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Retinal macroglia changes in a triple transgenic mouse model of Alzheimer's disease

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ABSTRACT

The retinas of Alzheimer's disease (AD) patients and transgenic AD animal models display amyloid beta deposits and degeneration of ganglion cells. Little is known, however, about the glial changes in the AD retina. The present study used a triple transgenic mouse model (3xTG-AD), which carries mutated human amyloid precursor protein, tau, and presenilin 1 genes and closely mimics the human brain pathology, to investigate retinal glial changes in AD. AD cognitive symptoms are known to begin in the 3xTG-AD mice at four months of age but plaques and tangles are not seen until six to twelve months. Müller cells in 3xTG-AD animals were GFAP-positive, indicating activation, at the earliest time point investigated, nine months. Astrocyte activation was also suggested in the 3xTG-AD mice by an apparent increase in size and process number. Another glial marker, S100, was expressed by astrocytes in both the non-transgenic (NTG) controls and 3xTG-AD retinas. Labeling was predominantly nuclear in nine month non-transgenic (NTG) control mice but was also seen in the cytoplasm and processes at 18 months of age. Interestingly, the nuclear localization was not as prominent in the 3xTG-AD retina even at nine months with labeling observed in astrocyte processes. The diffusion of S100 suggests the possible secretion of this protein, as is seen in the brain, with age and, more profoundly, associated with AD. Several dense, abnormally shaped, opaque structures were noted in all 3xTG-AD mice investigated. These structures, which were enveloped by GFAP and S100-positive astrocytes and Müller cells, were positive for amyloid beta, suggesting that they are amyloid plaques. Staining control retinas with amyloid showed similar structures in 30% of NTG animals but these were fewer in number and not associated with glial activation. The results herein indicate retinal glia activation in the 3xTG-AD mouse retina.

1. Introduction

Astroglial cells in Alzheimer's disease (AD) undergo complex morphological and functional changes that may contribute to the evolution of this disease (Rodriguez and Verkhratsky, 2011; Rodriguez et al., 2009b; Verkhratsky et al., 2010, 2012). Astrocytes in the AD brain have increased expression of GFAP (Beach and McGeer, 1988), S100B (Marshak et al., 1992), and heme oxygenase 1 (Schipper et al., 2006), while glutamine synthetase is reduced (Robinson, 2001). These changes demonstrate not only the activation of astrocytes but also functional changes. In AD brains, S100B expression is increased in correlation with neuritic plaque density (Mrak et al., 1996; Sheng et al., 1994). Similarly, GFAP expression increases with disease severity (Wharton et al., 2009). This timeline suggests that the astrocyte changes are not simply a response to increases in amyloid beta. Given the dependency of neurons on astrocytes for normal functioning, it is easy to speculate that disruptions to normal astrocyte metabolism or protein expression would affect neurons as well (Steele and Robinson, 2012).

As an extension of the central nervous system, the retina is not spared in AD with patients experiencing optic nerve and ganglion cell degeneration (Blanks et al., 1989, 1996a, 1996b; Hedges et al., 1996; Hinton et al., 1986). In addition, an increase in the ratio of astrocytes to neurons has been noted, although it remains unclear

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whether this was due to an increase in astrocytes or a reduction of neurons (Blanks et al., 1996b). While some groups have reported amyloid beta plaques in the retina of AD patients (Koronyo-Hamaoui et al., 2011; Tsai et al., 2014), others have not found plaques (Blanks et al., 1989; Ho et al., 2013). Retinal degeneration and amyloid deposition have also been reported in single and double transgenic mouse models of AD, primarily in the ganglion cell and inner nuclear layers (Liu et al., 2009; Ning et al., 2008; Perez et al., 2009; Shimazawa et al., 2008). Amyloid plaques were also observed in the plexiform layers, outer nuclear layer, and choroid of a rat transgenic AD model (Tsai et al., 2014). Recently, senile plaques in the retina were detected in $APP_{SWE}/PS1_{\Delta E9}$ mice *in vivo*, providing a potential diagnostic tool for AD (Koronyo-Hamaoui et al., 2011). Importantly, in that study, retinal Aβ deposits appeared earlier than those in the brain, suggesting that retinal damage may be an early AD biomarker. Glial changes have also been noted in the retinas of AD patients (Blanks et al., 1996a).

Assuming retinal changes reflect those in the brain, investigation of retina represents a novel, less invasive portal for studying the AD pathology. The retina has the advantage of being readily accessible photographically for *in vivo* diagnosis. In addition, the retina of mouse models can be examined in its entirety *in vivo* or in flatmount postmortem preparations instead of cross sectional analysis needed for the brain.

Here, we extend previous research to investigate changes in retinal glia in a 3xTG-AD mouse model that mimics progression of human AD pathology (Oddo et al., 2003b; Shimazawa et al., 2008). The 3xTG-AD mice, which carry mutated human amyloid precursor protein, tau, and presenilin 1, demonstrate numerous functional impairments including reduced long term potentiation, altered spatial memory and deficient long-term memory (Oddo et al., 2003a, 2003b). These transgenic mice also show some neuronal loss accompanied by loss of spines on dystrophic dendrites (Bittner et al., 2010). The 3xTG-AD mouse mimics AD pathology more closely than other transgenic AD models (Olabarria et al., 2010).

