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Candesartan neuroprotection in rat primary neurons negatively correlates with aging and senescence; a transcriptomic analysis

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Abstract

Preclinical experiments and clinical trials demonstrated that Angiotensin II AT1 receptor overactivity associates with aging and cellular senescence, and that AT1 receptor blockers (ARBs) protect from age-related brain disorders. In a primary neuronal culture submitted to glutamate excitotoxicity, Gene Set Enrichment Analysis (GSEA) revealed expression of several hundred genes altered by glutamate and normalized by Candesartan correlated with changes in expression in Alzheimer's patient's hippocampus.

To further establish whether our data correlated with gene expression alterations associated with aging and senescence, we compared our global transcriptional data with additional published datasets, including alterations in gene expression in neocortex and cerebellum of old mice, human frontal cortex after age of 40, gene alterations in the Werner syndrome, rodent caloric restriction, Ras and oncogene-induced senescence in fibroblasts, and to tissues besides the brain such as muscle and kidney.

The most significant and enriched pathways associated with aging and senescence were positively correlated with alterations in gene expression in glutamate-injured neurons, and, conversely, negatively correlated when the injured neurons were treated with Candesartan. Our results involve multiple genes and pathways, including CAV1, CCND1, CDKN1A, CHEK1, ICAM1, IL-1B, IL-6, MAPK14, PTGS2, SERPINE1, TP53, encoding proteins associated with aging and senescence hallmarks, such as inflammation, oxidative stress, cell cycle and mitochondrial function alterations, insulin resistance, genomic instability including telomere shortening and DNA damage, and the senescent-associated secretory phenotype.

Our results demonstrate that AT1 receptor blockade ameliorates central mechanisms of aging and senescence. Using ARBs for prevention and treatment of age-related disorders has important translational value.

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Compliance with Ethical Standards

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Keywords

Angiotensin receptor blockers; Glutamate excitotoxicity; p53 Neuroprotection; Aging; Senescence

1. INTRODUCTION

Aging is a universal process affecting the whole organism and leading to senescence, the loss of cell replication and enhanced apoptosis. Mayor processes compromising cells in the aging brain and leading to senescence include dysregulated inflammation, enhanced oxidative stress, activation of p53, cell cycle and mitochondrial dysfunction, insulin resistance, genomic instability with telomere shortening and DNA damage, cerebrovascular alterations and activation of the senescent-associated secretory phenotype [1–9]. In the brain, senescence impairs cognitive and motor skills and is the major risk factor for neurodegenerative disorders such as Parkinson disease and Alzheimer's disease [10].

Angiotensin II, through AT1 receptor activation, contributes to homeostasis by regulating multiple brain functions [11–14]. However, brain AT1 receptor overactivity is associated with major alterations accelerating aging and leading to senescence, such as increased brain inflammation and oxidative damage, disruption of the mitochondrial respiratory chain, enhanced glutamate excitotoxicity and reduction of cerebral blood flow, major factors in the development and progression of age-related disorders [11–13, 15–17].

Consequently, inhibition of AT1 receptors by administration of Angiotensin Receptor Blockers (ARBs) has been shown to be strongly neuroprotective in neuronal, astrocyte, microglia and endothelial cerebrovascular cell cultures [18–21]. When administered *in vivo*, ARBs ameliorate early injury mechanisms including uncontrolled inflammation, age-related cognitive decline, prevent impairments in metabolic function and protect the cerebral vasculature [13, 17, 23, 23] Long-term inhibition of AT1 receptors by oral ARB administration in rats or life-long deletion of AT_1 receptors in AT_1 receptor knock out mice extend their lifespan [24–28].

ARB administration shares common protective and anti-aging mechanisms with calories restriction [20, 25] an intervention that extends the lifespan, reduces biomarkers of cellular senescence and retards several aspects of aging [29]. In addition, when administered systemically *in vivo*, ARBs ameliorate brain injury in rodent models of brain disease, particularly age-related cerebrovascular and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [17, 19, 21, 27, 30–36]. These preclinical reports are being substantiated by controlled clinical studies, revealing that ARBs protect cognition after stroke and during aging, and by cohort analyses reporting that ARBs reduce the incidence and progression of Alzheimer's disease [11, 12, 34, 37–40].

To address molecular mechanisms of ARB neuroprotection, we have performed a global gene analysis of rat primary neurons injured by glutamate [19]. The expression of hundreds of genes altered by glutamate was normalized when the neurons were incubated in the presence of Candesartan. When our findings were compared with those found in independently published datasets from Alzheimer's disease autopsy brains [19], we

discovered positive correlations in the expression of multiple genes altered by glutamate, and, conversely, negative correlations with the gene expression of glutamate-injured neurons treated with Candesartan. This study revealed common disease mechanisms between our *in vitro* results and those occurring in Alzheimer's disease [19].

Since aging is the most important risk factor for Alzheimer's disease, we asked the question whether Candesartan neuroprotection included additional pathways directly involved in aging and senescence. To this effect we run our global transcriptional data through Gene Set Enrichment Analysis (GSEA) in additional published datasets reflecting gene alterations during aging and senescence.

2. Methods

2.1 Gene expression analysis

We analyzed our own raw data from a previous experiment [19] submitted to Gene Expression Omnibus (GEO) under accession GSE67036. In this experiment we had incubated separated cultures of primary rat cerebellar granule cells (CGC) treated with either vehicle, Candesartan, glutamate or Candesartan and glutamate. Each group consisted on 5 independent experiments. Standard procedures extraction of total RNA, labeling, hybridization, washing and staining were as per manufacturer recommendation (Affymetrix, Santa Clara, CA). The raw data is submitted to GEO under accession GSE67036. Detailed procedures have been described [19].

