Do the retina and brain share similar molecular and cellular pathological footprints? Insights from an Alzheimer's disease animal model

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Research

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Abstract

Background: The concept 'the retina as a window to the brain' in Alzheimer's disease (AD) has been explored in recent years since patients sometimes present visual alterations before the first symptoms of dementia. The retina is an extension of the brain and can be assessed by non-invasive methods. However, assessing the retina for AD diagnosis is still a matter of debate. Using the triple transgenic mouse model of AD (3xTg-AD), this study was undertaken to investigate whether the retina and brain undergo similar molecular and cellular changes during the early stages of AD pathology, and if the retina could anticipate the pathological alterations occurring in the brain.

Methods: We used the 3xTg-AD and wild-type mice (C57BL6/129S), at 4 and 8 months of age, and assessed several parameters in the retina and brain (hippocampus and cortex): amyloid-beta ($A\beta$) and hyperphosphorylated tau (p-tau) levels, barrier permeability, cell death, neurotransmitter levels and glial changes.

Results: We detected increased $A\beta$ levels in the hippocampus and cortex and increased p-tau in the hippocampus, retina and cortex of 3xTg-AD mice. The brain and retinal barriers were unaffected. At 4 months, the content of some synaptic proteins increased in the brain but not in the retina. No cell death, including retinal ganglion cells loss, was detected in 3xTg-AD mice. Overall, no changes were observed in glutamate and GABA levels in all regions. There was an increase in astrogliosis in the hippocampus at 4 months and a decrease in the retina at 8 months. No changes were detected in Müller cells reactivity. Furthermore, we did not find changes in the number of microglia in 3xTg-AD mice, but we detected a different profile in microglia branching in the hippocampus and retina, at 4 months, where the number and length of the processes increased in the hippocampus and decreased in the retina.

Conclusions: At the early stages of pathology, the retina, hippocampus and cortex of 3xTg-AD are not significantly affected, but already present some molecular and cellular alterations. The retina did not mirror the changes detected in the brain in this animal model of familial AD, and these observations should be taking into account when using the retina as a potential diagnostic tool for AD.

Background

The retina and the brain have the same embryonic origin. The retina is part of the central nervous system (CNS) and is considered an extension of the brain being connected to the brain by the optic nerve. The concept of 'the retina as a window to the brain' has been increasingly explored in several brain neurodegenerative diseases, including Alzheimer's disease (AD). The potential use of the retina as a diagnostic tool has some advantages. We can assess the retina by non-invasive and inexpensive methods. Moreover, the retinal layers present an unique organization that easily enables the detection of structural changes (1).

Visual problems have been detected in some AD patients prior to the appearance of the first signs of dementia (2). However, the precise molecular, cellular and functional changes in the retina of AD patients

are still unclear and not completely elucidated yet (reviewed in (3)). The retinas of AD patients present increased glial reactivity (4–6) and cell death (7–10) (post-mortem analysis). However, the presence of AD hallmarks in the retina of AD patients is controversial, with some claiming their presence and others demonstrating their absence (11–14). Concordantly, in AD animal models there are also controversial findings and missing gaps that need to be filled in, such as those related with glial reactivity, cell death and blood brain barrier (BBB) and blood retinal barrier (BRB) permeability. There is a lack of studies assessing simultaneously the brain and retina at the early stages of the disease, in order to understand whether the pathology impacts the brain and retina with the same extension and also to clarify which region is firstly affected.

We recently described structural and functional changes in the brain and retina of the triple transgenic mouse model of AD (3xTg-AD) (15, 16), a well characterized model that develops both senile plaques and neurofibrillary tangles in a progressive and age-dependent manner (17). We reported that 3xTg-AD mice, as early as 4 and 8 months of age, already present impaired locomotion activity and recognition memory as well as a decrease of hippocampal volume and taurine levels (15). In the retina, at these early time points, 3xTg-AD mice present retinal thinning accompanied by an atrophy of the visual cortex (16). Thus, in the present study we aimed to disentangle the molecular and cellular changes that might occur at the early stages of the pathology, which may underlie the structural and functional changes described previously, both in the retina and brain of the 3xTg-AD mouse model (16), as well as to evaluate whether the molecular and cellular changes are similar in the retina and brain and if the retina can anticipate the changes observed in the AD brain. To achieve our goals, we assessed several molecular and cellular parameters, simultaneously in the retina and brain of 3xTg-AD mice, at the early stages of the pathology (4 and 8 months of age).

Material And Methods

Animals

Experiments were performed in 3xTg-AD mice harboring three human mutated genes (PS1M146V, APPSWE and TauP301L) (17) and in age-matched Wild Type (WT) animals (with C57BL6/129S genetic background), at 4 and 8 months of age. All animals were maintained under controlled light (12 h day/night cycle), relative humidity (68%), and temperature (21.8 ± 2 °C), with access to food and water ad libitum. All experiments using animals were approved by the Animal Welfare (Órgão Responsável pelo Bem-Estar Animal - ORBEA 16/2015) of the Coimbra Institute for Clinical and Biomedical research (iCBR), Faculty of Medicine, University of Coimbra, and by Direção Geral de Alimentação e Veterinária (DGAV 0421/000/000/2015) and conducted in accordance with the European Community directive guidelines for the use of animals in laboratory (2010/63/EU) transposed to the Portuguese law in 2013 (Decreto-Lei 113/2013), and in agreement with the Association for Research in Vision and Ophthalmology statement for animal use.

