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Research Article

Early Transient Neuronal CyclinD1 Expression Precedes Atrophy in the Frontal Cortex of APP23 Mice

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Abstract

Alzheimer's disease (AD), the most common form of dementia, is neuropathologically characterized by the deposition of Amyloid-β (Aβ) in plaques. Interestingly, a significant number of neurons in AD brains display aberrant re-entry of cell cycle and expression of associated proteins. While this has been observed in a number of Aβ Precursor Protein (APP) transgenic mouse models of AD, the temporal and spatial profile of neuronal cell cycle protein expression was unclear. Here, we show that neuronal expression of the cell cycle protein CyclinD1 together with pro-apoptotic Caspase-3 is limited to the frontal cortex of young transgenic APP23 mice, an area that displays with atrophy in aging mice. Expression of the cell cycle proteins CyclinD1, Cyclin-Dependent Kinase 4 (CDK4) and Proliferating Cell Nuclear Antigen (PCNA) in the brains of aging APP23 mice were limited to microglia and astrocytes, while we did not observe neuronal cell cycle protein expression in neurons. Taken together, our data supports that aberrant neuronal cell cycle events contribute early on to AD, while later cell cycle activation in the CNS associated with Aβ is linked to reactive gliosis.

Introduction

The most common abnormalities found in Alzheimer's disease (AD) brains are extracellular amyloid-β (Aβ) plaques and intracellular Neurofibrillary Tangles (NFTs), latter formed by aberrantly phosphorylated microtubule-associated protein tau. NFTs are predominantly found in the hippocampus and entorhinal cortex, and with lower densities elsewhere [1]. In addition to the Aβ plaques and NFTs, there is significant neuronal cell death with distinct regional variability [2-4]. The exact cellular events leading to neuronal cell death remain unclear, but several neuropathological studies linked neuronal death to unexpected reappearance of cell cycle events [5-7]. Ectopic neuronal cell cycle re-entry has been implicated in several other neurodegenerative conditions, including Parkinson's disease [8], frontotemporal dementia [9], amyotrophic lateral sclerosis [10], and temporal lobe epilepsy [11], suggesting a possible common pathogenic mechanism. Ectopic activation of the cell cycle in post-mitotic neurons is thought to lead to neurodegeneration and cell death [9,12]. A crucial role of Aβ in cell cycle re-activation is supported by *in vitro* studies, and by expression of cell cycle proteins in mouse and rat cortical primary neurons challenged with Aβ [13-15]. Other studies support a role of tau in neuronal cell death induced by cell cycle re-entry *in vitro* and in animal models [16-19]. Different transgenic mice expressing human Aβ Precursor Protein (APP) gene variants have been used to study the association of cell cycle-related protein expression and cell death in AD, with divergent results [20-22]. Because aberrant neuronal cell cycle re-entry is closely associated with sites of neuronal degeneration in human brains and mouse models of AD, we examined the expression of cell cycle markers in an APP transgenic mouse line (APP23) with Aβ plaque formation and cell loss. Accordingly, it has been reported that APP23 mice display neuronal death in specific areas of the brain that corresponds to those most affected in Alzheimer's disease [23,24]. Our data show that neuronal cell cycle protein expression was focal in the frontal cortex of APP transgenic mice before amyloid plaque deposition, and it was associated with a subsequent volume reduction of the frontal cortex together with a disappearance of neuronal cell cycle protein expression. We also demonstrated that amyloid depositions in older APP23 mice were closely associated with cell cycle protein expression in astrocytes and microglia, but not neurons, consistent with reactive gliosis and inflammation linked to Aβ toxicity.

Material and Methods

Mice

APP23 mice have been previously described [25-27]. Mice were maintained on a C57Bl/6 background. All experiments have been approved by the Animal Care and Ethics Committee of the University of New South Wales.

Tissue preparation

Mice were anesthetized and transcardially perfused with ice cold Phosphate Buffered Saline (PBS) followed by 4% Paraformaldehyde (PFA) in PBS as previously described [28]. Brains were removed, hemispheres separated and immersion-fixed in 4% PFA overnight at 4°C for histological analysis. Fixed brains were processed using an automated system (Excelsior, Thermo, USA), embedded in paraffin and sagitally sectioned at the level of the mid-hippocampus into 3 µm thick sections using a microtome (Thermo, USA). Sections were mounted on Superfrost Plus slides (Menzel, Germany) and dried overnight followed by incubation at 60°C for 1.5 h.