2.2 Datasets description and microarray data mining

We used GSEA (http://www.broadinstitute.org/gsea/) [41] to compare our data to published datasets [19]. For a more comprehensive description of the GSEA and the Broad Molecular Signatures Database v5.0 (MSigDB) [42] see [43, 44]. Alternatively, we used published microarray datasets from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) [45] to derive gene sets that we then used for GSEA analysis. All of the differentially expressed genes were included in the analysis: GSE8150 [46], GSE60652 [47], GSE28464 [48], GSE11882 [49], GSE17757 [50] and GSE11697 [51], were downloaded from the National Center for Biotechnology Information (NCBI) GEO database and imported into Partek Genomics Suite software (Partek, Inc., St. Louis, MI). The geneset names, accession numbers and website links are all described in Table 1 and Online Resources; 1 for aging, 2 for senescence and 3 for combined brain regions). Ingenuity pathway analisis (IPA). (http://www.ingenuity.com) [52] (Ingenuity Systems, Redwood City, CA) was used to identify canonical pathways associated with the differentially expressed genes.

To identify pathways associated with differentially expressed genes we used the CluePedia plugin of Cytoscape (v3.7.1) (http://www.cytoscape.org/) [53]. Datasets downloaded from GEO were Robust Multichip Average (RMA) normalized and differential gene expression was accessed by one-way ANalysis Of VAriance (ANOVA). For microarrays cross platform comparisons, we used a p value of <0.05 and a 1.2-fold change cutoff. Raw data from these datasets were analyzed with Partek Genomics Suites under similar conditions used for our

neuronal culture data. To avoid cross platform heterogeneity, we focused only on datasets generated on the Affymetrix platform.

3. RESULTS AND DISCUSSION

We run our global transcriptional data (GSE67036) [19] through the GSEA (http://www.broadinstitute.org/gsea/ [42]. Using this functional enrichment analysis, we identified multiple genes altered in our study that were over-represented and most significantly associated with alterations in gene expression found in many aging and senescence datasets. Multiple genes in these selected genesets, that were associated and upregulated with aging and senescence (Fig 1 and Online Resources 1 and 2) showed a statistically significant positive correlation with genes upregulated by glutamate in our study and a negative correlation with the expression of those genes that, when upregulated by glutamate, were reversed or normalized by Candesartan. Based on these associations, we postulate that administration of Candesartan could significantly ameliorate or reverse the effects of aging and senescence in gene expression.

The results obtained with our cell culture were compared with many transcriptome analysis studies in mice. Analysis of gene expression profiles of neocortex and cerebellum in adult and old mice indicated alterations in the expression of multiple genes with aging, that were similar in these two regions and that were indicative of inflammatory responses, microglial activation, complement cascade genes, stress response, oxidative stress, and reduced neurotrophic support [54] (Fig 1a; Online Resource 1). The association with changes in the expression of genes in our study were similar in all cases. There were 38 out of 85 enriched genes when comparing those found upregulated in the aged neocortex including NDRG1, FOS, CRYAB, APOD, MPEG1, C1QC, IFI27, C1QA, PTGS1, CTSZ, CTSS, (Online Resource 1), and 26 out of 80 genes upregulated in the aged cerebellum (LEE_AGING_CEREBELLUM_UP) including FOS, IRF7, MPEG1, C1QC, BCL2A1, IFI27, CIQA, AXL, CTSZ, and CTSS. (Fig 1a, Online Resource 1). This study also noted that alterations in gene expression in brains from aged mice correlated with those found in autopsy brains from patients who suffered from neurodegenerative disorders [54].

A comparison of the gene expression profiling of fibroblast cell lines from young and old human donors with those obtained from patients suffering from Werner Syndrome or progeria, a premature aging disorder, indicated that over 90% of the annotated genes were similarly changed in normal aging [55]. Our data showed a striking correlation with 19 genes out of 83 altered in both Werner syndrome and normal aging. Genes up regulated in Werner Syndrome and normal aging are also upregulated by glutamate and normalized by Candesartan in our neuronal culture, including CHEK1, NDRG1, TNFRSF10B and NR1H3. (Fig 1b, Online Resource 1; KYNG_WERNER_SYNDROM).

These results are supported by a meta-analysis of multiple age-related gene expression profiles consistently identified with age [56]. In this study, 29 out of 49 identified genes were enriched in our microarray analysis including LITAF, APOD, MPEG1, C1QC, C1QA. CTSS, CTSZ (Online Resource 1). Another study analyzed the transcriptional profile from the neocortex of young, adult and old mice treated with vitamin E supplementation [46]. In

this study, 122 out of 422 genes upregulated in the cortex of aged mice correlated with our microarray analysis including GFAP, ZCCHC24, EFS, HSPA2, LITAF, CCND1, C1QA, IFI27, C1QC, GRN, APOD and MPEG1 (Online Resource 1).

The correlation of our neuronal culture results and those revealed in aging is not limited to findings in the brain, but they are also consistent with gene alterations reported in other tissues such as muscle including TGFB1I1, FOS and TGIF1 [57] (Fig 1c, Online Resource 1), and NRG1, FGFR2, TFAP2A, CNP, GRN, CCL2, CDKN1A, PIK3CG and SERPINE1 [58] Online Resource 1, and kidney, including RAB31, ZCCHC24, TIMP2, MPEG1, GRN, C1QC, MYOF, BCL2A1, AXL, C1QA, CCL2, VCAM1 and SLC6A6 [59] (Fig 1d, Online Resource 1).

Similarly, two analysis of genes upregulated in general cellular aging (From the Gene Ontology (GO) database http://www.ncbi.nlm.nih.gov/geo/) [45] revealed that 90 of 252 genes, (Fig 1e, GO_AGING, Online Resource 1) and 22 out of 62 genes are enriched when compared with our microarray analysis (GO_CELL_AGING. Online Resource 1), respectively. This includes TP53, HMGA1, CHEK1, LITAF, FOS, TIMP2, CRYAB, MME, TNFRSF10B, APOD, SERPINE1, IL6 and IL1β.

A transcriptional profile in human frontal cortex was compared in human autopsy samples ranging from ages from 26 to 106 years. This study reported that the expression of a set of genes important for synaptic plasticity, vesicular transport, mitochondrial function, calcium homeostasis and neuronal signaling and involved in learning, memory and neuronal survival was reduced, and that DNA damage was enhanced, with reduced DNA repair in their promoters after age 40. These findings correlated with those damaged by oxidative stress in cultured human neuroblastoma cells and are indicative of a genetic signature of human cortical aging [60]. A comparison of these results with our global gene analysis revealed that of the 244 genes altered in the aged human frontal cortex, the expression of 129 genes negatively correlated with our findings in neuronal cultures [60] (Fig 1f, LU_AGING_BRAIN_UP, Online Resource 1).