Tissue homogenization

The hippocampus, cortex and retina of 3xTg-AD and age-matched WT mice were dissected in ice-cold Hank's balanced salt solution (HBSS: 137 mM NaCl, 5.4 mM KCl, 0.45 mM KH_2PO_4 , 0.34 mM Na_2HPO_4 , 4 mM, NaHCO_3 , 5 mM glucose; pH 7.4), and then frozen in liquid nitrogen and stored at -80 °C, until further use.

i) Standard homogenization protocol

Samples were lysed in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with cOmplete™ Mini, ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail tablets (Roche, Basel, Switzerland), PhosSTOP™ phosphatase inhibitor tablets (Roche, Basel, Switzerland), 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Roche, Basel, Switzerland) and 2 mM of dithiothreitol (DTT; Fisher, Hampton, NH, EUA). The hippocampus and cortex were homogenized, frozen in liquid nitrogen, and thawed three times. The retinas were sonicated with 3 pulses of 2 s each. Samples were then centrifuged at 16 100 x g during 10 min, at 4 °C, and the supernatant was collected. After protein quantification using the bicinchoninic acid assay (BCA assay) (Pierce Biotechnology, Rockford, IL, USA), samples were denatured by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% sodium dodecyl sulfate (SDS), 0.6 M DTT, 0.012% bromophenol blue), and heating for 5 min, at 95 °C.

ii) Specific homogenization protocol for A β detection in the retina

For A β detection, the retinas were lysed as previously described (18). Briefly, retinas were lysed in tissue homogenization buffer (THB: 2 mM Tris, 250 mM sucrose, 0.5 mM EDTA, 0.5 mM Ethylene Glycol Tetraacetic Acid (EGTA)) supplemented with cOmplete™ Mini and sonicated with 10 pulses of 1 s each. Then, 0.4% diethylamine (DEA) solution (0.4% DEA in 100 mM NaCl) was added to the homogenate in the proportion 1:1. Samples were centrifuged at 128 000 x g during 1 h, at 4 °C, and the supernatant was collected and neutralized with 0.5 Tris-HCl (pH 6.8). After protein quantification (BCA assay), samples were denatured by adding 6x concentrated sample buffer and heating for 5 min, at 95 °C.

Western blotting

Samples were separated in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred (750 mV, 1h30) onto polyvinylidene difluoride (PVDF) membrane (Immobilon®-P, Merck Millipore, Cork, Ireland), as previously described (19). Then, the membranes were blocked in 5% bovine serum albumin (BSA), or 5% skim milk, and incubated overnight at 4 °C with primary antibody diluted in 5% BSA or 1% skim milk. After rinsed in Tris-buffered saline buffer (137 mM NaCl, 20 mM Tris-HCl; pH 7.6) containing 0.1% Tween-20 (TBS-T), the membranes were incubated during 1 h at room temperature (RT) in Horseradish Peroxidase (HRP)- or Alkaline Phosphatase (AP)-conjugated secondary antibodies diluted in 5% BSA or 1% skim milk (Table 1). Immunoreactive bands were visualized using Clarity™ Western enhance chemiluminescence (ECL) Substrate (Bio-Rad, Hercules, CA, USA) in Image Quant LAS 500 (GE Healthcare, Chicago, IL, USA), or enhanced chemifluorescence (ECF) substrate (ECF™ substrate; GE Healthcare, Chicago, IL, USA) in Thyphoon FLA 9000 (GE Healthcare, Chicago, IL, USA). Digital quantification of band intensity was performed using Quantity One software (Bio-Rad, Hercules, CA, USA).

The stripped membranes were then reprobed and tested for β -actin immunoreactivity to confirm that similar amounts of protein were used in each lane. Values were obtained by calculating the ratio between the target protein band intensity and β -actin band intensity. Values are presented as percentage of age-matched WT mice.

Table 1
Primary and secondary antibodies used for Western blotting

	Antibody	Host	Dilution	Protein (µg)	Supplier
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	Antibody	Host	Dilution	Protein (µg)	Supplier
	Antibody	Host	Dilution	Protein (µg)	Supplier
Primary Antibodies	Anti-APP	Rabbit	1:1000	20	Sigma-Aldrich
	Anti-Aβ (D54D2)	Rabbit	1:1000	20	Cell Signaling Technology
	Anti-p-tau ^(ser396)	Rabbit	1:1000	10	Santa Cruz Biotechnology
	Anti-BACE	Rabbit	1:1000	20	Cell Signaling
	Anti-Claudin-5	Rabbit	1:250	20	Invitrogen
	Anti-Occludin	Rabbit	1:250	20	Invitrogen
	Anti-ZO-1	Rabbit	1:250	40	Invitrogen
	Anti-ChAT	Goat	1:1000	20	Millipore
	Anti-Syntaxin 1	Mouse	1:5000	10	SYSY
	Anti-Synaptophysin	Mouse	1:1000	10	Sigma-Aldrich
	Anti-Synapsin 1	Mouse	1:40000	10–20	SYSY
	Anti-PSD95	Rabbit	1:5000	10–20	Cell signaling
	Anti-GFAP	Mouse	1:5000	20	Millipore
	Anti-Vimentin	Rabbit	1:1000	15	Abcam
	Anti-β-Actin	Mouse	1:5000	-	Sigma-Aldrich
	Anti-β-Actin	Rabbit	1:5000	-	Sigma-Aldrich
Secondary Antibodies	HRP Anti-Rabbit		1:10000	-	Bio-Rad
	HRP Anti-Mouse		1:10000	-	Bio-Rad
	HRP Anti-Goat		1:10000	-	Invitrogen

Antibody	Host	Dilution	Protein (μ g)	Supplier
AP Anti-Mouse		1:10000	-	Sigma-Aldrich
AP Anti-Rabbit		1:10000	-	Sigma-Aldrich

APP: amyloid precursor protein; A β : amyloid beta; p-tau: phosphorylated tau protein; BACE: beta secretase 1; ZO-1: zonula occludens-1; ChAT: Choline acetyltransferase; PSD95: postsynaptic density protein 95; GFAP: Glial fibrillary acidic protein; AP: alkaline phosphatase; HRP: horseradish peroxidase.