Staining procedures

Staining was carried out as previously described [29,30] briefly, sections were deparaffinized in xylene and then gradually re-hydrated in 100%, 96% and 70% ethanol and then water for 10 min each. Following hydration the sections were pretreated in 10 mM citrate buffer (0.1 M citric acid, 0.1 M sodium citrate tribasic dihydrate) pH 5.8 for 7 min at 95 degrees under pressure in antigen retrieval microwave vacuum histoprocessor (Milestone, USA). Slides were cooled down for 15 min under running water, and for Immunohistochemistry (IHC) only, slides were placed in 3% H_2O_2 in 50% ethanol for 45 min. After rinsing in PBS sections were incubated for 1 h in blocking buffer (3% goat serum, 2% BSA in 0.1 M PBS), then transferred to a humid chamber and incubated overnight with primary antibodies at 4°C. Following rinsing in PBS for Immunofluorescence (IF), slides were incubated with Alexa Fluor-labelled secondary antibodies (dilution 1:250, Thermo Fisher, USA) for 1 h at room temperature, then rinsed in PBS and mounted with Fluoromount Aqueous Mounting Medium (Sigma). Nuclei were strained with 4′6-Diamidine-2′-phenylindole dihydrochloride (DAPI, Molecular probes, Carlsbad CA, USA). For IHC, sections were incubated with biotinylated secondary antibody antibodies (1:500 in blocking buffer) for 1 h, at room temperature and then washed. Then, sections were incubated with Avidin-Horseradish Peroxidase (HRP) coupled complexes (1:00 Vectastain Elite ABC HRP Kit) diluted in 0.1 M PBS for 1 h at room temperature. Following washing, sections were incubated in a DAB/H_2O_2 solution (dilution of one DAB tablet in 15 ml PBS as per the manufacturer's instructions; Sigma) for approximately 10 min. Finally, sections were washed, counterstained with haematoxylin, rinsed in running water for 15 minutes, dehydrated to xylene, and mounted in DPX (Sigma). Images were taken with an Olympus BX51 microscope equipped with a DP70 CCD camera, digitized and processed by means on Fiji-imageJ (NIH). To measure cortical layer thickness sagittal brain sections were deparaffinized in xylene, re-hydrated and stained with 0.1% Cresyl Violet. For each brain, the thickness of the cortical layers was measured using the Cell Sens Image Tool software (Cell Sens standard 1.9 Olympus corporation, USA).

Antibodies

The following primary antibodies to specific protein targets were used: proliferating cell nuclear antigen (PCNA; 1:250, BD Bioscience), Cyclin-Dependent Kinase 4 (CDK4) and CyclinD1 (both 1:150, Santa Cruz Biotechnology), cleaved Caspase-3 (1:50, abcam), glial fibrillary acidic protein (GFAP; 1:100, Sigma), neuronal nuclei (NeuN; 1:100, abcam) and ionized calcium binding adaptor molecule 1 (Iba1; 1:100, Wako).

Statistics

Statistical analysis was done with the Prism6 software (GraphPad), using or linear regression as specified in the figure legends. All value shown are mean ± Standard Error of the Mean (SEM).

Results

Transient neuronal expression of CyclinD1 in APP23 transgenic mice prior to amyloid plaque formation

Previous studies on expression of active cell cycle proteins in neurons in different models of Alzheimer's disease have reported diverging findings [19-21]. In the present study we addressed ectopic cell cycle protein expression in the APP23 mouse line, which expresses K670N/M671L (Swedish) mutant human APP in neurons [25]. We first determined expression of CyclinD1 in brains of young APP23 mice at different ages (Figure 1). Ectopic expression of CyclinD1 in APP23 mice was limited to frontal cortical neurons commencing at 3 and being most abundant at 4.5 months of age. Prior to 3 and after 4.5 months we did not observe CyclinD1 expression in APP23 mice. No CyclinD1 expression was detectable in non-transgenic control mice. Taken together, neuronal CyclinD1 is transiently expressed prior to plaque formation in APP23 mice.