Genes reduced in the aged cortex and damaged by oxidative stress reduce DNA repair, leading to decreased expression of genes involved in learning, memory and neuronal survival. These include genes such as NDRG1, GSN, C1QC and C1QA, involved in defects of the DNA-repair system, the tumor suppressor pathway, the telomere maintenance system, the insulin/Akt pathway, and other metabolic pathways [60].

Of special interest is that alterations in gene expression in our neuronal culture dataset affected by glutamate and normalized by Candesartan strongly associate with those found in two mice strains subjected to calorie restriction [57]. It is already known that enhanced calorie intake is a risk factor for diabetes and hyperinsulinemia, and that alterations of the insulin pathway induce cellular senescence in vitro [61]. In turn, calorie restriction, like long-term ARB administration, extends the lifespan of various species, favors lipid metabolism, protects from renal disease, decreases biomarkers of cellular senescence *in vivo*, and retards several aspects of aging [15–17,29]. Calorie restriction reduced the age-associated up-regulation of genes regulating inflammation, stress, microglial activation and

the complement cascade [54]. We have recently shown that changes in gene expression during calorie restriction associate with the expression of genes altered by inflammation in microglia cultures and normalized by the ARB Telmisartan [20]. The effects of calorie restriction are not restricted to the brain, since it reduces age-dependent oxidative damage in muscle [57, 58] Online Resource 1). These results support the hypothesis that ARB administration and calories restriction share common protective and anti-aging mechanisms, and they are both associated with longevity.

The correlation of our neuronal culture results is not limited to published aging-related databases but extend to databases reporting alterations in gene expression involved in senescence. A review of studies on gene expression profiling to identify those genes involved in senescence, some with supporting functional data, revealed universal genes regulating senescence, across cell types and model systems, including the cell cycle pRB/p53, cytoskeletal, interferon-related, insulin growth factor-related, MAP kinase and oxidative stress pathways [62], In this dataset, of the 75 genes upregulated by senescence, 37 genes up regulated by glutamate and down regulated by Candesartan were found enriched in our glutamate-candesartan study (Fig 1g, Online Resource 2). They include STAT1, SERPINE1, RAB31, TFAP2A, TGFB1/1, HSPA2, IRF7, CRYAB, CTGF, GSN, CDKN2B, THBS1, CYP1B1, THBS1 and CDKN1A.

We found additional correlations between our neuronal culture results and a microarray analysis of human IMR90 fibroblasts differentiated into macrophages submitted to Ras-induced senescence [48]. From the many genes (378) with enhanced expression by senescence, the expression of 135 genes was positively correlated in our study with glutamate-induced injury and negatively correlated to the effects of Candesartan in our system (Fig 1h), including TGFB1/1, HSPA2, LPP, CTGF, MYOF, THBS1 and PTGS1 (Online Resource 2 GSE60652_IMR90_RAS_SENESCENCE_R)

Similarly, microarray analysis of IMR90 fibroblasts subjected to oncogene-induced senescence with reduced glycolytic pathway and down regulation of the retinoblastoma protein identified 82 genes out of 254 are enriched in our neuronal cultured study [47] (Fig 1i). (Online Resource 2 GSE28464_IMR90CELLS_RAS_SENESCENCE_UP)

Gene Ontology analysis of genes associated with senescence identified that out of 30 senescence genes, 18 genes were enriched in our study (GO_CELLULAR_SENESCENCE, Online Resource 2). Genes upregulated by glutamate, normalized by Candesartan and enriched in this study include CDKN1A, TP53, MAPK14, CAV1, THBS1 and HMGA2.

Moreover, using proliferating and senescent WI-38 cells, [63] identified 31 and of those 8 were enriched in our glutamate-Candesartan analysis, including CDKN1A that encodes for p21, a major target of p53 and linking DNA damage to cycle arrest [64], LCAT, CCND2, stimulated by oxidative stress inducing cellular senescence [65] and ADAMTS5, a disintegrin and metalloproteinase with thrombospondin motifs 5, upregulated in extracellular matrix destruction and osteoarthritis [66] (Online Resource 2, TANG_SENESCENCE_TP53_TARGETS_UP).

Given the large number of aging genesets negative correlated with glutamate + Candesartan, we sought to identify common aging genesets so we could define pathways associated with Candesartan effects. We used the GEO database to compare our dataset with up-regulated genes in 6 different published aging datasets from different human and primate brain areas. GSE11882 included samples from the human posterior cingulate cortex (PCG), superior frontal gyrus (SFG), Hippocampus (Hippo), and Entorhinal Cortex (IEC) [49]. GSE11697 included primate Hippocampus CA1 region (Hippocampus CA1) [51], and GSE17757 included primate and human SFG [50].

We defined 116 genes consistently up-regulated in all 6 datasets from aged brains by at least 1.2 folds (Online Resource 3_Age up-regulated in 6 datasets). Of these 116 aged brain genes, 99 genes were present in our neuronal array study. Of those 99 genes, 70 genes were up-regulated by glutamate (p-value<0.01) and down-regulated or normalized by Candesartan in our study. (Online Resource 3: GSEA AGE_UP-REGULATED_6DATASE). Moreover, when we used GSEA to run these 116 genes comnmonly up-regulated by aging over our entire neuronal microarray, we found a highly significant enrichment with genes up-regulated by glutamate cytotoxicity and down-regulated by Candesartan.(Fig 2 and Online Resource 3_Age up-regulated in 6 datasets). This is highly significant, considering that these results have been obtained from different tissues, different array platforms and different species.

Interestingly, two of the genes up regulated by glutamate in the 6 aging datasets and reversed by Candesartan encode for glutamate transporters and play a role in excitatory signaling. SLC25A18 is a mitochondrial glutamate transporter [67] and SLC7A11, encoding for the cystine/glutamate transporter xCT, has been found upregulated in Alzheimer's disease patients [68].