Tissue preparation

Animals were anaesthetized using a combination of ketamine (90 mg/kg) (Imalgene 1000, Merial, Lyon, France) and xylazine (10 mg/kg) (Rompum®, Bayer, Leverkusen, Germany), administered with intraperitoneal injection, and then were intracardially perfused with 0.1 M phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 KH₂PO₄; pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS.

The brain was collected, post-fixed in PFA solution overnight, and then dehydrated in 30% sucrose solution in PBS. The samples were frozen in dry ice and stored at -80 °C until further use. Coronal sections (30 μ m-thick) were obtained using a cryostat (Leica CM3050S, Leica Biosystems, Nussloch, Germany), and stored in cryoprotectant solution (50 mM phosphate buffer; pH 7.2; 48.7 mM NaH₂PO₄·H₂O, 56.2 mM KHPO₄, 30% sucrose, 30% ethylene glycol) at 4 °C, until further use.

The enucleated eyes were fixed in PFA solution for 2 h at RT, and dehydrated in 15% sucrose solution for 1 h, followed by 30% sucrose solution overnight. The samples were then embedded in a 1:1 30% sucrose and embedding resin (Shandon™ Cryomatrix™, Thermo Fisher Scientific, Waltham, MA, USA) solution, for 15 min, before freezing in dry ice. The samples were stored at -80 °C, until further use. Retinal sections (16 μ m-thick) were obtained using a cryostat, and mounted on adhesive slides (Superfrost Plus™, Thermo Fisher Scientific, Waltham, MA, USA). The sections were air-dried at RT and stored at -20 °C, until further use.

Immunofluorescence

i) Brain cryosections

Free-floating brain sections located at the stereotaxic coordinates of intraneural + 1.98 mm and - 1.82 mm from bregma (20) were blocked in 5% BSA and 0.1% Triton X-100 in PBS, during 2 h at RT, and then incubated with the primary antibody, anti-ionized calcium-binding adapter molecule 1 (Iba-1; 1:1000; Wako Chemicals, North Chesterfield, VA, USA) diluted in blocking solution for 48 h at 4 °C. After washing in PBS, the slices were incubated with the secondary antibody, Alexa Fluor® 488 goat anti-rabbit,

Invitrogen™ (1:1000; Thermo Fisher Scientific, Waltham, MA, USA) diluted in blocking solution for 2 h at RT, and then with 1:5000 4',6-diamidino-2-phenylindole (DAPI, Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA), during 10 min at RT. After washing, the slices were mounted using mounting medium (Glycergel, Dako, Carpinteria, CA, USA), and kept at 4 °C until being visualized.

For counting cells and 3D reconstructions, z-stack images were acquired with a confocal microscope (LSM 710 Axio Observer, Zeiss, Oberkochen, Germany), using EC Plan-Neofluar 40x/ 1.30 oil Dic M27 and Plan-Apochromat 63x/ 1.40 Oil Dic M27 objective, respectively.

ii) Retina cryosections

Retina cryosections were fixed in ice-cold acetone at -20 °C for 10 min, followed by rehydration in PBS. The slices were permeabilized in 0.25% Triton X-100 in PBS for 30 min, and blocked in 10% normal goat serum and 1% BSA in PBS, for 30 min at RT. The slices were then incubated overnight at 4 °C with the primary antibodies (Table 2) diluted in 1% BSA in PBS. After washing, the slices were incubated with the secondary antibodies (Table 2) and DAPI (1:5000), for 1 h at RT, and then mounted with mounting medium and kept at 4 °C until being visualized.

Table 2
Primary and secondary antibodies used for immunofluorescence

	Antibody	Host	Dilution	Supplier
Primary Antibodies	Anti-Brn3a	Mouse	1:500	Millipore
	Anti-Iba-1	Rabbit	1:1000	Wako
	Anti-Vimentin	Rabbit	1:500	Abcam
Secondary Antibodies	Alexa Fluor® 568 Goat Anti-Mouse IgG1		1:500	Invitrogen
	Alexa Fluor® 488 Goat Anti-Rabbit (H + L)		1:500	Invitrogen
Brn3a: brain-specific homeobox/POU domain protein 3A; Iba1: calcium-binding adapter molecule 1.				

iii) Retina whole mounts

Retina whole mounts were blocked in 0.1% Triton X-100, 3% BSA and 10% goat serum in PBS for 1 h at RT, and then incubated with the primary antibody (anti-Iba-1; 1:500; FUJIFILM Wako Chemicals, Richmond, VA, EUA), for 72 h at 4 °C. After washing, the retina whole mounts were incubated with the secondary antibody (Alexa Fluor® 488 Goat Anti-Rabbit; 1:500; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4 °C, and then washed again and incubated with DAPI (1:1000). Upon rising with PBS, the retinas were mounted using fluorescence mounting medium. Z-stack images were acquired using a confocal microscope (LSM 710, Zeiss, Oberkochen, Germany), equipped with a Plan-Apochromat 63x/ 1.40 Oil Dic M27 objective.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay

Apoptotic cells were assessed in the retina and brain cryosections using DeadEnd™ Fluorometric TUNEL System following the manufacturer's instructions (Promega, Madison, WI, USA). The nuclei were stained with DAPI (1:5000). A positive control was also performed using DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to cause DNA fragmentation.

