The choroid plexus transcriptome reveals changes in type I and II interferon responses in a mouse model of Alzheimer's disease

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A R T I C L E   I N F O

Article history:
Received 19 March 2015
Received in revised form 29 May 2015
Accepted 10 June 2015
Available online 16 June 2015

Keywords:
Alzheimer's disease
Aging
Memory
Choroid plexus
Transcriptome
Neuroinflammation
Interferons
Cerebrospinal fluid
Hippocampus
Glial cells

A B S T R A C T

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a marked decline in cognition and memory function. Increasing evidence highlights the essential role of neuroinflammatory and immune-related molecules, including those produced at the brain barriers, on brain immune surveillance, cellular dysfunction and amyloid beta (Aβ) pathology in AD. Therefore, understanding the response at the brain barriers may unravel novel pathways of relevance for the pathophysiology of AD. Herein, we focused on the study of the choroid plexus (CP), which constitutes the blood–cerebrospinal fluid barrier, in aging and in AD. Specifically, we used the PDGFB-APPSwInd (J20) transgenic mouse model of AD, which presents early memory decline and progressive Aβ accumulation, and littermate age-matched wild-type (WT) mice, to characterize the CP transcriptome at 3, 5–6 and 11–12 months of age. The most striking observation was that the CP of J20 mice displayed an overall overexpression of type I interferon (IFN) response genes at all ages. Moreover, J20 mice presented a high expression of type II IFN genes in the CP at 3 months, which became lower than WT at 5–6 and 11–12 months. Importantly, along with a marked memory impairment and increased glial activation, J20 mice also presented a similar overexpression of type I IFN genes in the dorsal hippocampus at 3 months. Altogether, these findings provide new insights on a possible interplay between type I and II IFN responses in AD and point to IFNs as targets for modulation in cognitive decline.

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1. Introduction

Alzheimer’s disease (AD) is the most prevalent form of dementia and is predicted to affect eighty million people worldwide by 2040 (Ballard et al., 2011; Querfurth and LaFerla, 2010). The major constraint of AD patients is the severe loss of cognitive abilities (Ballard et al., 2011). Moreover, this neurodegenerative disease is characterized by two main brain pathological hallmarks: the extra-cellular deposition of amyloid beta (Aβ) peptides, in the form of senile plaques, and the formation of intracellular neurofibrillar tangles, as a result of increased aggregation of hyperphosphorylated Tau protein (Gotz et al., 2001; Hardy and Selkoe, 2002; Lewis et al., 2001; Scheuner et al., 1996). The amyloidogenic hypothesis of AD considers that amyloid pathology may result from increased Aβ production, through the amyloidogenic processing of the amyloid precursor protein (APP), and/or decreased excretion of Aβ out of the brain (Hardy and Selkoe, 2002; Roberson and Mucke, 2006). In fact, oligomerization of Aβ peptides in the brain has been described as an early pathogenic event that is responsible for behavior alterations (Mucke et al., 2000; Palop and Mucke, 2010). However, regardless of the attention given to increased Aβ formation and toxicity in the brain, neuroinflammation is also recognized as an early and essential mediator of brain pathology and behavior alteration in AD (Lucin and Wyss-Coray, 2009; Wyss-Coray, 2006). In this context, two relevant emerging concepts should be considered: (I) the age-associated changes in the blood composition and (II) the levels/ratio of type I and II interferon (IFN) cytokines. Regarding the first concept, recent observations showed the involvement of the systemic milieu in memory formation and the role of specific inflammatory molecules in age-related decreased synaptic plasticity and memory function (Baruch et al., 2013; Villeda et al., 2011). Regarding the second concept, an enhanced type I IFN signaling at the brain barriers, and
decreased type II IFN response, have recently been implicated in cognitive decline in aged mice (Baruch et al., 2014).

Of relevance, the brain barriers, namely the blood–brain barrier, composed by the endothelial cells of the blood capillaries, and the blood–cerebrospinal (CSF) barrier, formed by the choroid plexus (CP) epithelial cells, seems to influence both the excretion of Aβ peptides out of the brain (Carro et al., 2002, 2005; Zlokovic, 2008) and the inflammatory response (Marques et al., 2009, 2013; Zlokovic, 2008). Particularly, besides its role in the formation and secretion of the CSF, recent studies clearly indicate that the CP is able to modulate the cognitive function, through changes in the neuroinflammatory response and in brain immune surveillance (Baruch et al., 2013, 2014; Baruch and Schwartz, 2013). Interestingly, the role of the CP in the neuroinflammatory response in AD, as well as its connection with impaired memory and cognition, is still poorly understood and is addressed in the present study. Specifically, we took advantage of a mouse model of AD that presents early cognitive deficits and brain amyloid pathology, the PDGFB-APPSwInd (J20) (Mucke et al., 2000), and respective wild-type (WT) age-matched littersmates, to study changes in the CP transcriptome and in the composition of the CSF. Furthermore, we analyzed the extension of the changes observed at the level of the CP, by assessing the neuroinflammatory response at the dorsal hippocampus (dHPC), a brain region that is seriously affected in AD (Palop et al., 2007; Villeda et al., 2011). Altogether, the results suggest that alterations in IFN signaling may contribute to neuroinflammation, amyloid pathology and cognitive impairment in aging and in AD.

2. Materials and methods

2.1. Ethics statement

Animal handling and experimental procedures were conducted in accordance with the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457). Animals were kept in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and the Council. The animals were housed and maintained in a controlled environment at 22–24 °C and 55% humidity, on 12 h light/dark cycles and fed with regular rodent’s chow and tap water ad libitum.

