



## UvA-DARE (Digital Academic Repository)

### Activity- and pharmacology-dependent modulation of adult neurogenesis in relation to Alzheimer's disease

Marlatt, M.W.

**Publication date**  
2012

[Link to publication](#)

**Citation for published version (APA):**

Marlatt, M. W. (2012). *Activity- and pharmacology-dependent modulation of adult neurogenesis in relation to Alzheimer's disease*.

**General rights**

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

**Disclaimer/Complaints regulations**

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

**Chapter 4: Iba1+ microglia proliferate in the aged human hippocampus at sites of amyloid deposition**

Michael W. Marlatt, Jan Bauer, Eleonora Aronica, Elise S. van Haastert, Jeroen J.M. Hoozemans, Marian Joels, Paul J. Lucassen

*Submitted*

**Abstract**

Microglia and astrocytes are thought to contribute to Alzheimer's disease (AD) by responding to early disease features and contributing to chronic neuroinflammation. Despite the potentially pivotal role these cells play in disease progression, little is known about proliferation of these cell types during AD. Previously, increased cell proliferation was identified in glia-rich regions of the AD hippocampus however the phenotype of these proliferating cells was not determined. We addressed this earlier finding by developing two triple-immunohistochemical stainings to identify astrocytic and microglial proliferation in coordination with amyloid beta (A $\beta$ ) plaque deposition. Accordingly, three cohorts of age-matched individuals (aged >70 years) were studied for proliferative changes by co-localizing proliferating cell nuclear antigen (PCNA) with GFAP+ astrocytes and Iba1+ microglia. PCNA+ cells were found across hippocampus indicating ongoing proliferation. Iba1+ cells, found in the same areas, co-labeled with PCNA, indicating microglial proliferation within the hippocampus of aged individuals. Proliferating Iba1+ cells were specifically seen within the borders of A $\beta$  plaques indicating an active response to plaque accumulation however Iba1+ cell density and morphology remained unchanged between disease cohorts. Our data indicates that microglial proliferation occurs within the aged human hippocampus in a process spurred by A $\beta$  plaques. This process appears in various A $\beta$  plaque subtypes and may contribute to known mechanisms of chronic neuroinflammation.

**List of Abbreviations**

antibody (Ab)

amyloid- $\beta$  (A $\beta$ )

Alzheimer disease (AD)

blood-brain barrier (BBB)

Central Nervous System (CNS)

Control (Con)

Cornus Ammonis (CA)

Dementia (Dem)

dentate gyrus (DG)

glial fibrillary acidic protein (GFAP)

GFAP immunopositive (GFAP+)

glia maturation factor (GMF)

Herpes Simplex Virus (HSV)

Iba1 immunopositive (Iba1+)

interleukin-1 (IL-1)

neurofibrillary tangles (NFT)

P2X<sub>7</sub> receptor (P2X<sub>7</sub>R)

paired helical filament (PHF-1)

proliferating cell nuclear antigen (PCNA)

PCNA immunopositive (PCNA+)

Receptor of Advanced Glycation Endproducts (RAGE receptor)

subgranular zone (SGZ)

**Introduction**

Alzheimer disease (AD) is an age-associated chronic neurodegenerative disease and the most common form of dementia. The two main pathological lesions of AD, amyloid- $\beta$  (A $\beta$ ) plaques and neurofibrillary tangles (NFTs) advance through the brain in a hierarchical manner, with the hippocampus affected early in the disease[1]. These protein accumulations are not directly correlated with cognitive decline as NFTs have been found in the hippocampus of elderly individuals irrespective of their cognitive status[2]. This brain structure is however highly relevant to the disease due to its critical role in learning, memory, and executive behavioral functions[3]. In addition to A $\beta$  plaques and NFTs, neuroinflammation and glial changes are known to occur during AD[4]. Indeed, mixed epidemiological evidence has shown that anti-inflammatory drugs reduce the risk for AD[5].

Neuroinflammation during AD is highly complex[6, 7] but microglia have been specifically implicated in aging and neurodegeneration[8, 9]. Multiple roles have been established for these immune cells during health and disease (reviewed by Graeber)[10]. While a number of endogenous protein markers exist, activated microglia express Iba1, an inducible protein that regulates actin remodeling[11]. Iba1 immunopositive (Iba1+) cells exist in different activated states in the CNS that are morphologically identifiable. Activated cells typically have an amoeboid or rod-like morphology, as opposed to minimally activated cells that have short ramified processes. Microglia have been found to migrate to A $\beta$  lesions[12] and have been identified in the periphery of A $\beta$  plaques[13-15]. Microglia can degrade A $\beta$  peptide *in vitro*[16, 17] and participate in clearance of A $\beta$  from the brain[18], therefore responding for the aberrant protein accumulations. This initial activity may however have deleterious effects as microglia are broadly implicated in neuroinflammation during AD (reviewed by McGeer)[19]. These cells should be additionally noted as having multiple age-related changes in morphology and function. Most notably, it has been shown that microglia decline in proliferative function in aged and AD brains tissue; microglia exposed to repeated challenges to over time have a diminished capacity for proliferation that corresponds with cellular deterioration [20]. A proinflammatory signature has nonetheless been identified in AD hippocampus [21]. Activation and recruitment of

microglia occurs in concert with astrocytes as they secrete interleukin-1 (IL-1), a major cytokine and potent activator of astrogliosis [22, 23].

Astrocytes change with aging and AD showing hypertrophy with A $\beta$  plaque pathology, but their active role in disease progression is unknown. Amyloid plaques can show intense astrocytic participation that is thought to correspond to ongoing degradation [24]. Astrocytic degradation of A $\beta$ 1-42 is known to depend on the aggregation state of the peptide [25], consistent with reports showing that cultured astrocytes can degrade A $\beta$ 1-42 [26] and N-terminal truncation of A $\beta$  occurs in vivo [27, 28]. We previously documented increased proliferation in glia-rich regions of the hippocampus in pre-senile AD patients ( $\leq 70$  years old) [29]. However, the phenotype of these proliferating cells, and their relationship to A $\beta$  plaques, was unknown. Given the role of microglia and astroglia in disease, we anticipated proliferation of these cell types could explain the increased proliferation in AD.

In the current study we therefore developed two triple-immunohistochemical stainings to detect astrocyte or microglial proliferation in combination with A $\beta$  pathology and examined three cohorts of age-matched individuals that differed in cognitive and neuropathological staging. We assessed; a) whether elderly cohorts ( $>70$  years old) of demented or AD subject exhibited increased proliferation, b) if astrocytes (GFAP+) or microglia (Iba1+) contribute to these proliferative changes; and c) if proliferation has a direct relationship with A $\beta$  pathology. Control and dementia cohorts were matched for AD pathology (Braak stages 1-2), but distinguished by cognitive status, i.e. demented or non-demented, and were compared to an AD cohort with severe pathology (Braak stages 4-5).