2. Materials and methods

2.1. Animal generation and care

The 3xTG-AD mice and non-transgenic background-matching controls (NTG) were bred and housed at IKERBASQUE in Bilbao, Spain as previously described (Olabarria et al., 2010; Rodriguez et al., 2008). All animals were used according to ARVO guidelines. Tails from both 3xTG-AD and NTG mice were sent to *Transnetyx* to test for the presence of the retinal degeneration 8 (rd8) mutation in *Crb1*. All tails analyzed were negative for this mutation.

2.2. Tissue collection

All mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and perfused with 3.75% acrolein/2% PFA followed by 2% PFA alone as previously described (Olabarria et al., 2010). After perfusion, eyes were enucleated and either washed in phosphate buffer and retinas then dissected or stored in cryopreservation buffer (25% sucrose and 3.5% glycerol in 0.05 M PB at ph 7.4). Tissue was collected from NTG or C57BL/6J controls and 3xTG-AD mice from 9 to 24 months of age (M). A minimum of three control and 3xTG-AD mice were analyzed for each age group.

2.3. Cryopreservation

Whole eyes and fluorescently labeled flatmount retinas were cryopreserved as previously described (Edwards et al., 2011). Briefly, tissues were exposed to increasing concentrations of sucrose, from 5 to 20%, then incubated in 20% sucrose for 2 h, and placed in biopsy molds containing a 2:1 ratio of 20% sucrose to Optimal Cutting Temperature (OCT; Tissuetek/Sakura, Torrance, CA) solution for 30 min. After infiltration, blocks were frozen in isopentane and dry ice. Finally, 8 μm sections were cut using a *Leica* cryostat (Wetzlar, Germany).

2.4. Immunohistochemistry

Flatmounts were used to investigate the GFAP and S100α/β (herein referred to as S100) expression in the retinas of 3xTG-AD and NTG or C57BL/6J mice from 9 to 24 M. Flatmount retinas were blocked in 5% goat serum in Tris-buffered saline (TBS) containing 0.1% BSA and 0.1% Triton ×100 (TBS-T/BSA) for 6 h at 4 °C. Retinas were incubated in a cocktail containing rabbit-anti-GFAP (1:200; Dako, Carpinteria, CA USA) and mouse anti-S100 (1:200; Santa Cruz, Dallas, TX USA) or mouse anti-amyloid beta (1:100; Covance) prepared in 2% goat serum in TBS-T/BSA for 24 h at 4 °C. Following washes, retinas were incubated in fluorescent conjugated secondary antibodies (1:300; goat anti-rabbit cy3 or cy5, goat anti-mouse cy3; Jackson Immunoresearch, West Grove, PA USA), prepared in TBS-T containing $CaCl_2$ for 24 h at 4 °C. Along with secondary antibodies, isolectin from *Griffonia simplicifolia* (GS isolectin 1:100; Invitrogen, USA) was applied to label retinal vessels. Immunohistochemistry was performed on 8 μm cryosections as previously described (Edwards et al., 2011). In addition to the primary antibodies used for flatmount immunohistochemistry, mouse anti-glutamine synthetase (1:1000; Millipore) was used to label cryosections. Images were taken using a *Zeiss* 710 Meta confocal microscope equipped with *Zen* software (Carl Zeiss, Jena, Germany). A minimum of three mice from each group was analyzed for each antibody.

2.5. Counting of GFAP-positive Müller cells

Müller cells are normally GFAP-negative or express very low levels of this protein (Sarthy et al., 1991) but express this protein upon activation (Eisenfeld et al., 1984; Fisher and Lewis, 2003; Sarthy and Egal, 1995). When imaging a flatmount retina, GFAP-positive Müller cell processes can be seen aligning with the ganglion cell nerve fibers. When imaged at the base of the superficial retinal vessels or below, GFAP-positive Müller cell processes are visible as dots of fluorescence seen throughout the depth of the retina. In order to assess the number of GFAP-positive Müller cell processes (punctate dots), individual images from 20× confocal Z-stacks at the base of the primary retinal vasculature where astrocytes are not in focus (ex. Fig. 1D, H, L, P), were collected from both NTG and 3xTG-AD retinas. Images were opened in Image J software (National Institute of Health, Bethesda, MD USA) and channels split to isolate the GFAP channel. The threshold was then adjusted to accurately represent the number of GFAP-positive Müller cell processes and the image made binary. The “analyze particle” tool with the particle size set at 10–100 and 0–1 circularity was used to count cells. The total number of GFAP-positive cell processes per 20× image was plotted and unpaired *T*-tests used to compare NTG and 3xTG-AD retinas at 9 M and 18–24 M. Images were counted from three animals per group.

3. Results

3.1. Glial activation is noted at 9 M of age in the 3xTG-AD retinas

At 9 M, flatmount analysis revealed astrocytes labeled with GFAP and S100 in both NTG and 3xTG-AD retinas (Fig. 1). The S100 observed in astrocytes of the NTG retinas at 9 M was most intense