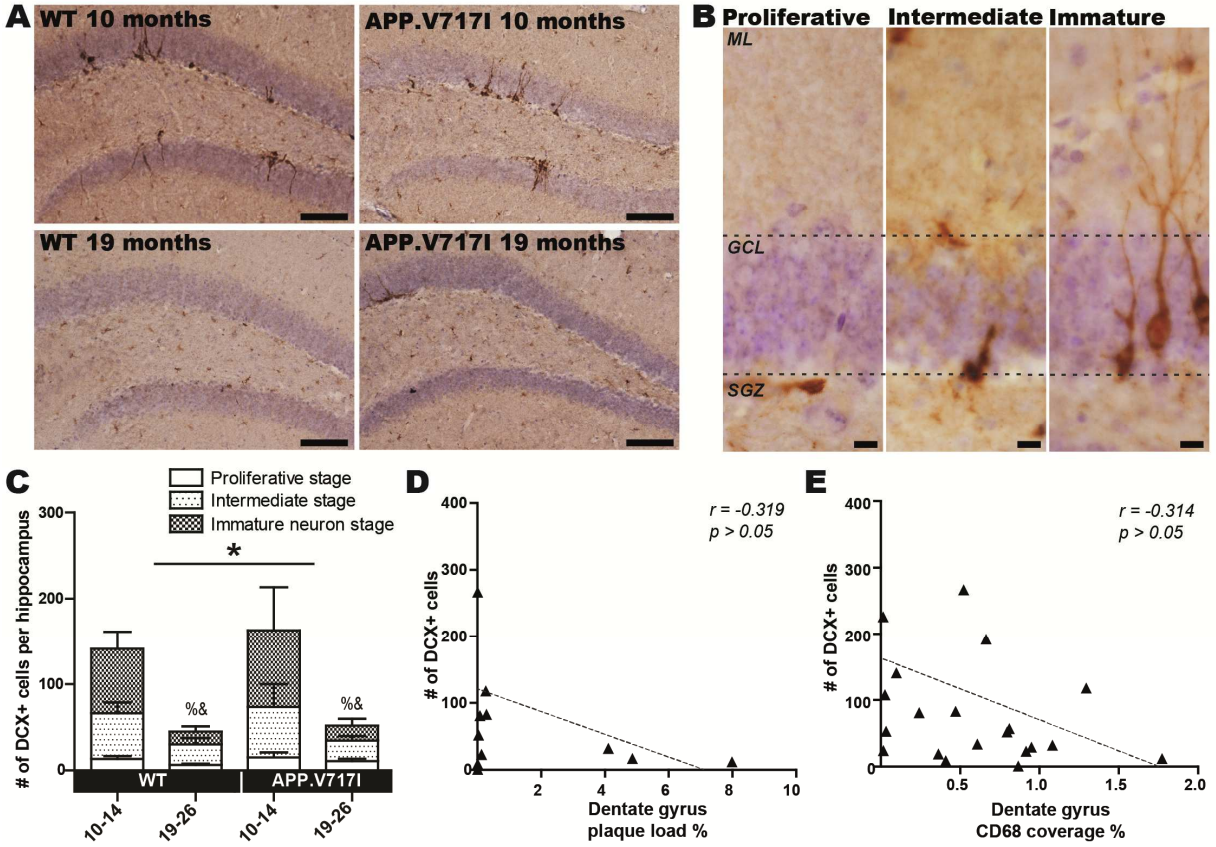
Figure 5: Neurogenic CR+ cells in WT and APP.V717I are not affected by aging

