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Transcriptomic Analyses of Neurotoxic Astrocytes Derived from Adult Triple Transgenic Alzheimer's Disease Mice

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Abstract

Neurodegenerative diseases such as Alzheimer's disease have been classically studied from a purely neuronocentric point of view. More recent evidences support the notion that other cell populations are involved in disease progression. In this sense, the possible pathogenic role of glial cells like astrocytes is increasingly being recognized. Once faced with tissue damage signals and other stimuli present in disease environments, astrocytes suffer many morphological and functional changes, a process referred as reactive astrogliosis. Studies from murine models and humans suggest that these complex and heterogeneous responses could manifest as disease-specific astrocyte phenotypes. Clear understanding of disease-associated astrocytes is a necessary step to fully disclose neurodegenerative processes, aiding in the design of new therapeutic and diagnostic strategies. In this work, we present the transcriptomics characterization of neurotoxic astrocytic cultures isolated from adult symptomatic animals of the triple transgenic mouse model of Alzheimer's disease (3xTg-AD). According to the observed profile, 3xTg-AD neurotoxic astrocytes show various reactivity features including alteration of the extracellular matrix and release of pro-inflammatory and proliferative factors that could result in harmful effects to neurons. Moreover, these alterations could be a consequence of stress responses at the endoplasmic reticulum and mitochondria as well as of concomitant metabolic adaptations. Present results support the hypothesis that adaptive changes of astrocytic function induced by a stressed microenvironment could later promote harmful astrocyte phenotypes and further accelerate or induce neurodegenerative processes.

Keywords Adult 3xTg-AD mice · Astrocytes · Neurodegeneration · Alzheimer's disease · Transcriptomics

Introduction

Astrocytes are abundant and ubiquitous glial cells of the central nervous system (CNS) originated from neural progenitors. Once considered bystander cells, astrocytes are now being increasingly recognized as crucial players in brain function specially due to its involvement in supportive and regulatory processes including maintenance of the blood–brain barrier (BBB), neuronal metabolic support, production of antioxidants, water and ion homeostasis (pH, K^+ buffering), dendrite outgrowth, synapse development and plasticity, gap-junction communication, neurotransmitter regulation (glutamate and GABA, ATP, D-serine, among others), and immune responses (Sofroniew and Vinters 2010; Verkhratsky and Nedergaard 2017). Increasing evidence shows that astrocytes present a diversity of subtypes and states closely related to their role in health and disease processes. Considering their anatomical distribution astrocytes can be broadly classified into white

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matter astrocytes (fibrous), grey matter astrocytes (protoplasmic), ependymal astrocytes, radial glia, and perivascular astrocytes (Verkhratsky and Nedergaard 2017). In addition, as part of the brain defense and repairing processes, astrocytes can differentiate into a number of states broadly called astrocyte reactivity. Structural and functional changes associated with astrocyte reactivity are observed during aging, infection, trauma, ischemia, and neurodegenerative diseases among other processes (Dossi et al. 2018; Sofroniew 2020). Morphological changes classically associated with its reactivity include increased expression of structural proteins such as glial fibrillary acidic protein (GFAP) and vimentin, hypertrophy of the soma and cell projections, and proliferation. It is worth noting that these features are a narrow representation of context-dependent processes. More detailed studies regarding structural, developmental, and functional diversity of astrocytes from distinct brain regions are an ongoing area of research (Chai et al. 2017; Sofroniew 2020).

Reactive astrogliosis provide adaptive functions that help preserve neuronal tissue in multiple disorders, even though in contexts like excessive neuro-inflammation it can also be associated with loss of astrocyte homeostatic functions or even gain of detrimental functions (Glass et al. 2010; Heneka et al. 2014; Sofroniew 2020). In this sense, astrocyte dysfunction could be involved in many diseases of the nervous system, such as Alzheimer's disease (Heneka et al. 2015; Acosta et al. 2017). Alzheimer's disease (AD) is a neurodegenerative disorder manifested by progressive cognitive impairment and memory loss. The underlying neuropathology includes brain deposition of aggregates of misfolded amyloid-β (Abeta) peptide, hyperphosphorylated microtubule-associated protein tau, and neuronal loss (Selkoe and Hardy 2016). It is postulated that stress conditions associated with the disease microenvironment may trigger reinforcing interactions between astrocytes and other glial cells resulting in a compromise of astrocyte and neuronal function as well as chronic neuroinflammation (Wheeler et al. 2019; Joshi et al. 2019; Sofroniew 2020). This could result in astrocyte detrimental effects like altered homeostasis of neurotransmitters glutamate and GABA, K^+ and Ca^+ ion homeostasis, release of pro-inflammatory mediators, build-up of intracellular Abeta, and a reduced proliferative capacity of reactive astrocytes during aging (Nagele et al. 2003; Grimaldi et al. 2019; Sofroniew 2020). Deleterious effects of astrocytic populations have been observed in other diseases and animal models (Varcianna et al. 2019; Spanos and Liddelow 2020). Some examples include the "aberrant" astrocytes isolated from SOD1G93A transgenic mouse model of amyotrophic lateral sclerosis, the ischemia-derived astrocytes isolated from early ischemic penumbra and core of adult rats, or the more broadly defined inflammatory type "A1" astrocytes (Díaz-Amarilla et al. 2011; Zamanian et al. 2012; Villarreal et al. 2016). These astrocytes present an increased proliferative capacity and neurotoxic effects associated with the secretion of toxic factors in the conditioned media. In our previous study, we isolated and cultivated astrocytes from cortex and hippocampus of symptomatic 9–10-month-old 3xTg-AD mice (old 3xTg-AD astrocytes), a model of AD which replicates most of the disease features including plaque and tangle pathology, age cognitive deficits, astrocyte reactivity, and brain atrophy (Oddo et al. 2003a, b; Bittner et al. 2010; Giuliani et al. 2013, 2014; Belfiore et al. 2019; Chiquita et al. 2019; Javonillo et al. 2022). These old 3xTg-AD astrocytes showed differential expression of astrocytic markers compared to controls. Likewise, in line with the previous examples, they presented a high rate of proliferation and were neurotoxic to primary neuronal cultures both in neuron-astrocyte co-cultures and when their conditioned medium (ACM) was added to the neuronal cultures (Diaz-Amarilla et al. 2022).

Characterizing the cellular and molecular mechanisms underlying the effects of reactive astrocytes in AD represents a key research goal. Thus, in the present work, we further studied these old 3xTg-AD neurotoxic astrocytes through transcriptomics lens. The observed transcript profile suggested a particular reactivity state, produced from underlying stress responses such as an unfolded protein response related to endoplasmic reticulum (ER) stress. Old 3xTg-AD neurotoxic astrocytes presented signals of metabolic adaptation to cellular stress at the mitochondria, glycolysis, and other energetic routes as well as alteration of the extracellular matrix and release of pro-inflammatory and proliferative factors. These alterations would constitute adaptive microenvironmental-induced changes in astrocyte function during disease progression that could later promote or accelerate neurodegeneration through synergic mechanisms.

Methods

Materials

All reagents were acquired from Sigma unless otherwise stated.

Animals

All procedures using laboratory animals were performed in accordance with the international guidelines for the use of live animals and were approved by the Institutional Animal Ethics Committee (CEUA-CUDIM) as established by the National Committee of Animal Experimentation (CNEA). Mice of female sex homozygous 3xTg-AD (B6;129-Tg(APPSwe, tauP301L)1Lfa Psen1$^{(tm1Mpm)}$/Mmjax strain) (Oddo et al. 2003b) and wild-type non-transgenic female mice (Non-Tg)

(C57BL/6 J strain) (The Jackson Laboratory) were bred and housed in CUDIM SPF centralized animal facility with a 12-h light–dark cycle and ad libitum access to food and water.

Astrocyte Cultures from Adult and Neonatal Mice

Old 3xTg-AD astrocytes were prepared from the cerebral cortex and hippocampus of 9–10-month-old 3xTg-AD mice according the methods described previously (Diaz-Amarilla et al. 2022). Cells were isolated from female mice, since in the 3xTg-AD model, sexual differences have been observed in the development of the pathology, such as expression of plaques and tangles and behavioral deficits, with a greater expression of transgenes in females (Clinton et al. 2007; Carroll et al. 2010; Belfiore et al. 2019). Processed cells were plated in tissue flasks and then maintained at 37 °C in a humidified 5% CO_2 incubator. Culture medium was removed after 7 days *in vitro* (DIV), then replaced every 3–4 days, and at 21–28 DIV were passaged for the first time. Cells were amplified and maintained at 37 °C in a humidified 5% CO_2 incubator.

As previously reported, the low yield achieved from astrocytes isolation of 9–10-month-old non-Tg mice prevented their use with statistical value in our studies (Diaz-Amarilla et al. 2022). Thus, astrocytic cultures derived from neonatal non-Tg mice as well as astrocytes from neonatal 3xTg-AD mice were used as controls. The latter non-toxic controls allowed us to assess whether astrocytes from this mice model of AD exhibit alterations when isolated before the disease onset. To do so, primary neonatal astrocytes were prepared from the cerebral cortex and hippocampus of new-born (postnatal day 0–2) 3xTg-AD, and C57BL/6 J mice according the methods described by Cassina et al. with minor modifications (Cassina et al. 2002; Diaz-Amarilla et al. 2022). Briefly, the tissues were minced and then dissociated both enzymatically (in trypsin 0.25%/EDTA 0.02% for 10 min at 37 °C) and mechanically to obtain single cell suspension. After that, remains of not disaggregated tissue were allowed to decant for 2 min without disturbing the cell suspension. Once plated, cell culture medium was changed once every 2–3 days. When cells grew to confluence after approximately 10 DIV, flasks were vigorously shaken to remove the loosely attached contaminant cells such as microglia and oligodendrocyte progenitor cells (OPCs). They were later incubated for another 48 h with 10 µM cytosine arabinoside, and then amplified and maintained at 37 °C in a humidified 5% CO_2 incubator.

RNA-seq Analysis

Sample Processing

Total RNA was extracted from adult 3xTg-AD, neonatal 3xTg-AD, and non-Tg astrocytic cultures plated in 35-mm Petri dishes by a three-stage process with Trizol (TRI Reagent® Sigma-Aldrich T9424), column purification (PureLink™ RNA mini kit Cat. 12183018A), and removal of contaminant DNA with DNAse treatment (PureLink™ DNase Set Cat. 12,185,010) according to the manufacturer-recommended procedures. Three biological replicate samples were used for RNA isolation for each experimental group. RNA sample quality was verified using a Bioanalyzer (RNA Integrity Number > 8 for all samples). Samples were stored in RNAStable (RNAstable® Tube Kit Ref. 93,221–001) before shipping. Library preparation with poly-A selection (NEBNext) and high throughput sequencing were performed at by GENEWIZ Inc. (South Plainfield, NJ) using an Illumina HiSeq (2 × 150 bp, ~ 38 M depth).

Bioinformatics

Raw data was processed according to previous described protocols (Love et al. 2015; Chen et al. 2016; Smyth et al. 2018). Preliminary quality control was performed with FastQC. Adapters, low quality reads, etc. were trimmed with SeqPurge (phred score > 33) (Sturm et al. 2016). Read alignment, count, and annotation were performed in R with package Rsubread (Liao et al. 2019) and function featureCounts (Liao et al. 2014) using mouse reference genome GRCm38, paired-end reads, and multi mapping allowed. Reads were annotated sing Rsubread mm10 in-built annotations from NCBI-RefSeq. Around 16,000 transcripts were detected with at least 0.5 counts per million bases in at least two samples. Statistical analyses were performed with package edgeR (Robinson et al. 2010). Differential gene expression analysis was performed with limma-voom (p-value 0.05, 5% FDR) (Law et al. 2014). A comprehensive and lenient approach for gene ontology and pathway analysis was performed by joining results of GAGE, camera, and goana package and functions (Luo et al. 2009; Young et al. 2010; Wu and Smyth 2012). Top GO terms were selected if p-value < 0.01 for either GAGE or camera functions or < 1E −04 for goana function. Enriched GO terms were further prioritized using GOxploreR function which considers each term position in the ontology hierarchy (Manjang et al. 2020). Plots of the relative importance of each GO term were generated with REVIGO web server using minimum p-value observed (Supek et al. 2011). Enriched KEGG pathways were gathered if p-value < 0.01 for any of the previous functions. Colored KEGG maps of selected pathways were generated with the aid KEGG Mapper color tool (Kanehisa et al. 2021). Network interactions of selected differentially expressed genes (DEGs) were plotted with STRING database web server (Szklarczyk et al. 2019).