To define the molecular pathways associated with these common aging geneset we used the DAVID Gene Onthologies. Using GO_BP, we identified 39 biological processes significantly associated with our findings including extracellular matrix disassembly, integrin-mediated signaling pathway, negative regulation of apoptotic process and inflammatory response (Online Resource 3, GO_BP). To identify key genes and pathways we used GOTERM_MF_DIRECT. We identified 17 associated pathways. Our results included receptor activity, cadherin binding involved in cell-adhesion and protein binding (Online Resource 3, GO_MF). Similar significant associations were found by GO_CC analysis of cellular components, including extracellular exosome, cell surface, plasma membrane and blood microparticles (Online Resource 3, GO_CC). Using Go_Diseases, we found 13 types of associated disorders. The most significantly associated diseases were immune, cardiovascular and pharmacogenomic disorders (Online Resource 3, GO_Diseases). To discover gene-disease associations, we compared our data base with gene ontology. We identified 19 GO_GAD diseases, including Type2 diabetes, asthma, systemic lupus erythematosus and chronic renal failure (Online Resource 3, GO_GAD_Disease).

We then used the Ingenuity Pathways Analysis (IPA) upstream regulator analysis for both the genes upregulated by glutamate and downregulated or normalized by Candesartan and the genes upregulated in 6 different aged brain. The list of the 116 genes most upregulated in

6 aging datasets and the list of the genes upregulated by Glutamate and downregulated by Candesartan (525 genes) were put in the IPA upstream regulators to determine if the same pathways were shared between aging and Candesartan action. We were able to show that both datasets share over 90% of their upstream regulators and that the z-scores for these common age-related upstream regulators are reversed when the CGC neurons injured by glutamate are treated with Candesartan (Online Resource 4: table IPA_ Combined GSE11882_CGC-Candesartan). This application yielded transcription regulators that are known from the literature to target the genes associated with aging and general inflammation and compared their z-score direction to weight in the predicted effect of the regulator on a subset of the genes (Table 2).

Many upstream-regulators associated with general inflammation pathways such as Il-6, TNF, the NFkB complex, insulin, TGFβ-SMAD and P38 MAPK-MEK-ERK1/2 show a positive z-score in aging, meaning that these upstream regulators can have a positive regulation (upregulation) on many downstream genes. These z-scores are then reversed for the same upstream regulators for the glutamate-up/Candesartan-down regulated genes. Reciprocally, resveratrol, a known anti-inflammatory and anti-aging compound and its positive partners SIRT1, TSC2 and PTEN [69–71], show a positive z-score for the glutamate-up/Candesartan-down regulated genes and negative z-score for aging. (Online Resource 4: Table IPA_ Combined GSE11882_CGC-Candesartan, Representative upstream regulators; Table 2).

To further demonstrate the common pathways shared between these two genelists, we used the CluePedia plugin of Cytoscape (v3.7.1) [53]. As expected, these two lists show many pathways associated with signal transduction, cellular response to external stimuli, immune response, secretion and exocytosis, T-cell differentiation and activation, cell motility migration and proliferation-morphogenesis. (Fig 3 a). Then we asked the question of how many genes from each list are associated with each Gene Ontology pathway. Fig 3 b shows that each pathway shares 40–60% genes from each gene list. This mean that aging and glutamate cytotoxicity normalized by Candesartan share almost the same pathways.

Using Gene Set Enrichment Analysis (GSEA), over 70 genes significantly upregulated by glutamate in our neuronal culture dataset and normalized by exposure to Candesartan are enriched in several aging and senescence data sets. These genes are involved in all the major pathways that are considered hallmarks of aging and senescence, (Online Resource 5) (Fig 4). The defined hallmarks of aging are closely interrelated, and many genes play essential roles in several major signaling systems. For example, telomere dysfunction alters mitochondrial function, enhances oxidative stress and upregulates the inflammasome. abnormality, oxidative stress, and hyperactivation of the NLRP3 inflammasome [72]. Many genes, including those encoding for transforming growth factor beta (TGFβ) or tumor necrosis factor-alpha (TNFα) exert pro-longevity and anti-longevity effects, and their activity may enhance neuroprotection or promote neurodegenerative disorders, depending on the biological and environmental context [73].

A pathway of major importance in aging is that controlled by TP53. The TP53 gene encodes the tumor protein p53, regulating the cell cycle arrest, mediates DNA damage leading to cell

senescence, cell death and many other functions. Unrestrained and excessive p53 activation is detrimental to healthy aging [74–77]. p53 is activated by multiple stressors, including glutamate excitotoxicity [78]. Nine genes from these studies are part of the p53 signaling pathway (TP53, CDKN1A, CDKN2A, CASP9, CHEK1, CCND2, SERPINE1, CDK1 and ATM (Table 3, Fig 4) and 24 genes have a protein interaction with p53 protein (CDKN2A, VIM, KAT6A, IRF7, BCL2, CHEK1, HMGA1, MAPKAPK5, TFAP2A, SIRT1, GSN, CRYAB, TGM2, MAPK14, AGT, HRAS, H2AFX, PRKCD, CDK1, ATM, HSPA2, TP53 and PML) (Table 3, Online Resource 5; Fig 4)

Members of the p53 pathway include genes reported to be enhanced in by inflammatory stimuli, in aging and in Alzheimer's disease: BCL2A1, encoding BCL-2-related protein A, a direct transcription target of NF-κB in response to inflammatory mediators [79], CDKN1A and CDKN2B, encoding for p21, a cyclin-dependent kinase inhibitor protein, a major target of p53 associated to DNA damage and a senescence associated gene [80–83] (Table 3). CHEK1, encoding for the CHK1 checkpoint homolog, increased in aging and Alzheimer's disease [84], TNFRSF10B encoding for the tumor necrosis factor receptor superfamily member 10B or death receptor 5 (DR5) that mediates age-related p53-induced apoptosis [85]; ICAM1, encoding for the intracellular adhesion molecule 1, participating in the inflammatory phenotype of senescent cells [86], MAPK14, encoding for the mitogen-activated kinase 14, is activated by environmental stressors and pro-inflammatory cytokines, regulates p53 inducing senescence, plays a key role in stress factors affecting genomic integrity, and participates in Alzheimer's disease [87, 88], THBS1, encoding for thrombospondin 1, leads to enhanced ROS and increased p53 transcription with advancing age [89–91], LIF, encoding for the leukemia inhibitory factor, a protein regulated by p53, stimulated by pro-inflammatory cytokines and with a major role in inflammation [92], and TFAP2A, encoding for the transcription factor AP-2 alpha and associated with DNA hypermethylation that directly interacts with p53 [93, 94] (Fig 4).