2.2. Animal model and experimental groups

Male hemizygous B6.Cg-Tg(PDGFB-APPSwInd)20Lms/2J/Mmjax (J20) mice, in a C57BL/6J background, first developed by Professor Lennart Mucke, of The J. David Gladstone Institutes (San Francisco, CA, USA), were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The J20 mice express a mutant form of the human APP bearing both the Swedish (K670N/M671L) and the Indiana (V717F) mutations (APPSwInd). Expression of the transgenic insert is directed by the human platelet-derived growth factor (PDGF) promoter (Mucke et al., 2000). Hemizygous mice present the highest expression of the transgene product in neurons of the neocortex and HPC. At 5–7 months of age diffuse Aβ peptides deposition is detected in the dentate gyrus (DG) and neocortex. Amyloid deposition is progressive with all stages of Aβ pathology, particularly before and after Aβ plaque formation. The value of achieved power (1−β) of the sample was calculated using the IBM SPSS Statistics for Windows (Version 23.0. Armonk, NY: IBM Corp, USA), on the basis of the sample size (N = 80) and effect size (partial eta squared = 0.044) associated with the Morris water maze (MWM) test, which was analyzed by a parametric repeated measures two-way ANOVA (x = 0.05). Taking that the Mauchly’s Test revealed a violation of sphericity for the analyzed groups, the Huynh–Feldt correction was applied and a final value of 1−β = 0.841 was obtained, which is considered to be statistically powerful (Mazen et al., 1985).

2.3. MWM paradigm

Memory was evaluated during the light phase of the diurnal cycle using the MWM paradigm. The MWM was performed in a white circular pool (170 cm in diameter and 50 cm in height), filled with tap water (23 ± 1 °C; 30 cm of depth) and placed in a poorly lit room. The water tank was divided into four imaginary quadrants (north, east, south, and west), each corresponding to a fixed extrinsic clue visible to the mouse. A transparent escape platform (14 cm in diameter and 30 cm high), invisible to the mouse, was placed in the center of one of the quadrants and was maintained in that same position during the five days of the acquisition. For spatial reference-memory acquisition, a HPC-dependent task, the mice were placed randomly in one of the quadrants in each trial, and allowed to search for the hidden platform. During the five days of the acquisition phase, each mouse performed four trials per day. Each trial was concluded when the platform was reached within the time-limit of 120 s. If failing to reach the platform within this time-period, the mouse was guided to the platform and allowed to stay in it for 30 s. The four random consecutive trials of each day were video-captured with a video-tracking system (Viewpoint, Champagne au Mont d’or, France) and the mean values of the time (latency to platform) and speed to reach the platform were calculated. To analyze the MWM performance, mice were divided into groups according to their age and genotype. After two independent experiments with identical results, the final number of mice per group was the following: at 3 months, N (WT) = 16 and N (J20) = 8; at 5–6 months, N (WT) = 8 and N (J20) = 10; at 11–12 months, N (WT) = 23 and N (J20) = 15.

2.4. Tissue sample collection and storage

During the light phase of the diurnal cycle, specifically in the morning, mice were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (150 mg/kg; Imalgene® 1000) and medetomidine hydrochloride (0.3 mg/kg, Dorben®). Under deep anesthesia, CSF samples were collected from the cisterna magna and checked for blood contamination using a hemocytometer. Samples were stored at –80 °C until further use. After this, mice were transcardially perfused with 0.9% saline and the brains were removed from the skull. For gene expression analysis by microarray and qRT-PCR, the CP samples from each brain ventricle of the same mouse were rapidly removed under a conventional light stereomicroscope (SZX7, Olympus, Hamburg, Germany), pooled, snap-frozen and stored at –80 °C. Specific brain areas, namely the dHPC, were obtained by macrodissection, snap-frozen and stored at –80 °C for RNA extraction and gene expression analysis. Whole brains were fixed in 4% paraformaldehyde (PFA, Panreac Química S.L.U., Barcelona, Spain) for 48 h and kept in paraffin blocks until further sectioning. Alternatively, brains were immediately embedded in Richard-Allan Scientific™ Neg-50™ Frozen Section Medium (Thermofisher Scientific, Waltham, MA, USA), snap-frozen and kept frozen at –20 °C until further sectioning.
2.5. Microarray and Ingenuity® Pathway Analysis

Total RNA was extracted from CP tissue using the RNeasy® Plus Micro Kit (Qiagen, Hamburg, Germany), following the manufacturer’s instructions. After quality assessment using an Agilent Bioanalyzer (Agilent Technologies, CA, USA), 500 ng of total RNA were used for T7-based mRNA amplification using the manufactured kit TargetAmp™ 2 – Round Biotin aRNA Amplification Kit 3.0 (Epicentre, Illumina, Wisconsin, USA), according to the manufacturer’s instructions. The quality of amplified RNA was assessed using an Agilent Bioanalyzer. Amplified RNA was labeled and hybridized to Illumina Mouse Ref 8 v2.0 gene expression BeadChips, querying the expression of approximately 25,600 RefSeq-curated gene targets, for microarray analysis by the Southern California Genotyping Consortium (SGCC) core using the Illumina BeadStation platform. Statistical microarray analysis and gene expression analysis were performed at the Informatics Center for Neurogenetics and Neurogenomics (ICNN) core at UCLA using R (www.r-project.org) and Bioconductor (www.bioconductor.org) packages as described before (Coppola, 2011; Coppola et al., 2008). The raw data was deposited in the GEO database, accession number GEO: GSE66598. Gene pathway and functional analysis was performed using the Ingenuity Pathway Analysis 6.0 (IPA; Ingenuity® Systems, www.ingenuity.com). The CP transcriptome of WT mice at 3 (N = 3), 5–6 (N = 4) and 11–12 (N = 6) months, and of J20 mice at 3 (N = 4), 5–6 (N = 4) and 11–12 (N = 3) months, was analyzed and compared. The individual data sets consisted on: (I) the comparison between the CP transcriptome of J20 mice and the CP transcriptome of WT mice, at different ages; (II) the comparison between the CP transcriptome of 11–12 months-old mice and that of 3 months-old mice, for each genotype independently. The data sets containing the accession numbers, the log ratio and the p-value for each gene, whose expression was significantly changed (p-value < 0.05), were uploaded into the IPA database. Using IPA and its applications, each gene identifier was mapped and the genes were used to build networks associated with a specific canonical pathway or with a relevant biological function or disease, according to the IPA database library. The Ingenuity® Upstream Regulator Analysis computed an activation z-score and a Fisher’s exact p-value (overlap p-value) for the most altered genes. Furthermore, we used the IPA Upstream Regulator application to assemble an interactome using the five upstream regulators with the highest activation z-scores and the five upstream regulators with the lowest activation z-scores, along with the corresponding downstream target genes/molecules whose expression was significantly altered in the CP of J20 mice at each age. To be included in the interactome, the networks had to present at least one connection with an upstream regulator or with a downstream gene of a different adjacent network; orphan networks were not presented.