Western Blotting

Astrocyte confluent monolayers were washed with PBS and then lysed with appropriate buffer containing 50 mM Hepes (pH 7.5), 50 mM NaCl, 1% Triton X-100, and complete protease inhibitor mixture (Roche cOmplete™ Ref. 11,697,498,001). Protein quantification was achieved using a Bicinchoninic Acid (BCA) kit. Protein samples were prepared in Laemmli sample buffer 5x, resolved on 12% SDS-PAGE gels, and transferred onto PDVF membranes (BioRad) using a wet transfer system. Membranes were blocked in 5% dried milk in TBS-T (0.5 mM Tris base, pH 8, 75 mM NaCl, and 0.2% (vol/vol) Tween-20) for at least 2 h at room temperature. Membranes were incubated with primary antibody diluted in 2% BSA in TBS-T and then with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 2% BSA in TBS-T for 1 h. Membranes were developed using chemiluminescent substrates (Thermo). Primary antibodies used in the study include as follows: anti-β-actin (1–4000) (A5441 Sigma), anti-GLT1 (1–500) (PA5-17,099 Thermo Fisher), anti-Nestin (ab11306 Abcam), OxPhos Rodent WB Antibody Cocktail (1–1000) (45–8099 Thermo Fisher).

Results and Discussion

Transcriptomic Profile Distinguishes Adult Symptomatic 3xTg-AD Mice-Derived Astrocytes from Those Obtained from 3xTg-AD Neonates

Neurotoxic astrocytes obtained from adult symptomatic 3xTg-AD mice (Tg.Old) were studied and compared against neonatal astrocytes obtained from 3xTg-AD mice (Tg.Neo). Multivariate analysis, PCA and clustering analysis, showed a clear distinction between neurotoxic astrocytes and non-toxic control astrocytes (Fig. 1). Notably most of the variation was observed on the first principal component, leaving a minor variation in the second component where the neonatal control group was distributed. Analysis of differential gene expression showed around 2500 upregulated and 3300 downregulated genes from a total of around 16,400 transcripts that passed the expression threshold criteria. Although the focus of the present study is to assess astrocytic changes that have arisen from age and disease progression but not from transgenesis per se, our experimental design also included astrocytes isolated from neonatal wild-type mice (Wt.Neo). A comparison including this group showed that it clusters together with the Tg.Neo astrocytes on the PCA scores plot and in the hierarchical clustering plot (see Online Resource 1, Fig. S1). No differentially expressed genes were found either under the current criteria. These results further corroborate our previous findings where both Tg.Neo and Wt.Neo astrocytes behaved equally and showed no signs of neurotoxicity, thus supporting the idea that old 3xTg-AD astrocytes are a product of age and disease progression (Diaz-Amarilla et al. 2022).

Astrocyte diversity extends through both anatomical and developmental dimensions, as well as differentiating into reactive states under stress conditions (Khakh and Sofroniew 2015; Sofroniew 2020). Multiple markers have been associated with the astrocyte lineage and its different states, each with different degree of specificity, being the most classical markers GFAP, glutamine synthetase (GS, Glul), Aldh1l1,

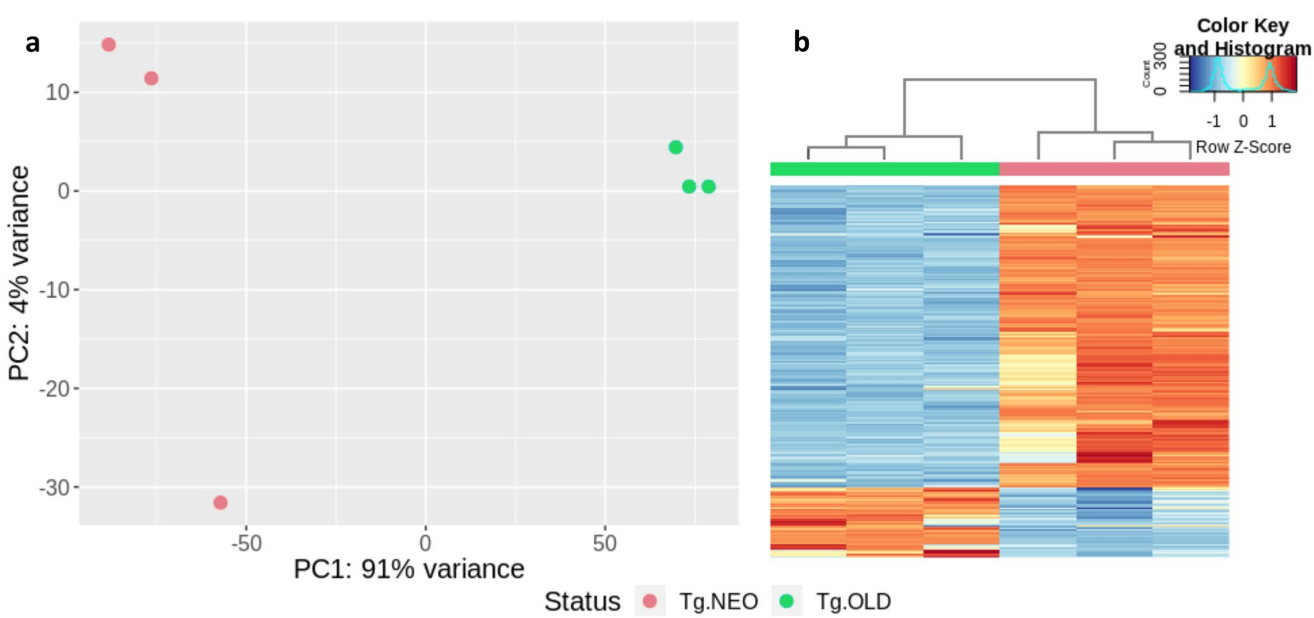


Fig. 1 Multivariate statistical analysis of astrocyte transcriptomes. **a** PCA analysis. **b** Hierarchical clustering of top 500 most variable transcripts

and S100b among others (Cahoy et al. 2008; Boisvert et al. 2018; Zhang et al. 2019). As an approach to characterize 3xTg-AD neurotoxic astrocytes and place them into astrocyte diversity evidenced so far, we assessed the expression of broad astrocyte markers on the transcriptomics data. A list of markers was gathered from literature sources and online databases (Cahoy et al. 2008; Molofsky et al. 2012; Zhang et al. 2014; Boisvert et al. 2018) (see full list of markers and associated references in Online Resource 2). From the transcriptomics data analyzed, we found a group of typical astrocyte markers not differentially expressed (Glul, S100b, Cd44, Gjb6/Cx30, Slc4a4, etc.); a second group of downregulated markers (Gfap, Slc1a2/GLT1/EAAT2, Nes/Nestin, Aldh1l1, Sox9, Aqp4, Slc1a3/GLAST/EAAT1, etc.); and a third subset of upregulated astrocyte markers (Fig. 2). Considering expression log fold change (logFC), some of the most remarkable astrocytic markers in Tg.Old cells would be Dio2, Slc7a11, Aqp1, and Slc1a6/EAAT4 with logFC above 4. Dio2 is an enzyme related to thyroid hormone metabolism (Morte and Bernal 2014). The cystine-glutamate exchanger (xCT, Slc7a11) is a membrane transporter that promotes glutathione synthesis by catalyzing cysteine uptake and glutamate release. It appears to be expressed in scattered astrocytes throughout the mouse brain and more strongly in select blood–brain-CSF interface areas (Ottestad-Hansen et al. 2018). Slc1a6 is a glutamate transporter expressed both in astrocytes and cerebellar Purkinje cells (Hu et al. 2003). Similarly water channel Aqp1 is expressed in both neurons and astrocytes but with some regional specificity (Gao et al. 2012). Other interesting astrocyte markers upregulated in the transcriptomics data are Tumor protein D53 (Tpd52l1) and frizzled homolog 2 (Fzd2). Tpd52l1 is associated with calcium signaling and cell cycle and Fzd2 is a Wnt receptor found selectively and highly expressed in mouse astrocytes (Boutros and Byrne 2005; Cahoy et al. 2008; McKenzie et al. 2018).

Noteworthy some of the suggested upregulated markers are related with astrocyte function in disease (Fig. 2). Calmodulin-regulated spectrin-associated protein 1 (Camsap1) is a cytoskeleton-associated protein expressed on both GFAP-negative and GFAP-positive astrocytes (Yoshioka et al. 2012). In rats, Camsap1 immunoreactivity was observed in ependymal cells bearing various astrocyte markers, and increased after injury, suggesting that this could be a marker of newly born astrocytes (Yoshioka et al. 2012). Milk fat globule epidermal growth factor 8 (Mfge8) is a secreted phagocytic protein that might increase with age in

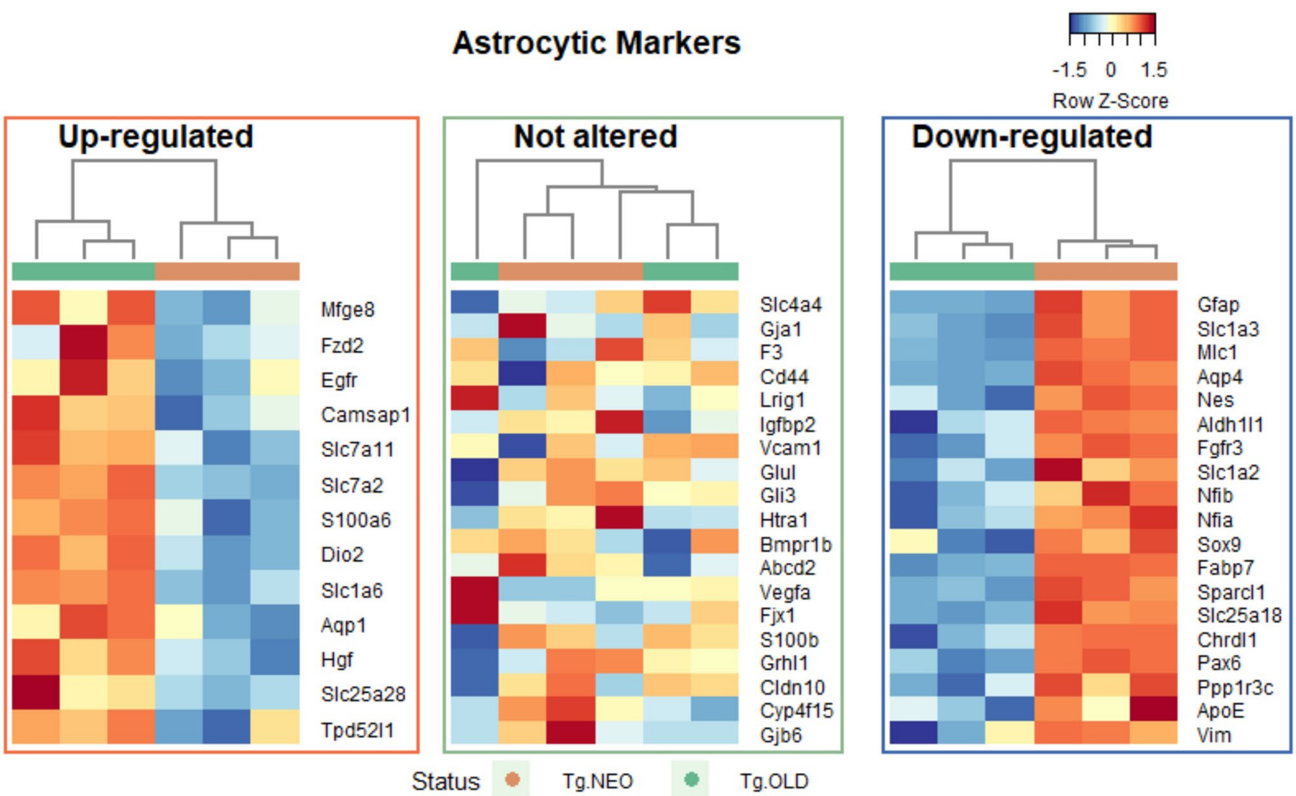


Fig. 2 Astrocyte markers upregulated, not altered, and downregulated in Tg.Old astrocytes transcriptome. Markers broadly associated with astrocytes were gathered from literature and online databases (Cahoy et al. 2008; Molofsky et al. 2012; Zhang et al. 2014; Boisvert et al. 2018). For brevity, only a marker subset is represented (see full marker list and associated literature references in Online Resource 2)

mouse and possibly has a role in astrocyte reactivity regulation (Clarke et al. 2018; Xu et al. 2019; Li et al. 2019). S100a6 (Calcyclin) is calcium binding protein involved in proteolytic degradation, cytoskeletal dynamics, and cell proliferation and associated with neural and astrocyte precursors in the mouse hippocampus (Hoyaux et al. 2002; Yoshioka et al. 2012). Noteworthy, expression of this protein is linked to impaired axons from both amyotrophic lateral sclerosis (ALS) mouse model and human patients, and it is induced in mouse astrocytes after stroke (Hoyaux et al. 2002; Zamanian et al. 2012). Another interesting marker is the carrier protein Slc25a28, which could also be associated with mitochondrial dysfunction (Babenko et al. 2018).