## **Materials and methods**

### **Subjects**

Hippocampal brain tissue was obtained via the Netherlands Brain Bank rapid autopsy program in accordance with all local ethical legislation and in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Signed informed consent was available for all patients in the study. Brain tissue was dissected and fixed in 10% buffered formalin for 1-2 months, then dehydrated before paraffin embedding. 8  $\mu$ m sections were mounted on Superfrost Plus slides. Hippocampal tissue was obtained from 8 age-matched non-

demented controls (Con), 8 clinically demented (Dem) cases, and 8 confirmed Alzheimer disease (AD). Braak staging of the three groups were respectively: Con:  $1.4 \pm 0.2$ , Dem:  $1.5 \pm 0.2$ , AD:  $4.8 \pm 0.3$ . Age (yrs): Con:  $80 \pm 3$  years of age, Dem:  $84 \pm 2$ , AD:  $81 \pm 2$ . Brain weight (g): Con:  $1208 \pm 65$ , Dem:  $1238 \pm 87$ , AD:  $1125 \pm 50$ . No significant differences in postmortem delay or fixation duration were present and the groups were further balanced male: female in a 1:1 ratio (Supplementary Table 1).

### **Immunohistochemistry**

Sections were deparaffinized in xylene and rehydrated through graded ethanol solutions, then washed in 0.05 M PBS prior to antigen retrieval by heating in a food steamer (MultiGourmet FS 20, Braun, Kronberg/Taunus, Germany) for 60 minutes at  $100^\circ\text{C}$  in citrate buffer (pH = 6.0). Sections were cooled to room temperature prior to incubation with 0.3%  $\text{H}_2\text{O}_2$  to quench endogenous peroxidase activity. Primary antibodies were diluted in Dako Washing Buffer (Dako S3006, Glostrup, Denmark) supplemented with 10% fetal calf serum and incubated for 1 hr at room temperature (RT) and then overnight at  $4^\circ\text{C}$ .

Triple immunostaining was performed through sequential development steps. First, anti-A $\beta$  antibody (Ab) (1:2,000 MAB1561, clone 4G8, Millipore, Billerica, MA, USA) was reacted with biotinylated sheep anti-mouse (1:500 Jackson ImmunoResearch, West Grove PA, USA), followed by incubation with avidin-peroxidase (Sigma, Germany) and development with Diaminobenzidine (DAB, Sigma, Germany) substrate. After washing, sections were incubated with anti-PCNA Ab (1:25,000 Dako M0879, Glostrup, Denmark) overnight, followed by donkey anti-mouse Alkaline Phosphatase conjugated Ab, and NBT/BCIP substrate to produce a blue stain. In order to inactivate the binding properties of the first round of antibodies and to retrieve additional GFAP and Iba1 epitopes, sections were then treated with EDTA (10 mM, pH = 9.0) in TRIS buffer for 30 minutes followed by washing and final incubation with rabbit polyclonal anti-GFAP (1:1,500 Dako Z0334, Glostrup, Denmark) or anti-Iba1 Ab (1:1,500 kindly provided by Dr. S. Kohsaka, National Institute of Neuroscience, Tokyo, Japan). Subsequently, sections were incubated with Alkaline Phosphatase-conjugated donkey anti-rabbit (Jackson ImmunoResearch, USA) and developed with Fast Red substrate to stain, in separate series, astrocytes and microglia dark red.

All secondary antibodies were incubated for 1 hr at room temperature. The anti-A $\beta$  4G8 antibody was found previously to provide the most consistent staining of diffuse plaques in FFPE human tissue. 4G8 Ab (recognizing amino acids 17-24 of A $\beta$  peptide) recognizes extracellular APP domains containing the A $\beta$  epitope and thus labels granule and pyramidal neurons as well as A $\beta$  plaques. The anti-PCNA antibody has been validated before and identifies proliferating cell profiles in human brain. Iba1 has been validated to identify activated microglia in various CNS disorders like Creutzfeldt-Jacob disease, brain tumor and influenza encephalitis. Glial fibrillary acidic protein (GFAP) is a classic member of the intermediate filament protein family involved in astrocyte cytoarchitecture and generally considered a sensitive marker of reactive astrogliosis in the CNS.

### **Morphometry and quantification**

Cross sectional areas for the dentate gyrus and CA subregions were determined using StereoInvestigator software (Microbrightfield Inc, USA) linked to a Zeiss Axiophot microscope (Carl Zeiss AG, Germany) to outline the appropriate hippocampal subregions. No significant differences were observed across subregions, however surface areas of the CA1/2 trended toward being smaller in the AD cohort (one-way ANOVA  $p = 0.14$ ). Cell count values were normalized to the surface area of each anatomical subregion.

Quantification of PCNA+ and Iba1+ cells was obtained from the PCNA/Iba1/A $\beta$  stained slides, while quantification of GFAP+ cells was obtained from the PCNA/GFAP/A $\beta$  triple stained sections. All phenotypic quantification was performed in mid-level sections of the hippocampus.

## **Results**

### **PCNA expression found across disease cohorts and hippocampal subregions**

We first studied GFAP+ and PCNA+ cells across cohorts to evaluate any association between the two protein markers. We found PCNA+ cells at a low density throughout the main subregions of the hippocampus including the dentate gyrus (DG) and subgranular zone (SGZ) (Fig 1a, b), areas populated with granule neurons and neural stem cells respectively. PCNA+ cells were seen adjacent to GFAP+ cells in the hilus and Cornu Ammonis (CA) regions of the hippocampus.