Within this pathway, SERPINE1, encoding for the plasminogen activator inhibitor-1 (PAI-1) [95, 96] is of great interest. PAI-1 is the major inhibitor of endogenous thrombolysis, promoting thrombosis, it is strongly activated by Angiotensin II [97], and inhibited by ARBs, including Candesartan [98]. SERPINE1 is both a marker and a mediator of senescence and many age-related disorders [99–103] and it is much higher in p16-positive senescence cells [104]. It is activated by ROS, oxidative stress, the DNA Damage Response (DDR), the pro-senescence TGFβ, glutamate excitotoxicity and stress-induced anxiety-like behavior [105, 106].

Inflammation and oxidative stress are major determinant of aging and senescence. Genes involved in inflammatory pathways include BCL2A1, ICAM-1, LIF, CAV1 encoding calveolin 1, upregulated by oxidative stress [107], CCL2, encoding chemokine (C-C motif) ligand 2, associated with neurodegenerative disorders [108]; CSF2, encoding the colony stimulating factor 2, part of the inflammatory cascade controlled by NF-κB [109], CTGF, encoding for connective tissue growth factor, induced by oxidative stress and in accelerating aging models [110], CTSS, encoding cathepsin S, associated with olfactory dysfunction [111] and CTSZ, encoding for Cathepsin Z involved in dopamine neuron death [112], and RAB31, encoding for Ras-related protein Rab-31, stimulating high levels of oxidative stress

[113] and THBS1, encoding for thrombospondin 1, a protein enhancing ROS and contributing to coronary ischemia in advancing age [90].

TGFβ also contributes to cellular senescence. TGFB1I1, encoding for transforming growth factor beta-1-induced transcript 1 protein, is involved in multiple functions including apoptosis, the immune system and increasing IL-1-beta, and TGFBR2, encoding for transforming growth factor, beta receptor II is a TGF beta receptor increased in rodent models and in patients with Alzheimer's Disease linking neuroinflammation and amyloidosis [114] (Online Resource 5).

Some additional inflammatory genes are upregulated by Angiotensin II and reduced by AT1 receptor blockade, such as VCAM-1 encodes the cell adhesion molecule vascular cell adhesion protein 1. Its production is enhanced in response to tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), that is involved in high-glucose endothelial cell senescence, [115, 116], and MPEG1, encoding for the macrophage expressed gene 1 [117] an inflammatory gene that reduces necessary angiogenic responses to ischemia in aged rats [118].

Candesartan normalizes the glutamate-upregulated Senescence-Associated Secretory Pathway (SASP). The SASP increases in multiple tissues with aging and is triggered by senescence cells experiencing DNA damage [119], and Candesartan normalizes the upregulated SASP as a consequence of glutamate toxicity. Normalized genes include including IL1β, encoding for the pro-inflammatory cytokine interleukin 1β, [120, 121] and IL6, encoding for IL-6, that stimulates inflammation and auto-immune processes in multiple age-related diseases [122, 123] and SERPINE1.

Abnormal expression of a number of genes included in our analysis is genotoxic. They are directly involved in DNA alterations leading to genome instability during aging, and in particular telomere dysfunction, leading to mitochondrial abnormality, oxidative stress and hyperactivation of the NLRP3 inflammasome. These genes include CHEK1 [84], NDRG1, encoding for the NDRG1 protein, strongly associated with age and disorders of DNA methylation and with multiple functions in stress responses, immunity myelination and cell adhesion [124, 125], NFKBIZ encoding for NF-kappa-B inhibitor zeta a nuclear protein induced by inflammation and TNF-α, associated with senescence and cell death [126–129], and TIMP2, encoding for the tissue inhibitor of metalloproteinases 2 (TIMP2), increasing after genotoxic stress and part of the SASP [130]. ZCCHC24 encodes for zinc finger, CCHC domain containing 24. This protein binds nucleic acids and is upregulated in aging, contributing to genomic instability and telomere attrition [7, 131, 132]. Other genotoxic-related genes include those encoding histones such as HIST1H2AC, encoding for histone 1, H2ac, a protein fundamental for DNA function [7, 104, 133], HMGA2, encoding for high mobility group AT-hook 2, promoting stem cell aging [134] (Online Resource 5).

Genes with roles in cell-cell adhesion and tight junctions include CLDN1 encodes for Claudin-1, involved in tight junction deterioration [135] and LPP, encoding for LIM domain containing preferred translocation partner in lipoma or lipoma-preferred partner, involved in cell-cell adhesion and cell motility [136] (Online Resource 5).

Genes involved in myelin and mitochondrial function and enhanced during aging include CNP, encoding for 2',3'-cyclic nucleotide 3'phosphodiesterase [137, 138], EREG, encoding for epiregulin [139], GDF15, encoding for growth differentiation factor 15, a protein that contributes to radiation-induced senescence mediated by the p16 pathway [140, 141], IFI27, encoding for interferon, alpha-inducible protein 27, a protein that destabilizes mitochondrial membrane function sensitizing cells to apoptotic stimuli [142, 143], IRF7, encoding for interferon regulatory factor 7, involved in the transcriptional activation of virus-induced cellular genes, and increased expression of interferon type 1, an mRNA signature in the aging brain [144–148], LPIN1 and RAC2 encoding for Ras-related C3 botulinum toxin substrate 2, associated with decline in mitochondrial respiration and prostaglandin metabolism, that delays post-stroke angiogenesis in the aging brain [117].