Downregulation of GFAP was also observed at the protein level by western blot in our previous work (Diaz-Amarilla et al. 2022). In order to further validate our transcriptomic data, here we explored the protein expression of additional cell markers associated with astrocyte function that were observed altered in the transcriptomic profile of Tg.Old astrocytes: Nestin and GLT-1 (Fig. 3). Nestin is a type VI intermediate filament protein expressed in radial glia multipotent progenitors in the embryonic brain, but mature astrocytes do not show Nestin expression and it is also associated with reactive astrocytes (Cho et al. 2013; Potokar et al. 2020; Jurga et al. 2021). GLT-1 (Glutamate transporter-1) is a Na^{+}-dependent transporter that plays a key role in glutamate homeostasis by removing excess glutamate in the CNS. Glutamate excitotoxicity is associated with various neurodegenerative processes (Pajarillo et al. 2019), and reduced expression of this transporter is observed in AD (Monterey et al. 2021). Furthermore, GLT-1 has been proposed as one potential marker for reactive astrocytes in different pathological contexts (Díaz-Amarilla et al. 2011; Escartin et al. 2021), with a decrease in ARNm and protein expression, in accordance with the results described in our present manuscript.

Reactive Astrocytic Marker Profile of Tg.Old Astrocytes

Astrocytes respond to CNS injury and disease through a mechanism referred as reactive astrogliosis that can lead to a wide range of changes in their molecular expression, morphology, and function. These changes can in turn influence diverse outcomes in the CNS, from neuroprotection, and preservation of homeostasis and neurological functions, to contribution to detrimental effects through loss of beneficial functions or gain of abnormal functions. Such dysfunctions are regulated by molecular signaling events that are context-specific (Zamanian et al. 2012; Sekar et al. 2015; Sun et al. 2015; Itoh et al. 2017; Henrik Heiland et al. 2019; Diaz-Castro et al. 2019; Wheeler et al. 2020; Al-Dalahmah et al. 2020; Habib et al. 2020; Escartin et al. 2021). Thus, we focus on astrocytic reactivity markers as a way to characterize molecular expression patterns of Tg.Old astrocytes in the context of CNS disorders. In this sense, a list of around 300 markers associated with reactivity was gathered from literature sources (Zamanian et al. 2012; Liddelow and Barres 2017; Escartin et al. 2021; Jurga et al. 2021) (see full list of markers and associated literature references in Online Resource 2). From these, a fraction of around 70 markers was found differentially expressed in the transcriptomics data in a direction consistent with reactivity (see a sample subset in Fig. 4a). Considering a logFC threshold of 4, some of the most remarkable upregulated markers would be Timp1 and Plaur, while from those expected to be downregulated, Slc1a2 (GLT-1, EAA2), Slc1a3 (GLAST), and Kcnj10 (Kir4.1) would be the most remarkable. Alteration of some of these markers has been associated with harmful effects in the CNS. As previously stated, this is the case of the Slc1a2 gene (GLT-1, EAAT2 glutamate transporter) that has been proposed as a potential marker of reactive astrocytes in different pathological contexts (Díaz-Amarilla et al. 2011;

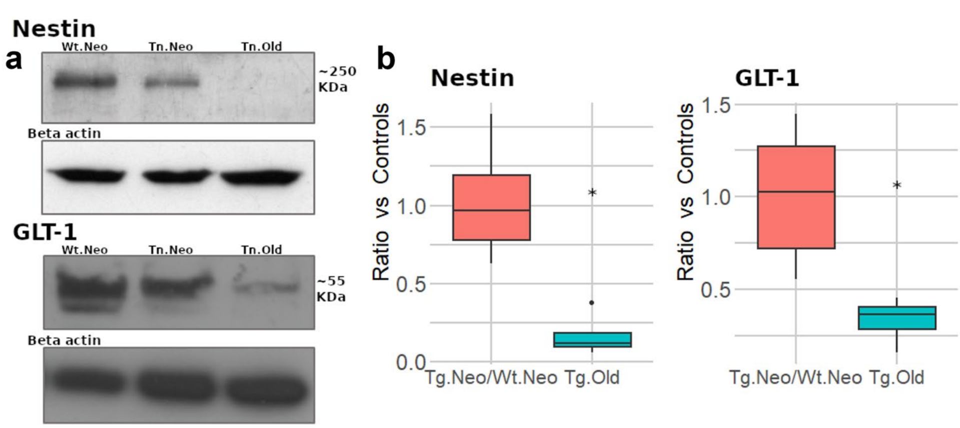


Fig. 3 Protein expression of GLT-1 and Nestin markers in Tg.Old astrocytes. A lenient approach was followed to detect trends in the protein expression: Tg.Old group samples were compared against control samples Tg.Neo and Wt.Neo joined as a single group, values were normalized against Beta actin expression and additionally by the average expression of controls from each assay (Ratio). Under this approach, downregulation of both proteins was observed: Nestin (p.adj = 0.004), GLT-1 (p.adj = 0.01) (Wilcoxon test)

Escartin et al. 2021), showing a decrease in mRNA and protein expression which is in agreement with our results (Figs. 2, 3, and 4). In addition to GLT-1, GLAST is another astrocyte glutamate transporter with an important role in glutamate homeostasis, also showing reduced expression in AD (Monterey et al. 2021). Kir4.1 is an inwardly rectifying K^+ channel exclusive of glial cells that regulates astrocyte resting membrane potential, cell volume, and facilitates glutamate uptake (Nwaobi et al. 2016). Reduced expression is associated with AD and ALS among other pathologies (Wilcock et al. 2009; Olsen et al. 2015). In particular, decreased astrocytic Kir4.1 current may promote cell cycle progression and proliferation and has been associated with brain trauma, ischemia, and inflammation (Nwaobi et al. 2016). Plaur gene encodes a membrane protein that binds the serine proteinase, urokinase-type plasminogen activator (uPA), and activates a cascade of extracellular proteinases that function in tissue remodeling. It has been associated with axonal damage and neuroinflammation in multiple sclerosis as well as glioblastoma cell proliferation (Gveric et al. 2001; Gilder et al. 2018). Timp1 is a cytokine released from astrocytes that has been associated with a neuronal protective effect upon Abeta exposure (Saha et al. 2020).

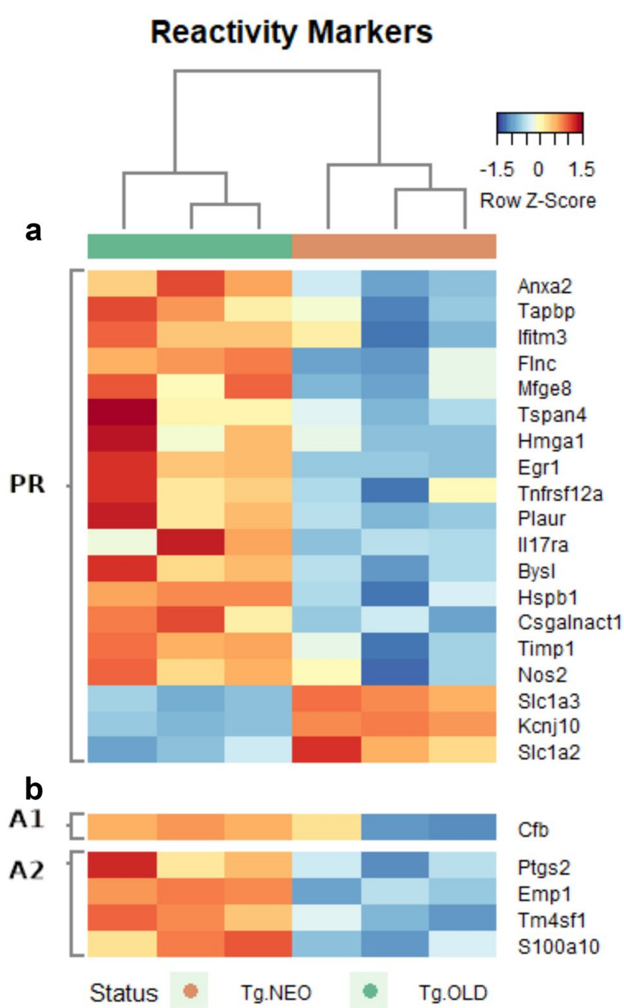


Fig. 4 Reactive astrocyte markers in Tg.Old astrocytes. **a** Markers broadly associated with astrocyte reactivity and "pan reactive" markers ("PR"). **b** Type "A1" and type "A2" reactive astrocyte markers. Markers were gathered from multiple literature references (Zamanian et al. 2012; Liddelow and Barres 2017; Escartin et al. 2021; Jurga et al. 2021). For brevity, only a subset of markers of those present among DEGs with an expression pattern consistent with reactivity is depicted (see full marker list and associated literature references in Online Resource 2)

Other noteworthy upregulated reactive astrocytic markers include interferon-induced transmembrane protein 3 (Ifitm3), heat-shock protein B1 (Hspb1), bystin (Bysl), and annexin A2 (Anxa2), which are related with Abeta production, inflammation, and proliferation. Ifitm3 promotes Abeta production though interaction with gamma secretase (Hur et al. 2020). Increased Hspb1 is associated with neuroinflammation and can be liberated by astrocytes after exposure to Abeta (Nafar et al. 2016; Dukay et al. 2021). Bystin is an adhesion molecule upregulated under brain damage, and promotes proliferation though phosphorylation of glycogen synthase kinase-3β (Gsk-3β) (Sha et al. 2020). Anxa2 is a calcium-dependent multifunctional phospholipid-binding protein that promotes glioma proliferation (Chen et al. 2019a).

Recent attempts to molecularly classify reactive astrocytes include the inflammatory type "A1" and the ischemic related type "A2" (Zamanian et al. 2012; Liddelow and Barres 2017). A2 reactive astrocytes have been associated with protective functions while A1 with neurotoxicity and loss of trophic support. Several markers have been proposed to distinguish these cell types *in vitro* and *in vivo* (Liddelow et al. 2017; Clarke et al. 2018). Figure 4b presents type "A1" and "A2" associated markers found among DEGs upregulated in Tg.Old astrocytes. Most of the detected DEGs illustrate possible roles of astrocytes in disease even though they cannot be associated with harmful effects a priori. Complement factor b, Cfb, is part of the alternative pathway of complement activation. Polymorphisms of this gene have been associated with AD (Proitsi et al. 2012), and its expression pattern is consistent with the neurotoxic subtype, "A1". Various upregulated transcripts have been found among the "A2" astrocyte type set. Prostaglandin-endoperoxide synthase 2 (Ptgs2), also known as cyclooxygenase-2 (Cox-2), appears to be the most relevant with logFC 6.8 (remaining DEGs show a logFC above 2). Ptgs2 is a pro-inflammatory enzyme responsible for the conversion of arachidonic acid to prostaglandins and it can be stimulated by Il-1α or inhibition of oxidative phosphorylation (Astakhova et al. 2019). Enhanced expression of this enzyme has been found in affected regions of brains from patients with AD (Yasojima et al. 1999; Ho et al. 2001). Emp1 and S100a10 have been associated with a particular reactivity state caused by astrocyte unfolded protein response (Smith et al. 2020). Epithelial membrane protein 1, Emp1, is a tight junction-associated protein involved in the BBB (Bangsow et al. 2008). Plasma membrane protein S100a10 forms a heterotetramer with annexin A2 contributing to the plasminogen activation pathway and other processes like exocytosis (Hedhli et al. 2012). Transmembrane 4 L six family 1 (Tm4sf1) protein is overexpressed in gliomas and other malignant cells (Fu et al. 2020). Beyond these interesting results related to proposed “A1” and “A2” astrocytic types, caution should be taken as these classifications rely mainly in correlative associations (Sofroniew 2020).

In the following sections, we resort to gene ontology (GO) and pathway analysis using free resources such as KEGG to achieve a more comprehensive assessment of the transcriptomics profile of Tg.Old astrocytes. In particular, some key alterations will be further discussed in the context of astrocyte physiology and its role in pathology.

Tg.Old Astrocyte Phenotype Illustrated by Gene Ontology and Pathway Analyses

Normal astrocyte function in the CNS involves processes such as neuronal synapse regulation (tripartite synapse), neuronal metabolic support, ion homeostasis, and BBB (Sofroniew and Vinters 2010). Gene ontology and pathway analysis in Tg.Old detected altered processes that could impair astrocyte homeostatic role. From enriched GO terms associated with downregulated transcripts, various attenuated processes are suggested (Fig. 5). These include ontologies related with nervous system development, cell adhesion and cell projection development, synapse function and formation, and ion transport through the cell membrane. Analysis of the downregulated KEGG pathways detected in the neurotoxic astrocytes portraits a complementary picture (Fig. 6). In this sense, suggested pathways can be grouped based on their overlap in those associated with synaptic transmission and cell adhesion molecules (“*neuroactive ligand receptor interaction*”, “*GABAergic synapse*”, “*glutamatergic synapse*”, etc.), and those associated with ion transport, calcium signaling, and cAMP signaling (“*cAMP signaling pathways*”, “*calcium signaling pathway*”, “*gastric acid secretion*”, etc.) (see Online Resource 1, Fig. S10A). Taken together, the observed changes suggest a decrease in the neuron-supportive activities offered by astrocytes, in particular ion homeostasis, cell adhesion, and synaptic regulation.