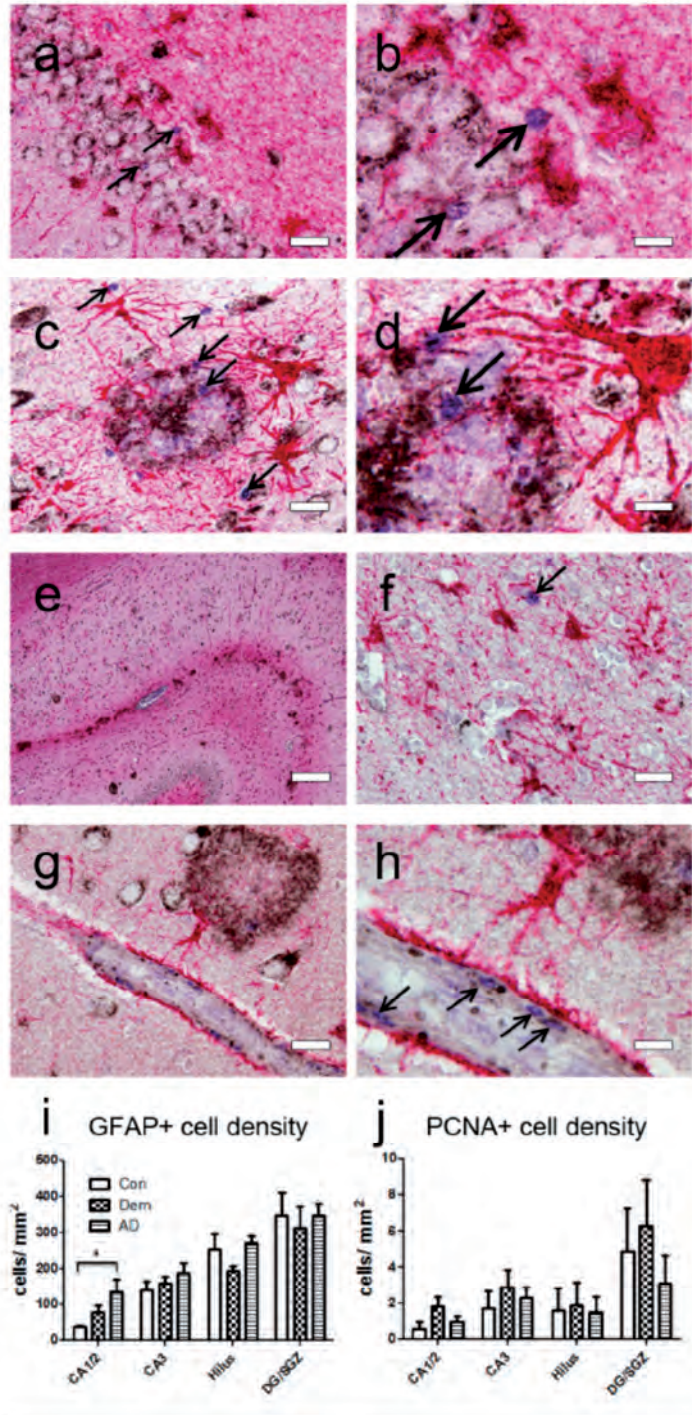


These cells were typically small and did not display membrane-bound APP (labeled by 4G8 antibody) as observed in adjacent granule and pyramidal neurons. Hypertrophic GFAP+ astrocytes infiltrating A $\beta$  plaques did not co-label with PCNA in any hippocampal subregion (Fig 1c,d). Regardless of astrocyte morphology, location, or plaque association, these cells did not co-label with PCNA.

Within the CA regions, PCNA+ cells were found in astrocyte-rich regions that lacked A $\beta$  pathology (Fig. 1f), and, consistent with our previous findings, they were also present in the vascular epithelium (Fig. 1g,h). The population of PCNA+ cells was not significantly elevated in the AD cohort; rather the dementia cohort showed the greatest amount of proliferation (Fig. 1j). Quantification of PCNA+ cells trended toward a significant increase in the dementia cohort: Con:  $3 \pm 1$  cells, Dem:  $18 \pm 5$  cells, and AD:  $12 \pm 3$  cells within the CA1/2 subregion (one-way ANOVA  $p=0.07$  excluding dementia case 97-004 with an exceptionally high density of 232 PCNA+ cells in the CA1/2).

GFAP+ astrocytes showed a characteristic, spider-like cellular morphology. They were seen in close proximity to neurons and blood vessels with endfeet clearly visible, contacting blood vessel walls. In the CA regions and subiculum hypertrophic astrocytes were observed with processes infiltrating diffuse, primitive, and dense-core A $\beta$  plaques (Fig 1c,d). These cells had larger cell bodies and processes compared to astrocytes not associated with plaques, however, not all astrocytes close to plaques (within 50  $\mu\text{m}$ ) showed this hypertrophic morphology. GFAP expression was enriched in the stratum moleculare and stratum lacunosum of the AD hippocampus, where astrocytes are found preferentially with A $\beta$  plaques (Fig 1e). A significantly increased population of GFAP+ cells was observed in the CA1/2 area in the AD patient cohort compared to controls (Fig 1i) (one-way ANOVA  $p = 0.02$ ).

The population of A $\beta$  plaques with invasive hypertrophic GFAP+ astrocytes was increased in the AD population (One-way ANOVA  $p<0.001$ ). However, we did not observe any indication that mature astrocytes proliferate in the presence of A $\beta$  plaques.