CyclinD1 positive neurons express pro-apoptotic cleaved caspase-3

Nuclear translocation of CyclinD1 peaks before DNA fragmentation during neuronal-programmed cell death after ischemia in rat brains [31]. Furthermore, neuronal cell cycle reactivation leading to apoptosis has been proposed as a pathomechanism in AD [15,32]. Therefore, we determined whether CyclinD1-positive neurons in 4.5 month old APP23 mice also expressed Cleaved Caspase-3 (CC3), a marker of apoptosis [13]. Co-stained serial sections of brains from APP23 transgenic mice and control mice revealed abundant staining of CC3 in APP23 mice, with approximately 40% of CC3-positive cortical neurons also expressing CyclinD1 (Figure 2). No CC3 staining was observed in other brain regions of APP23 mice at 4.5 months of age, and in non-transgenic controls. Hence, CyclinD1 expression in neurons of APP23 mice may be associated with apoptosis.

CyclinD1 expression is associated with atrophy in the frontal cortex of APP23 mice

Next we determined whether neuronal CyclinD1expression was associated with neurodegeneration, since CyclinD1 expression was limited to frontal cortex in 4.5 month-old (Figure 1) and co-expressed with the pro-apoptotic cleaved caspase-3 (Figure 2). We measured the total cortical thickness (i.e. measured from the pial surface to the white matter) in Nissl-stained sections of frontal and occipital cortex at different ages (Figure 3). The mean frontal cortical thickness reduced in aged APP23 mice compared with non-transgenic mice. No differences between APP23 and non-transgenic mice were found in the occipital cortex. Taken together, we found selective atrophy within the frontal cortex of APP23 mice.

PCNA is expressed in microglia but not neurons in aging APP23 mice

While neuronal CyclinD1 expression in the frontal cortex seized at 6 months of age, we found progressively increasing expression of

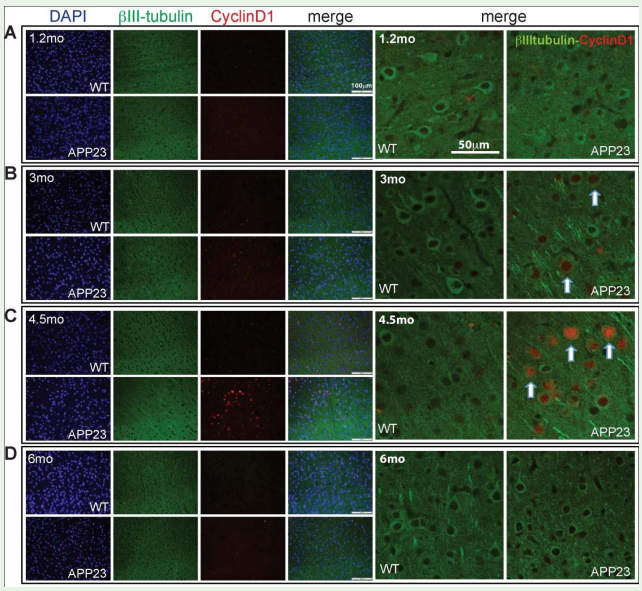

Figure 1: Transient neuronal CycinD1 expression in the frontal cortex of young mutant APP transgenic APP23 mice.
Sagittal brain sections from mice of the indicated genotype and age were stained with antibodies to CyclinD1 (red) and neuronal β III-tubulin (green), as well as nuclear staining with DAPI (blue). Higher magnification of merged channels is provided on the right for each age. (**A**) Wild-type (WT) and APP23 brains at 1.2 months lacked CyclinD1 expression. (**B**) Appearance of CyclinD1-positive neurons started at 3 months in APP23 but not WT mice, and (**C**) was most abundant at 4.5 months. Arrows indicate cells with co-labeling of CyclinD1 and neuronal β III-tubulin. (**D**) At 6 months neuronal Cyclin D1 was no longer expressed in the cortex of APP23 mice. Scale bars, 100 μm and 50 μm.