Enriched GO terms associated with upregulated transcripts suggest an alteration of process related to protein synthesis and processing, with a clear involvement of the endoplasmic reticulum (Fig. 7). Moreover, ontologies such as “*regulation of cytokine production*”, “*cellular response to unfolded protein*”, and “*cell redox homeostasis*” suggest an enhancement of processes related to cell proliferation and inhibition of apoptosis as well as activation of stress responses (see full schematic in Online Resource 1, Fig. S8). In regard of signaling pathways from KEGG, similar processes are suggested (Fig. 8). Among the most confidently upregulated pathways are those related to protein synthesis and processing such as “*ribosome biogenesis*” and “*protein processing*

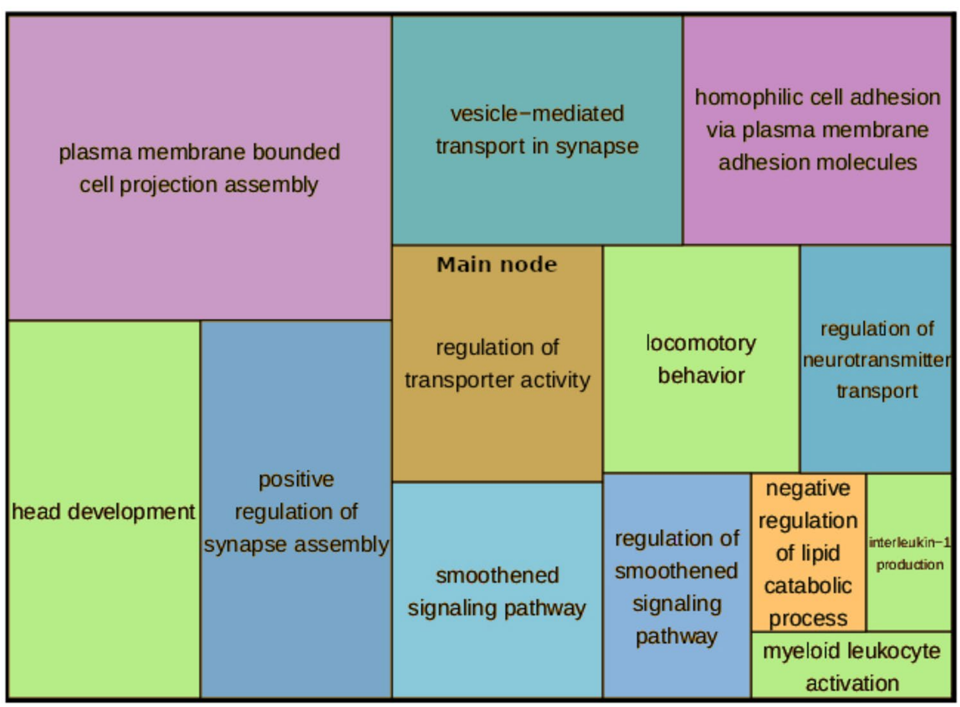


Fig. 5 Enriched GO terms associated with downregulated transcripts in Tg.Old astrocytes. Only main node is presented, see full schematic in Online Resource 1, Fig. S7. Each rectangle is a representative of a single group of GO terms, which are then associated into “superclusters” and displayed in different colors. The size of the rectangles is adjusted to reflect the *p*-value

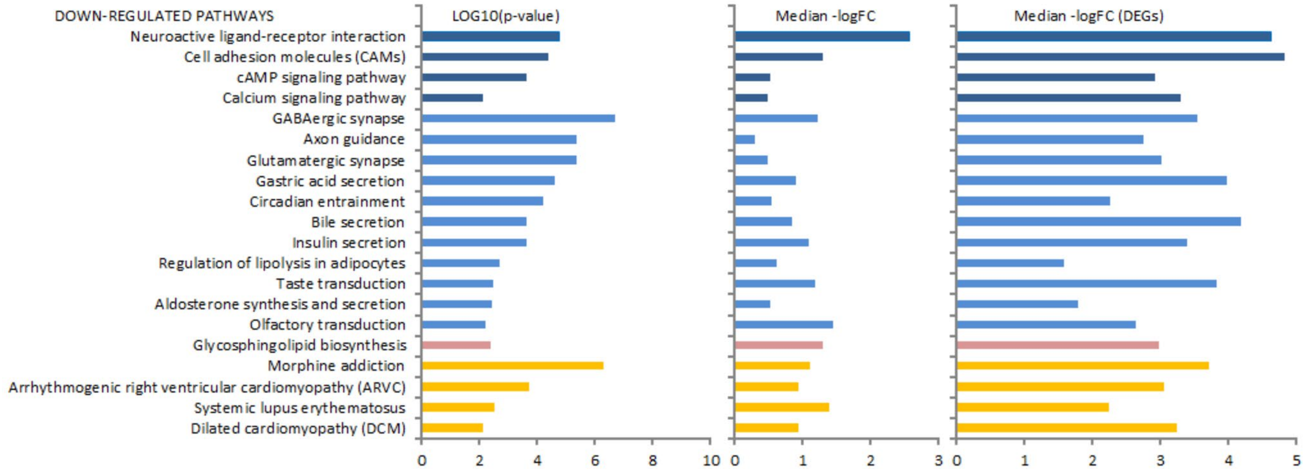


Fig. 6 Enriched KEGG pathways derived from downregulated transcripts in Tg.Old astrocytes. Pathways are organized according to KEGG class; pathways in blue belong to the following classes: Signal transduction; Signaling molecules and interaction; light blue: Organismal Systems (Nervous system, Development and regeneration; etc.); purple: Metabolism; and yellow: Human diseases. For brevity, only the most representative pathways inside each group are included. See Online Resource 1, Fig. S9–10, Table S1–2 for the full list and analysis of pathway overlap. Log10(p-value) takes the minimum p-value among GAGE, camera, and goana results, Median logFC considers the median log fold change of all the genes detected in the pathway, and Median logFC (DEGs) is restricted to differentially expressed genes (DEGs). The total amount of DEGs involved in downregulated pathways is 494 (354 effectively down), with a median fold change of − 2.92

in endoplasmic reticulum." In another group, we find pathways related to cell proliferation, pro-inflammatory pathways, and in general response to stress. Metabolic perturbations are also suggested at the energetic level and in the metabolism of amino acids, pyrimidines, and glycans. Altered pathways show a considerable overlap of DEGs (see Online Resource 1, Fig. S9). For example "*protein processing in endoplasmic reticulum*" links many of the previously suggested alterations, like protein synthesis and catabolism, stress responses, and metabolic

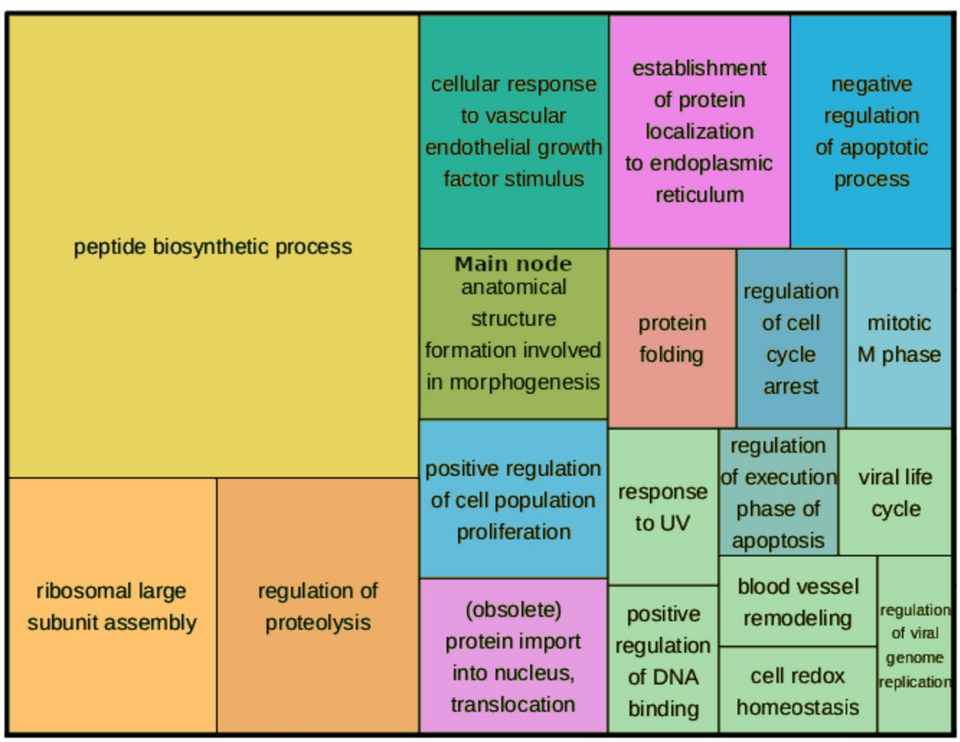


Fig. 7 Enriched GO terms associated with upregulated transcripts in Tg.Old astrocytes. Only main node is presented, see full schematic in Online Resource 1, Fig. S8. Each rectangle is a representative of a single group of GO terms, which are then associated into "super-clusters" and displayed in different colors. The size of the rectangles is adjusted to reflect the p-value

alterations in the frame of an unfolded protein response. As detailed in the following sections, these processes can reinforce each other, such that metabolic alterations and pro-inflammatory responses could manifest as adaptations to increased cell proliferation, protein synthesis, or even be an additional source of stress, such as perturbations in the mitochondria. Altogether these findings are consistent with the increased proliferative capacity that we previously described for these astrocytes (Diaz-Amarilla et al. 2022).

Transcriptional Alterations Associated with Pro-inflammatory and Proliferative Features

As indicated from the pathway analysis and reactivity markers evaluated, Tg.Old astrocytes show a pro-inflammatory and proliferative profile (Figs. 4 and 8). In addition to previously identified enzymes such as Ptgs2 and Nos2, Tg.Old astrocytes show transcriptional alterations associated with other proinflammatory proteins such as Pla2g4a, recently described as part of a pan-injury astrocyte response (Viejo et al. 2021). Moreover, transcriptomics results suggest the alteration of various cell adhesion molecules, growth factors and trophins, cytokines, matrix metalloproteases, and transcription factors such as p65 (Rela, Nuclear Factor-kappa-B p65 subunit) that have a prominent role in regulating neuroinflammation, proliferation, and related metabolic adaptations. These alterations illustrate both protective and harmful roles of astrocyte reactivity. For example, cytosolic phospholipase A2 (Pla2g4a, cPLA2) activity has important roles in health and disease (Leslie 2015). It catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid which is subsequently metabolized into eicosanoids (Leslie 2015). Expression of this enzyme in astrocytes has been associated with harmful pro-inflammatory effects in mouse models (Chao et al. 2019).

Altered cytokine profiles (Fig. 9) have been previously associated with astrocyte reactivity in AD including interleukin-6 (Il-6), interleukin-33 (Il-33), C–C motif chemokine ligand 2 (Ccl2), and stromal cell-derived factor 1 (Cxcl12) (Viejo et al. 2021). These cytokines can promote beneficial effects such as Il-34 and Il-33, dual or even harmful effects like Il6 and Ccl11 depending on the disease context. Il-6 for example is one of the most well-known mediators of astrocyte inflammatory processes and has been implicated in protective but also in harmful effects like promotion of neuronal death and deregulation of synapse formation (Wei et al. 2012; Hou et al. 2016; Kummer et al. 2021). Chemokine Ccl11 is a potent eosinophil chemoattractant in the peripheral immune system and has also been found associated with neuroinflammation and neurodegeneration, even though the evidence is conflicting. Ccl11 levels in plasma and CSF increase with aging even though in AD patients it has only been found increased in plasma and in ALS patients is correlated with slow disease progression (Villeda et al. 2011; Leung et al. 2013; Furukawa