High-performance liquid chromatography (HPLC)

The hippocampus, cortex and retina of 3xTg-AD and age-matched WT mice were homogenized in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate (DOC) and 0.1% SDS in PBS (pH 7.4), supplemented with complete mini EDTA-free protease inhibitor cocktail tablets (Roche, Basel, Switzerland), PhosSTOP™ phosphatase inhibitor tables (Roche, Basel, Switzerland), 0.1 mM PMSF (Roche, Basel, Switzerland) and 2 mM DTT (Fisher, Hampton, NH, EUA). Samples were frozen three times in liquid nitrogen, and centrifuged at 13 200 x g for 10 min at 4 °C, before the collection of the supernatants. After protein quantification, the levels of gamma-aminobutyric acid (GABA) and glutamate were measured by separation in a reverse-phase high-performance liquid chromatography (HPLC). Briefly, amino acids were separated by a reverse-phase GilsonASTED HPLC system, composed of a Spherisorb ODS column (particle size: 5 μ m; 150 mm long; 4.6 mm) at 25 °C, and a Gilson model 121 fluorescence detector set at excitation and emission wavelengths of 340 nm and 410 nm, respectively. A linear gradient elution program was applied for amino acid elution: eluent A (30 mmol/l sodium acetate buffer; pH 6.8) from 100–50%, and eluent B (methanol) from 0–50%, with a flow rate of 2.5 ml/min. The integration of the amino acid peak area and further calculations were carried out by the Gilson system software. The amino acid quantification was carried out using standard amino acids solutions under the same conditions, and normalized for protein levels.

3-D Neurolucida Software

Tridimensional morphometric analysis of microglial cells in retina whole mounts and brain slices was performed by manual reconstruction using the Neurolucida software (MBF Bioscience, Williston, VT, USA), as previously described (21). The morphometric data (number and length of processes) were extracted through Neurolucida explorer. Microglial cells (8–10 cells) were drawn per animal and per condition.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). The assessment of normality of the values was performed using the Shapiro-Wilk test. Values normally distributed were analyzed using the parametric Student's t-test; values that failed the assumption of normality were analyzed using the nonparametric Mann-Whitney U test. Differences were considered significant for $p < 0.05$. Values were presented as mean $\pm$ SEM.

Results

Since the retina and brain share the same embryonic origin, the concept 'The retina as a window to the brain' in neurodegenerative diseases has been explored. Here, we investigated which molecular and cellular changes can be detected in the retina and brain of the 3xTg-AD mouse model. Moreover, we assessed if those changes show a similar profile in the retina and brain and if some changes are firstly detected in the retina. Since we are particularly interested in identifying the initial changes that might occur in the retina and brain, we focused only in the early stages of the pathology.

Assessment of the molecular hallmarks of AD in the retina and brain of 3xTg-AD mice

Amyloid precursor protein (APP), A β , p-tau and beta-secretase (BACE) protein expression levels were assessed by Western blotting in the hippocampus, cortex and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age (Fig. 1).

The APP levels in 3xTg-AD mice were significantly higher in the hippocampus, at 8 months ($218.0 \pm 33.0\%$ of WT, $p < 0.01$), whereas in the cortex and retina of 3xTg-AD mice no changes were found at 4 and 8 months of age. Concerning A β , significantly higher protein levels were detected in the hippocampus of 3xTg-AD mice, at both 4 and 8 months of age ($439.5 \pm 79.8\%$ of WT, $p < 0.001$; and $614.1 \pm 163.6\%$ of WT, $p < 0.05$, respectively). Likewise, in the cortex of 3xTg-AD mice, A β expression levels were significantly higher, at 4 and 8 months of age ($514.5 \pm 128.6\%$ of WT, $p < 0.01$; and $5649.7 \pm 848.5\%$ of WT, $p < 0.01$, respectively). Conversely, in the retina of 3xTg-AD mice no changes were detected in A β levels, neither at 4 nor at 8 months. The p-tau protein levels were significantly increased in the hippocampus of 3xTg-AD mice, at 4 and 8 months of age ($328.8 \pm 88.1\%$ of WT, $p < 0.01$; and $259.6 \pm 37.1\%$ of WT, $p < 0.01$, respectively), and in the cortex and retina of 3xTg-AD mice, but only at 4 months (cortex: $198.6 \pm 31.3\%$ of WT, $p < 0.01$; retina: $162.1 \pm 38.8\%$ of WT, $p < 0.01$). At 8 months of age, the p-tau levels in the retina of 3xTg-AD mice remained increased ($162.1 \pm 38.8\%$), but without reaching statistical significance, whereas in the cortex p-tau protein levels returned to values similar to the control. Regarding BACE expression levels, no statistically significant differences were determined in all regions analyzed at both time points.

Assessment of blood-retinal and brain barrier integrity of 3xTg-AD mice

It has been reported an impairment of BBB integrity associated with AD (22). The control of permeability of BBB and BRB integrity is assured by the correct assembly of tight junctions (TJ) protein complexes. We investigated the brain and retinal barrier integrity by assessing the content of the tight junction proteins claudin-5, occludin and zonula occludens-1 (ZO-1), in the hippocampus, cortex and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age (Fig. 2).

The protein levels of claudin-5, occludin and ZO-1 remained unchanged in all regions analyzed at both time points, with an exception of a decrease of occludin levels in the retina of 3xTg-AD-mice at 4 months

($56.0 \pm 16.7\%$ of WT), although not significantly (Fig. 2). The integrity of BRB and BBB was also assessed using the Evans blue assay. However, no vascular permeability changes were detected in 3xTg-AD mice, at both 4 and 8 months of age (data not shown).

Evaluation of changes in cholinergic neurons, synaptic proteins and cell loss in 3xTg-AD mice

In AD several types of neurons degenerate in the brain (23, 24), but cholinergic neurons are particularly affected (24).

We investigated the protein expression of: (i) choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of the neurotransmitter acetylcholine, (ii) syntaxin 1, synaptophysin, and synapsin 1 (presynaptic proteins), and (iii) postsynaptic density protein 95 (PSD95; post-synaptic protein), in the hippocampus, cortex and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age, by Western blotting (Fig. 3A).