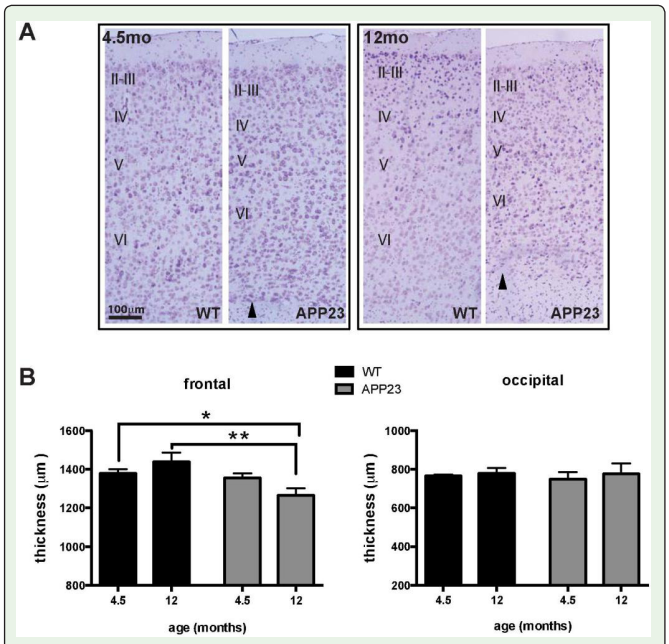

Figure 3: Atrophy of the frontal cortex in aged APP23 mice.
The thickness of the frontal cortex was measured on matching, Nissl-stained sagittal brain sections from 4.5 and 12 month-old Wild-Type (WT) and APP23 mice. (A) Representative images of Nissl-stained sections of the frontal cortex of WT and APP23 mice at 4.5 and 12 months of age, with labeling of the neural layers II-VI and the white matter (arrowhead). (**B**) Thickness of the frontal cortex was significantly reduced in 12 months-old APP23 mice compared to 4.5 and 12 months-old WT animals (n=5-7; *, $P<0.05$; **, $P<0.01$ [on-way ANOVA; F(5,17)=2.628]).

the cell cycle protein PCNA from 8 months of age in APP23, but not non-transgenic mice (Figure 4). Co-staining with cell-type specific markers showed that PCNA staining was limited to Iba1-positive microglia, while there was no co-localization with NeuN-positive neurons at 8 months of age in APP23 mice (Figure 4A and 4B). PCNA-positive cells in APP23 brains were frequently found in the proximity of neurons, possibly resembling activated microglia interacting with compromised neurons (Figure 4B). In 12 month-old APP23 mice, microglia surrounding forming Aβ plaques were mostly positive for PCNA (Figure 4C). At this age, few PCNA-positive cells did not co-localize with any of the cell-specific markers NeuN, Iba1 and GFAP (Figure 4C – inset). Taken together, the majority of PCNA-positive cells in the cortex of aging APP23 mice were microglia.

CyclinD1- and CDK4-positive cells surrounding amyloid plaques in aged APP23 mice

APP23 mice develop amyloid plaques predominantly in the cortex and in the hippocampus as they age. These plaques are accompanied by neuritic changes, reactive astrocytes, activated microglia and hyperphosphorylated tau [25,33]. Staining for aberrant expression of cyclins in cells surrounding amyloid-β plaques in aged