2.6. Primary cultures of CP epithelial cells and incubation with Aβ1–42

Primary cultures of CP epithelial cells were prepared from the CP tissue of rat pups due to the higher cellular yield of the isolation procedure when compared to the mouse CP. This procedure was performed as described before (Marques et al., 2009), with minor modifications. Briefly, neonates (P5–P7) were sacrificed and the CP dissected from the four brain ventricles, under a conventional light stereomicroscope (SZX7, Olympus). The tissue was rinsed in phosphate buffer saline (PBS, Invitrogen, Carlsbad, CA, USA) without Ca²⁺ and Mg²⁺, followed by a 25 min digestion with 0.1 mg/ml pronase (Sigma–Aldrich, St. Louis, MO, USA) at 37 ºC. Predigested tissue was recovered by sedimentation and briefly shaken in 0.025% trypsin–EDTA (Invitrogen) containing 12.5 μg/ml DNase I (Roche, Amadora, Portugal). The supernatant was then withdrawn and kept on ice with 10% fetal bovine serum (FBS, Invitrogen). This step was repeated five times. Cells were collected by centrifugation and re-suspended in culture media consisting of Ham’s F-12 and Dulbecco’s modified Eagle’s medium (DMEM-F12, Invitrogen) supplemented with 10% FBS, 2 mM glutamine, 50 mg/ml gentamycin, 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml sodium selenite, 10 ng/ml epidermal growth factor, 2 mg/ml hydrocortisone and 5 ng/ml basic fibroblast growth factor (all from Sigma–Aldrich). For further enrichment, cells were incubated on plastic dishes for 2 h at 37 ºC. The supernatant containing the CP epithelial cells was collected and placed for seeding on 0.4 μm pore size transwells (Corning Life Sciences, Lowell, MA, USA) coated with laminin (BD Biosciences, Bedford, MA, USA) at a density of 1 x 10⁶ cells/cm². The culture medium was changed every two days and CP epithelial cells were maintained in culture for 5–7 days, at 37 ºC in a humid atmosphere (5% CO₂), before performing the experiment. To assess the cellular purity and confluence after 7 days in culture, an immunofluorescent staining was performed, using a primary antibody for transthyretin (TTR) (kindly provided by Dr. Maria Joao Saraiva, Institute for Molecular and Cell Biology, Porto, Portugal), a specific marker of CP epithelial cells (Sousa et al., 2007). Cell counting revealed that > 95% of the cells stained positive for TTR. Stock Aβ1–42 (American Peptide Company, Sunnyvale, CA, USA) was prepared by dissolving the peptides in Tris–NaCl buffer (150 mM NaCl and 50 mM Tris–HCl pH 7.4, all from Sigma–Aldrich) to a final concentration of 221.5 μM and kept at –80 ºC. After reaching confluence, CP epithelial cells were incubated for 60 h with DMEM-F12 alone (vehicle) or a solution of 1 μM monomeric/dimeric Aβ1–42, by dissolving stock Aβ1–42 in DMEM–F12 medium.

2.7. Primary cultures of astrocytes and neurons

This procedure was performed as described before (Mesquita et al., 2014). Details are provided in Supplementary methods (Appendix A).

2.8. Gene expression analysis by qRTPCR

The quality assessment and quantification of the total RNA extracted from CP samples was performed in the NanoDrop® ND-1000 and 350 ng of RNA from each sample were amplified using the MessageAmp™ II aRNA Amplification Kit (Ambion, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA was also extracted from primary cultures of CP epithelial cells, using the RNeasy® Plus Micro Kit (Qiagen), or from samples of dHPC, using the Trizol reagent (Invitrogen), following the manufacturer’s instructions. Then, after quantification in the NanoDrop®, 500 ng of total or amplified RNA from each sample was reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio–Rad Laboratories, Hercules, CA, USA) following the manufacturer’s instructions. Primers used to measure the expression levels of selected mRNA transcripts by qRTPCR were designed using the Primer-BLAST tool of the National Center for Biotechnology Information (Bethesda, MD, USA) on the basis of the respective GenBank accession numbers. The reference gene hypoxanthine guanine phosphoribosyl transferase (Hprt) was used as internal standard for the normalization of the expression of selected transcripts. All GenBank accession numbers for Mus musculus (in vivo studies) or Rattus norvegicus (in vitro experiments) gene transcripts and primer DNA sequences are provided in Supplementary data (Appendix A). Annealing temperatures are available on request. The qRTPCR was performed on a CFX 96TM real–time system instrument (Bio–Rad), with the SoFast™ EvaGreen® Supermix (Bio–Rad) according to the manufacturer’s instructions, using equal amounts of cDNA from each sample. The cycling parameters were...
1 cycle at 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, annealing temperature (primer specific) for 30 s and 72 °C for 30 s, finishing with 1 cycle at 65 °C to 95 °C for 5 s (melting curve). Product fluorescence was detected at the end of each elongation cycle. All melting curves exhibited a single sharp peak at the expected temperature.