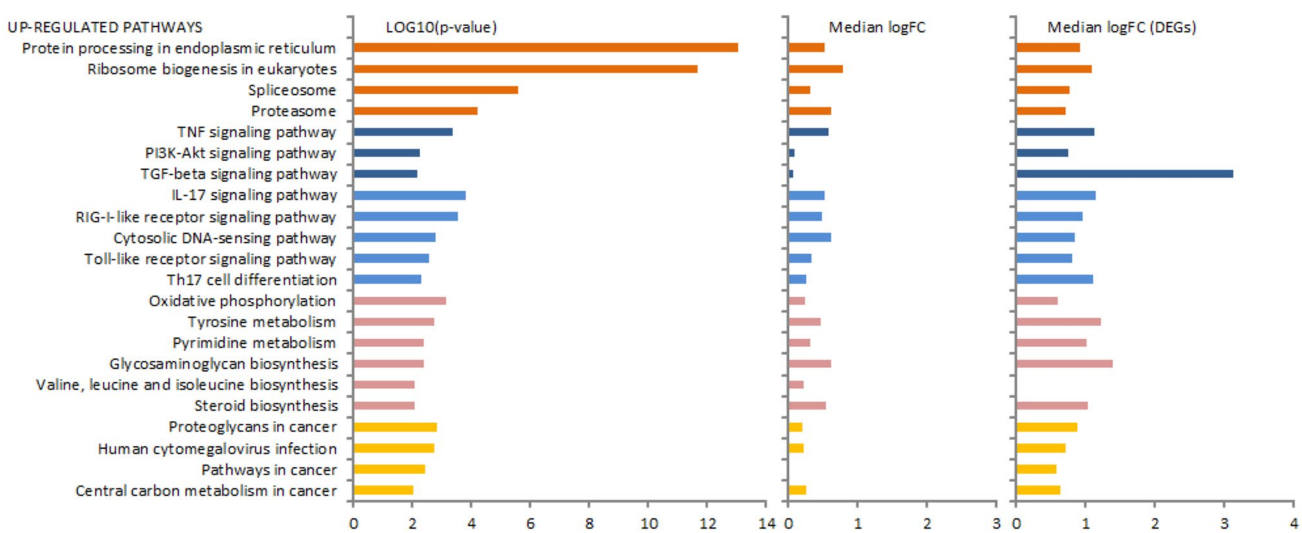


Fig. 8 Enriched KEGG pathways derived from upregulated transcripts in Tg.Old astrocytes. Pathways are organized according to KEGG class; pathways in orange belong to the following classes: Transcription; Translation; Folding, sorting and degradation; blue: Signal transduction; Signaling molecules and interaction; light blue: Organismal Systems (Immune system); purple: Metabolism; and yellow: Human diseases. For brevity, only the most representative pathways inside each group are included. See Online Resource 2 for the full list and analysis of pathway overlap. Log10(p-value) takes the minimum p-value among GAGE, camera, and goana results, Median logFC considers de median log fold change of all the genes detected in the pathway, and Median logFC (DEGs) is restricted to differentially expressed genes (DEGs). The total number of DEGs involved in upregulated pathways is 803 (567 up), with a median fold change of 0.92

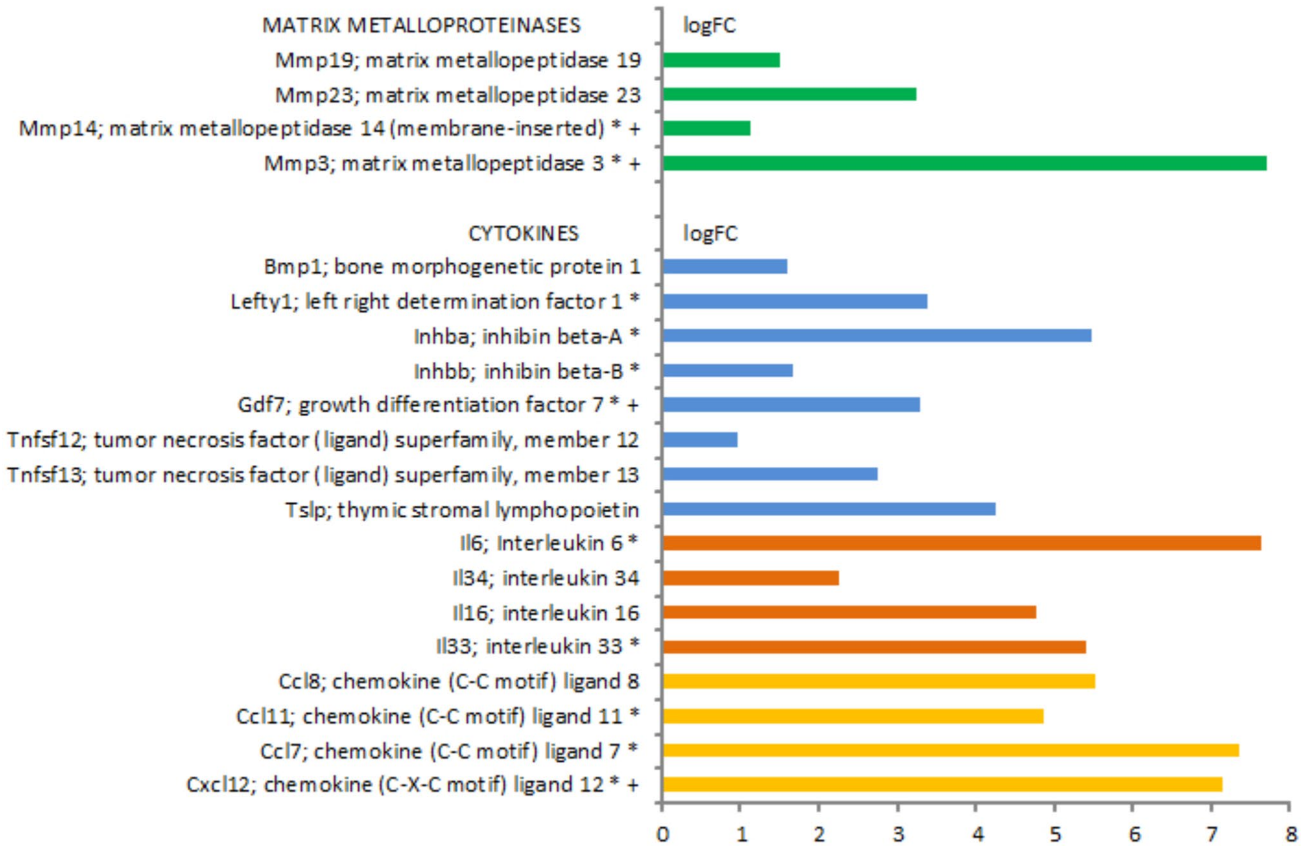


Fig. 9 Upregulated cytokines and matrix metalloproteinases examples in Tg.Old astrocytes. * indicates factors involved in upregulated KEGG pathways, and + indicates factors involved in downregulated KEGG pathways. Upregulated pathways include "*Pathways in cancer*", "*PI3K-Akt signaling pathway*", "*TNF signaling pathway*", and "*IL-17 signaling pathway*", Downregulated pathways include "*Axon guidance*", "*Calcium signaling pathway*", and "*cAMP signaling pathway*", *q*-value for interleukin 6 is 0.0506, and considering its functional importance, it was also included

et al. 2015; Cherry et al. 2017; Sirivichayakul et al. 2018). Studies in a transgenic AD mouse model, mouse neuronal cultures, and microglia-neuronal co-cultures suggest that Ccl11 could promote neurodegeneration activating Gsk-3β as well as induce microglia to produce reactive oxygen species and neurotoxicity (Parajuli et al. 2015; Zhu et al. 2016).

Alternatively evidence regarding other factors like Il-34, Il-33, and Cxcl12 illustrates a protective or counterbalancing role of reactive astrocytes in neurodegeneration. For example, Il-34 is associated with neuroprotective effects *in vitro* by regulating microglia phenotype (Mizuno et al. 2011; Ma et al. 2012). Similarly, Il-33, initially described as a cellular alarmin released after tissue damage, is involved in synapse refinement by microglia and in the regulation of immune responses (Liew et al. 2016; Vainchtein et al. 2018). Moreover, it has a role in injury healing and has been found neuroprotective in multiple models by regulating microglia and astrocyte phenotype (Gadani et al. 2015; Fu et al. 2016; Yang et al. 2017; Carlock et al. 2017; Chen et al. 2019c). Genetic and transcriptional deficiencies of Il-33 have been associated with AD (Chapuis et al. 2009). Similarly, Il-33 deficiency can cause tau abnormality, neurodegeneration, and AD-like symptoms in aged mice and Il-33 administration can reduce Abeta load and plaque formation as well as improve synapse plasticity and cognitive symptoms (Fu et al. 2016; Carlock et al. 2017). Even though, Il-33 activity also appears to facilitate glioma cell proliferation and migration through upregulation of Mmp2 and Mmp9 via NF-κB signaling (Zhang et al. 2017). Cxcl12 plays a fundamental role in neurogenesis, axon guidance, neurite outgrowth, and synapse regulation, in addition to being reactivated after brain inflammation or injury (reviewed in (Guyon 2014)). *In vitro* it promotes astrocyte proliferation (Bonavia et al. 2003). Reduced levels have been found in AD patients and mouse models of the disease (Parachikova and Cotman 2007; Laske et al. 2008). Beneficial effects have also been reported in mouse models (Shin et al. 2011; Raman et al. 2011; Wang et al. 2012; Capsoni et al. 2017). Cxcl12 expression can be upregulated by HIF-1α, being associated with tumor growth (De Falco et al. 2004).

Release of trophic factors and regulation of extracellular matrix (ECM) are part of normal astrocyte function and reaction, specifically affecting proliferation, neuronal support, synapsis formation, and axon growth, as well as tissue reconstitution and scar formation. In this sense, when compared respect to control, transcripts of various matrix metalloproteinases (MMPs) are altered in Tg.Old astrocytes (Fig. 9). MMP are a group of proteases capable of processing ECM components and other proteins, contributing to wound healing, angiogenesis, and regulation of BBB permeability, among other processes (Löffek et al. 2011; Rempe et al. 2016). Evidence regarding their role in AD shows that even though they may help to degrade Abeta and reduce its deposit, they could also participate in the amyloidogenic pathway, promote harmful inflammatory process, neuronal loss, axonal damage, and dysfunction of the BBB (Paumier et al. 2019; Rivera et al. 2019). Mmp3 for example is associated with both beneficial functions and harmful effects like contributing to synapse plasticity or apoptosis (Kim and Hwang 2011; Van et al. 2012). In addition to MMPs, transcripts coding members of other protease families were found upregulated, such as Adam12, Adam19, and Bmp1. Noteworthy, Adam12 protein levels have been found reduced in AD even though its proteolytic activity might be increased and *in vitro* evidence indicates that it may mediates toxic effects of Abeta (Malinin et al. 2005).

Furthermore, transcripts coding multiple ECM proteins are found altered in neurotoxic astrocytes including collagen (Col1a1, Col1a2), laminin (Lamb1, Lama4), and integrin (Itgb1, Itgb3, Itgb7, Iga10) forms. See Fig. 10 for an example list of growth factors and ECM proteins whose transcripts are upregulated by neurotoxic astrocytes. These adhesion molecules can be upregulated in reaction processes and intervene in axonal growth and scar formation (Wiksten et al. 2007; Lei et al. 2012; Wang et al. 2015; Neo and Tang 2017). Particular functions suggested by the altered transcripts include proliferation (Itgb1), neurogenesis (syndecan-1), BBB repair (thrombospondin 2), and anti-inflammation (Cd47, Efemp1, and galectin-1) (Qu et al. 2010, 2011; Tian et al. 2011; Starossom et al. 2012; Wang et al. 2015; Thorén et al. 2019; Mouthon et al. 2020; Gheibihayat et al. 2021).

Released growth factors like Igf1, Hgf, Vegf, and Fgf can have neuroprotective effects or promote the proliferation of astrocytes and other cells (Beilharz et al. 1998; Madathil et al. 2013; Pitt et al. 2017; Calvo et al. 2018; Savchenko et al. 2019; Chen et al. 2019b; Desole et al. 2021). Alteration of the corresponding transcripts is consistent with our previous observations regarding an increased proliferative capacity but also suggest that old 3xTg-AD neurotoxic astrocytes conserve some neurotrophic effects (Diaz-Amarilla et al. 2022). For example, Manf is a neurotrophin with a role in neuronal differentiation (Tseng et al. 2017, 2018) that can also be secreted in response to injury and/or cellular stress showing protective functions such as downregulation of NF-kB-dependent inflammatory responses (reviewed in Pöyhönen et al. (2019) and Linnerbauer and Rothhammer (2020)). On the contrary, Bdnf and Ngf can promote in some contexts detrimental effects on neurons. Astrocytes upregulate Bdnf in response to injury and other cellular stresses (Dougherty et al. 2000; Qu et al. 2010; Fulmer et al. 2014). Bdnf signaling though TrkB receptors has been shown to promote neuronal survival and neurite outgrowth while signaling through p75NTR receptors can induce apoptosis in cultured neurons (Lee et al. 2001; Arévalo and Wu 2006; Cragnolini and Friedman 2008). Nonetheless, Bdnf can promote astrocyte reactivity and microglia activation, as well as secretion of excessive nitric oxide (NO), Tnf-α, and Il1-β contributing to harmful neuroinflammation (Colombo et al. 2012; Ding et al. 2020; Jeon et al. 2020). A similar behavior is displayed by Ngf. Transcriptional or protein upregulation of Ngf is observed in injury, ischemia, and Parkinson's disease models (Lee et al. 1998; Goss et al. 1998; Nakagawa and Schwartz 2004). Remarkably, imbalance of Ngf signaling through TrkA and p75NTR receptors has been associated with Alzheimer's disease and other disorders (Tiveron et al. 2013; Mysona et al. 2015; Fahnestock and Shekari 2019). For instance, pro-Ngf has a higher affinity to p75NTR which can induce neuronal and oligodendrocyte apoptosis (Beattie et al. 2002; Marchetti et al. 2019). Particularly, pro-Ngf appears to be associated with astrocyte reactivity in spinal cord injury and neuronal death after exposure to reactive astrocyte conditioned media (Domeniconi et al. 2007; Cheng et al. 2020).