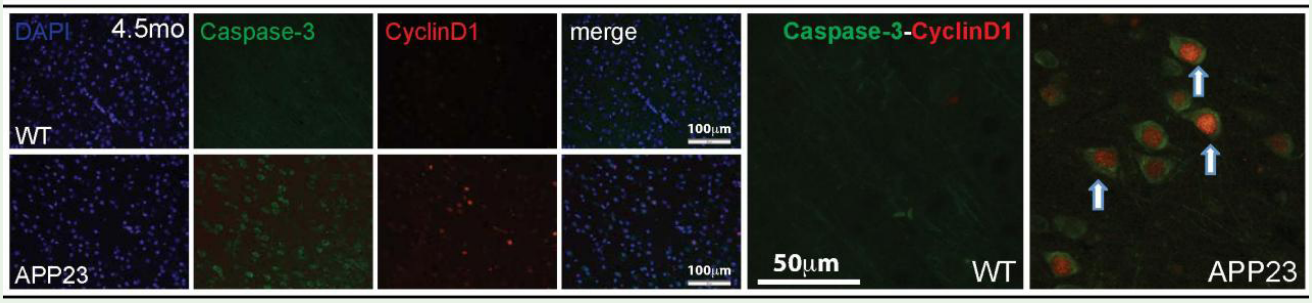

Figure 2: Co-expression of CyclinD1 and pro-apoptotic cleaved Caspase-3.
Immunofluorescence staining with antibodies to CyclinD1 (red), cleaved Caspase-3 (green) and nuclear DAPI (blue), showed neurons ectopically expressing CyclinD1 also stained for the pro-apoptotic cleaved form of Caspase-3 in the frontal cortex of APP23, but not wild-type (WT) mice. Scale bars, 100 μm and 50 μm.

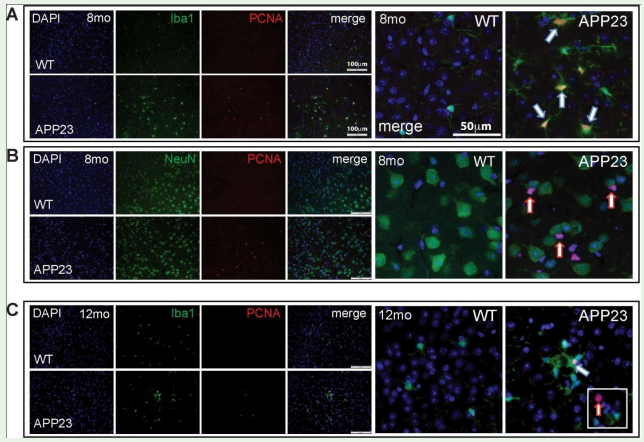

Figure 4: Microglia but not neurons express PCNA in the cortex of aging APP23 mice.
Immunofluorescent staining with antibodies to PCNA (red), microglial Iba1 (green), neuronal NeuN (green) and nuclear DAPI (blue) of Sagittal cortex sections from 8 and 12 month-old Wild-Type (WT) and APP23 mice. Higher magnification of merged channels is provided on the right for each age. (**A**) Staining of PCNA co-localized with increased microglial Iba1 staining (white arrows) in the brains of APP23 but not WT mice at 8 months of age. (**B**) No co-staining of PCNA and neuronal NeuN was found in 8 months old APP23 and WT mice. Note that PCNA-positive cells were frequently in close proximity to neurons in APP23 brains (red arrows). (C) At 12 months of age, PCNA-positive microglia (Iba1) was surrounding plaques (white arrow) in APP23 but not WT mice. At this age few PCNA positive-cells could not be identified by any of the used cell-type markers (red arrow; inset). Scale bars, 100 µm and 50 µm.

APP23 mice showed abundance of cells positive for both CyclinD1 and its dependent kinase CDK4 (Figure 5A and 5B). Quantification of CyclinD1-positive cells surrounding amyloid-β plaques in APP23 from 12 months of age onwards showed a significant and progressive increase in numbers over the following year (Figure 5C). Numbers of CyclinD1 positive cells in wild-type brains remained low over time. Hence, while we found a transient neuronal expression of CyclinD1 in younger APP23 mice, there are progressively increasing number of cells expressing CyclinD1 surrounding Aβ plaques in aged APP23 mice.