Alterations in prostaglandin metabolism play significant roles in aging and the development of neurodegenerative disorders, including alterations in expression of PLA2G4A, encoding for Cytosolic phospholipase A2, associated with decline in mitochondrial respiration in the female aging brain and Alzheimer's disease [149], PTGS1, encoding for Cyclooxygenase 1 (COX-1), prostaglandin-endoperoxide synthase 1, increased in older subjects with schizophrenia [150] and PTGS2 encoding for prostaglandin-endoperoxide synthase 2, cyclooxygenase-2 or COX-2, involved in premature aging [151].

Additional senescence-associated genes include CYP1B1, encoding for Cytochrome P450, family 1, subfamily B, polypeptide 1 [152], ENC1, encoding for ectodermal-neural cortex (with BTB-like domain) with a proposed role in the cognitive decline during aging [153], GFAP, encoding for the glial fibrillary acidic protein, enhanced in models of Alzheimer's disease and a marker of neurological damage [154], GNG11, encoding for guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-11 and inducing cellular senescence [155], . GRN, encoding for granulin and associated with hippocampal sclerosis [156, 157], HSPA2, encoding for heat shock 70kDa protein 2 that has been associated with increased amyloid-beta and tau in Alzheimer's disease [158]. TGM2 encodes for tissue transglutaminase, a protein with a role in apoptosis and inflammation that increases resistance to proteolysis resulting in abnormal aggregation of proteins and involved in Alzheimer's and Parkinson's disease [159–162] (Online Resource 5). MME encodes for membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10) or neprilysin. This protein is associated with Alzheimer's disease and renal damage; it is a downstream effector of PI3K mediating the induction of senescence, and a compound combining neprilysin and AT1 receptor blockade is beneficial for the treatment of heart failure [163, 164]. MYOF, encoding for fer-1-like 3, myoferlin, that has been associated with age in human populations [165] and PLXNB1, encoding for Plexin B1 and associated with cognitive decline and amyloid neuropathology [166] (Online Resource 5).

A number of genes that regulate glucose metabolism and insulin sensitivity, with enhanced expression in aging and diabetes, include GFPT2, encoding for glutamine-fructose-6-phosphate transaminase and associated with type2 diabetes [167], GM2A, encoding for GM2 ganglioside activator and enhancing insulin resistance [168], LITAF, encoding for the lipopolysaccharide-induced TNF factor, enhanced in diabetes, aging and inflammatory processes [169], LPIN1, encoding for lipin 1, a protein associated with insulin resistance,

inflammation and myelin disease [170] and PIK3CG, encoding for phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma, increasing oxidative stress and upregulating MAPK, playing a role in Angiotensin II-induced NADPH oxidase activation and leading to cardiomyopathy through AT1 receptor activation, that is downregulated by AT1 receptor inhibition [171, 172] (Online Resource 5).

The expression of some genes, upregulated by glutamate and normalized by Candesartan, may represent compensatory mechanisms to injury. For example, overexpression of MCTP1, encoding for multiple C2 domains, transmembrane 1, inhibits oxidative stress produced by glutamate excitotoxicity [173]. NRG1 encodes for Neuroregulin 1, a protein that attenuates stress-induced vascular senescence [174]. TGIF1, encoding for Homeobox protein TGIF1, binds to the retinoid X receptor responsive element. When expression is reduced, TGFβ signaling is increased leading to DNA damage and premature senescence [175, 176], and TNFAIP3, encoding for tumor necrosis factor, alpha-induced protein 3, a protein induced by tumor necrosis factor that inhibits TNF-mediated apoptosis; it is critical for limiting inflammation by terminating TNF-induced NF-κ B responses and critical for the homeostatic role of telomeres [72] (Online Resource 5).

4. CONCLUSIONS

Our initial global gene analysis of our neuronal culture revealed that multiple alterations in gene expression resulting from glutamate excitotoxicity were reversed or normalized by incubation in the presence of the ARB Candesartan, and that these alterations in gene expression significantly correlated with findings in autopsy brains from patients with Alzheimer’s disease. We had now compared our findings with those reported in multiple aging and senescence datasets, and we found significant correlations with alterations in expression of multiple genes reported in aging and senescence datasets.

In our study we used a culture of primary neurons, the cerebellar granule cells, established as the best model to determine mechanisms of neuronal survival, apoptosis and aging [177–180]. In addition, our results support the hypothesis of the importance of the cerebellum in cognitive behavior and its influence in the aging process [181–183].

We found enriched alterations in gene expression related to all major mechanisms associated with aging and senescence, including defects of the DNA-repair and telomere maintenance systems, the tumor suppressor pathway, reduction of synaptic plasticity, vesicular transport and mitochondrial function, alterations in glucose metabolism, excessive oxidative stress and inflammation, and the SASP. These alterations have been associated with reduction on neuronal survival, decreased learning and memory characteristic of neurodegenerative and many other age-related disorders, and common not only for brain disorders but for age-related diseases of the whole organism. Interestingly, most of the molecules that influence the phenotypic changes of aging also regulate cellular senescence, suggesting a causative link between cellular senescence and aging.

Our results support the hypothesis of a major negative, pro-aging and pro-senescence, influence of excessive Angiotensin II AT1 receptor activity not only in the brain but also in

the periphery, and demonstrate the overall protective, anti-aging effects of ARB administration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	ANalysis Of Variance
ARBs	Angiotensin Receptor Blockers
CGC	cerebellar granule cells
COX-1	cyclooxygenase 1
COX-1	cyclooxygenase-2
GEO	Gene Expression Omnibus
GO	Gene Ontology database
GSEA	Gene Set Enrichment Analysis
IL-1	interleukin-1
IPA	Ingenuity pathway analisis
MSigDB	Broad Molecular Signatures Database v5.0
NCBI	National Center for Biotechnology Information
PAI-1	plasminogen activator inhibitor
PCG	posterior cingulate cortex
RMA	Robust Multichip Average
SASP	Senescence-Associated Secretory Pathway
SFG	superior frontal gyrus
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor-alpha