The protein levels of ChAT were assessed in the three regions, as an indicator of potential cholinergic neuronal loss. ChAT content remained unchanged in 3xTg-AD mice at 4 and 8 months in all regions analyzed, being observed just a tendency for higher protein levels of ChAT in the hippocampus, at 8 months. Regarding synaptic proteins' levels, It was detected a significant increase in syntaxin 1 ($258.8 \pm 48.0\%$ of WT, $p < 0.05$) in the cortex and in synapsin 1 in the hippocampus ($170.2 \pm 13.7\%$ of WT, $p < 0.05$) and cortex ($140.7 \pm 14.1\%$ of WT, $p < 0.05$) at 4 months. There was also a significant increase in the levels of PSD95 in the hippocampus ($163.9 \pm 19.9\%$ of WT, $p < 0.05$) at 4 months. These alterations in the hippocampus and cortex did not persist until 8 months. In the retina of 3xTg-AD mice no changes were found in synaptic protein levels.

The number of retinal ganglion cells (RGCs) was assessed in retina cryosections of 3xTg-AD mice and age-matched WT mice, at 4 and 8 months of age, by immunofluorescence. Despite a tendency for lower values, we did not find significant decrease in the number of Brn3a-positive cells (RGCs) in 3xTg-AD mice compared to age-matched WT mice (Figs. 3B,C). The number of TUNEL-positive (apoptotic) cells was also evaluated by TUNEL assay. At these early stages, we did not find any cell death by apoptosis (Fig. 3D) in the three regions studied.

Assessment of glutamate and GABA levels in the retina and brain of 3xTg-AD mice

Changes in the content of neurotransmitters were already reported in the brain of AD animal model and patients (25, 26). However, there is lack of information about this topic in the retina. We evaluated the levels of gamma-aminobutyric acid (GABA) and glutamate in the hippocampus, cortex and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age, by HPLC (Fig. 4).

The levels of GABA and glutamate did not change in the hippocampus of 3xTg-AD mice comparing with age-matched WT, at 4 and 8 months (Fig. 4A). In the cortex, at 4 months, 3xTg-AD mice presented a

significant decrease in the GABA levels (WT: 0.006 ± 0.0002 nmol/mg protein; AD: 0.004 ± 0.0006 nmol/mg protein; $p < 0.05$) and a tendency for a decrease in the levels of glutamate. At 8 months no changes were detected in the cortex (Fig. 4B). In the retina, no statistically significant changes were detected in the levels of GABA and glutamate, but at 8 months there was a tendency for increased levels of glutamate (Fig. 4C).

Evaluation of glial protein immunoreactivity in the retina and brain of 3xTg-AD mice

The contribution of glial cells for AD pathophysiology has been widely explored (reviewed in (27)). The protein levels of glial fibrillary acidic protein (GFAP), a common marker of astrocytes, and Müller cells end-feet in reactive state, was assessed in the hippocampus, cortex and retina homogenates of 3xTg-AD mice and age-matched WT mice, at 4 and 8 months of age, by Western blotting (Fig. 5A). At 4 months, the levels of GFAP in the hippocampus of 3xTg-AD increased ($276.7 \pm 40.3\%$ of WT, $p < 0.001$), while no changes were detected in the cortex and retina of the same animals. At 8 months, the increase of GFAP in the hippocampus found at 4 months did not persist ($78.6 \pm 12.4\%$ of WT). Again no changes in the content of GFAP were detected in the cortex. Moreover, a decrease in GFAP protein content was detected in the retina of 3xTg-AD mice ($39.2 \pm 15.5\%$ of WT; $p < 0.05$).

Müller cells are the principal glial cells in the retina. The protein levels of vimentin, a selective marker of these cells in the retina, were also evaluated by Western blotting (Fig. 5A). The distribution and morphology of Müller cells in the retina was assessed by immunofluorescence (Fig. 5B). At 4 and 8 months, no changes were observed in the protein levels of vimentin in the retina of 3xTg-AD mice ($117.3 \pm 18.8\%$ of WT and $93.1 \pm 15.5\%$ of WT, respectively). Likewise, no clear changes in the distribution and morphology of Müller cells were detected in the retina of 3xTg-AD mice compared to age-matched WT mice, at the early stages (4 and 8 months of age).

Assessment of changes in microglial cells in the retina and brain of 3xTg-AD mice

Microglial cells are the immunocompetent cells of the CNS. Under pathological conditions, such as AD, microglial cells assume an activated state characterized by cell proliferation, migration, alterations in morphology and release of pro-inflammatory mediators (28). We assessed the number of microglial cells in the CA1 hippocampal region (hippocampal region most affected in AD) and in the retina of 3xTg-AD mice and age-matched WT mice, at 4 and 8 months of age (Fig. 6A-D). No significant changes were observed in the number of microglial cells neither in the hippocampus nor in the retina of 3xTg-AD mice comparing with age-matched WT. Under an insult, retinal microglial cells can migrate to different retinal layers. Therefore, we also evaluated the distribution of these cells in several retinal layers. No differences between 3xTg-AD and age-matched WT mice were found in the distribution of microglial cells in each retinal layer (data not shown).

The 3D morphology of microglial cells (number and length of processes) was also assessed in the CA1 hippocampal region and in the retina of 3xTg-AD mice and age-matched WT mice, at 4 and 8 months of age (Fig. 6E-P). At 4 months, morphological changes were detected in microglial cells of 3xTg-AD mice in the CA1 region and in the retina. However, while in CA1 region microglial cells of 3xTg-AD mice presented a hypertrophic morphology, with increased number and length of processes (Figs. 6E-G), in the retina microglia featured an atrophic appearance, i.e., less number and length of processes (Figs. 6H-J), compared to age-matched WT mice. At 8 months, changes detected in microglial cells in the CA1 region of 3xTg-AD mice at 4 months did not persist. Microglia presented a morphological structure similar to the microglia morphology in the age-matched WT mice (Fig. 6K-M). In the retina, microglia presented a hyper-ramification in the more proximal branch orders and an atrophy in the more distal branches, comparing with the age-matched WT (Fig. 6N-P).