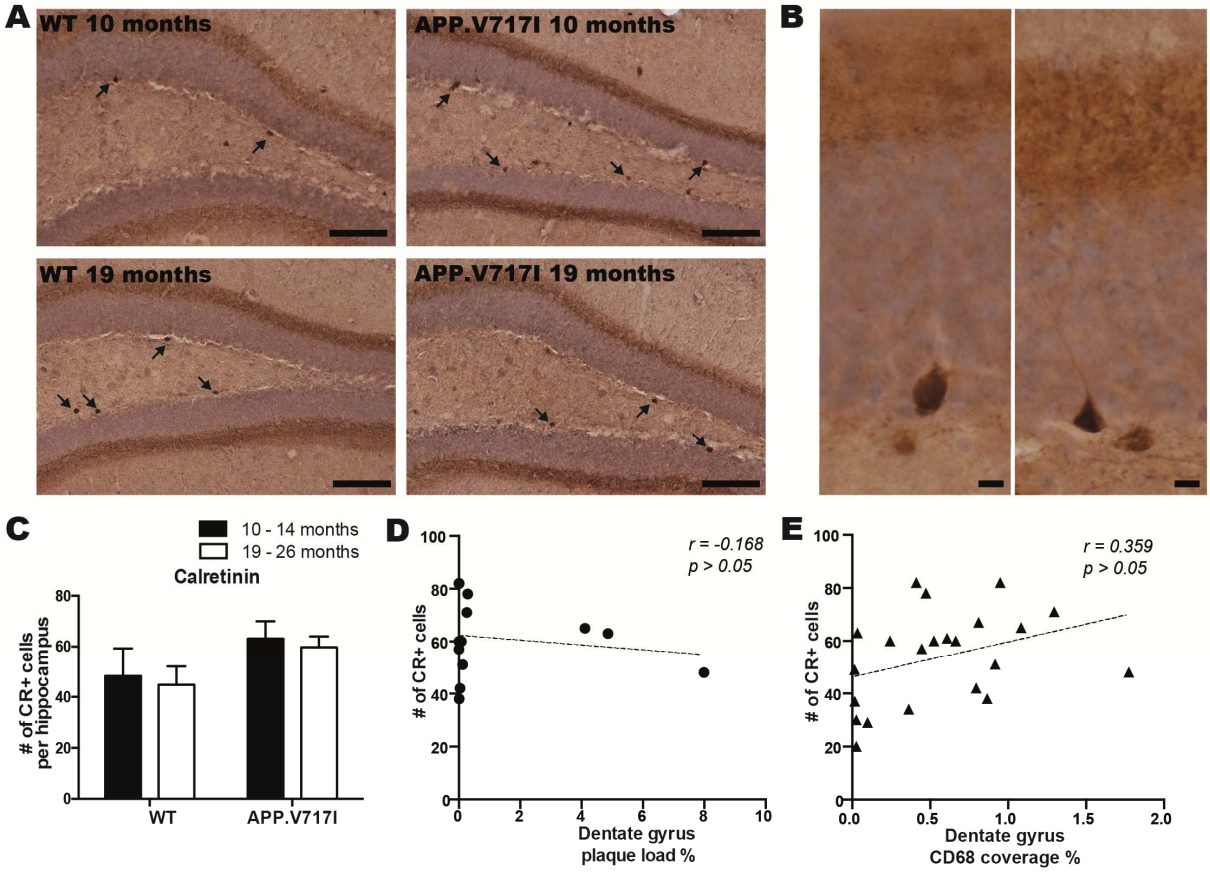
(A) Representative images of CR immunoreactivity in the hippocampus of 10-month-old WT and APP.V717I, and 19-month-old WT and APP.V717I mice. Black arrows point to a couple of CR+ cells in the DG. (B) Two example images of CR+ cells in the SGZ and GCL. (C) The number of new-born CR+ cells in the SGZ and GCL of the DG are not significantly affected by either age or genotype, although CR+ cell numbers tended to be increased in APP.V717I. CR+ cell counts are not correlated with (D) plaque pathology in the DG or (E) CD68 coverage in the DG. *Scale bars: (A) 100 μm, (B) 10 μm. Abbreviations: CR, calretinin.*




A
10 months
sub
DG
CA
26 months
A'
10 months
sub
26 months
sub
Plaque load
B
Subiculum
% plaque load
#$
#$
age in months
C
DG
% plaque load
#$%
age in months
D
CA
% plaque load
#$%
age in months
Cell - associated amyloid
E
CA pyramidal cell layer
# of cells / 0.1 mm²
age in months
F
Subiculum
# of cells / 0.1 mm²
#
age in months
G
Subiculum
r = -0.667
p < 0.05
% plaque load
cell-associated amyloid


A
WT 10 months
CA
sub
DG
APP.V717I 10 months
WT 26 months
APP.V717I 26 months
A'
WT 10
sub
APP.V717I 10
WT 26
APP.V717I 26
10 - 14 months
19 - 26 months
B
Subiculum
*#
% of area covered CD68
WT
APP.V717I
C
CA
#
D
DG
E
Subiculum CD68+ cell numbers
# of CD68+ cells/mm2
F
Subiculum CD68 expression individual cell level
&
%@$
Average CD68+ cell surface (µm²)
G
CA1 CD68 expression individual cell level
H
Subiculum
r=0.597
p<0.01
CD68+ cell surface (µm²)
% plaque load
I
Subiculum
r=-0.718
p<0.01
% of area covered CD68
Cell-associated amyloid
J
CA
r=0.038
p>0.05




A
WT 10 months
APP.V717I 10 months
WT 19 months
APP.V717I 19 months
B
C
10 - 14 months
19 - 26 months
Calretinin
# of CR+ cells per hippocampus
WT
APP.V717I
D
r = -0.168
p > 0.05
# of CR+ cells
Dentate gyrus plaque load %
E
r = 0.359
p > 0.05
# of CR+ cells
Dentate gyrus CD68 coverage %

Highlights

* Increased CD68+ microgliosis parallels amyloid pathology in aging APP.V717I mice

* Aging reduced hippocampal neurogenesis in wildtype and APP.V717I mice

* The reduced neurogenesis is not modulated by amyloid pathology or CD68+ microgliosis