Gliosis Precedes Amyloid-β Deposition and Pathological Tau Accumulation in the Neuronal Cell Cycle Re-Entry Mouse Model of Alzheimer’s Disease

Kevin H.J. Parka,b,c,d,∗ and Tomás Barretta

aNeuroscience Program, Central Michigan University, Mount Pleasant, MI, USA
bBiochemistry, Cellular & Molecular Biology Graduate Program, Central Michigan University, Mount Pleasant, MI, USA
cDepartment of Psychology, Central Michigan University, Mount Pleasant, MI, USA
dMichigan Alzheimer’s Disease Center, University of Michigan, Ann Arbor, MI, USA

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Abstract.

Background: The presence of cell cycle markers in postmortem Alzheimer’s disease (AD) brains suggest a potential role of cell cycle activation in AD. It was shown that cell cycle activation in postmitotic neurons in mice produces Aβ and tau pathologies from endogenous mouse proteins in the absence of AβPP or tau mutations.

Objective: In this study, we examined the microglial and astrocytic responses in these mice since neuroinflammation is another key pathological feature in AD.

Methods: Our neuronal cell cycle re-entry (NCCR) mouse model are bitransgenic mice heterozygous for both Camk2a-tTA and TRE-SV40T. Using this tet-off system, we triggered NCCR in our animals via neuronal expression of SV40T starting at 1 month of age. TRE-SV40T Tg mice were used as SV40T transgene controls. The animals were examined at following time points: 2, 3, 4, 6, and 12 months of age. The microglia and astrocyte responses in our mice were determined by image analysis and stereology on brain sections immunofluorescently labeled using the following antibodies: Iba1, CD45, CD68, MHCII, and GFAP. Cellular senescent marker p16 was also used in this study.

Results: Our NCCR mice demonstrate early and persistent activation of microglia and astrocytes. Additionally, proinflammatory and senescent microglia phenotype and brain leukocyte infiltration is present at 12 months of age.

Conclusion: In the absence of FAD gene mutations, our NCCR mice simultaneously display many of the pathological changes associated with AD, such as ectopic neuronal cell cycle re-entry, Aβ and tau pathologies, neuroinflammation, and neurodegeneration. These animals represent a promising alternative AD mouse model.

Keywords: Alzheimer’s disease, amyloid-β, cell cycle, leukocyte infiltration, mouse model, neuroinflammation, senescence, sporadic AD, tau

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia and is clinically defined by memory loss and cognitive impairment. The main pathologi-
The hallmarks of AD are amyloid-β (Aβ) plaques, neurofibrillary tangles, and neuroinflammation. Neuroinflammation in AD is characterized by gliosis as well as brain infiltration of leukocytes. The AD cases are categorized as either familial or sporadic, with sporadic form representing 95% of the AD cases. A subset of familial AD (FAD) cases are characterized by disease causing gene mutations in amyloid-β protein precursor (AβPP) and presenilin (PSEN) genes. The discovery of causative familial AβPP and PSEN gene mutations in FAD patients highlighted Aβ as a potential pathogenic driver of AD. Transgenic expression of these gene mutations in mice helped determine early-on the significant role of AβPP and PSEN gene mutations on amyloidogenic process underlying Aβ deposition and plaque formation [1–3]. Despite the prevalence of Aβ plaque pathology in the AβPP mouse models, these mice do not develop tangle-like pathology [4]. Findings from various AβPP mouse models suggest that the Aβ pathology, however severe, is insufficient for generating tangle pathology.

AD is a complex disorder with many different abnormal pathological changes that manifest during the aging process. This poses a significant challenge in identifying pathogenic mechanisms in the context of sporadic AD. Furthermore, the commonly used AD model in the field represents the FAD pathogenic mechanism affecting a subset of the 5% of the AD cases while 95% of the AD cases are sporadic with no underlying genetic cause. The presence of various cell cycle markers in postmortem AD brains suggests a potential role of neuronal cell cycle re-entry as a possible non-genetic pathogenic process in AD [5–15].