2.9. Quantification of Aβ and IFN-α in the CSF

The quantification of Aβ in the CSF was performed by a direct enzyme-linked immunosorbent assay (ELISA) performed in Nunc MaxiSorp® flat-bottom 96-well plates (ThermoFisher Scientific). The wells were coated with 1 μL of CSF diluted in 99 μL of a KH2PO4/K2HPO4 buffer (pH 8.0) solution (1:100 dilution factor), for 2 h at 37 °C. After washing with PBS 0.05% Tween® 20 (Sigma–Aldrich), a blocking step with PBS 10% skim milk (Nestle S.A., Linda-a-Velha, Portugal) was performed for 1 h at room-temperature (RT). Then, consecutive incubations for 1 h at RT were performed: first with rabbit anti-human Aβ (1:500, D542D, Cell Signaling Technology, Danvers, MA, USA), second with biotinylated horse anti-rabbit (1:500, Vector Laboratories, Burlingame, CA, USA) and third with streptavidin-peroxidase (1:2500, Sigma–Aldrich). Each incubation step was separated by thorough washes with PBS 0.05% Tween® 20 and PBS. Finally, the detection of Aβ was achieved by adding a solution containing 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt (ABTS, Sigma–Aldrich), which induced a colorimetric reaction read at 405 nm. The standard curve was obtained using known concentrations of human Aβ1-42 (American Peptide Company, Sunnyvale, CA, USA) that ranged from 0.1 to 100 ng/μL (considering the linearity of the assay). The levels of IFN-α were measured in 1 μL of CSF, using the commercially available Mouse IFN alpha Platinum ELISA kit (Affymetrix, eBioscience, Vienna, Austria), according to the manufacturer’s instructions. The standard curve was obtained using known concentrations of mouse IFN-α that ranged from 31.3 to 2000 pg/mL.

2.10. Immunofluorescent staining for Aβ peptides

Serial sections of frozen brain (coronal sections) with a thickness of 20 μm were cut in the cryostat and collected to SuperFrost® Plus slides (ThermoFisher Scientific). Sections were then fixed for 20 min in 4% PFA (Panreac), at RT. A step of antigen retrieval was performed afterwards, by heating the tissue sections for 20 min in 10 mM acetic acid solution, pH 6.0 (Sigma–Aldrich). Next, tissue peroxidases were inactivated, by immersion in a 0.3% H2O2 solution for 30 min, and a blocking step was performed by incubation in a PBS-T 0.5% BSA solution. Sections were then incubated overnight, at RT, with rabbit anti-mouse GFAP (1:200, Dako, Glostrup, Denmark) or rabbit anti-mouse IBA1 (1:300, Wako Chemicals GmbH, Neuss, Germany) antibodies diluted in blocking solution and, in the following day, rinsed in PBS-T and incubated with biotinylated goat polyvalent antibody (UltraVision Large Volume Detection System, ThermoFisher Scientific) for 1 h at RT. Finally, after rinsing in PBS-T, sections were further incubated for 1 h with streptavidin- horseradish peroxidase (UltraVision, ThermoFisher Scientific), rinsed again in PBS and exposed to a 3,3-diaminobenzidine solution (Sigma–Aldrich) for 10 min. After a thorough wash with PBS, sections were dried and mounted in Entellan (Merck, Darmstadt, Germany). The morphology of 10 astrocytes (GFAP+ cells) located in the DG of the dHPC of each mouse (N = 30 astrocytes/group) was assessed using an optical microscope (BX51TF, Olympus) coupled to a camera (U-TV1X-2, Olympus) and with the NeuroLucida software (Olympus). The samples were given a code name so that the morphology could be assessed by an investigator who was blinded to the experiment. Data was analyzed with the NeuroExplorer software (Olympus). The number of microglia/macrophages in the DG of the dHPC was assessed in serial brain sections and the number of cells per mm² was calculated (N = 4/group at 3 and 5–6 months, and N = 5/group at 11–12 months).

2.12. Statistical analysis

Values are reported as mean ± standard error of mean (SEM). The number of biological replicates (N) of the representative independent experiments is specified in the legend of each figure. Statistical significant differences between groups were determined using the parametric repeated measures two-way ANOVA with Bonferroni post hoc test (two-tailed) or the non-parametric Mann Whitney test and Kruskal–Wallis with Dunns’Bonferroni post hoc test (two-tailed). Simple correlations were obtained by regression analysis, using the Pearson’s correlation test, and results were considered to be statistically significant for p < 0.05 (** and p < 0.001 (***)). Multiple simple correlations were obtained by regression analysis, using the Pearson’s correlation test, and results were considered to be statistically significant for p < 0.05 (two-tailed). Regression models (two-tailed) were designed and run in Matlab R2009b (7.9.0.529, Mathworks, Natick, MA, USA). To avoid collinearity, the independent variables age and IFN-α were centered, considering for that the respective mean values. To verify the statistical significance of the interactions between the different predictors, four interaction terms were created (age*genotype, age*IFN-α, genotype*IFN-α and age*genotype*IFN-α). No animals were excluded from the analysis.

3. Results

3.1. Age-associated memory decline was anticipated in j20 mice

The MWM was used to test the performance of WT and J20 mice in a spatial reference-memory task at 3, 5–6 and 11–12 months of age (Fig. 1A and B). At 3 months, WT mice took significantly less time to find the hidden platform, when compared to age-matched J20 mice (Fig. 1B). At the ages of 5–6 months and 11–12 months, the differences between WT and J20 mice became smaller; still, at 11–12 months, J20 mice presented a significantly worst performance than WT mice at day 5 of the MWM (Fig. 1B). No statistical significant differences were observed in terms of swimming speed in the MWM, when comparing mice of different
genotypes at each age (Supplementary Fig. 1). To assess brain amyloid pathology in J20 mice, we measured the levels of Aβ in the CSF and evaluated the degree of Aβ aggregation and deposition in the dHPC (Fig. 1C and D). There were no statistically significant differences between the levels of Aβ in the CSF of J20 mice at different ages (Fig. 1C); yet, there was a progressive accumulation of Aβ and increased plaque burden in the dHPC of J20 mice with aging that was more obvious at 11–12 months (Fig. 1D). No Aβ was detected in the CSF or in the brain of WT mice (Fig. 1C and D).