Transcriptional Alterations Associated with Biosynthetic and Energy Metabolism

Transcriptomics data suggest alterations in old 3xTg-AD neurotoxic astrocyte energy metabolism, including glycolysis, pentose phosphate pathway (PPP), TCA cycle, and mitochondrial activity. Glucose enters the cell though specific glucose transporters (Glut1), and is phosphorylated by hexokinase (and glucokinase) to produce glucose-6-phosphate (G6P), a common precursor of glycolysis for energy and lactate production and of the pentose–phosphate pathway (PPP) (Bélanger et al. 2011). The PPP is a fundamental component of cellular metabolism, necessary for carbon homeostasis, synthesis of nucleotide, amino acid precursors, and reducing factors for anabolism and regulation of oxidative stress (Stincone et al. 2015). The rate-limiting step in glycolysis is catalyzed by 6-phosphofructokinase (Pfk1) and in PPP activity by glucose-6-phosphate dehydrogenase (G6pd). Glucose entrance steps do not appear to be altered in Tg.Old astrocytes. Even though some glucose transporters isoforms are downregulated, Glut1 transcript (Slc2a1) is not altered, as well as transcripts coding some glycolysis

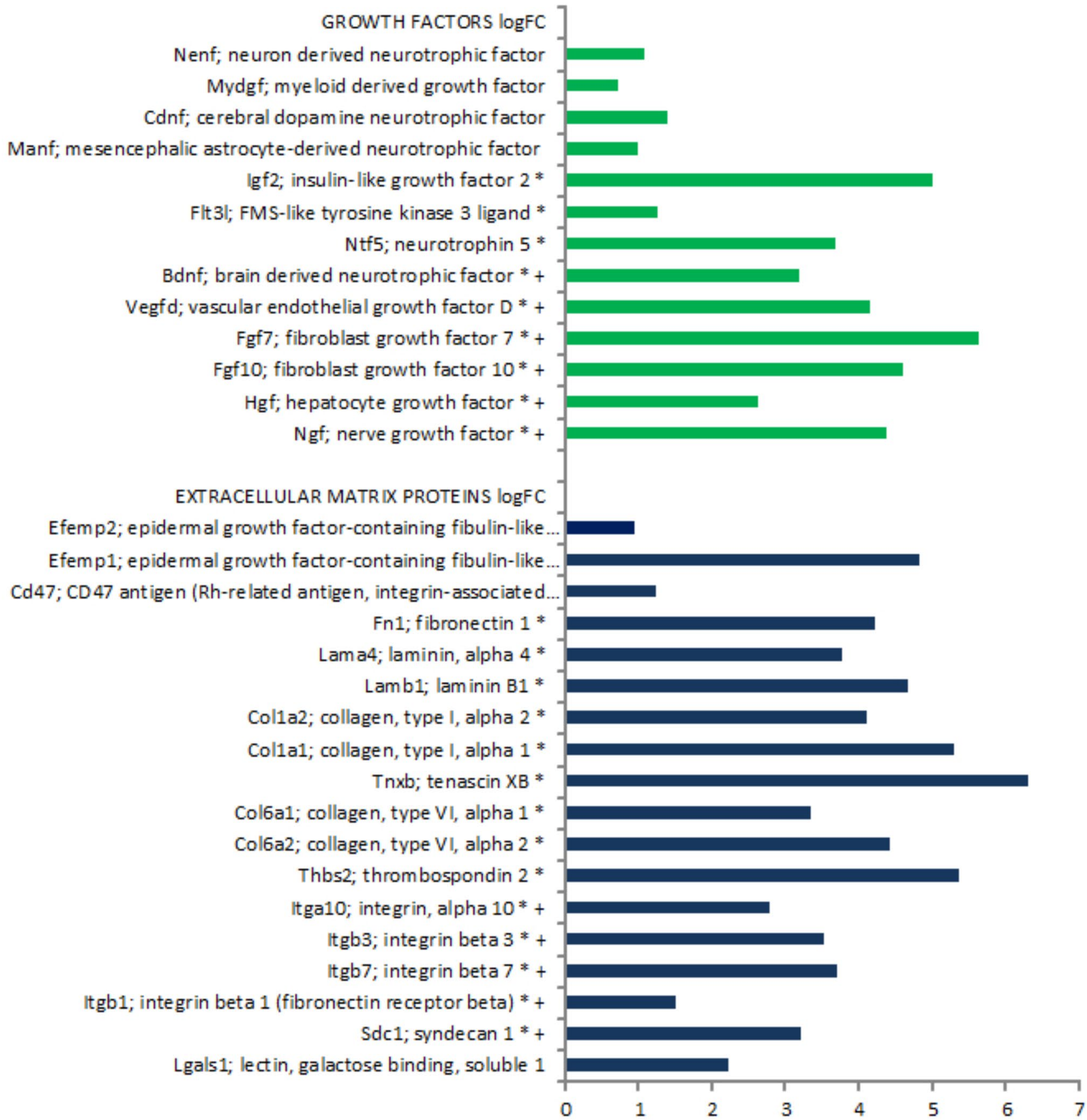
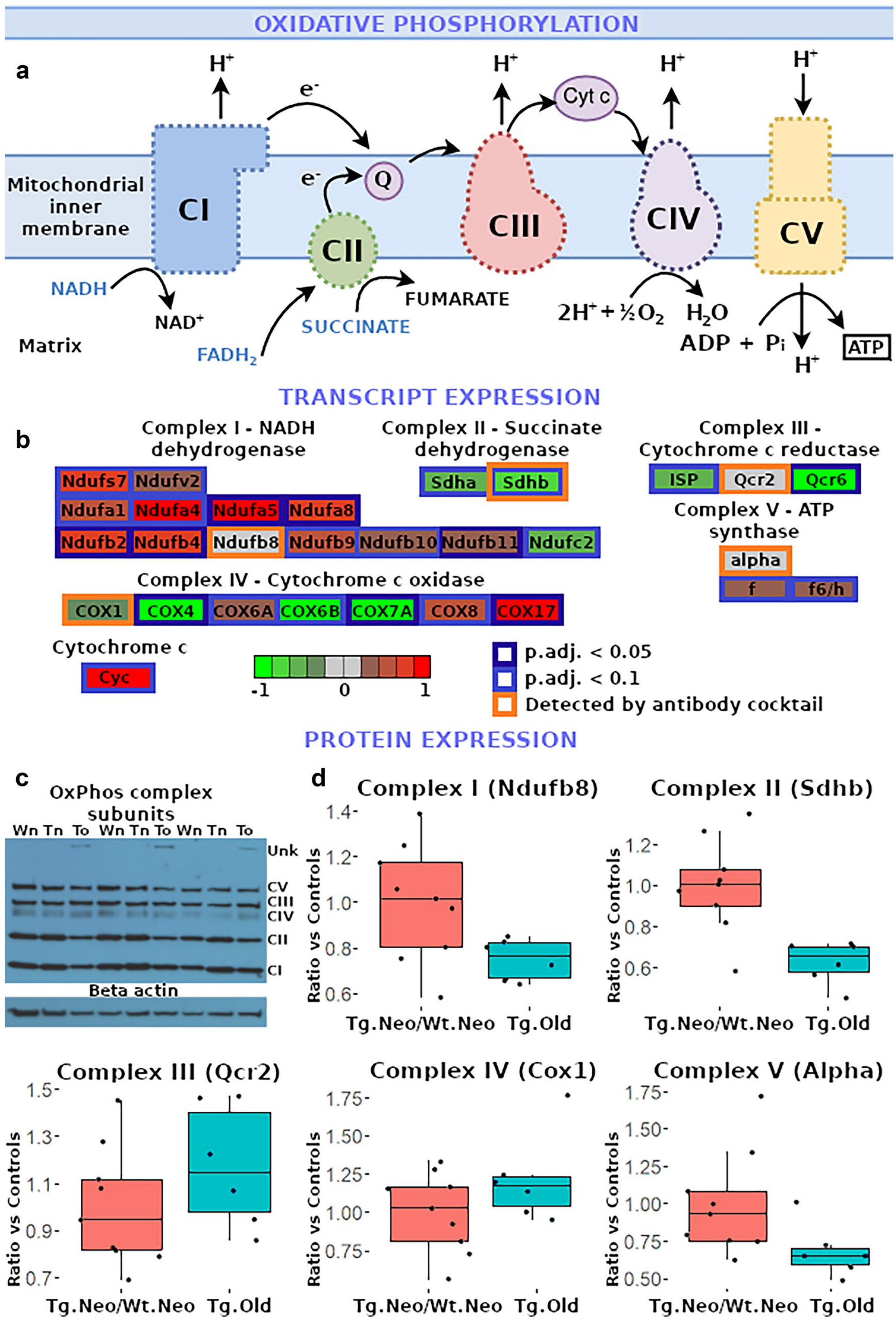


Fig. 10 Upregulated growth factors, extracellular matrix, and related protein examples in Tg.Old astrocytes. * indicates factors involved in upregulated KEGG pathways, and + indicates factors involved in downregulated KEGG pathways. Upregulated pathways include "*Pathways in cancer*", "*PI3K-Akt signaling pathway*", "*TNF signaling pathway*", and "*IL-17 signaling pathway*", Downregulated pathways include "*Axon guidance*", "*Calcium signaling pathway*", and "*cAMP signaling pathway*"

enzymes, such as hexokinase and glucokinase (Hk1, Hk2, Hk3, Gck). Subsequent steps of this route suggest a relative shift from glycolysis to PPP. Glycolysis branch might be reduced as two Pfk1 subunit transcripts (Pfkm and Pfkp) are downregulated (Pfkl is not altered). In the PPP branch, G6pd presents two isoform transcripts, being H6pd upregulated. Noteworthy in cancer cells, Pfk1 inhibition drives PPP flux and supports cell growth (Yi et al. 2012). Another important alteration suggesting the same shift is the downregulation of Pfkb3 transcript. Pfkb3 suppression increases the flux through PPP to foster cell defense and repair mechanisms (Stincone et al. 2015; Franklin et al. 2016). As indicated


OXIDATIVE PHOSPHORYLATION
a
H+
e-
Q
CI
CII
CIII
Cyt c
CIV
CV
Mitochondrial inner membrane
NADH
NAD+
Matrix
FADH2
SUCCINATE
FUMARATE
2H+ + ½O2
H2O
ADP + Pi
ATP
TRANSCRIPT EXPRESSION
b
Complex I - NADH dehydrogenase
Ndufs7 Ndufv2
Ndufa1 Ndufa4 Ndufa5 Ndufa8
Ndufb2 Ndufb4 Ndufb8 Ndufb9 Ndufb10 Ndufb11 Ndufc2
Complex II - Succinate dehydrogenase
Sdha Sdhb
Complex III - Cytochrome c reductase
ISP Qcr2 Qcr6
Complex V - ATP synthase
alpha
f f6/h
Complex IV - Cytochrome c oxidase
COX1 COX4 COX6A COX6B COX7A COX8 COX17
Cytochrome c
Cyc
-1 0 1
p.adj. < 0.05
p.adj. < 0.1
Detected by antibody cocktail
PROTEIN EXPRESSION
c
OxPhos complex subunits
Wn Tn To Wn Tn To Wn Tn To
Unk
CV
CIII
CIV
CII
CI
Beta actin
d
Complex I (Ndufb8)
Complex II (Sdhb)
Complex III (Qcr2)
Complex IV (Cox1)
Complex V (Alpha)
Ratio vs Controls
Tg.Neo/Wt.Neo
Tg.Old

◂**Fig. 11** Transcript and protein expression of oxidative phosphorylation complexes in Tg.Old astrocytes. **a** Oxidative phosphorylation schematic (created with diagrams.net and Gimp tools). **b** Transcript color map adapted from KEGG (mmu00190). Differentially expressed subunits with a strict (p.adj < 0.05) and a lenient criteria (p.adj < 0.1) are depicted, as well as subunits detected at the protein level. **c** Example western blot scan. Protein expression of different mitochondrial complex subunits was assessed by western blot using an antibody cocktail. In old 3xTg-AD neurotoxic astrocytes, an additional band (Unk) was detected at high molecular weight (~ 135 kDa). **d** Individual protein expression plots. A lenient approach was followed to detect trends in the protein expression: Tg.Old (To) group samples were compared against control samples Tg.Neo (Tn) and Wt.Neo (Wn) joined as a single group, and values were normalized against Beta actin expression and additionally by the average expression of controls from each assay (Ratio). Under this approach, trends in the alteration of subunits from the following complexes were observed: CI (p.adj = 0.07), CII (p.adj = 0.005), and CV (p.adj = 0.04) (Wilcoxon test)

previously, these alterations allow cell survival under metabolic stress and are commonly found in malignant cells. Snail (Snai1) appears to be one of the transcription factors involved in this metabolic shift and its transcript is upregulated in neurotoxic astrocytes (Kim et al. 2017).