Discussion

The concept 'the retina as an additional tool for AD diagnosis' has been increasingly explored, since some AD patients present visual alterations before the appearance of the first cognitive symptoms. Moreover, the retina and the brain share the same embryonic origin.

We recently reported the existence of structural and functional changes in the brain and retina of 3xTg-AD mice (15, 16). These alterations occur as early as 4 months of age. Because of the detection of these early changes in this animal model of AD, in the present work we investigated the presence of potential early molecular and cellular alterations in the retina and brain that may underlie the structural and functional changes detected in this model. Moreover, we intended to check whether the potential changes in the retina and brain were similar, as well as to check whether some changes could eventually occur in the retina before they appear in the brain.

Increased A β levels were detected in the hippocampus and cortex of 3xTg-AD mice at 4 and 8 months, despite no changes detected in APP and BACE at both time points, with the exception of increased levels of APP in the hippocampus at 8 months. These results suggest that the increased levels of A β detected in the brain may result from an increase of BACE activity or an impairment of A β removal. Several evidences point out mechanisms of A β clearance as a potential therapeutic strategy to modulate AD (29–31). In the retina, no changes were detected in A β levels. Actually, the presence of A β in the retina of animal models of AD is still a matter of controversy (reviewed in (3)). In fact, we tried different approaches and techniques to detect A β in the retina and we were only successful by using a specific protocol of homogenization abovementioned. It was recently reported A β staining in retinal slices of 3xTg-AD mice, at 5 post-natal weeks (32). We also found a similar result, however, when we incubated both retinal slices and whole mounts only with the secondary antibody, we observed the same staining pattern. Thus, the A β staining previously reported may actually result from precipitation of the secondary antibody or unspecific binding. In fact, 3xTg-AD mice are known to be one of the animal models of AD with lower A β expression in the retina (33).

We also checked the hyperphosphorylated tau protein in the hippocampus, cortex and retina of 3xTg-AD mice. For that, we used an antibody that recognizes tau protein phosphorylated at serine 396, a phosphorylation site associated with early AD (34). We detected increased p-tau levels in the hippocampus and retina of 3xTg-AD mice, at both time points, while in the cortex this increase was only observed at 4 months. Similarly, others reported increased p-tau in the retinas of 3xTg-AD mice already at pre-symptomatic stages (32). However, it is important to highlight that these authors used a different antibody that recognizes the protein tau phosphorylated at serine 202 and threonine 205, which is more associated with the later stages of AD.

Regarding barriers integrity, our results indicate that claudin-5, occludin and ZO-1 levels are not affected in the retina and brain of 3xTg-AD mice at early stages (4 and 8 months of age). Additionally, we did not detect albumin extravasation from the blood to brain or retinal parenchyma. In summary, our results show that at early time points the BRB and BBB are not compromised in 3xTg-AD mice. From the best of our knowledge, this is a pioneering study assessing simultaneously the integrity of BBB and BRB in an AD animal model. Previous studies were mainly focused in BBB, and still no consistent results were reported (35–38). In the retina, attenuated and disorganized ZO-1 and occludin staining was reported in 5xFAD mice, an AD animal model with a more aggressive phenotype, at 8 months (39).

We also assessed cell loss, and synaptic and neurotransmitters changes. Cholinergic neurons are the most affected in AD, and their degeneration contributes for the memory loss (24). We did not observe changes in the protein levels of ChAT in any region analyzed of 3xTg-AD mice at 4 and 8 months, suggesting that cholinergic neurons and the synthesis of acetylcholine are not affected in this animal model at these early stages. In 3xTg-AD mice, others demonstrated a decrease in the number of ChAT-positive cells in the basal forebrain already at 4 months (40). These apparent contradictory results could be due to the assessment of distinct regions: basal forebrain versus hippocampus and cortex. Additionally, others also reported a decrease in the number of ChAT⁺ cells in the retina of APP/ PS1 mice, associated with an increase of cell death by apoptosis, but this was observed at 13–16 months (41). In fact, we cannot discard the possibility that the 3xTg-AD mice can also present changes in the number of ChAT⁺ cells at an older age.

We assessed cell death in the hippocampus, cortex and retina to clarify whether cell death could occur simultaneously in the retina and brain of 3xTg-AD mice at the early stages. We did not detect any evidence of cell death by apoptosis in all regions analyzed. Formerly, it was described an increase in the number of cells stained for cleaved caspase-3 both in the retina and hippocampus of 3xTg-AD mice at 5 weeks of age that disappear at 30–40 weeks (7–10 months) (32). However, caspase-3 has been also implicated in the regulation of synaptic plasticity (42, 43) and in mitochondria function (44). Grimaldi and colleagues claimed that increased staining for caspase-3 indicates neuronal apoptosis (32), but they did not show any evidence of cell death in the retina and brain of 3xTg-AD mice.

Loss of RGCs in AD patients has also been described (reviewed in (3)). We did not find a significant decrease in the number of RGCs, at 4 and 8 months. Additionally, we did not find changes in the function

of RGCs in 3xTg-AD mice (16), therefore suggesting that RGCs are not affected in this animal model at the early stages of pathology.