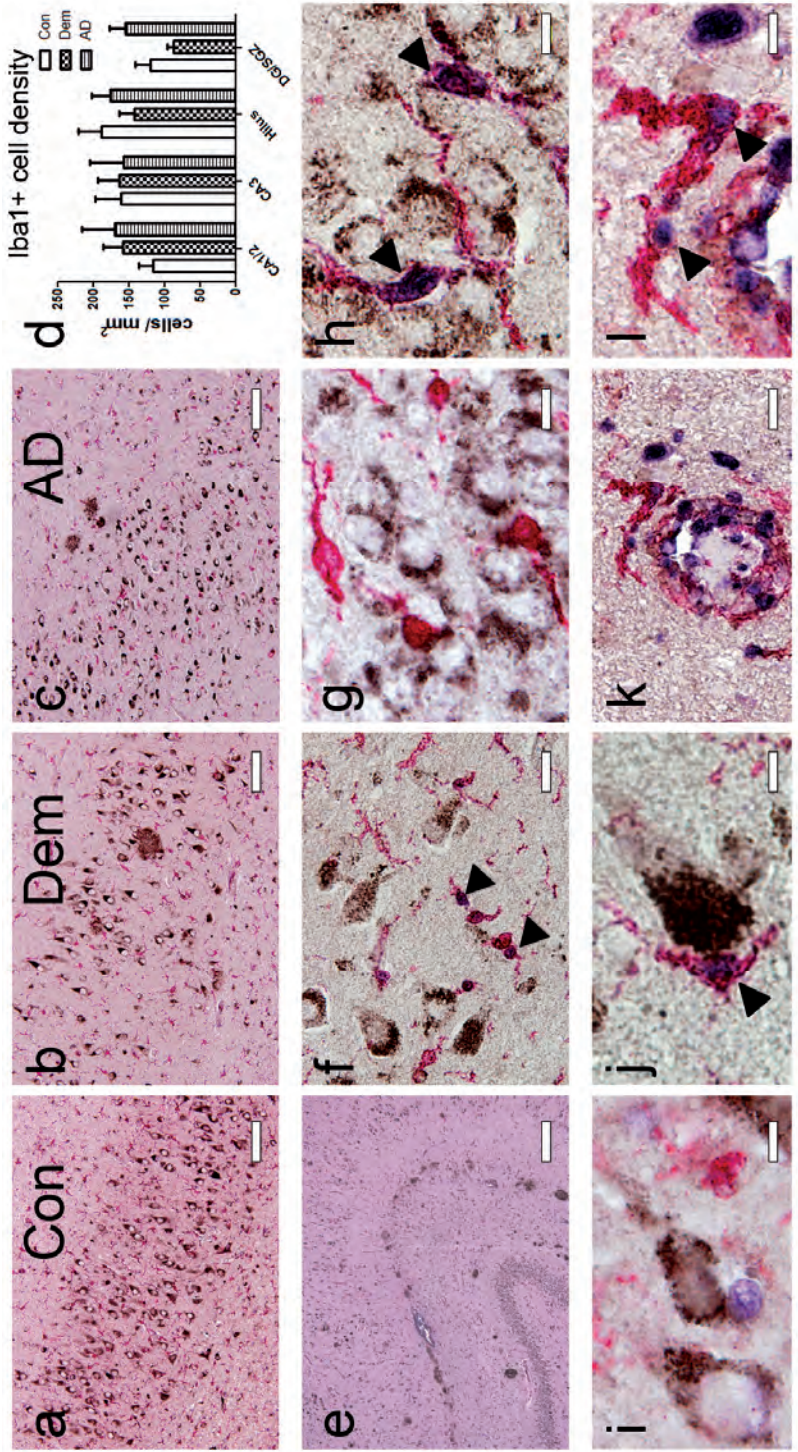


**Figure 1. GFAP and PCNA expression in the human hippocampus** A) Immunolabeled GFAP+ (red) and PCNA+ (blue) did not co-localize in the SGZ or other areas of the hippocampus. B) PCNA+ cells (arrows) were found in glia-rich regions and C) and blood vessels of the hippocampus. D) PCNA+ cells were observed in the presence of hypertrophic astrocytes with processes infiltrating A $\beta$  plaques (black). E) A low-magnification image representative of GFAP and A $\beta$  plaques distributed through the CA areas, with heavy staining in the stratum moleculare and stratum lacunosum F) GFAP+ cells were found at higher density in the CA1/2 region in the AD versus Control subjects (One-way ANOVA  $p < 0.001$ ). Scale bars: left panels A and D, B = 50  $\mu\text{m}$ . right panels A and D, C = 20  $\mu\text{m}$ ; panel E = 500  $\mu\text{m}$ .

**Iba1 morphology unchanged across conditions, microglia proliferate in all hippocampal subregions.**

Since GFAP+ astrocytes did not co-label with PCNA, we next used combined Iba1-PCNA immunocytochemistry to test microglial proliferation. In the brains of aged subjects, Iba1+ microglia had a ramified morphology with observable processes. There were neither morphological alterations nor quantitative changes in Iba1 expression across patient cohorts (one-way ANOVA  $p = 0.43$ )(Figs 2a-d). Furthermore, local expression of Iba1 was not associated with areas enriched for GFAP expression or A $\beta$  deposits, like the stratum moleculare and stratum lacunosum (Fig 2e).

Microglia within the CA subregions co-labeled with PCNA+ (Fig 2f) and Iba1+/PCNA+ co-expression was also seen in the DG and SGZ (Fig 2g,h). We closely inspected mature pyramidal neurons for evidence of PCNA expression, but never observed neurons co-labeled with PCNA although clear examples of proliferating and non-proliferating microglia were found adjacent to pyramidal neurons (Fig 2i, j). A Herpes Simplex Virus (HSV) encephalitis brain sample was included as positive control (Fig 2k,l). HSV encephalitis is observed as an acute focal, necrotizing inflammation; infection of neurons is thought to occur after infection of vascular endothelium[39]. In agreement, frequent PCNA+/Iba1+ cells were found in blood vessels. These Iba1+ cells had an amoeboid morphology, unlike the ramified cells found in the aged patient cohort, indicating active clearance of cellular debris.





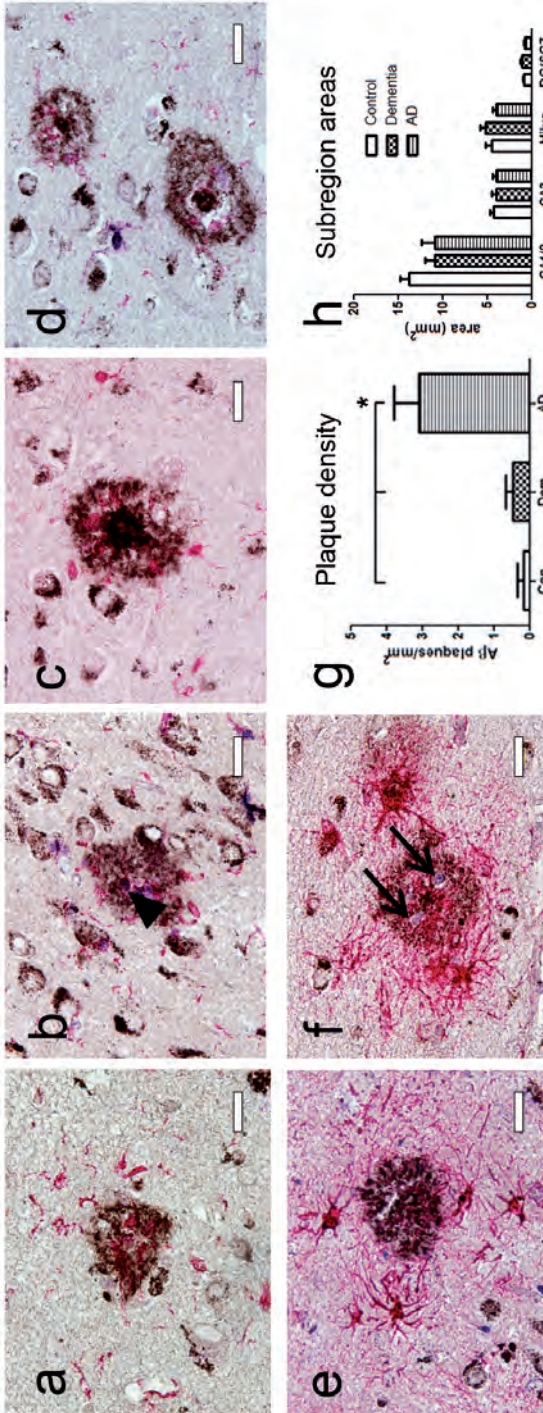
**Figure 2. Iba1 morphology and proliferation in human hippocampus** A-C) Representative images for Con, Dem, and AD subjects respectively, illustrating no morphological changes in the appearance of Iba1+ (red) cells. D) Quantitative analysis of Iba1+ cell density showed no significant difference between cohorts. Ramified Iba1+ cells throughout the hippocampus coexpress PCNA (arrowheads): E) representative A $\beta$  and Iba1 distribution, F) ramified and proliferating Iba1+ cells the in CA3 in the absence of A $\beta$  plaques, G-H) in the GCL across patient cohorts, I-J) near pyramidal neurons expressing APP (black). K-L) Positive control section from a brain infected with Herpes Simplex Virus encephalitis (HSVE). Infected vessels show activated Iba1+ microglia and co-expression of PCNA (arrowheads). Scale bars A-C= 100  $\mu$ m, E= 500  $\mu$ m, F= 50  $\mu$ m, G-J = 20  $\mu$ m, K = 50  $\mu$ m, L=20  $\mu$ m.