Mitochondrial oxidative phosphorylation is one of the main cellular sources of ATP. In this process, ATP synthesis at the FOF1-ATP synthase (complex V or CV) is coupled to the transport of electrons through the electron transport chain (ETC), constituted by a series of multimeric enzymes (complexes I to IV or CI-CIV) and two electron carriers (coenzyme Q and cytochrome c) located at the inner mitochondrial membrane (Zhao et al. 2019; Tang et al. 2020; Gonzalez-Franquesa et al. 2021). Mitochondrial dysfunction including impairment of the ETC complexes or of the ATP synthase leads to reduced ATP production, Ca^{2+} dyshomeostasis, and ROS generation being one of the contributing factors of AD (Wang et al. 2020b). Particularly, in the 3xTg-AD mouse model, decline in mitochondrial respiration and enzymes required for bioenergetics in the brain can be observed at 3 month of age (Yao et al. 2009; Adlimoghaddam et al. 2019). Mitochondrial dysfunction can also impair astrocyte function (Fiebig et al. 2019; Bantle et al. 2021). Energy production in astrocytes is largely based on mitochondrial oxidative metabolism, even though they can remarkably increase their glycolytic and glycogenolytic metabolism in response to neuronal activity (Hertz et al. 2007). In the present work, we observed that old 3xTg-AD neurotoxic astrocytes present numerous transcript alterations of mitochondrial complexes illustrating a complex scenario (Fig. 11). Complementary information was assessed by western blot analysis using an antibody cocktail directed to specific mitochondrial complex subunits, as an approach to confirm transcriptomic alterations at the mitochondrial level found in Tg.Old astrocytes. For complex I (CI), catalytic subunit transcripts do not show differential expression but transcripts coding a few accessory subunits are altered. Ndufa5 subunit transcript is upregulated, pointing towards an increase in efficiency of CI activity (Chadwick et al. 2011). Contrarily, alteration trends in the expression of accessory subunit transcripts Ndufc2 and Ndufa4l2 suggest a reduction of CI activity (Sekar et al. 2015; Rubattu et al. 2016; Raffa et al. 2019; Forte et al. 2020; Chen et al. 2021). Noteworthy, overexpression of Ndufa4l2 was observed in AD astrocytes (Sekar et al. 2015) as well as in glioblastoma (Chen et al. 2021). Similarly, a trend towards downregulation of the accessory CI subunit Ndufb8 (not significant) was observed by western blot (Fig. 11). Taken together, downregulation of these accessory subunits suggests a minor decrease in CI activity but a compensatory role of the other subunits cannot be discarded.

Complex II (CII) is also a component of the TCA cycle where it catalyzes oxidation of succinate to fumarate, while in the ETC facilitates electron transfer from flavin adenine nucleotide (FAD) to electron carrier ubiquinol (Zhao et al. 2019; Tang et al. 2020). CII downregulation has been associated with AD (Patro et al. 2021) and senescent process *in vitro* (Yoon et al. 2003). At the transcriptomics level, we observed a trend towards downregulation of catalytic subunits Sdha and Sdhb, the latter being consistent with protein expression (Fig. 11). These evidences indicate a deficiency in CII activity in old 3xTg-AD neurotoxic astrocytes.

In the case of complex III (CIII), some contradictory trends are observed. At the protein level, the metal binding subunit Qcr2 (Uqcrc2) is not altered (trends to be upregulated), while the transcript of catalytic subunit ISP/Rieske (Uqcrfs1) trends to be downregulated suggesting a deficiency of this complex. Qcr6 transcript coding a structural and regulatory subunit is also downregulated. Complex IV (CIV) shows a similar behavior. Metal binding subunit Cox1 is not altered at the protein or transcript level. Transcripts Cox6a1 and Cox8a of catalytic subunits trend to be upregulated. Fine tuning of alternative isoforms of Cox4 and Cox7a is also observed but these alterations would not necessarily translate into loss of CIV activity. Alteration of transcripts coding other structural and regulatory subunits like Cox6b2 and chaperone Cox17 suggests a reorganization of CIII as discussed below.

Regarding complex V (CV), transcript Atp5a1 coding the alpha subunit (catalytic) is not altered but the corresponding protein appears to be downregulated suggesting a diminished mitochondrial capacity to produce ATP. Downregulation of this subunit has been associated with cancer progression (Zhang et al. 2021). Another important alteration observed is the upregulation of cytochrome c transcript. Cytochrome c serves as an electron carrier between CIII and CIV of the ETC, as a ROS scavenger and in initiation of apoptosis once it leaks from mitochondria into the cytosol (Wenzel et al. 2019). Recently, it has been found that it can act extracellularly and modulate astrocyte functions through toll-like receptor 4 (Wenzel et al. 2019).

Beyond individual mitochondrial complex activity, global arrangement of the ETC adds another layer of regulation. Current evidences indicate that ETC complexes can be organized freely in the membrane or assemble into supramolecular structures known as supercomplexes (SCs). In particular, functional arrangements of CI-III-IV called “respirasomes” have been confirmed (Acín-Pérez et al. 2008). CII appears to not be involved in supercomplex formation but it might have a regulatory role (Jang and Javadov 2018). Supercomplex arrangements are thought to improve control of ROS production, maintain the structural integrity of ETC complexes (assisting for example CI assembly and stability), and prevent protein aggregation in the inner mitochondrial membranes (Acín-Pérez et al. 2008; Genova and Lenaz 2014) (reviewed in Lobo-Jarne et al. (2020)). In the CNS, these arrangements contribute to the metabolic specialization of different cell types. In contrast with neurons, astrocytes appear to have higher levels of free CI than assembled into super complexes, which might be associated with increased ROS production in astrocytes (Lopez-Fabuel et al. 2016). Transcripts of some important subunits involved in supercomplex formation appear to be altered in old 3xTg-AD neurotoxic astrocytes, including CI subunits Ndufb4 (upregulated) and Ndufb9 (trend to be upregulated), CIII subunits Rieske (Uqcrfs1) (trend to be downregulated) and Qcr6 (Uqcrh) (downregulated), as well as various isoforms of CIV subunit Cox7a (Schägger 2002; Garone et al. 2018). Remarkably interchange between Cox7a2l, Cox7a1, and Cox7a2 isoforms could potentially determine whether CIV stays as a monomer, forms oligomers, or associates with CIII (Garone et al. 2018). Interestingly western blot analysis detected an additional band at high molecular weight (~135 kDa) only in Tg.Old samples that could relate to the formation of supercomplex arrangements. Further analyses are needed to confirm the existence of these alterations and its impact on mitochondrial activity. Furthermore, in some studies, it has been observed that important reductions of activity of CI, CIII, and CIV (greater than 70%) were necessary to produce a significant decrease in ATP production, so a mild impairment in the ETC complexes might not be sufficient to cause bioenergetics impairment as seen in AD brain (Letellier et al. 1994; Barrientos and Moraes 1999).

The tricarboxylic acid cycle (TCA) which supplies the ETC with reduced factors NADH and FADH can be another source of alterations (Zhou et al. 2018). Cytoplasmic metabolites to sustain TCA function are provided from either a canonical oxidative pathway or anaplerotic pathways. The canonical oxidation pathway is a continuation from glycolysis, where pyruvate enters the TCA as acetyl-CoA, carried out by pyruvate dehydrogenase complex (Pdh), a key rate-limiting enzyme (Zhou et al. 2018). Deficiency of TCA enzymes pyruvate dehydrogenase, and alpha-ketoglutarate dehydrogenase appear to be present in AD (Sorbi et al. 1983; Gibson et al. 2012). Compromised Pdh function has also been observed in 3xTg-AD neurons and leads to a deficit in acetyl-CoA, and subsequent decrease of OxPhos activity (Yao et al. 2009). Transcripts corresponding to the main catalytic subunits of the pyruvate dehydrogenase complex are not found altered in old 3xTg-AD neurotoxic astrocytes, but the regulatory subunit Pdp1 (pyruvate dehydrogenase phosphatase catalytic subunit 1) appears to be downregulated. The anaplerotic pathways involve metabolic enzymes such as pyruvate carboxylase (Pcx), phosphoenolpyruvate carboxykinase (Pck1, Pck2), malic enzyme (Me1, Me2, Me3), glutaminase (Gls), and glutamate dehydrogenase (Glud1, Glud2). Even though anaplerotic pathways are thought to not contribute to ATP production directly, some studies have suggested that glutamine supports NADH production from the Gls/Glud anaplerotic pathway similarly to the canonical pathway (Reitzer et al. 1979; Le et al. 2012). While Pcx transcript is downregulated, Gls transcript appears to be upregulated suggesting that old 3xTg-AD neurotoxic astrocytes have an increased use of glutamine as a carbon source compared with neonatal astrocytes.

Finally, perhaps the clearest perturbation observed in old 3xTg-AD neurotoxic astrocytes involves activity at the endoplasmic reticulum and the stress responses that emerge from it (see simplified scheme in Fig. 12). Disruption of ER homeostasis, such as accumulation of misfolded proteins in the ER lumen, can trigger an adaptive mechanism named the unfolded protein response (UPR) resulting for example in reduced protein synthesis, increased protein clearance, and chaperone expression (Schmitz et al. 2018; Ghemrawi et al. 2018; Zhang et al. 2020b). A transcriptional alteration of various elements directly associated with the canonical UPR like Grp78 (Hspa5), Ire1α, and Atf4 or its regulation such as Creb3l1 can be observed suggesting some level of ER stress occurring in the old 3xTg-AD neurotoxic astrocytes (Kondo et al. 2005; Schmitz et al. 2018; Ghemrawi et al. 2018; Zhang et al. 2020b). Noteworthy, transcription factor Atf4 ties these alterations with subsequent metabolic changes like aminoacid metabolism. Many enzymes and transporters related to amino acid and central carbon metabolism are altered, like Adh1, Adh7, Aldh3a1, Fah, Got1, Got2, Maoa, Aoc2, Cad, Nos2, Ldha, Slc1a5, Slc7a, Enpp1, and B4galt1. Another remarkable alteration is the enzyme Gls, a central hub of brain metabolism. The shift of these metabolisms could have detrimental effects on neuronal homeostasis by lack of support or be directly neurotoxic through excessive liberation of glutamate.

Endoplasmic reticulum and mitochondrial stress responses as well as inflammatory pathways and metabolic derangements are mutually regulated and converge through few major transcription factors (Hotamisligil 2010; Salvadó

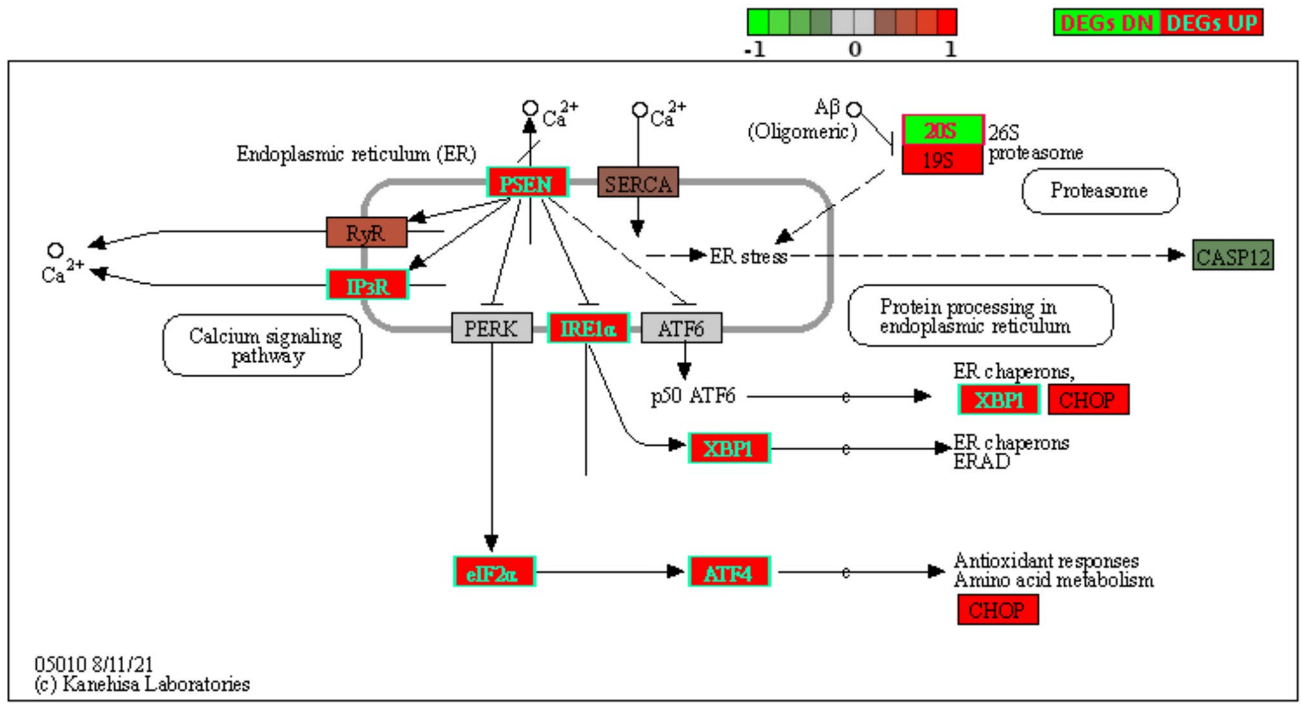


Fig. 12 Signaling related to protein processing in the endoplasmic reticulum. Simplified excerpt taken from "*Alzheimer's disease*" KEGG colored map (mmu05010). Color pattern expressed according to the observed logFC in Tg.Old astrocytes. Nodes corresponding to DEGs were highlighted

et al. 2015; Martin-Jiménez et al. 2017; Schmitz et al. 2018; Ghemrawi et al. 2018). Beyond its protective role (Cissé et al. 2016; Wang et al. 2018), prolonged ER stress can result in harmful inflammation and cell death which contribute to the progression of various diseases including cancer, diabetes, and neurodegenerative diseases (Duran-Aniotz et al. 2017; Martin-Jiménez et al. 2017; Schmitz et al. 2018). In particular, astrocytic ER appears to be an element linking metabolic and neurological diseases and has been associated with a distinct reactivity state that alters the astrocytic secretome, leading to loss of synaptogenic function *in vitro* and harmful effects *in vivo* (Martin-Jiménez et al. 2017; Smith et al. 2020). Similarly to metabolic diseases (Contreras et al. 2014), ceramide activation in astrocytes can promote pro-inflammatory responses, increased ROS, and ER stress which could contribute to the progression of neurodegenerative diseases (Prasad et al. 2008; de la Monte et al. 2012; Gu et al. 2013).