3.2. Age and Aβ impacted differently on the CP transcriptome of WT and of J20 mice

The changes in the CP transcriptome of J20 mice were assessed by microarray at 3, 5–6 and 11–12 months of age and normalized to the changes observed in age-matched WT mice (Fig. 1A). The highest number of up- and down-regulated genes in the CP of J20 mice was observed at 11–12 months (Fig. 1E). Of notice, we found no commonly altered genes after overlapping the CP transcriptomic changes of J20 mice at 3, 5–6 and 11–12 months (Fig. 1F). Moreover, we found few commonly altered genes when comparing the transcriptomic changes in the CP of J20 mice two ages at a time (Fig. 1F). The ten most up- or down-regulated genes in the CP of J20 mice (Table 1) revealed alterations in genes that are involved in the functions classically attributed to the CP, such as the modulation of the CSF's composition, and that regulate the survival of CP cells and the maintenance of the barrier integrity (Table 1). We also analyzed the effect of aging, per se, in the CP transcriptome, by normalizing the changes observed in WT or J20 mice at 11–12 months to that of 3 months-old mice of the same genotype (Supplementary Figs. 2 and 3). Interestingly, we found a consistent alteration in the expression of genes that regulate the cellular circadian rhythm in the 11–12 months-old mice, which is describe in detail in the Supplementary data (Supplementary Figs. 2 and 3 and Supplementary Tables 1 and 2). Surprisingly, when considering all the analyzed data sets and comparisons
### Table 1
Most altered genes in the CP of J20 mice (normalized to age-matched littermate WT mice).

<table>
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<tr>
<th>Accession number</th>
<th>Genes symbol</th>
<th>Fold change</th>
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<th>Genes symbol</th>
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<th>p-value</th>
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<td><strong>Gene symbol</strong></td>
<td><strong>Fold change</strong></td>
<td><strong>p-value</strong></td>
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<td>NM_008947</td>
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<td>Gste1</td>
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<td>Adenosine A2b receptor</td>
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<td>1.43</td>
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<td>NM_008504.2</td>
<td>Granzyme M (lymphocyte metase 1)</td>
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performed, the fold-changes in gene expression are small and varied to a maximum fold-increase of 3.01 and fold-decrease of 3.45 (Table 1 and Supplementary Tables 1 and 2).

3.3. The pattern of changes in the CP transcriptome of J20 mice varied with age

Using the gene network analysis computed by the IPA software, we were able to establish a list of the 5 most altered gene networks, and associated biological functions, at each age in the CP of J20 mice (Fig. 2A–F). Of notice, this analysis revealed that the most altered gene network is different at each age (Fig. 2B, D and F). At 3 months, at least two of the altered gene networks were involved in the regulation of one of the following biological functions: molecular transport, metabolism of small molecules, cell-to-cell signaling/endocrine system function and cell death/survival (Fig. 2A). At 5–6 months of age, in addition to the alterations in the genes of network 5 (Fig. 2C) already affected at 3 months (Fig. 2A), the main biological functions and processes found to be altered were related with the hematological system, inflammatory and humoral immune responses and cellular morphology and motility (gene networks 1–4, Fig. 2C). Of notice, those alterations in inflammation/immune-related pathways at 5–6 months were sustained at 11–12 months, since the most altered gene network in the CP of 11–12 months-old J20 mice regulated the inflammatory and antimicrobial responses (gene network 1, Fig. 2E) and included, as central modulators, the genes interferon beta 1 (Ifnb1) and signal transducer and activator of transcription 1 (Stat1) (Fig. 2F). The gene networks 2–5, affected at 11–12 months, showed again alterations in processes such as small molecule biochemistry, molecular transport and cellular signaling, movement and survival (Fig. 2E).

3.4. Type I and II IFN responses were altered in the CP of J20 mice

Taking into account the previous alterations, particularly those regarding gene pathways involved in the regulation of the inflammatory/immune responses in the CP of J20, we next pursued the underlying transcriptional regulators that could be responsible for such alterations in gene expression (Fig. 3 and Supplementary Table 3). For that analysis, we used the IPA Upstream Regulator application, that computed an activation z-score predictive of the activation status of a given transcriptional regulator, taking into account the expression level of the corresponding downstream target gene. This approach allowed the assembly of gene interactomes (Fig. 3B–D). At 3 months, this approach discriminated the genes Toll-like receptor 9 (Tlr9), interferon regulatory factor 7 (Irf7) and interferon (alpha and beta) receptor 1 (Ifnar1), all of them predicted to be up-regulated in the CP of J20 mice, when compared with age matched WT mice (Fig. 3A and B). Surprisingly, at the ages of 5–6 and 11–12 months, interferon gamma (Ifng) presented a low activation z-score that suggested a possible down-regulation of this gene in the CP of J20 mice (Fig. 3A, C and D). Noticeably, the inactivation of the Ifng-regulated pathway was predicted to affect the expression of 18 target genes at 5–6 months (Fig. 3C and Supplementary Table 3), an effect that was even more marked at 11–12 months, affecting 25 downstream genes/molecules (Fig. 3D and Supplementary Table 3).

To confirm and complement the array data, the expression of type I IFN-related genes [Tlr9, Ifnar1, Irf7, interferon alpha 1 (Ifna1) and interferon beta 1 (Ifnb1)] and of type II IFN-related genes [Igf2 and interleukin-1 beta (Ilb)] was measured by qRT-PCR (Fig. 4A–G). Once again, we observed an overexpression of Tlr9, Ifnar1, Irf7 and Ifnb1, in the CP of J20 mice at 3 months, when compared with WT mice; together with Ifna1, these genes remained highly expressed in the CP of J20 mice at 5–6 months when compared to 3 months-old WT mice (Fig. 4A–E). In the CP of WT mice, despite the unaltered expression of Irf7, Ifna and Ifnb1 (Fig. 4C–E) at different ages, we observed a significant overexpression of Tlr9 and Ifnar1 at 11–12 months, when compared to the 3-months-old mice of the same genotype (Fig. 4A and B). The genes Igh and Icam1, which are related with a type II IFN response, were also significantly overexpressed at 3 months in the CP of J20 mice, when compared to age-matched WT mice (Fig. 4F and C). On the other hand, at 5–6 months, there was a significant down-regulation of Ifng in the CP of J20 mice, when compared to the expression levels at 3 months in J20 and at 5–6 months in WT mice, which was maintained at 11–12 months (Fig. 4F).

Next, to discriminate the specific contribution of CP epithelial cells to the expression of type I or type II IFN genes in AD, we performed primary cultures of CP epithelial cells on a transwell system (to mimic the in vivo tight and polarized arrangement that these epithelial cells present in the blood-CSF barrier, Fig. 4H), and stimulated these cells at the apical membrane, which mimics the CSF-side, with 1 μM Aβ1–42. We observed that in response to Aβ1–42, the CP epithelial cells significantly overexpressed the type I IFN genes Tlr9 and Ifnar1, but not the type II IFN gene Igh (Fig. 5I–K).