The presence of neuronal cell cycle markers in AD prompted us to develop a mouse model for directly testing the pathogenic role of chronic neuronal cell cycle re-entry in AD. Our conditional transgenic mice express SV40 large T antigen (SV40T) regulated by tetracycline response element (TRE) (TRE-SV40T or “TAg” mice) [16]. SV40T is a powerful oncoprotein that activates the cell cycle by perturbing the retinoblastoma protein (pRb) and p53-mediated tumor suppressor pathways [17]. Using the tet-off system, we induced ectopic cell cycle activation in postmitotic neurons via neuronally targeted SV40T expression [16]. This was accomplished by crossing the TAg mice with mice expressing the tetracycline-controlled transactivator (tTA) under the control of CamKinase IIα (Camk2a) promoter (Camk2a-tTA mice or “OFF” mice) [18]. When combined with the tet-off gene expression system, SV40T-mediated aberrant cell cycle can be induced in the resulting neuronal cell cycle re-entry (NCCR) mice by removing doxycycline from the diet post-weaning [16].

Our previously published work demonstrate that SV40T expression in postmitotic neurons increases the brain expression of various cell cycle regulatory proteins and neuronal DNA synthesis [16]. Furthermore, we demonstrated that these mice also produce AD-like amyloid and tau pathologies with aging [16]. Importantly, the Aβ and tau pathologies in these animals were generated from endogenous mouse proteins in the absence of FAD mutations. It has also been demonstrated that SV40T-mediated forced cell cycle re-entry in primary neuron cultures can promote hyperploidy [19] similar to what has been observed in AD and aged brains [10, 20].

Chronic neuroinflammation is thought to be a risk factor for AD [21, 22]. It has been shown that microglia activation is observed in the prodromal and preclinical stages of AD [23] and microglia activation is positively correlated with tau aggregation and amyloid deposition [24]. The role of chronic inflammation in AD is highlighted by identification of APOE4, TREM2, and CD33 as genetic risk factors for sporadic AD [25, 26]. Although these gene mutations by themselves do not produce pathological hallmarks of AD in mice, the presence of these gene mutations modulate Aβ plaque formation and clearance in the AβPP mice [27–30]. Thus, a large body of research suggests a significant role of inflammation process in AD. A network analysis using epigenetic and transcriptomic datasets from AD brains identified a major hub genes that are associated with molecular pathways involved in cell cycle re-entry and inflammation [31]. Others have shown that inducing neuronal cell cycle re-entry in vivo using either c-Myc or c-Abl leads to gliosis and neuronal loss [32, 33]. Here we demonstrate that early and persistent gliosis is also observed in our NCCR mouse model of AD. In comparison to AβPP mouse models and other cell cycle re-entry models using either c-Myc or c-Abl, our SV40T-mediated NCCR mouse model of AD simultaneously display many of the pathological changes associated with AD in the absence of FAD gene mutations. These animals represent an alternative mouse model for sporadic AD.
MATERIALS AND METHODS

Animals

All mice were maintained on a C57BL/6N genetic background. Camk2a-tTA mice are available from the Jackson Laboratory (Bar Harbor, ME). TRE-SV40T mice were previously generated and characterized [16]. Camk2a-tTA mice and TRE-SV40T mice were maintained as heterozygotes in separate colonies. Camk2a-tTA mice were crossed with TRE-SV40T mice to generate bitransgenic mice heterozygous for both Camk2a-tTA and TRE-SV40T (NCCR mouse model). Camk2a-tTAXTRE-SV40T breeding pairs were maintained on doxycycline (Dox) diet. The offspring was also maintained on dox diet until 1 month of age, at which time they were switched to standard (std) diet to induce cell cycle re-entry in postmitotic neurons via SV40T expression. TRE-SV40T litter mate mice were used as controls in this study. All animals were housed in individually ventilated cages under specific-pathogen-free condition and maintained on a 12-h light cycle with access to food and water ad libitum. PCR analysis was performed on genomic DNA isolated from tail tissue collected at the time of weaning and again at the animal’s end point. All animal protocols were approved by the Institutional Animal Care and Use Committee at Central Michigan University.