### A $\beta$ plaque load, morphology and degradation

Similar to earlier studies, the 4G8 antibody labeled diffuse, primitive, dense-core, and remnant A $\beta$  plaque profiles[24]. Remnant plaques show intense astrocytic participation with ragged edges indicative of degradation. The control and dementia groups had a similar degree of A $\beta$  staining in the hippocampus; A $\beta$  plaques in control and dementia cases were mainly confined to the subiculum and parahippocampal gyrus. As expected, total A $\beta$  plaque load was significantly increased in the hippocampus in the AD cohort compared to the control and dementia groups (one-way ANOVA  $p < 0.001$ ) (Fig 3g). Morphologically, the plaque pathology in the AD cohort was distinct from the control and dementia sections; AD cases had a significant increase in dense-core (One-way ANOVA  $p < 0.01$ ) and GFAP-associated, i.e. degraded plaque subtypes (One-way ANOVA  $p < 0.001$ ).

Interestingly, Iba1+ cells often formed a concentric ring around A $\beta$  dense-cored plaques and were generally found in close association to plaques (Fig 3a-d). Iba1+/PCNA+ double-labeled cells were visible within the borders of A $\beta$  plaques (Figs 3b). Also, PCNA+ single-labeled cells were found associated with degraded plaques, often within 200  $\mu$ m of all plaque subtypes (Figs 3e,f). The majority of plaques however, did not show evidence of proliferating PCNA+/Iba1+ microglia.

Despite this evidence that microglia proliferate directly at sites of A $\beta$  deposition and in proximity to plaque-laden areas, there was no quantitative increase in overall Iba1+ cell numbers in the AD cohort. Similarly, no statistically significant increases were found in the number of Iba1+ cells co-localized with A $\beta$  plaques in AD compared to the control and dementia cohorts (one-way ANOVA  $p = 0.49$ ).



**Figure 3. Glia Participate in plaque morphology and degradation.** Please note that chromogen Fast Red was used to visualize either microglia (top panels) or astrocytes (bottom panels) in these images; A-D) Iba1+ cells participate in the formation of dense-core plaques; proliferating Iba1+ cells (arrowheads) are seen within plaques. (E-F) GFAP+ astrocytes participate in degradation of multiple plaque subtype; proliferating cells (arrowheads) within the borders of plaques degraded by GFAP+ astrocytes. Scale bars A-F = 50  $\mu$ m. G) Plaque load was significantly increased in the AD cohort compared to Con and Dem cohorts (One-way ANOVA  $p < 0.001$ ) H) Hippocampal cross sectional areas were not significantly different.

## **Discussion**

We investigated proliferative changes in glia of the human hippocampus with a specific focus on cognitive status and A $\beta$  pathology. Proliferating cells were found throughout the hippocampal subregions including the CA areas and astrocyte-rich regions. In contrast to a group of younger cases we studied before [29], proliferation was not increased in either the senile dementia or AD cohorts when compared to age-matched controls. PCNA+ profiles were never observed in GFAP+ astrocytes indicating that mature astrocytes do not proliferate or re-enter the cell cycle during dementia or AD. Iba1+ microglia however, did co-express PCNA across cohorts. They were specifically present within the borders of A $\beta$  plaques, indicating that microglia proliferate directly at the site of A $\beta$  deposition. Our observations agree with previous findings regarding microglial participation in formation and maintenance of A $\beta$  plaques, specifically dense-core subtypes identified by a concentric ring of Iba1+ microglia.

In contrast to our previous study that utilized a group of younger, i.e. pre-senile AD patients (mean age 66 years) [29], the present study was conducted with an older cohort of AD and dementia subjects (mean age 81 years). Proliferation was highest, although not statistically significant, in the dementia cohort when compared to the control subjects and AD cases suggesting that proliferation and microgliosis may impact cognitive function.

The morphology of the microglial cells indicated that they were minimally activated, when compared to the hyper-ramified and rod-like Iba1+ morphologies observed e.g. in pediatric epilepsy patients [40]. Our present Iba1 stainings highlight that compared to other severe neurological disorders, microglia do not show dramatic changes in quantity or morphology during AD. Whereas aged microglia in general do not show maximal activation when studied morphologically, there is



evidence that these cells in functional terms, are highly pro-inflammatory. Ex-vivo microglia cultures isolated from aged mice have shown elevated production of proinflammatory molecules Il-6 and TNF- $\alpha$ , while microglia from old animals further have a decreased ability to internalize A $\beta$  compared to young animals[41].

An interesting concept in this respect is that microglial and astroglial responses may be coordinated in a concerted manner that increases neuroinflammation. As recently described, increased levels of glia maturation factor (GMF) were detected in the periphery of A $\beta$  plaques[42]. GMF is primarily expressed in astrocytes and responsible for enhanced production of TNF- $\alpha$ , Il-1 $\beta$ , Il-6, and IP-10 by microglia [43]. Hence, a combination of primary astrocytic and microglial responses may occur during plaque deposition. As each cell type responds to A $\beta$  plaques, microglial proliferation and astrocytic GMF expression may be complementary processes that enhance local inflammation at A $\beta$  plaque sites. It will be of interest to address the relationship between these events in future studies.

Our data showing microglial proliferation agrees with previous findings regarding P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) in the AD brain. This membrane-bound, purigenic, ATP binding receptor is expressed in both monocytes and microglia. Microglia isolated from AD brain have shown elevated P2X<sub>7</sub>R expression where the receptor was found to facilitate A $\beta$  induced cytokine production [44]. Interestingly, transfection of cells with P2X<sub>7</sub>R leads to increased proliferation rates[45]. In primary hippocampal cultures, overexpression of P2X<sub>7</sub>R, in the absence of pathological insults, is sufficient to drive microglial activation and proliferation[46]. In an AD mouse model, P2X<sub>7</sub>R was up regulated in microglia cells that surrounded plaques[47].