Regarding synaptic changes, our results suggest that the retina and brain are not similarly affected, at 4 months, in this animal model. At 8 months, we did not detect any changes in the levels of the synaptic proteins evaluated in the three regions analyzed. Although others detected decreased synaptophysin and PSD95 levels in the hippocampus and cortex of 3xTg-AD mice at 4 and 7 months (45), most studies did not observe changes in synaptic proteins at 6, 8 and 9 months in this AD animal model (46–48). At 4 months we detected an increase in the content of several synaptic proteins, which has been corroborated by other studies, at early stages (57). This increase might be due to a compensatory mechanism. Also, in postmortem brains from AD patients it was described an increase in several synaptic proteins at early stages of the pathology (Braak 3 and 4) that did not persist at later stages (Braak 5 and 6) (49). These studies corroborate our findings, suggesting that a biphasic synaptic protein response during AD progression is likely to occur. These evidences lead us to hypothesize that compensatory mechanisms (increase of synaptic proteins) might occur to keep homeostasis and cell survival. However, with the disease progression the synaptic deficit reaches a certain threshold where the brain cannot compensate anymore.

Several reports have demonstrated that AD patients present a disturbance in glutamate and GABA levels, leading to a neuronal circuitry disruption, and consequently affecting memory (50–55). Changes in neurotransmission occur prior to cognitive decline (25). We detected a decrease of GABA in the cortex of 3xTg-AD mice at 4 months, but no more significant changes were observed in the GABA and glutamate levels in all regions analyzed. Others reported a decrease in vesicular glutamate transporter type I (vGluT1) density, without neuronal damage, in the hippocampus of 3xTg-AD mice, at 4 months (56). At the early stages the total glutamate levels do not change, but with the alterations in vGluT1 the glutamate transport could be compromised and therefore the glutamate levels at the synaptic cleft.

Evidences point out the glial cells as key elements in the AD pathophysiology (reviewed in (57)). Regarding GFAP, our results suggest the occurrence of differential glial fluctuations over time in the areas analyzed. It was recently reported an increase of astrocytic reactivity in the retina of 3xTg-AD mice at 5–10 and 30–40 weeks (32), which is not in agreement with our findings. However, others also reported inconsistent glial changes in the brain and retina in APP/PS1 mice (58). Moreover, we did not find significant changes in vimentin, suggesting no significant changes in Müller cells, at the early stages. Others also reported no changes in Müller cells in 3xTg-AD (59) and in APP/PS1 mice (60), at early stages. Conversely, evidence of an increase of GFAP staining in the Müller cells end-feet in the retina of 3xTg-AD mice at 9 months of age (32) suggests an increase of Müller cells reactivity. Taking into account our results and previous findings, it appears that Müller cells reactivity may occur in AD, but at later stages.

During an insult microglial cells increase their proliferative rate, change their morphology, and acquire a reactive state in order to reestablish the homeostasis (61). No changes were detected in the number of microglial cells neither in the hippocampus nor in the retina of 3xTg-AD mice at early stages. Former

studies also reported no changes in the number of microglial cells in the retina (32) and brain of 3xTg-AD mice, at early stages (62). We also assessed microglial morphology, i.e., the number and length of processes. In the hippocampus of 3xTg-AD mice we observed a hypertrophy of microglial cells (only at 4 months), while in the retina microglial cells display an atrophic morphology. Moreover, at 8 months, retinal microglial cells featured a biphasic morphological structure since presented atrophy in the last orders and a hypertrophy in the initial ones. These results suggest a complex microglial remodeling in AD and also indicate there is a differential microglia remodeling in the retina and hippocampus during AD pathology. To our knowledge, this study is the first one that simultaneously analyzed the microglia structure in the brain and retina in the context of AD. It was reported an increase of microglial cells complexity in the retina of 3xTg-AD mice at early stages (5–10 weeks) (32). This apparent contradictory results could be explained because they used a different approach for 3D microglia reconstruction (imageJ plug-in) and the time point assessed was different (5–10 weeks versus 4 months in our work). We recently reported changes in retinal microglia of 3xTg-AD mice at older ages. Microglia acquired a different morphology and orientation along the retina, and their localization changed from a parallel to a perpendicular position relative to the retinal surface (63). In the brain of AD patients, it was found a reduction in the length of processes and arborized area of microglial cells, indicative of microglia reactivity (64). This group of findings led us to hypothesize that in the early stages microglial cells adopt a hyper-ramified morphology trying to maintain the appropriate brain surveillance. However, with disease progression, microglia acquire a less complex morphology accompanied with a senescent state.

Conclusion

Our results show that at the early stages there are no significant and consistent molecular and cellular changes in 3xTg-AD mice in the regions analyzed. Moreover, some of the few alterations detected in the brain and retina of 3xTg-AD mice do not match, suggesting that at molecular and cellular level the retina does not seem to mirror the brain changes associated with AD phenotype.

List Of Abbreviations

A β - amyloid beta; AD - Alzheimer's disease; AP - alkaline phosphatase; APP - amyloid precursor protein; BACE - beta-secretase; BBB - blood-brain barrier; BCA - bicinchoninic acid; BRB - blood-retinal barrier; BSA - bovine serum albumin; CNS - central nervous system; ChAT - choline acetyltransferase; DAPI - 6-diamidino-2-phenylindole; DOC - sodium deoxycholate; DTT - dithiothreitol; ECF - chemifluorescence; ECL - chemiluminescence; EDTA - ethylenediaminetetraacetic acid; EGTA - ethylene glycol tetraacetic acid; GABA - gamma-aminobutyric acid; GFAP - glial fibrillary acidic protein; HBSS - Hank's balanced salt solution; HPLC - high-performance liquid chromatography; HRP - horseradish peroxidase; Iba-1 - ionized calcium-binding adapter molecule 1; PAGE - polyacrylamide gel electrophoresis; PBS - phosphate buffer saline; PFA - paraformaldehyde; PMSF - phenylmethylsulfonyl fluoride; PSD95 - postsynaptic density protein 95; p-tau - phosphorylated tau; PVDF - polyvinylidene difluoride; RGCs - retinal ganglion cells; RT - room temperature; SDS - sodium dodecyl sulfate; THB - tissue homogenization buffer; vGlut1 - vesicular

glutamate transporter type I; WT - wild-type; TJ - tight junctions; TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling; ZO-1 - zonula occludens-1