Antibodies

The following antibodies were used in this study: Iba1 and p16 (ab178846 and ab108349, respectively, abcam, Cambridge, MA, USA); CD68 (MCAA1957GA, Bio-Rad, Hercules, CA, USA); MHCII (#556999, BD Biosciences, San Jose, CA, USA); CD45 (YW62.3, Thermo Fisher Scientific, Waltham, MA, USA); SV40T and PCNA (Pab 101 and PC10, respectively, Santa Cruz Biotechnology, Dallas, TX, USA); GFAP (MAB360) (Millipore, Billerica, MA, USA); Alexa Fluor conjugated goat-anti mouse 488, goat-anti rabbit 594, and goat-anti-rat 594 (Thermo Fisher Scientific). PHF1 antibody (generously provided by Dr. Peter Davies, The Feinstein Institute for Medical Research, Manhasset, NY); 4G8 (aa17-24 Aβ, Biolegend, San Diego, CA); Aβ42 (C42, C-terminal human Aβ42, IBL-America, Minneapolis, MN)

Immunofluorescence

Immunofluorescence was performed as previously reported [34]. The images were captured on a Zeiss AxioCam M2 microscope (Carl Zeiss Inc., Thornwood, NY) using a 20X objective and digitized using the ZEN software (Carl Zeiss Inc.). Confocal images were captured on a 60X objective, using a Nikon Eclipse Ti inverted microscope on a Nikon A1R confocal, using NIS-Elements software (version 5.20.00, Nikon, Melville, NY, USA).

Image analysis using Image J

For neuroinflammation quantification, a total of 20 sites were sampled for each animal and used as independent data points. Image J software was used for quantifying area fraction of GFAP, Iba1, and MHCII immunofluorescent labeled sections [35]. For each immunofluorescence stain, images were converted to 8-bit grey scale, and then thresholded. Images of GFAP immunofluorescent and MHCII immunofluorescent sections were thresholded to minimize background and artifact. Threshold intensity was adjusted similarly for each sample and the area fraction occupied by the GFAP thresholded and MHCII thresholded signal was reported. For the analysis of Iba1 immunofluorescence labeled sections, threshold intensity of each image was adjusted so that just the soma of Iba1 stained cells were included. The area fraction occupied by the Iba1 thresholded signal was reported as Iba1 somatic area. The dimensions of each of the 20× images (region of interest) evaluated were 991.59 μm by 795.58 μm.

Immunohistochemistry

Prior to staining, heat-induced epitope retrieval was done on tissue being probed for SV40T and PCNA. Sections were incubated in 10mM sodium citrate, pH 6.0, in a 95°C water bath for 10 min, then let cool at room temperature (RT) for 20 min. Following this, sections were rinsed in 1X TBS three times for 15 min, followed by permeabilization in a 3% H2O2/0.3% triton-x100/1X TBS solution for 30 min at RT. Following a rinse three times in 1X TBS for 5 min, the sections were blocked in blocking solution for 1 h. Sections were incubated overnight at 4°C on a shaker with primary antibody in the blocking solution. On the following day, the sections were rinsed one time in 0.015% triton-x100/1X TBS for 5 min, then washed in 1X TBS three times for 1 min. Following this, sections were incubated in 1X TBS for 20 min, followed by wash three times in 1X TBS for 1 min. Following this, sections were incubated in 1X TBS for 10 min, followed by wash three times in 1X TBS for 1 min. Following this, sections were incubated in 1X TBS for 10 min, followed by wash three times in 1X TBS for 1 min.
TBS solution followed by two rinses in 1X TBS for 15 min at RT. The sections were incubated with goat anti-mouse biotin conjugated secondary antibody (1:500; Southern Biotechnology, Birmingham, AL, USA) for 2 h at RT. Next, they were rinsed one time in 0.015% triton-x100/1X TBS solution followed by two rinses in 1X TBS for 15 min. Sections were then incubated in VECTASTAIN® Elite® ABC HRP solution (Vector Laboratories, Burlingame, CA, USA) for 1 h. Following a rinse in 1XTBS 4 times for 10 min, sections were Pierce™ DAB Substrate solution (Thermo Fisher Scientific), which was prepared following manufacturer instructions. Tissue incubation times varied depending on the antibody reaction and background development. Finally, tissue was rinses twice in 1XTBS for 10 min and mounted on Super frost Plus microscope slides (Thermo Fisher Scientific) and left to air dry overnight. The next day, slides were incubated in xylene for 2 min and coverslipped using Permount® (Thermo Fisher Scientific). Bright field images were captured on a Zeiss AxioCam M2 microscope (Carl Zeiss Inc.) using a 20X objective and digitized using the ZEN software (Carl Zeiss Inc.).