While proliferating microglia are seen in the hippocampus, the source of these cells remains to be determined. CNS microglia are unique because two populations exist, namely resident cells present since development, and infiltrating microglia that are derived from circulating bone marrow-derived monocytes that pass the blood-brain barrier (BBB). Iba1 identifies microglia in the brain regardless of their source. Determining the kinetics of microglial infiltration during a chronic disease is challenging, however hints have been provided. During the early stages of experimental autoimmune encephalitis resident microglia incorporate thymidine analog BrdU+ when no monocytic infiltration is present, indicating that proliferation of local

cells expands the microglial population during the early disease phase [48][49]. It is not clear if these models can be expanded to AD as experimental evidence suggests circulating cells may be involved in AD. In vitro experiments indicate that monocyte recruitment across the BBB is increased by A $\beta$  peptide interaction with RAGE receptor[50]. Additionally, bone marrow-derived microglia have been shown to drastically reduce plaque burden in AD models[51]. As such, the source and kinetics, i.e. mitotic and apoptotic regulation of each population remains to be determined during AD.

Proliferation in brain microvessels may also serve as an indicator of progressive disease. In agreement with earlier studies, we found PCNA+ cells associated with the vasculature [29, 52]. These cells may represent perivascular macrophages, or monocytes becoming perivascular macrophages [52]. Alternatively, PCNA+ expression associated with the vasculature may correspond with damaged blood vessels. Stroke-associated conditions and oxidative endothelial injury are well known to induce PCNA expression in vascular smooth muscle cells isolated from brain arterioles[53, 54] but from the patients records, there are no indications that this has played a role in our cohort.

PCNA+ profiles were clearly missing from mature hippocampal neurons, which are known to re-enter the cell cycle during AD. Expression of PCNA in mature hippocampal neurons is part of a cascade of events preceding neuronal death[55]. In agreement, other cell-cycle makers, including cyclin B1, cyclin D, and cdk4 have been observed in neurons during prodromal or later stages of AD[56]. These neurons are often also positive for intraneuronal accumulations of paired helical filament (PHF-1) tau [57]. Cell cycle protein expression during AD is supported by reports of increased neuronal aneuploidy in early AD[58], reviewed by Bonda et al.[59]. Our study does not conflict with the literature regarding neuronal cell cycle re-entry. Our triple-staining protocol was specifically developed to identify proliferation in glia; a number of methodological differences exist between our protocol and those identifying neuronal cell cycle re-entry. We employed a unique antigen retrieval technique with a low concentration of PCNA antibody to create a sensitive method for detecting proliferating glia and perivascular macrophages. Our results agree with previous neuropathological studies showing that microglia proliferate and change morphology consistent with activation[60].

Despite its limitations, postmortem histological investigation of human brain remains important, particularly since modeling glial participation in transgenic mice has so far provided conflicting results. For instance, microglia played almost no role in the formation and maintenance of A $\beta$  plaques in transgenic mice that lacked microglia[61]. However, others have shown microglial engagement in dense-cored plaques, more closely mirroring human pathology[62]. Bone marrow-derived microglia found inside the brain have been shown to drastically reduce plaque burden in animal models of disease[51]; a number of studies have found that increased microglia activation reduced A $\beta$  accumulation in APP transgenic mice[63-65]. Real time, in vivo 2-photon imaging further showed that plaques remain stable over time despite a rapid microglia response[12], which was confirmed in a follow-up study of study of longer duration [66]. However, the fact that transgenic mice seldom recapitulate the regional variability of A $\beta$  plaques seen in human AD suggests that these mouse models are not well suited for studying the complex in situ microglial responses that occur in the human brain[67].

We have refined earlier findings regarding cell proliferation, showing that Iba1+ microglia proliferate in the hippocampus of aged individuals. The data and discussion here, while focused on A $\beta$  plaques, does not rule out stimulation through additional mechanisms. Collectively it appears that neuroinflammation plays an important role in the AD phenotype, this work further establishes microglia as an important player in the balance of health and disease.

## **Acknowledgements**

We thank Dr. I. Huitinga, Ms. J. Wouda (SILS-CNS) and Mr. M. Kooreman from the Netherlands Brain Bank for their help with organizing the human brain material, and Mrs. Marianne Leisser for histological assistance. MWM and PJJ are supported by the European Union (NEURAD consortium, MEST-CT-2005-020013). PJJ is additionally supported by the Dutch Brain Foundation, IPF and the Internationale Stichting Alzheimer Onderzoek (ISAO). EA is supported by the Stichting Epilepsie Instellingen Nederland (SEIN) Heemstede, The Netherlands. JJMH is supported by the ISAO.

## **References**

- 1 Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. *Acta neuropathologica* 1991; 82: 239-59
- 2 Haroutunian V, Purohit DP, Perl DP, Marin D, Khan K, Lantz M, Davis KL, Mohs RC. Neurofibrillary tangles in nondemented elderly subjects and mild Alzheimer disease. *Archives of neurology* 1999 Jun; 56: 713-8
- 3 van der Flier WM, Scheltens P. Alzheimer disease: hippocampal volume loss and Alzheimer disease progression. *Nat Rev Neurol* 2009 Jul; 5: 361-2
- 4 Eikelenboom P, Bate C, Van Gool WA, Hoozemans JJ, Rozemuller JM, Veerhuis R, Williams A. Neuroinflammation in Alzheimer's disease and prion disease. *Glia* 2002 Nov; 40: 232-9
- 5 McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. *Neurology* 1996 Aug; 47: 425-32
- 6 Wyss-Coray T. Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat Med* 2006 Sep; 12: 1005-15
- 7 McGeer EG, McGeer PL. Neuroinflammation in Alzheimer's disease and mild cognitive impairment: a field in its infancy. *Journal of Alzheimer's disease : JAD* 2010; 19: 355-61
- 8 Perry VH, Nicoll JA, Holmes C. Microglia in neurodegenerative disease. *Nature reviews* Apr; 6: 193-201
- 9 Perry VH, Nicoll JA, Holmes C. Microglia in neurodegenerative disease. *Nat Rev Neurol* 2010; 6: 193-201
- 10 Graeber MB, Streit WJ. Microglia: biology and pathology. *Acta neuropathologica* 2010 Jan; 119: 89-105
- 11 Ohsawa K, Imai Y, Kanazawa H, Sasaki Y, Kohsaka S. Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia. *J Cell Sci* 2000 Sep; 113 ( Pt 17): 3073-84
- 12 Bolmont T, Haiss F, Eicke D, Radde R, Mathis CA, Klunk WE, Kohsaka S, Jucker M, Calhoun ME. Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. *J Neurosci* 2008 Apr 16; 28: 4283-92