3.5. Aging altered the pattern of type I and II IFN gene expression in the dHPC of WT and of J20 mice

To further investigate the IFN response at the brain parenchyma, we measured the expression of the same type I and II IFN genes in the dHPC of WT and J20 mice at different ages (Fig. 5A–G). As observed before in the CP of J20 mice, there was an

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**Table 2**

Main effects of age, genotype and IFN-α levels in the CSF, alone (model 1) and taking into account the interactions between independent variables (model 2), as predictors of the latency to platform in the MWM test.

<table>
<thead>
<tr>
<th>Independent variables and interactions</th>
<th>Latency (model 1)</th>
<th>Latency (model 2)</th>
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<td></td>
<td>( \beta )</td>
<td>( p )</td>
</tr>
<tr>
<td>Age</td>
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<td>Genotype*IFN-α</td>
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</table>

WT mice: \( N \) (3 months) = 12; \( N \) (5–6 months) = 7; \( N \) (11–12 months) = 9.

J20 mice: \( N \) (3 months) = 5; \( N \) (5–6 months) = 9; \( N \) (11–12 months) = 8.

No colinearity was observed between independent variables.
overexpression of type I IFN genes in the dHPC of J20 mice at 3 months, when compared to age-matched WT mice, that was statistically significant for Tlr9, Ifna1 and Ifnb1 (Fig. 5A, D and E). Additionally, we observed a progressive increase in the expression of all type I IFN genes, with aging, in the dHPC of WT mice, which was statistically significant at 11–12 months, when compared to the expression levels of WT mice at 3 months (Fig. 5A–E).

Regarding the expression of type II IFN genes in the dHPC (Fig. 5F and G), even though there was an increased expression of Icam1 in 11–12 months-old WT mice (Fig. 5F), no changes were observed on the levels of Ifng expression at the different ages (Fig. 5F).

In an attempt to unravel the specific contribution of different brain cell populations to the overexpression of type I and II IFN genes, we measured the expression of Tlr9, Ifna1 and Ifng in primary cultures of astrocytes and of neurons, at different time points upon stimulation with vehicle or 1 μM Aβ1–42. However, no statistically significant differences were observed when comparing

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**Figure 2.** Top five gene networks, and respective biological functions, specifically altered in the CP of J20 mice at different ages. The Ingenuity® Pathway Analysis setup computed the five most altered gene networks in the CP of J20 mice at 3 (A), 5–6 (C) and 11–12 (E) months, on the basis of the significantly up- (red) and down-regulated (green) genes, obtained after normalization to the expression levels in age-matched WT mice. An interaction scheme was assembled with the genes that composed the most altered network in the CP of J20 mice at 3 (B), 5–6 (D) and 11–12 (F) months.
vehicle- or Aβ1–42-treated cells at 24 and 72 h (Supplementary Fig. 4).

3.6. Early activation of astrocytes and microglia was observed in the dHPC of J20 mice

To have a different readout of the inflammatory response in the dHPC of WT and J20 mice at different ages, we measured the expression of Gfap and Iba1, as well as the morphology of astrocytes and the number of microglia/macrophages from the DG of the dHPC, based on the staining for GFAP and IBA1, respectively (Fig. 5H–K). There was a significant overexpression of Gfap and Iba1 in the dHPC of J20 mice, at 3 months, when compared to the expression levels in age-matched WT mice (Fig. 5H and J). The expression of Gfap was also significantly increased in the dHPC of WT mice at 11–12 months (Fig. 5H). Regarding the morphological characterization of astrocytes, overall, we observed that astrocytes from the dHPC of J20 mice presented a more ramified phenotype at 3 months, whereas, in the WT mice, these morphological alterations were observed at the age of 5–6 months (Fig. 5I). In that sense, except for the number of processes, a significant increase in the values of the analyzed astrocytic morphological parameters (length of individual processes, total surface and number of endings) was observed in astrocytes from the DG of J20 mice at 3 months, when compared to 3 months-old WT mice and to 5–6 months-old J20 mice (Fig. 5I). Noticeably, at 5–6 months, the astrocytes from the DG of WT mice presented more processes and were larger and more ramified than those of 5–6 months-old J20 mice and of 3 months- and 11–12 months-old mice of the same genotype (Fig. 5I). Concerning the quantification of microglia/macrophages, there was a significant increase in the number of IBA1⁺ cells per mm² in the DG of the dHPC of J20 mice at 11–12 months, when compared to all other groups (Fig. 5K).