RESULTS

SV40T expression induces neuronal cell cycle re-entry and early microglia activation

Our previous work demonstrated AD-like Aβ and tau pathologies generated from endogenous mouse proteins in the NCCR mouse model of AD [16]. Examination of the neuropathology in the NCCR mice confirms the presence of Aβ deposits and PHF-1-positive pathological tau accumulation in the 12-month-old NCCR mouse model (Fig. 1A, B). Aβ double-labeling immunofluorescence was done using 4G8 (recognizes aa17–24 of Aβ domain) and C42 (recognizes C-terminal domain of Aβ42) to identify Aβ42. Aβ was detected in the dentate gyrus of 12-month-old NCCR mouse brain sections (Fig. 1A, arrow), but not in 6-month-old NCCR mice and in age-matched TAg control littermates (TRE-SV40T mice) (Fig. 1A). PHF-1 antibody labels pSer396/404 tau, which is associated with paired helical filaments in AD [36]. PHF-1-positive pathological phospho-tau labeled neurons were observed in the dentate gyrus of 12-month-old NCCR mice (Fig. 1B, arrow), but not in 6-month old NCCR mice and in age-matched TAg control littermates (Fig. 1B).

The gliosis in the brains of these animals were not examined in our previously published study [16]. Since the neuronal SV40T expression in NCCR mice is regulated by doxycycline (dox), we used this conditional expression system to determine whether SV40T-mediated neuronal cell cycle re-entry induces astrocyte and microglia activation. Examination of 4-month-old NCCR animals show neuronal expression of SV40T (Fig. 2A) and PCNA (Fig. 2B). SV40T and PCNA expression was not detected in the TAg control mice (Fig. 2A, 2B). The neuronal expression of PCNA in 4-month-old animals following dox removal at 1 month of age suggests persistent neuronal cell cycle activation in the NCCR animals.

Examination of 3-month-old NCCR animals \((n = 3)\) maintained on std diet starting at 1 month of age show chronically induced neuronal SV40T expression and persistent neuronal cell cycle re-entry as demonstrated by proliferating cell nuclear antigen (PCNA) expression (Fig. 2C, a; 2E, a). Iba1 immunofluorescence show rod-like microglia in the hippocampus in the same animals (Fig. 2D, a). Rod-like microglia are activated microglia associated with neurodegenerative and neurological conditions [37]. It has been demonstrated that rod-like microglia is also associated with astrogliosis and chronic neuroinflammation response [38]. When a separate group of NCCR animals are put back on dox diet at 2 months of age (1-month std diet, then 1 month back on dox diet, \(n = 3\)), the SV40T and PCNA expressions are abolished at 3 months of age (Fig. 2C, b; 2E, b). With the halting of the SV40T expression and neuronal cell cycle activation, the rod-like microglia in the hippocampus become morphologically less pronounced (Fig. 2D, b). On the other hand, when the NCCR animals are constantly maintained on dox diet (never put on Std diet, \(n = 3\)), neuronal SV40T and PCNA expressions are chronically suppressed (Fig. 2C, c; 2E, c). Under this naïve condition, the microglia morphology and distribution display typical features of resting microglia observed in TAg control littermates (Fig. 2D, c).