- 13 Dickson DW, Farlo J, Davies P, Crystal H, Fuld P, Yen SH. Alzheimer's disease. A double-labeling immunohistochemical study of senile plaques. *The American journal of pathology* 1988 Jul; 132: 86-101
- 14 Haga S, Akai K, Ishii T. Demonstration of microglial cells in and around senile (neuritic) plaques in the Alzheimer brain. An immunohistochemical study using a novel monoclonal antibody. *Acta neuropathologica* 1989; 77: 569-75
- 15 Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. *J Neuroimmunol* 1989 Oct; 24: 173-82
- 16 Terry RD, Gonatas NK, Weiss M. Ultrastructural Studies in Alzheimer's Presenile Dementia. *The American journal of pathology* 1964 Feb; 44: 269-97
- 17 Yang CN, Shiao YJ, Shie FS, Guo BS, Chen PH, Cho CY, Chen YJ, Huang FL, Tsay HJ. Mechanism mediating oligomeric Abeta clearance by naive primary microglia. *Neurobiol Dis* 2011 Jan 8; 42: 221-30
- 18 Rogers J, Strohmeyer R, Kovelowski CJ, Li R. Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. *Glia* 2002 Nov; 40: 260-9
- 19 McGeer PL, McGeer EG. Local neuroinflammation and the progression of Alzheimer's disease. *Journal of neurovirology* 2002 Dec; 8: 529-38
- 20 Miller KR, Streit WJ. The effects of aging, injury and disease on microglial function: a case for cellular senescence. *Neuron glia biology* 2007 Aug; 3: 245-53
- 21 Colangelo V, Schurr J, Ball MJ, Pelaez RP, Bazan NG, Lukiw WJ. Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. *Journal of neuroscience research* 2002 Nov 1; 70: 462-73
- 22 Lee SC, Liu W, Dickson DW, Brosnan CF, Berman JW. Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. *J Immunol* 1993 Apr 1; 150: 2659-67
- 23 Giulian D, Li J, Li X, George J, Rutecki PA. The impact of microglia-derived cytokines upon gliosis in the CNS. *Dev Neurosci* 1994; 16: 128-36
- 24 Oide T, Kinoshita T, Arima K. Regression stage senile plaques in the natural course of Alzheimer's disease. *Neuropathol Appl Neurobiol* 2006 Oct; 32: 539-56
- 25 Nielsen HM, Mulder SD, Belien JA, Musters RJ, Eikelenboom P, Veerhuis R. Astrocytic A beta 1-42 uptake is determined by A beta-aggregation state and the presence of amyloid-associated proteins. *Glia* 2011 Aug; 58: 1235-46
- 26 Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC, Husemann J. Adult mouse astrocytes degrade amyloid-beta in vitro and in situ. *Nat Med* 2003 Apr; 9: 453-7
- 27 Funato H, Yoshimura M, Yamazaki T, Saido TC, Ito Y, Yokofujita J, Okeda R, Ihara Y. Astrocytes containing amyloid beta-protein (Abeta)-positive granules are associated with Abeta40-positive diffuse plaques in the aged human brain. *The American journal of pathology* 1998 Apr; 152: 983-92
- 28 Akiyama H, Mori H, Saido T, Kondo H, Ikeda K, McGeer PL. Occurrence of the diffuse amyloid beta-protein (Abeta) deposits with numerous Abeta-containing glial cells in the cerebral cortex of patients with Alzheimer's disease. *Glia* 1999 Feb 15; 25: 324-31

- 29 Boekhoorn K, Joels M, Lucassen PJ. Increased proliferation reflects glial and vascular-associated changes, but not neurogenesis in the presenile Alzheimer hippocampus. *Neurobiol Dis* 2006 Oct; 24: 1-14
- 30 Alafuzoff I, Pikkarainen M, Arzberger T, Thal DR, Al-Sarraj S, Bell J, Bodi I, Budka H, Capetillo-Zarate E, Ferrer I, Gelpi E, Gentleman S, Giaccone G, Kavantzias N, King A, Korkolopoulou P, Kovacs GG, Meyronet D, Monoranu C, Parchi P, Patsouris E, Roggendorf W, Stadelmann C, Streichenberger N, Tagliavini F, Kretschmar H. Inter-laboratory comparison of neuropathological assessments of beta-amyloid protein: a study of the BrainNet Europe consortium. *Acta neuropathologica* 2008 May; 115: 533-46
- 31 Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtas S, van Roon-Mom WM, Bjork-Eriksson T, Nordborg C, Frisen J, Dragunow M, Faull RL, Eriksson PS. Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science (New York, NY)* 2007 Mar 2; 315: 1243-9
- 32 van den Berge SA, Middeldorp J, Zhang CE, Curtis MA, Leonard BW, Mastroeni D, Voorn P, van de Berg WD, Huitinga I, Hol EM. Longterm quiescent cells in the aged human subventricular neurogenic system specifically express GFAP-delta. *Aging cell* Jun; 9: 313-26
- 33 Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. Microglia-specific localisation of a novel calcium binding protein, Iba1. *Brain research* 1998 Jun 1; 57: 1-9
- 34 v Eitzen U, Egensperger R, Kosel S, Grasbon-Frodl EM, Imai Y, Bise K, Kohsaka S, Mehraein P, Graeber MB. Microglia and the development of spongiform change in Creutzfeldt-Jakob disease. *J Neuropathol Exp Neurol* 1998 Mar; 57: 246-56
- 35 Tran CT, Wolz P, Egensperger R, Kosel S, Imai Y, Bise K, Kohsaka S, Mehraein P, Graeber MB. Differential expression of MHC class II molecules by microglia and neoplastic astroglia: relevance for the escape of astrocytoma cells from immune surveillance. *Neuropathol Appl Neurobiol* 1998 Aug; 24: 293-301
- 36 Mori I, Imai Y, Kohsaka S, Kimura Y. Upregulated expression of Iba1 molecules in the central nervous system of mice in response to neurovirulent influenza A virus infection. *Microbiol Immunol* 2000; 44: 729-35
- 37 Ito D, Tanaka K, Suzuki S, Dembo T, Fukuuchi Y. Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. *Stroke* 2001 May; 32: 1208-15
- 38 Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. *Acta neuropathologica* 2010 Jan; 119: 7-35
- 39 Tyler KL. Update on herpes simplex encephalitis. *Rev Neurol Dis* 2004 Fall; 1: 169-78
- 40 Wirenfeldt M, Clare R, Tung S, Bottini A, Mathern GW, Vinters HV. Increased activation of Iba1+ microglia in pediatric epilepsy patients with Rasmussen's encephalitis compared with cortical dysplasia and tuberous sclerosis complex. *Neurobiol Dis* 2009 Jun; 34: 432-40
- 41 Njie EG, Boelen E, Stassen FR, Steinbusch HW, Borchelt DR, Streit WJ. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. *Neurobiol Aging* 2012 Jan; 33: 195 e1- e12