3.7. Increased level of IFN-α in the CSF was correlated with a worst performance in the MWM

The level of IFN-α was measured in the CSF of WT and J20 mice, at different ages (Fig. 6A). Although not significant, we observed a tendency for an increased level of IFN-α in the CSF of J20 mice, when compared to 3 months-old WT mice, which was sustained over time (Fig. 6A). Interestingly, we observed a significant increase in the level of IFN-α in the CSF of WT mice at 5–6 and at 11–12 months, when compared to 3 months-old WT mice.
overexpression of levels at 3 months. (H–K) Treatment of primary cultures of CP epithelial cells, which are composed by expression in age-matched WT. At 5–6 months, the overexpression of variance of latency which was of 67.12% when no interactions were comparing the two models, the inclusion of the interaction relation between age and latency (model 2, Table 2). Of relevance, however, when the interactions between the independent vari-
ables were considered, the genotype significantly moderated the decrease in the expression of type I IFN genes, but not of the type II IFN gene overexpression of type I IFN genes, but not of the type II IFN gene expression of genes involved in the type II IFN response. Interestingly, the specific contribution of CP epithelial cells to the overexpression of type I IFN genes, but not of the type II IFN gene Ifng, shown to occur in vitro in response to Aβ, led us to suggest that the early overexpression of Ifng in the CP of J20 mice is due to immune-related cells that inhabit and circulate in the CP stromal compartment, a subject that warrants further investigation. Interestingly, the strong activation of the type I IFN response both in the CP and in the dHPC of J20 mice in early phases of the disease, before marked amyloid deposition, together with the decreased expression of Ifng in the CP at 5–6 and at 11–12 months, suggest a possible interplay between type I and II IFN responses and Aβ plaque formation. Moreover, although there is an apparent relationship between the up-regulation of type I IFN genes, the early overexpression of Gfap and Iba1 and increased astrocytic branching in the dHPC, occurring first in J20 and later in WT mice, it is still unclear whether these observations are somehow connected. Of notice, together with a lower expression of type I IFN genes a decreased branching of astrocytes and increased number of microglia/macrophages was also found in the DG of the dHPC of J20 mice at 11–12 months. Intriguingly, also at 11–12 months, along with a decreased inflammatory gene expression in the dHPC, the behavioral impairment of J20 mice seems to become milder, when compared to 3 months-old mice of the same genotype. Once again, whether there is a causal relationship between these events in the brain of J20 mice and the deficits in memory is something that needs to be addressed in the future. In addition, an age-dependent increased level of IFN-α in the CSF, observed only in WT mice, is correlated with a poor performance in a spatial reference-memory task. On the other hand, the J20 mice presented no significant alterations in the level of IFN-α in the CSF, which is in agreement with their early and sustained memory impairment. In fact, it is still controversial whether an early induction of a type I IFN inflammatory response in the brain, followed by a reduced type II IFN response, is beneficial or detrimental for AD

In order to analyze the effects of age, genotype and IFN-α levels in the CSF as predictors of the spatial-reference memory performance, we designed two regression models, to take into consideration the main effects of the independent variables alone or all the possible interactions (Table 2). The genotype and the levels of IFN-α in the CSF, alone, were significantly associated with alterations in the performance in the MWM (model 1, Table 2). However, when the interactions between the independent variables were considered, the genotype significantly moderated the relation between age and latency (model 2, Table 2). Of relevance, when comparing the two models, the inclusion of the interaction effects only represented an increment of 4.65% in the explained variance of latency which was of 67.12% when no interactions were included in the analysis.

4. Discussion

A progressive dysfunction of the blood–CSF barrier, which is formed by the CP epithelial cells, is described in AD (Johanson et al., 2004; Marques et al., 2013; Serot et al., 2012). To better understand the specific alterations that occur at this barrier, we used the J20 mouse model of AD (Baron et al., 2014; Mucke et al., 2000) and investigated the changes in the CP transcriptome at the ages of 3, 5–6 and 11–12 months, in comparison with age-matched WT mice. We observed aging-induced alterations in the CP transcriptome involving gene networks that regulate central processes, such as the cellular circadian rhythm (Zhang and Kay, 2010), which are likely to affect the metabolic activity and cytoarchitecture of the blood–CSF barrier and to contribute to a defective turnover of the CSF (He et al., 2014; Serot et al., 2012). Regarding the CP of J20 mice, there was a sustained overexpression of genes that participate in the type I IFN response and a clear decrease in the expression of Ifng, the major type II IFN gene. In a later age, but following the same direction, the CP of WT mice displayed an aging-associated increased expression of genes that encode for two receptors that are involved in the type I IFN response, Tlr9 and Ifnar1, but no significant alterations in the expression of genes involved in the type II IFN response. Interestingly, the specific contribution of CP epithelial cells to the overexpression of type I IFN genes, but not of the type II IFN gene Ifng, shown to occur in vitro in response to Aβ, led us to suggest that the early overexpression of Ifng in the CP of J20 mice is due to immune-related cells that inhabit and circulate in the CP stromal compartment, a subject that warrants further investigation. Interestingly, the strong activation of the type I IFN response both in the CP and in the dHPC of J20 mice in early phases of the disease, before marked amyloid deposition, together with the decreased expression of Ifng in the CP at 5–6 and at 11–12 months, suggest a possible interplay between type I and II IFN responses and Aβ plaque formation. Moreover, although there is an apparent relationship between the up-regulation of type I IFN genes, the early overexpression of Gfap and Iba1 and increased astrocytic branching in the dHPC, occurring first in J20 and later in WT mice, it is still unclear whether these observations are somehow connected. Of notice, together with a lower expression of type I IFN genes a decreased branching of astrocytes and increased number of microglia/macrophages was also found in the DG of the dHPC of J20 mice at 11–12 months. Intriguingly, also at 11–12 months, along with a decreased inflammatory gene expression in the dHPC, the behavioral impairment of J20 mice seems to become milder, when compared to 3 months-old mice of the same genotype. Once again, whether there is a causal relationship between these events in the brain of J20 mice and the deficits in memory is something that needs to be addressed in the future. In addition, an age-dependent increased level of IFN-α in the CSF, observed only in WT mice, is correlated with a poor performance in a spatial reference-memory task. On the other hand, the J20 mice presented no significant alterations in the level of IFN-α in the CSF, which is in agreement with their early and sustained memory impairment. In fact, it is still controversial whether an early induction of a type I IFN inflammatory response in the brain, followed by a reduced type II IFN response, is beneficial or detrimental for AD