SV40T-mediated neuronal cell cycle re-entry induces neuroinflammation

Neuroinflammation is marked by activated and proliferating microglia and reactive astrocytes that are characterized by increased expression of glial fibrillary acidic protein (GFAP). It has been shown that GFAP immunoreactivity in astrocytes are increased
Fig. 1. NCCR mouse model of AD display Aβ and pathological tau at 12 months of age. A) Immunofluorescence image showing Aβ deposits in the dentate gyrus of 12-month-old animals. Aβ was detected via double immunofluorescence using C42 (c-terminal Aβ42, rabbit antibody) and 4G8 (aa17–24 Aβ, mouse antibody) (arrow). Aβ stain was not detected in 6-month-old NCCR mice and age-matched Tag control mice (TRE-SV40T Tg mice littermates) maintained on the same diet regimen. B) Immunofluorescence labeling shows PHF-1-positive neurons in the dentate gyrus of 12-month-old NCCR mice (arrow). PHF-1 immunolabeling was not detected in 6-month-old NCCR mice and age-matched Tag control mice (TRE-SV40T Tg mice littermates) maintained on the same diet regimen. Number of animals examined; 6 months of age: NCCR (n= 2), TAg control (n= 2); 12 months of age: NCCR (n= 2), TAg control (n= 2).

with neuroinflammatory signals [39]. Furthermore, astrocyte activation is associated with microglia activation [39]. Therefore, we quantified the percent of area covered by GFAP and Iba-1 immunofluorescence as a measure of neuroinflammation in the NCCR mice using Image J [35]. Evaluation of different regions of interest (Fig. 3A) to determine the percent area covered by GFAP immunofluorescence at 4-, 6-, and 12-month-old NCCR and TAg control animals showed significant age effect (Fig. 3B, two-way ANOVA, F(2,162) = 22.6, p < 0.0001), genotype effect (Fig. 3B, two way ANOVA, F(1, 162) = 204.7, p < 0.0001), and interaction effect (Fig. 3B, two-way ANOVA, F(2,162) = 18.82, p < 0.0001). The mean values for NCCR animals at 4-, 6-, and 12-month-old animals show age-dependent increase (1.194% versus 2.076% versus 3.602%), although the increase is statistically significant only at 12 months of age (Fig. 3C). On the other hand, age-dependent increase is not observed in the TAg control animals (Fig. 3C). Therefore, GFAP and Iba-1 immunofluorescence time course data suggest the presence of chronic neuroinflammation in the NCCR mice.

To assess the activation phenotypes of the microglia in these animals at 12 months of age, we co-immunolabeled Iba1-positive microglia with various markers of pro-inflammatory microglia activation. It has been shown that pro-inflammatory microglia express more MHC-II, CD68, or CD45 [40–42]. Therefore, we assessed the microglia activation in 12-month-old animals using the following activation markers: CD45, CD68, or MHCII. A subset of Iba1-labeled microglia showed co-labeling with CD45, or CD68, or MHCII antibodies suggesting activated microglia in these mice (Fig. 4A, arrows). Furthermore, we also observed numerous cells that are strongly immunolabeled for CD45 but were not Iba1-positive (Fig. 4A, arrowheads). CD45 is a pan-leukocyte marker, and the presence
of these CD45-labeled cells are indicative of brain leukocyte infiltration in the NCCR mice [43]. We also stained the brain sections using p16 antibody. p16 is a tumor suppressor protein and a marker for senescence [44]. We hypothesized that the lack of mitosis in neurons despite chronic cell cycle activation would mimic a state of senescence and demonstrate increased p16 expression in neurons. Our DAB immunohistochemistry showed microglia-like stains (data not shown). Therefore, we performed immunofluorescence co-labeling on the brain sections using Iba-1 and p16, which helped identify a subset of microglia in the 12-month-old animals that show p16 expression (Fig. 4B, arrows). These p16-labeled microglia also displayed blebbing, a morphological hallmark of senescent microglia (Fig. 4B, arrows) [45]. Proinflammatory microglia in the 12-month old NCCR mice were quantified by measuring the percent of area covered by MHCII immunofluorescence. The MHCII fluorescence in the brain was restricted to microglia in the 12-month-old NCCR brains. MHCII-positive areas were 770% greater in the NCCR mice compared to TAg control mice at 12 months of age (Fig. 4C, unpaired...


**DISCUSSION**

The NCCR mice show Aβ deposits and pathological tau accumulation generated from endogenous mouse proteins in the absence of either AβPP or tau mutations. Furthermore, the NCCR mouse model simultaneously displays a number of pathological features that mirror the main pathological hallmarks of AD: Aβ and tau pathologies, neuroinflammation, and brain leukocyte infiltration. Chronic expression of SV40T induces persistent neuronal cell cycle activation and gliosis at all ages examined (2, 3, 4, 6, and 12 months of age). Aβ and tau pathologies, microglia activation and senescence, and brain leukocyte infiltration are observed around 12 months of age (Fig. 5).