- 42 Thangavel R, Stolmeier D, Yang X, Anantharam P, Zaheer A. Expression of Glia Maturation Factor in Neuropathological Lesions of Alzheimer's Disease. *Neuropathol Appl Neurobiol* 2011 Oct 28;
- 43 Zaheer A, Zaheer S, Sahu SK, Knight S, Khosravi H, Mathur SN, Lim R. A novel role of glia maturation factor: induction of granulocyte-macrophage colony-stimulating factor and pro-inflammatory cytokines. *Journal of neurochemistry* 2007 Apr; 101: 364-76
- 44 McLarnon JG, Ryu JK, Walker DG, Choi HB. Upregulated expression of purinergic P2X(7) receptor in Alzheimer disease and amyloid-beta peptide-treated microglia and in peptide-injected rat hippocampus. *J Neuropathol Exp Neurol* 2006 Nov; 65: 1090-7
- 45 Baricordi OR, Melchiorri L, Adinolfi E, Falzoni S, Chiozzi P, Buell G, Di Virgilio F. Increased proliferation rate of lymphoid cells transfected with the P2X(7) ATP receptor. *The Journal of biological chemistry* 1999 Nov 19; 274: 33206-8
- 46 Monif M, Reid CA, Powell KL, Smart ML, Williams DA. The P2X7 receptor drives microglial activation and proliferation: a trophic role for P2X7R pore. *J Neurosci* 2009 Mar 25; 29: 3781-91
- 47 Parvathenani LK, Tertysnikova S, Greco CR, Roberts SB, Robertson B, Posmantur R. P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *The Journal of biological chemistry* 2003 Apr 11; 278: 13309-17
- 48 Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat Neurosci* 2011; 14: 1142-9
- 49 Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci* 2007 Dec; 10: 1538-43
- 50 Giri R, Shen Y, Stins M, Du Yan S, Schmidt AM, Stern D, Kim KS, Zlokovic B, Kalra VK. beta-amyloid-induced migration of monocytes across human brain endothelial cells involves RAGE and PECAM-1. *Am J Physiol Cell Physiol* 2000 Dec; 279: C1772-81
- 51 Simard AR, Soulet D, Gowing G, Julien JP, Rivest S. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron* 2006 Feb 16; 49: 489-502
- 52 Williams K, Schwartz A, Corey S, Orandle M, Kennedy W, Thompson B, Alvarez X, Brown C, Gartner S, Lackner A. Proliferating cellular nuclear antigen expression as a marker of perivascular macrophages in simian immunodeficiency virus encephalitis. *The American journal of pathology* 2002 Aug; 161: 575-85
- 53 Gerzanich V, Ivanova S, Simard JM. Early pathophysiological changes in cerebral vessels predisposing to stroke. *Clinical hemorheology and microcirculation* 2003; 29: 291-4
- 54 Iadecola C, Goldman SS, Harder DR, Heistad DD, Katusic ZS, Moskowitz MA, Simard JM, Sloan MA, Traystman RJ, Velletri PA. Recommendations of the National Heart, Lung, and Blood Institute working group on cerebrovascular biology and disease. *Stroke* 2006 Jun; 37: 1578-81
- 55 Yang Y, Mufson EJ, Herrup K. Neuronal cell death is preceded by cell cycle events at all stages of Alzheimer's disease. *J Neurosci* 2003 Apr 1; 23: 2557-63

- 56 Busser J, Geldmacher DS, Herrup K. Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. *J Neurosci* 1998 Apr 15; 18: 2801-7
- 57 Smith TW, Lippa CF. Ki-67 immunoreactivity in Alzheimer's disease and other neurodegenerative disorders. *J Neuropathol Exp Neurol* 1995 May; 54: 297-303
- 58 Arendt T, Bruckner MK, Mosch B, Losche A. Selective Cell Death of Hyperploid Neurons in Alzheimer's Disease. In *American Journal of Pathology*. 2010: 15-20
- 59 Bonda DJ, Bajic VP, Spremo-Potparevic B, Casadesus G, Zhu X, Smith MA, Lee HG. Review: cell cycle aberrations and neurodegeneration. *Neuropathol Appl Neurobiol* Apr; 36: 157-63
- 60 Wierzba-Bobrowicz T, Gwiazda E, Kosno-Kruszewska E, Lewandowska E, Lechowicz W, Bertrand E, Szpak GM, Schmidt-Sidor B. Morphological analysis of active microglia--rod and ramified microglia in human brains affected by some neurological diseases (SSPE, Alzheimer's disease and Wilson's disease). *Folia Neuropathol* 2002; 40: 125-31
- 61 Grathwohl SA, Kalin RE, Bolmont T, Prokop S, Winkelmann G, Kaeser SA, Odenthal J, Radde R, Eldh T, Gandy S, Aguzzi A, Staufenbiel M, Mathews PM, Wolburg H, Heppner FL, Jucker M. Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. *Nat Neurosci* 2009 Nov; 12: 1361-3
- 62 Wirths O, Breyhan H, Marcello A, Cotel MC, Bruck W, Bayer TA. Inflammatory changes are tightly associated with neurodegeneration in the brain and spinal cord of the APP/PS1KI mouse model of Alzheimer's disease. *Neurobiol Aging* 2010 May; 31: 747-57
- 63 Wyss-Coray T, Lin C, Yan F, Yu GQ, Rohde M, McConlogue L, Masliah E, Mucke L. TGF-beta1 promotes microglial amyloid-beta clearance and reduces plaque burden in transgenic mice. *Nat Med* 2001 May; 7: 612-8
- 64 Wilcock DM, Munireddy SK, Rosenthal A, Ugen KE, Gordon MN, Morgan D. Microglial activation facilitates Abeta plaque removal following intracranial anti-Abeta antibody administration. *Neurobiol Dis* 2004 Feb; 15: 11-20
- 65 Herber DL, Roth LM, Wilson D, Wilson N, Mason JE, Morgan D, Gordon MN. Time-dependent reduction in Abeta levels after intracranial LPS administration in APP transgenic mice. *Experimental neurology* 2004 Nov; 190: 245-53
- 66 Hefendehl JK, Wegenast-Braun BM, Liebig C, Eicke D, Milford D, Calhoun ME, Kohsaka S, Eichner M, Jucker M. Long-term in vivo imaging of beta-amyloid plaque appearance and growth in a mouse model of cerebral beta-amyloidosis. *J Neurosci* 2011 Jan 12; 31: 624-9
- 67 Dickson DW. Microglia in Alzheimer's disease and transgenic models. How close the fit? *The American journal of pathology* 1999 Jun; 154: 1627-31