Other important feature associated with the ER stress response involves the transcription factors Hif-1α and c-Myc. An observed transcriptional upregulation of Hif-1α and c-Myc is consistent with previous findings and represent possible self-preserving adaptations of old 3xTg-AD neurotoxic astrocytes. Under hypoxic conditions and ER stress, Hif-1α activity increases glucose uptake and controls its flux through the glycolytic and pentose phosphate pathways, protecting neuronal cells and increasing cancer survival (Soucek et al. 2003; Chen et al. 2014; Lõpez-Hernández et al. 2015). Hif-1α is also associated with a shift in the expression of Ldh isoforms, observed in the transcriptomics profile, and that can reduce the usage of lactate as a fuel source in mitochondrial respiration and favor aerobic glycolysis (Papandreou et al. 2006). Similarly c-Myc gene can also be stimulated by ER stress response and enhance proliferation, glycolysis, glutathione biosynthesis from glutamine, and glutaminolysis (Le et al. 2012; Miller et al. 2012; Hart et al. 2012; Goetzman and Prochownik 2018).

Transcriptional Alterations Associated with Senescence-Like Features

Cellular senescence is a key feature of aging that contributes to tissue dysfunction (Baker et al. 2016; Kritsilis et al. 2018). Typically it involves permanent cell cycle arrest and resistance to apoptotic cell death dependent on the activity of p16ink4a and p53-p21-Rb (retinoblastoma) (Kritsilis et al. 2018; Zhu et al. 2021). Senescent cells exhibit increased unfolded protein response (UPR), indicative of endoplasmic reticulum (ER) stress (Cormenier et al. 2018). Various metabolic alterations have been associated with cellular senescence including a shift from oxidative phosphorylation to glycolysis, upregulation of tricarboxylic acid cycle, pentose

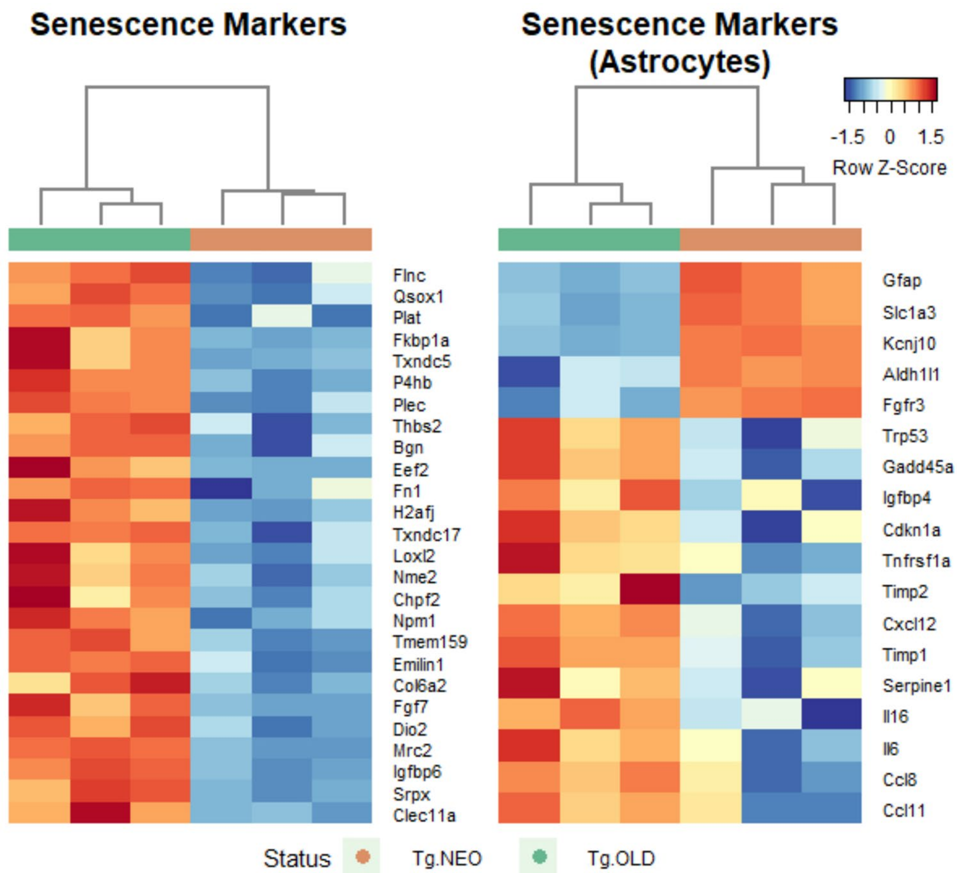


Fig. 13 Senescence markers in Tg.Old astrocytes. Markers were grouped in general senescence markers (left) and markers specifically identified in astrocytes (right). For general senescence markers, only top upregulated DEGs were considered (logFC > 1, p.adj < 0.01). Markers observed in astrocytes also include "negative" markers (expected to be downregulated), which comprise the first downregulated cluster. Considering its importance Il-6 was also included (p.adj = 0.0506). Marker and related proteins were gathered from literature sources (Bhat et al. 2012; Crowe et al. 2016; Casella et al. 2019; Basisty et al. 2020a, b; González-Gualda et al. 2021) (see full list and references in Online Resource 2)

phosphate pathway, and nucleotide synthesis pathways, as well as downregulation of fatty acid synthesis and altered mitochondrial function (Lee et al. 2006; Correia-Melo et al. 2016; Wu et al. 2017; Weichhart 2018; Kritsilis et al. 2018). Senescent cells release an array of factors called the senescence-associated secretory phenotype (SASP) that can contribute to harmful effects, including pro-inflammatory cytokines, chemokines, growth hormones, angiogenic factors, and matrix metalloproteinases (Kritsilis et al. 2018; Zhu et al. 2021).

Cellular senescence could be a contributing factor of neurodegenerative diseases. Senescent cells accumulate in brain tissue with age showing a pro-inflammatory secretion pattern (Bhat et al. 2012; Jurk et al. 2012; Kritsilis et al. 2018). Mouse and cell culture models of neurodegeneration indicate that senescent glia and in particular senescent astrocytes can promote pathology (Bhat et al. 2012; Chinta et al. 2018; Bussian et al. 2018). Senescent and reactive marker profiles reported in the literature show a considerable overlap, and specific features that distinguish both states are not clear even though some candidate genes have been proposed *in vitro* (Cohen and Torres 2019; Simmnacher et al. 2020). In this sense, it can be speculated that astrocyte senescence may be a form or a consequence of astrocyte reactivity. This interpretation may well apply to the similarities found between neurodegeneration and cancer. Is cell-transformation just senescence gone wrong? Is senescence reactivity gone too far?

An array of senescent markers was gathered from literature sources and compared against transcriptomics data (Bhat et al. 2012; Crowe et al. 2016; Casella et al. 2019; Basisty et al. 2020a, b; González-Gualda et al. 2021) (see full list and references in Online Resource 2). As expected based on the proliferative capacity of old 3xTg-AD neurotoxic astrocytes, some of the most typical senescence markers like β-galactosidase (Glb1), p16ink4a (Cdkn2a), lamin B1 (Lmnb1), and Vegfa were not differentially expressed, or expressed accordingly like Rb1 (downregulated) but a concordant profile emerged from others, including p21 (Cdkn1a) and Trp53 (Fig. 13). Transcripts coding proteins typically associated with astrocyte reactivity and injury processes including fibronectin, Il6, and Kcnj10 were detected. Noteworthy, a downregulation of Gfap, not typically expected in reactive astrocytes, matches a senescent profile. From the assessed profile, other interesting factors emerge. Insulin-like growth factor binding protein 4 and 6 (Igfbp4, Igfbp46) are associated with neuronal differentiation and function. Igfbp6 has been found elevated in the serum of AD patients and is one of the factors that astrocytes can produce to inhibit neuronal differentiation *in vitro* (Tham et al. 1993; Barkho et al. 2006). Similarly Igfbp4 can inhibit proliferation and promotes differentiation of neural progenitor cells *in vitro* (Niu et al. 2017). Thioredoxin domain-containing protein 5 (Txndc5), biglycan (Bgn), and plasminogen activator (Plat) are associated with pro-inflammatory effects (Adhami et al. 2008; Li et al. 2013; Horna-Terrón et al. 2014; Wang et al. 2017; Xie et al. 2020). Plat can be expressed by astrocytes after injury *in vivo*, excessive activity inhibits dendritic arborization and induces cell death in developing Purkinje cells (Adhami et al. 2008; Li et al. 2013). Noteworthy some factors have also been associated with cancer progression in the CNS, including nucleophosmin 1 (Npm1), filamin C (Flnc), and lysyl oxidase 2 (Loxl2) (Holmberg Olausson et al. 2015; Kamil et al. 2019; Zhang et al. 2020a).

Concluding Remarks and Perspectives

Neurotoxic astrocytes isolated from adult symptomatic 3xTg-AD mice showed multiple alterations associated with key regulatory processes, including energy metabolism, ion and neurotransmitter homeostasis, proliferation, and inflammation. The assessed alterations reassert the potential role of transcription factors like c-Myc and NF-κB as drivers of Tg.Old phenotype, which have been extensively studied in many homeostatic and disease contexts (Granic et al. 2009; Miller et al. 2012; Goetzman and Prochownik 2018; Jha et al. 2019; Zhang et al. 2020b). Other emerging factors include estrogen receptors which are increasingly being associated with protective roles regulating inflammatory process and Hif-1α associated with both harmful and beneficial effects in the CNS as well as with cancer progression (Wang et al. 2016, 2020a; Ashok et al. 2017; Arias-Cavieres et al. 2020; Grubman et al. 2021).

Noteworthy, multiple alterations related to glucose and amino acid metabolism were suggested. The net effect of these alterations on glycolytic rates, mitochondrial function, and aminoacid neurotransmitter homeostasis should be confirmed by functional assays.

Moreover, potential sources of neurotoxicity have been described among altered cytokine and growth factors. Finally, altered metabolism in the endoplasmic reticulum and mitochondrial could be a source of harmful metabolites like certain ceramides and fatty acids. In this regard, astrocyte release of saturated lipids has been recently reported as a neurotoxic mechanism (Guttenplan et al. 2021).

Remarkably, the observed profile is reminiscent of the behavior of transformed and senescent cells, pointing towards a common ground between metabolic disorders, neurodegenerative diseases, and cancer as other authors have suggested based on molecular and population studies (Sánchez-Valle et al. 2017; Martin-Jiménez et al. 2017). In this sense, some of the observed alterations might constitute a mechanism to counterbalance stress responses and promote proliferation: while in malignant cells, it results in increased aggressiveness, in reactive astrocytes and other glial cells, it can be initially protective. In a later disease stage, these alterations could impair astrocyte neuronal supporting role and instead, induce their neurotoxic potential in a self-sustained process.

Finally, an important caveat to note is that the lack of an age-matched control for the neurotoxic 3xTg-AD astrocytes prevents us from revealing the effect of aging as a separate factor in the development of this type of astrocyte, as well as the resulting effect of damage inflicted at the time of acquisition of an older brain. In this sense, in the present study, we assess astrocytic changes originated from both age and disease progression but not from transgenesis per se. It is worth noting that further proof-of-causality studies are required to attribute the specific neurotoxic effects observed in old neurotoxic 3xTg-AD astrocytes to any of the molecular changes described. Likewise, our *in vitro* study opened exciting questions that should be further evaluated: Can these neurotoxic astrocytes be isolated from male 3xTg-AD mice whose pathology is less consistent than in females? If so, is their neurotoxic effect as potent as in astrocytes isolated from female mice? What percentage of the total astrocytic population occupies *in vivo* this neurotoxic phenotype? In this sense, ongoing longitudinal studies in these mice (3 to 18 months) will provide complementary insights into this population of neurotoxic 3xTg-AD astrocytes and help us improve our understanding of astrocytic function throughout AD progression.

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

Declarations

Competing Interests The authors declare no competing interests.

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