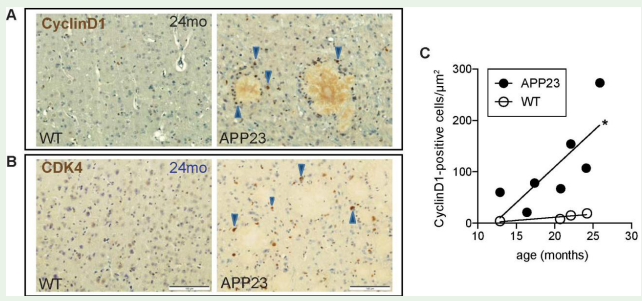

Figure 5: Cell cycle protein expression surrounding plaques in 24 months old APP23 mice.
Immunohistochemistry using antibodies to CyclinD1 and Cyclin-Dependent Kinase 4 (CDK4) on Sagittal brain sections from 2 years old Wild-Type (WT) and APP23 mice. (**A**) Frequent nuclear staining of CyclinD1 (brown) was detected in close proximity to a β plaques in APP23 mice. (**B**) Similarly, frequent nuclear staining of CDK4 (brown) was found in close proximity to A β plaquesin APP23 mice. (**C**) Linear regression analysis of numbers of CyclinD1 positive cells surrounding plaques in APP23 showed a significant progressive increase between 1 and 2 years of age (n=7; *,$P<0.05$ [y = 13.93x-169.1; R^2=0.5882]), compared to consistent low numbers in WT (n=4 [y=1.229x-13.05; R^2=0.8042]).

CyclinD1 and PCNA expressed in activated microglia and astrocytes in aged APP23 mice

To determine the type of cells with expression of cell cycle proteins surrounding Aβ plaques in aged APP23 mice, we co-stained brain sections of 24 month-old APP23 and non-transgenic mice with cyclinD1 or PCNA together with the different cell type markers NeuN, GFAP and Iba1 (Figure 6). Forty-five % of CyclinD1 positive cells co-stained with astrocytic GFAP, while 20% of CyclinD1 positive cells co-stained with microglial Iba1. Both GFAP-positive astrocytes

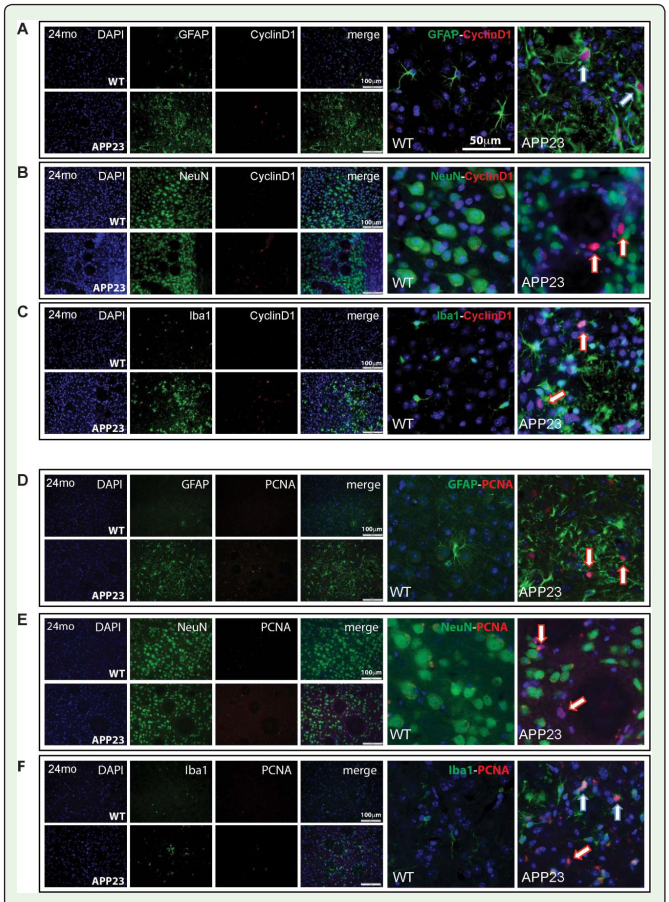

Figure 6: Cell cycle protein expression in microglia and in astrocytes of aged APP23 mice.
Immunonofluorescent staining of cortical brain section from wild-type (WT) and APP23 mice using antibodies to CyclinD1 (red; panels A-C) or PCNA (red; panels D-F) together with antibodies to the different cell-type specific markers (green) GFAP (astrocytes), NeuN (neurons) and Iba1 (microglia), and nuclear DAPI (blue). Higher magnification of merged channels is provided on the right for each age. (**A**) CyclinD1 staining co-localized with increased astrocytic GFAP staining in APP23 mice (white arrows). (**B**) CyclinD1 staining did not co-localize with neuronal NeuN (red arrows). (**C**) CyclinD1 staining did not co-localize with microglial Iba1 (red arrows). (**D**) Gfap staining did not co-localize with astrocytic GFAP (red arrows). (**E**) PCNA staining did not co-localize with neuronal NeuN (red arrows). (**F**) PCNA staining co-localized with increased microglial Iba1 staining in APP23 mice (white arrows). Note that there were also cells that did not co-localize with Iba1 (red arrow). Scale bars, 100 µm and 50 µm.