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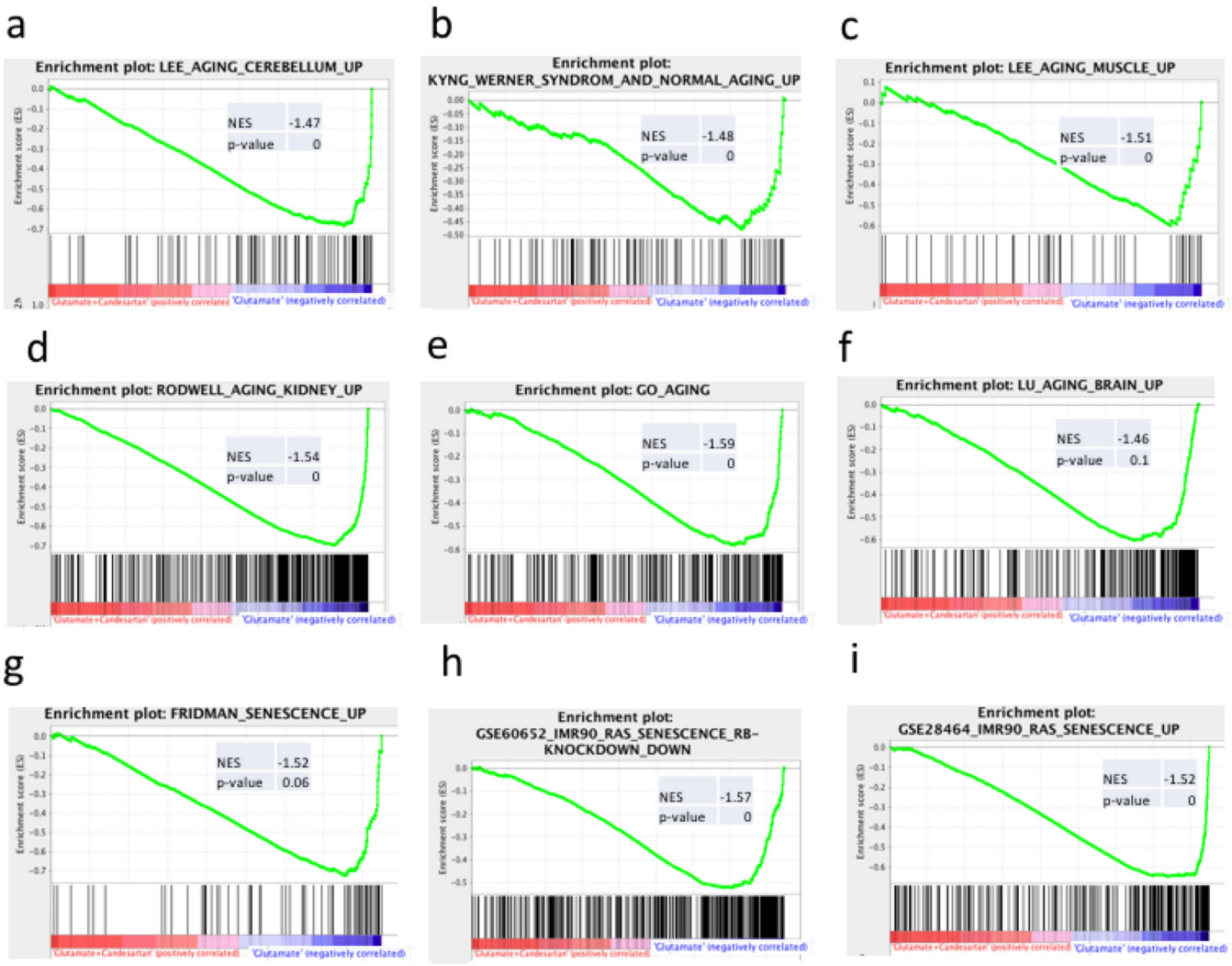

Fig 1. Gene Set Enrichment plots from representative aging pathway genesets.

The Fig represents Gene Set Enrichment Analysis plots showing the negative correlations of genes up-regulated by Glutamate and normalized by Candesartan in our neuronal cell culture with genesets associated with general aging in different brain areas and tissues (A-F, see Online Resource 1) and with senescence (G-I, see Online Resource 2). NES: Normalized Enrichment Score. Genesets with links and References are listed in this Table1.