Fig. 4. CP epithelial cells contributed differently to the overexpression of type I and II IFN genes in response to increased levels of Aβ peptides. (A–G) The expression of type I and II IFN genes in the CP was measured by qRT-PCR. Except for Ifna1, all the genes were significantly overexpressed in the CP of J20 mice at 3 months when compared to the expression in age-matched WT. At 5–6 months, the overexpression of Tlr9 (A), Ifnar1 (B) and Ifnb1 (E) was maintained in the CP of J20 mice, along with a significant overexpression of Ifna1 (D). Also in the CP of J20 mice at 5–6 months, there was a significant down-regulation of Ifng (F), when compared to expression levels in J20 mice at 3 months and in WT mice at 5–6 months. (F, G) Both the expression of Ifng and Icam1 were significantly decreased in the CP of J20 mice at 11–12 months, when compared to the expression levels at 3 months. (A, B) In the CP of WT mice, we observed a significant overexpression of Tlr9 and Ifnar1 at 11–12 months, when compared to the expression levels at 3 months. (H–K) Treatment of primary cultures of CP epithelial cells, which are composed by > 95% of TTR+ cells, with 1 μM Aβ<sub>42</sub> for 60 h induced a significant overexpression of Tlr9 (I) and Ifnar1 (J), but not of Ifng (K). Results regarding in vivo gene expression (A–G) are presented as mean ± SEM (N = 5/group); *p < 0.05 and “p < 0.01; Kruskal–Wallis with Dunn’s multiple comparison test (two-tailed). Results regarding in vitro gene expression (I–K) are presented as mean ± SEM (N = 4/group); “p < 0.05; two-tailed Mann Whitney test. Scale bar indicates 100 μm in (H).
pathogenesis (Baruch et al., 2014; Scholtzova et al., 2009, 2014; Taylor et al., 2014). Regarding the type II IFN response in AD, it has been previously shown that overexpression of IFN-γ boosts the amyloidogenic processing of APP both in vitro and in vivo (Blasko et al., 1999; Mastrangelo et al., 2009). In accordance, it was recently suggested that a higher frequency of IFN-γ-producing T-cells in the brain of AD transgenic mice is associated with increased glial activation and amyloid plaque formation, as well as with worst cognitive function (Brown et al., 2013). However, there is also evidence of a beneficial impact of increased levels of IFN-γ-producing T-cells in AD transgenic mice at 9 months of age, namely by increased microglial activation, increased Aβ phagocytosis and decreased plaque burden in the DG of the HPC (Monsonego et al., 2006). These divergent observations may be due to the specific effects of IFN-γ overproduction at different stages of brain pathology in AD, which prompts for a deeper characterization of the role of the type II IFN mediators in the AD brain at different ages. On the other hand, concerning the type I IFN response, a recent study showed that at the level of the CP of 22 months-old mice, increased levels of IFN-β signaling promote increased glial activation and impact on cognitive behavior (Baruch et al., 2014). Moreover, it was suggested that an aging-related central nervous system (CNS)-derived stimulus could be in the genesis of a shift from a type II IFN into a type I IFN response at the CP, and that this would compromise the immune-surveillance of the brain by decreasing the levels of IFN-γ (Baruch et al., 2014; Kunis et al., 2013). Interestingly, even in the absence of a viral infection, increased levels of type I IFN cytokines in the brain, particularly IFN-α, lead to massive encephalopathy, with marked meningoencephalitis, calcium mineralization, gliosis, and neurodegeneration (Akwa et al., 1998). In the context of AD, others have shown that the genes encoding IFN-α and IFN-β are overexpressed in cultured neurons, upon stimulation with Aβ, and in cortical cells extracted from the brains of AD patients, in a mechanism dependent on IRF7 and IFNAR1 (Akwa et al., 1998). In contrast, stimulation of the innate response with TLR9 agonists =cytosome-guanosine-containing DNA oligodeoxynucleotides (CpG DNA oligos), in two different mouse models of AD, ameliorated both the pathologic hallmarks and the
cognitive deficits presented by these mice, without any obvious inflammatory side-effects (Scholz et al., 2009, 2014). Also, increased production of IFN-β by astrocytes, upon specific TLR3 stimulation, was shown to affect the spontaneous activity of neurons from the CA1 region of the HPC. This effect of IFN-β on neuronal excitability was shown to be mediated by IFNAR1, due to the lack of changes in the HPC of Ifnra1-null mice (Costello and Lynch, 2013). Additionally, 9 months-old transgenic APP/PS1 mice present higher levels of IFN-α in the brain, when compared to age-matched WT mice (Taylor et al., 2014). Interestingly, our results also suggest that, along with a possible impact on behavior, an activation of a type I IFN response in the dHPC, in younger J20 mice and in 11–12 months-old WT mice may be linked with changes in the astrocytes’ morphology. It is widely accepted that increased levels of inflammatory molecules, whether in the circulation or in specific regions of the CNS, can affect the morphology and function of brain cells, especially of glial cells (Baron et al., 2014; Ghosh et al., 2013). Astrocytes in particular seem to play a central role in the inflammatory response and are good indicators of the level of brain inflammation (Medeiros and LaFerla, 2013; Wyss-Coray, 2006). Concurrently, the activation of astrocytes and microglia was shown to occur before Aβ plaque formation in the HPC of J20 mice (Baron et al., 2014; Beauquis et al., 2014; Wright et al., 2013) and that an increased microglial activation and clustering, observed at 4 weeks of age in the HPC of these mice, precedes neuronal atrophy, decreased neurogenesis and a decrease in the volume of the HPC (Fu et al., 2014). However, it is necessary to further investigate whether the early induction of type I IFN genes in the dHPC of J20 mice is directly promoting changes in glial activation and Aβ pathology. Moreover, it will be important to explore the impact of increased level of type I IFN cytokines, such as IFN-α, on the premature memory deficits observed in this AD mouse model and on the memory and cognitive impairments in older WT mice.

Overall, this study reveals that structures such as the CP and the dHPC may be key elements in the inflammatory signaling in the CNS, by modulating the levels of IFNs, with a possible impact on memory and cognition.

Competing interests

The authors have no conflicting financial interests to declare.

Database linking

According to the Minimum Information About a Microarray Experiment (MIAME), the microarray raw data has been deposited in the GEO database, with the accession number GEO: GSE66598.

Acknowledgments

The authors would like to acknowledge Pedro Moreira, Madalena Esteves and Dr. Patrício Costa (all from the ICVS/3B’s – PT Government Associate Laboratory) for comments and scientific input regarding the statistical analysis of this study. Sandro Dá Mesquita and Ana C. Ferreira are recipients of PhD fellowships by the Fundação para a Ciência e Tecnologia (FCT, Portugal)/FEDER and Programa Operacional Potencial Humano (POPH/FSE), references SFRH/BD/217/12013 and SFRH/BDE/51989/2012, respectively. Fernanda Marques is an assistant researcher and recipient of a FCT Investigator Grant with the reference IF/00231/2013. This work was supported by FCT and COMPETE through the project EXPL/NEU-OSD/2196/2013 and the Bial Foundation through the Grant 217/12.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.06.008.

References