and Iba1-positive microglia surrounding Aβ plaques presented with a tufted morphology, characteristic of activation. No significant co-staining of CyclinD1 and the neuronal marker NeuN was detected in aged APP23 mice (<4%). However, 30% of CyclinD1-positive cells surrounding Aβ plaques did not co-stain with any of the used markers. No CyclinD1-positive cells were detected in non-transgenic mice.

Thirty % of PCNA-positive cells co-stained with microglial Iba1 surrounding Aβ plaques. Neither neurons (NeuN) nor astrocytes (GFAP) stained with PCNA. Interestingly, the remaining over 60% of cells did no co-stain with any of the used cell type markers. No PCNA-positive cells were detected in non-transgenic mice of the same age. Furthermore, we found no PCNA expression in neurons of the hippocampus of APP23 mice at 12 and 24 months of age (Figure S1), different from a previous report [20]. Taken together, CyclinD1-positive cells surrounding Aβ plaques in aged APP23 mice were mostly astrocytes and to a lesser degree microglia, while only microglia stained positive for PCNA. Notably, a large number of cells with expression of cell cycle markers in APP23 brains remain uncharacterized.

Discussion

In the present study we have used immuno-staining to investigate neuronal cell cycle events in a mouse model of AD. We report that APP23 transgenic mice display ectopic neuronal expression of proteins related to the cell cycle before the deposition of Aβ plaques. The occurrence of neuronal cell cycle re-entry corresponded with the age of the onset of memory deficits and neuronal network aberrations in APP23 mice [27,34,35]. In addition, we showed that CyclinD1 expression that peaks at 4.5 months of age is accompanied by cleavage of pro-apoptotic caspase-3. There is ample evidence fora role of caspase-3 in AD; for example, exposure of cells to Aβ activates caspase-3 in cultured neurons [36] and cleaved caspase-3 is found in human AD supporting apoptotic neuronal death in AD [37]. Accordingly, we found that co-presence of CyclinD1 and cleaved caspase-3 precedes disappearance of cell cycle positive neurons in the frontal cortex after 4.5 months of age APP23 mice together with progressive atrophy of the frontal cortex. Our findings are in line with previous studies *in vitro* [38] and in the developing mouse brain *in vivo* [39] that showed that ectopically dividing neurons undergo apoptosis upon entering the cell cycle. Other studies suggested roles of caspase-3 in synaptic plasticity [40,41]. Cleaved caspase-3 may also be involved in synaptic dysfunction in AD, since pharmacological inhibition of caspase-3 restored glutamatergic synaptic transmission and hippocampal dysfunction in APP transgenic AD mice [42]. Taken together, ectopic neuronal cell cycle re-entry and apoptosis may contribute together to neuronal dysfunction and loss presented early in AD mouse models.

Post-mortem studies on both AD and MCI indicate that 5 and 10% of neurons are positive for cell cycle markers [43], and these localize to different brain areas, including the hippocampus, temporal cortex, subiculum, locus coeruleus, and dorsal raphe nuclei, but not infero-temporal cortex or cerebellum [7,44]. Further reports indicate that neurons in areas at risk for AD display cell cycle related proteins [45]. Interestingly, we found neuronal CyclinD1 expression limited to pyramidal neurons in the frontal cortex of APP23 mice (and in the hippocampus, which was around the onset of functional deficits and much prior to overt Aβ plaque pathology [25,27,34,35,46]. This was despite the fact that APP23 later develop Aβ plaques throughout the entire brain [27]. CyclinD1 expressing neurons were also described in the cortical forebrain of 6 month old human mutant APP transgenic J20 mice [13], which present with similar pathology but later onset compared to APP23 mice [47]. If CyclinD1-positive neurons also disappear as J20 mice age, similar to what we found in APP23 mice, remains to be tested. Importantly, we did not find neuronal CyclinD1 expression in older APP23 mice. To this end, it remains to be shown why neuronal CyclinD1 expression is both temporally and specially confined to the frontal cortex of young APP23 mice.