The simultaneous manifestation of these AD-related pathologies in the absence of FAD gene mutations highlights the usefulness of the model for studying AD in the context of sporadic AD, also referred to as late-onset AD.

The Aβ and tau pathologies in the NCCR mouse model are milder compared to either Aβ or tau pathologies in AβPP mouse models or mutant tau mouse models, respectively. This is likely due to the differences in AβPP and tau proteins between humans and mice. It has been shown that the difference in the three amino acid sequences between human and mouse Aβ makes human Aβ more pathological and prone to aggregation [46–50]. Adult mouse and human tau proteins are also different in amino acid sequences, resulting in differences in tau post-translational modification [51]. Additionally, adult mouse brains primarily express 4 microtubule binding repeat domain (4R) tau isoforms whereas adult human brains express 3R and 4R tau isoforms [52–56]. Tau inclusions in different tauopathies are
composed of different tau isoforms. For example, FTD is a 4R tauopathy where the tau inclusions are composed of 4R tau [57]. On the other hand, tau inclusions in Alzheimer’s disease are composed of 3R and 4R tau [57].

It is currently unclear what physiological events are mediating neuroinflammation in the NCCR mouse model. In addition to its ability to induce genes that modulate cell cycle activation, DNA repair, transcription, and chromatin structure [17], it has been shown that SV40T expression is sufficient to induce a panel of interferon stimulated genes in mouse embryonic fibroblasts [58]. A recent study has demonstrated that type I interferon response is present in AD brain samples and this response can drive neuroinflammation in various FAD APP mouse models [59]. Therefore, further study needs to be carried out to tease apart the roles of SV40T and neuronal cell cycle activation on neuroinflammation in the NCCR mice.

Although neuroinflammation is thought to be a risk factor in AD, acute neuroinflammation is thought to be neuroprotective while chronic neuroinflammation is thought to be detrimental [25, 60]. Studies using the FAD mouse models demonstrates that short-term (28 days) ablation of microglia using CSF-1R inhibitor in either APP/PS1 or 5xFAD mice did not reduce Aβ plaque load [61, 62]. On the other hand, longer-term (3 months) inhibition of microglia function either via CSF-1R inhibition mediated microglia ablation or NLRP3 inflammasome inhibition reduced Aβ plaque load in 5×FAD and APP/PS1 mice, respectively [62, 63]. Therefore, chronic neuroinflammation may help promote Aβ pathology in the context of FAD. However, the lack of tau pathology in FAD mouse models poses a challenge for studying the role of chronic inflammation on AD tau pathology, which is different from those of primary tauopathies such as frontal temporal dementia. It has been shown that microglia activation is observed in the prodromal and preclinical stages of AD [23] and microglia activation is positively correlated with tau aggregation and amyloid deposition [24]. However, the role of chronic inflammation in the context of sporadic AD is unclear. The presence of early and persistent gliosis that precede Aβ deposition and tau aggregation in the NCCR mice will allow us to examine the role of chronic neuroinflammation on Aβ and tau pathologies in the context of sporadic AD in our future studies.

Additionally, in order to increase the relevance of AD pathophysiological processes in the NCCR mice, we have humanized the Aβ domain of the endoge-
 nous mouse AβPP using the App knock-in mice [50]. Separately, we have also humanized the endogenous Mapt using the humanized Mapt knock-in mice, which express 6 human tau isoforms composed of 3R and 4R tau and represent a more physiologically relevant model for pathological tau modifications in humans [64]. These animals are currently being evaluated in our laboratory. The goal is to simultaneously humanize Aβ and tau in the NCCR mice, which are expected to develop age-dependent tau pathology formed from wild type human tau without seeding while simultaneously displaying other pathological hallmarks of AD such as ectopic neuronal cell cycle re-entry, humanized Aβ plaques, gliosis, neuroinflammation, and neurodegeneration. The NCCR mice represent a promising alternative AD mouse model for studying the interactions between crucial factors in AD and testing potential therapeutics in the context of sporadic AD.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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