Declarations

Ethics approval

All experiments using animals were approved by the Animal Welfare (Órgão Responsável pelo Bem-Estar Animal - ORBEA 16/2015) of the Coimbra Institute for Clinical and Biomedical research (iCBR), Faculty of Medicine, University of Coimbra, and by Direção Geral de Alimentação e Veterinária (DGAV 0421/000/000/2015) and conducted in accordance with the European Community directive guidelines for the use of animals in laboratory (2010/63/EU) transposed to the Portuguese law in 2013 (Decreto-Lei 113/2013), and in agreement with the Association for Research in Vision and Ophthalmology statement for animal use.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's information

ACRN, RC, AFA conceived and designed the experiments. ACRN, RC, SCC, CC, EJC, FIB performed the experiments. ACRN, RC, SCC, CC, EJC, FIB, PIM and AFA analyzed the results. PIM and AFA contributed with reagents/materials/analysis tools. ACRN wrote the first draft of the manuscript and all authors have read and approved the final version.

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Figures

Figure 1

Evaluation of molecular hallmarks of AD in the retina and brain of 3xTg-AD mice. Quantification of protein expression levels of amyloid precursor protein (APP), amyloid beta (A β), phosphorylated tau (p-tau) and beta-secretase 1 (BACE) ,in the hippocampus, cortex and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age, assessed by Western blotting. Values were normalized to β -actin (internal control), and are shown as percentage of age-matched WT mice $\pm$ SEM (n=5-12). Representative Western blots of APP, A β , p-tau, BACE and β -actin are presented below the graphs. *p < 0.05, **p < 0.01, ***p < 0.001, 3xTg-AD mice compared to age-matched WT mice.

Figure 2

Evaluation of the tight junction protein levels in the retina and brain of 3xTg-AD mice. Claudin-5, occludin and zonula occludens-1 (ZO-1) protein expression levels were evaluated by Western blotting in the hippocampus, cortex and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age. Values were normalized to β-actin (internal control), and are shown as percentage of age-matched

WT mice $\pm$ SEM (n=5-12). Representative Western blots of claudin-5, occludin and ZO-1 and β -actin are presented below the graphs.

Figure 3

Evaluation of choline acetyltransferase and synaptic proteins levels and cell loss in 3xTg-AD mice. (A) The protein levels of choline acetyltransferase (ChAT), syntaxin 1, synaptophysin, synapsin 1 and postsynaptic density protein 95 (PSD95) were evaluated by Western blotting in the hippocampus, cortex

and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age. Values were normalized to β -actin (internal control), and are shown as percentage of age-matched WT mice $\pm$ SEM (n=4-12). Representative Western blots of ChAT, syntaxin 1, synaptophysin, synapsin 1 and PSD95 and β -actin are presented below the graphs. *p < 0.05, 3xTg-AD mice compared to age-matched WT mice. (B) Brn3a-positive cells (RGCs) were assessed by immunofluorescence in retinal cryosections of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age, using an anti-Brn3a antibody (red). Nuclei were counterstained with DAPI (blue). Representative images are presented. Scale bar: 25 μ m. (C) Quantification of the number of Brn3a-positive cells in retinal cryosections of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age. Values were normalized to the retina length and are shown as mean $\pm$ SEM (n=4-6). (D) TUNEL-positive (apoptotic) cells were evaluated in retinal and brain cryosections of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age (n=3). Representative images showing the absence of TUNEL-positive cells are presented. A representative image of a positive control (brain slice incubated with Dnase I to cause DNA fragmentation) is also presented. Scale bar: 25 μ m. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

Figure 4

Glutamate and GABA levels in the retina and brain of 3xTg-AD mice. The glutamate and GABA levels were evaluated by HPLC in the hippocampus, cortex and retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age. Values were normalized to the amount of protein, and are shown as mean $\pm$ SEM (n=4-9). *p < 0.05, 3xTg-AD mice compared to age-matched WT mice.

Figure 5

Evaluation of changes in glial cells in the retina and brain of 3xTg-AD mice. (A) The protein expression levels of glial fibrillary acidic protein (GFAP) were evaluated by Western blotting in the hippocampus, cortex and retina homogenates, while the vimentin protein levels were assessed only in the retina homogenates of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age. Values were normalized to β -actin (internal control), and are shown as percentage of age-matched WT mice $\pm$ SEM ($n=6-12$). Representative Western blots of GFAP, vimentin and β -actin are presented below the graphs. * $p < 0.05$, *** $p < 0.001$, 3xTg-AD compared to age-matched WT mice. (B) The distribution of vimentin-positive cells (Müller cells) were assessed by immunofluorescence in retinal cryosections of 3xTg-AD and age-matched WT mice, at 4 and 8 months of age, using an anti-vimentin (green) antibody ($n=3$). Nuclei were counterstained with DAPI (blue). Representative images are presented. Scale bar: 25 μ m.

Figure 6

Evaluation of changes in microglial cells in the retina and brain of 3xTg-AD mice. Iba-1-positive cells (microglial cells) immunoreactivity was assessed by immunofluorescence in (A) the CA1 hippocampal region, and (B) retina cryosections of 3xTg-AD mice and age-matched WT mice, at 4 and 8 months of age, using an anti-Iba-1 (green) antibody (n=4-5). Nuclei were counterstained with DAPI (blue). Representative images are presented. Scale bar: 25 μm. Quantification of Iba-1-positive cells in (C) the CA1 hippocampal

region, and (D) retinal cryosections of 3xTg-AD mice and age-matched WT mice, at 4 and 8 months of age. Values were normalized to the CA1 area and to retina length, respectively, and are shown as mean $\pm$ SEM (n=4-5).