While there was no detectable neuronal cell cycle protein expression in APP23 mice older than 6 months of age, we found frequent expression of the cell cycle proteins Proliferating Cell Nuclear Antigen (PCNA), Cyclin-Dependent Kinase 4 (CDK4) and CyclinD1 in microglia and astrocytes as APP23 mice became older, in particular in the proximity of Aβ plaques. Our data is in line with the finding that aged APP23 mice present with significantly increased numbers of newly produced cells of the microglial and astrocytic linage [24]. Furthermore, our findings are consistent with a previous report that astrocytes, but not neurons express CyclinD1 in aged, 21.5 months-old APP23 mice [21], although, different from our study, no younger APP23 mice were examined or expression of cell cycle markers in microglia analyzed.

In AD the appearance of Aβ plaques is associated with increased numbers of microglia and astrocytes [48]. Studies in humans and in APP23 micereported a specific association between Aβ deposits and microglial markers in the cortex and in the hippocampus [49,50]. Already small Aβ plaques in APP23 mice that appear at about 6 months of age are associated with a microglial response [51]. Other mouse models with transgenic APP expression also show activated microglia in proximity of Aβ aggregates [52,53]. In AD brains, inflammation is believed to result in migration and proliferation of endogenous microglia [54]. Although we could readily detect PCNA in microglia of 8, 12 and 24 month-old APP23, suggesting that the used antibody was specific, we did not find detectable expression in neurons in different brain regions (including hippocampus) at any of the ages examined. This contrasts a previous report of neuronal PCNA expression in cortex and hippocampus of 1 year old APP23 mice, using immunohistochemistry [20]. Methodological differences may explain the diverting findings. Late cell cycle marker expression in microglia in APP23 in our study may suggest that recruitment (=migration) of microglia rather than proliferation contributes to early microgliosis, while microglia start proliferating only as the disease progresses. Interestingly, we found that a considerable number of cells that expressed cell cycle markers in 24 months old APP23 mouse brains did not stain with astrocyte or microglia (or neuron) markers. These cells may resemble infiltrating cells associated with progressed AD pathology [55,56]. Accordingly, a subpopulation of microglia-like cells associated with Aβ deposits originating from bone marrow-derived monocytes have been identified in double transgenic Alzheimer mice (APP/PS1) [57]. To this end the exact nature of dividing cells that did not stain for the markers used in our study remains to be identified.

In summary, we report that neuronal cell cycle protein expression in APP23 mice is temporally and spatially restricted to the frontal cortex at young ages, and associated with activation of

apoptosis and later atrophy. Cell cycle protein expression in aging APP23 mice, however is restricted to non-neuronal cells, including microglia, astrocytes and an unidentified cell population. To this end, the molecular mechanism that underline neuronal death in conjunction with cell cycle re-entry remain largely elusive. We have shown previously that Aβ toxicity is tau-dependent in AD mouse models [27]. This is regulated by p38γ-dependent phosphorylation of tau [46]. In an elegant study, the concept of tau-dependent Aβ toxicity has been extended to the role of neuronal cell cycle re-entry in disease, showing that crossing mutant APP transgenic mice on a tau deficient background prevents cell cycle re-entry and that tau phosphorylation is required for this process [13]. In addition, a role of tau in aberrant neuronal cell cycle re-entry is further supported by the findings that expression of human tau in mice results in cell cycle protein expression in human tau harboring neurons [20,58,59]. Further studies are required to elucidate the role(s) or tau and other factors in neuronal cell cycle re-entry in neurodegenerative diseases. In conclusion, our temporal profiling of cell cycle marker expression in APP23 mice supports that aberrant neuronal cell cycle events contribute early on to AD, while later cell cycle activation in the CNS associated with Aβ is linked to reactive gliosis.

Author Contributions

S.I. and L.I designed the study. S.I., C.S. and Y.K. performed experiments and analyzed data. L.I. and Y.K. obtained funding. S.I. and L.I. wrote the manuscript with input from all authors.

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