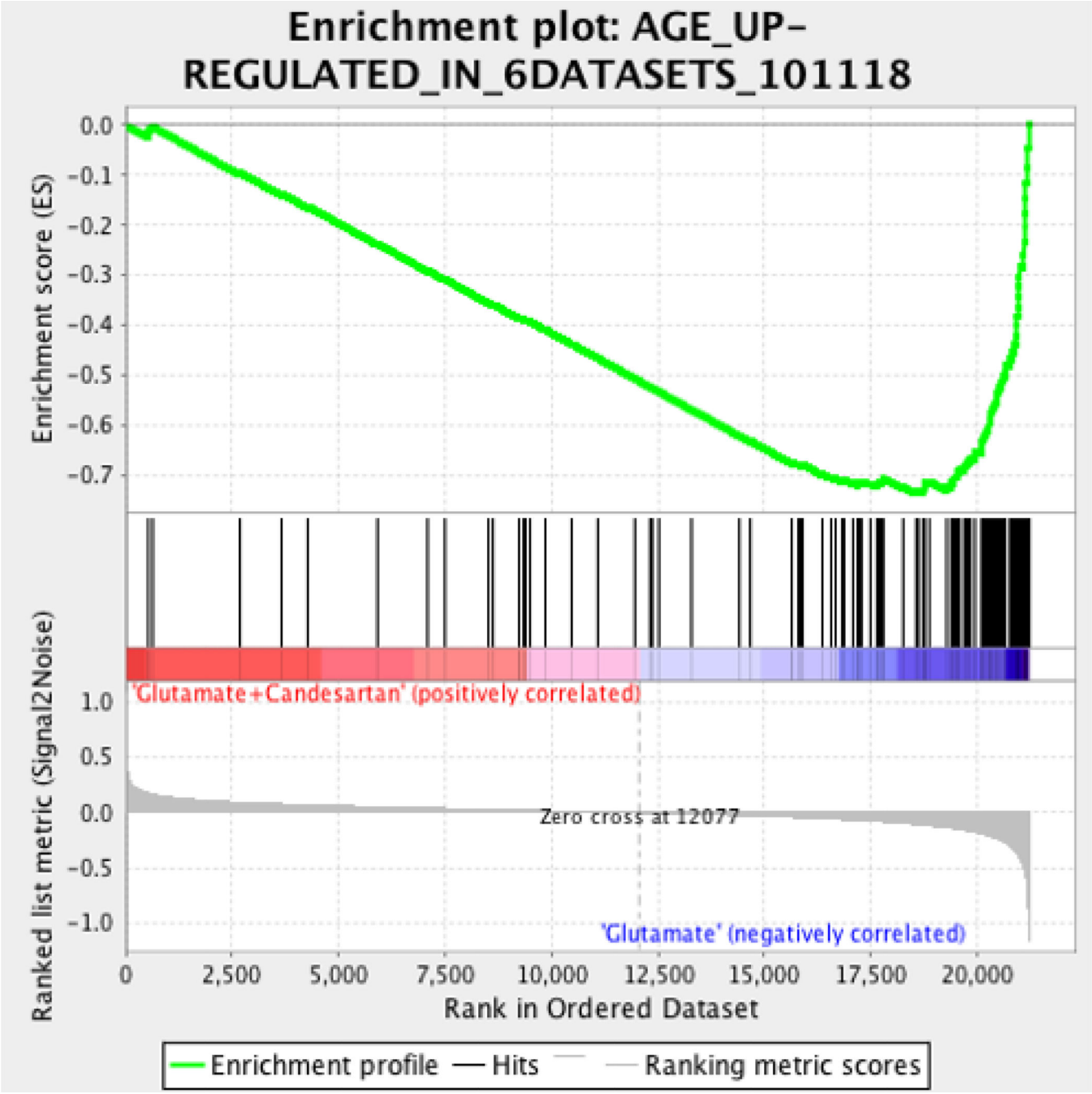

Fig 2. Negative Correlation of Candesartan effect with common senescence genesets.

Gene Set Enrichment Analysis plots showing the negative correlations of genes up-regulated by glutamate and down-regulated by Candesartan in our neuronal culture with a geneset of common consistently up-regulated genes in 6 different brain aging datasets including different brain areas (hippocampus, entorhinal cortex, superior frontal gyrus) (See Online Resource 3). Data were taken from: GSE11882_Aging (Berchtold et al., 2008, https://www.ncbi.nlm.nih.gov/pubmed/18832152), GSE11697_HippaCA1_Aging (Blalock et al.,

2010, https://www.ncbi.nlm.nih.gov/pubmed/20427664) and GSE17757_SFG_Aging (Somel et al., 2010, https://www.ncbi.nlm.nih.gov/pubmed/20647238).

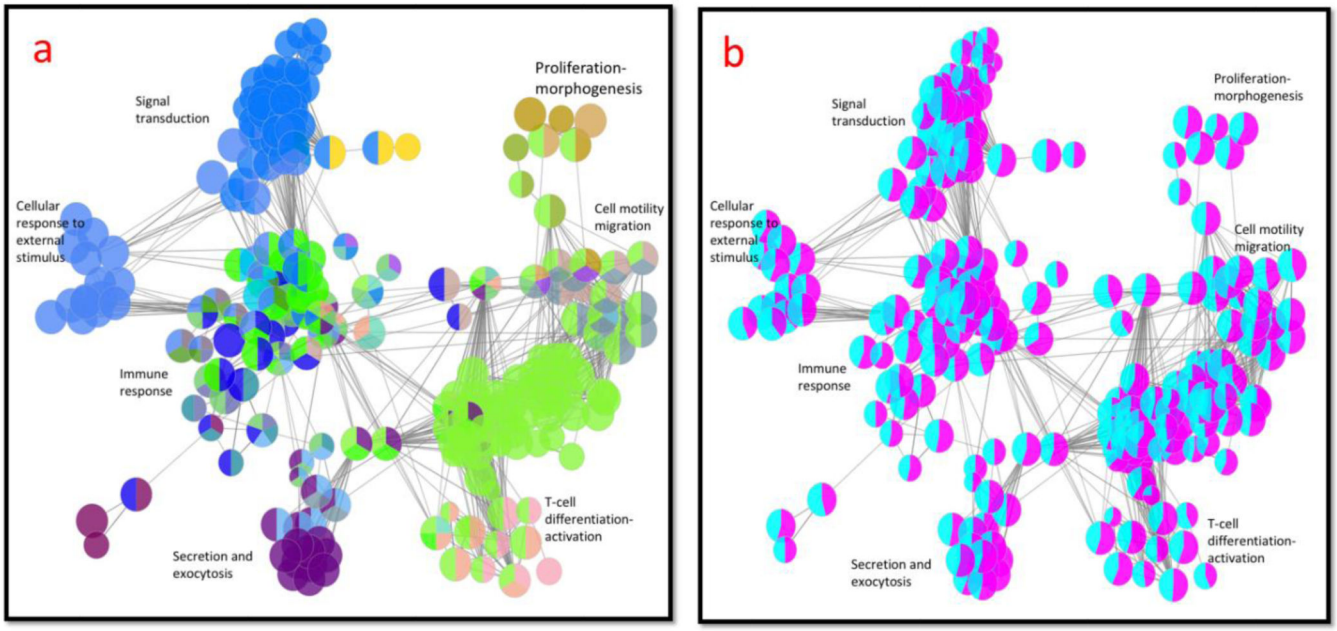

Fig 3. Pathways associated with differentially expressed genes.

Pathways associated with differentially expressed genes in 4 aging brain regions from GSE11882 (1.2 Fold change and p-value<0.05) and genes differentially expressed between rat cortical granule cells treated with cytotoxic glutamate versus cells pre-treated with Candesartan and then glutamate (1.2 Fold change and p-value<0.05). Figures were generated side-by-side using the CluePedia plugin of Cytoscape (v3.7.1) (http://www.cytoscape.org/). a: Color coded network of gene ontology pathways and their connection. b: The same network as in a. but this time showing the pie distribution of genes up-regulated in aging (purple) and genes up-regulated by glutamate and down-regulated by Candesartan(magenta).

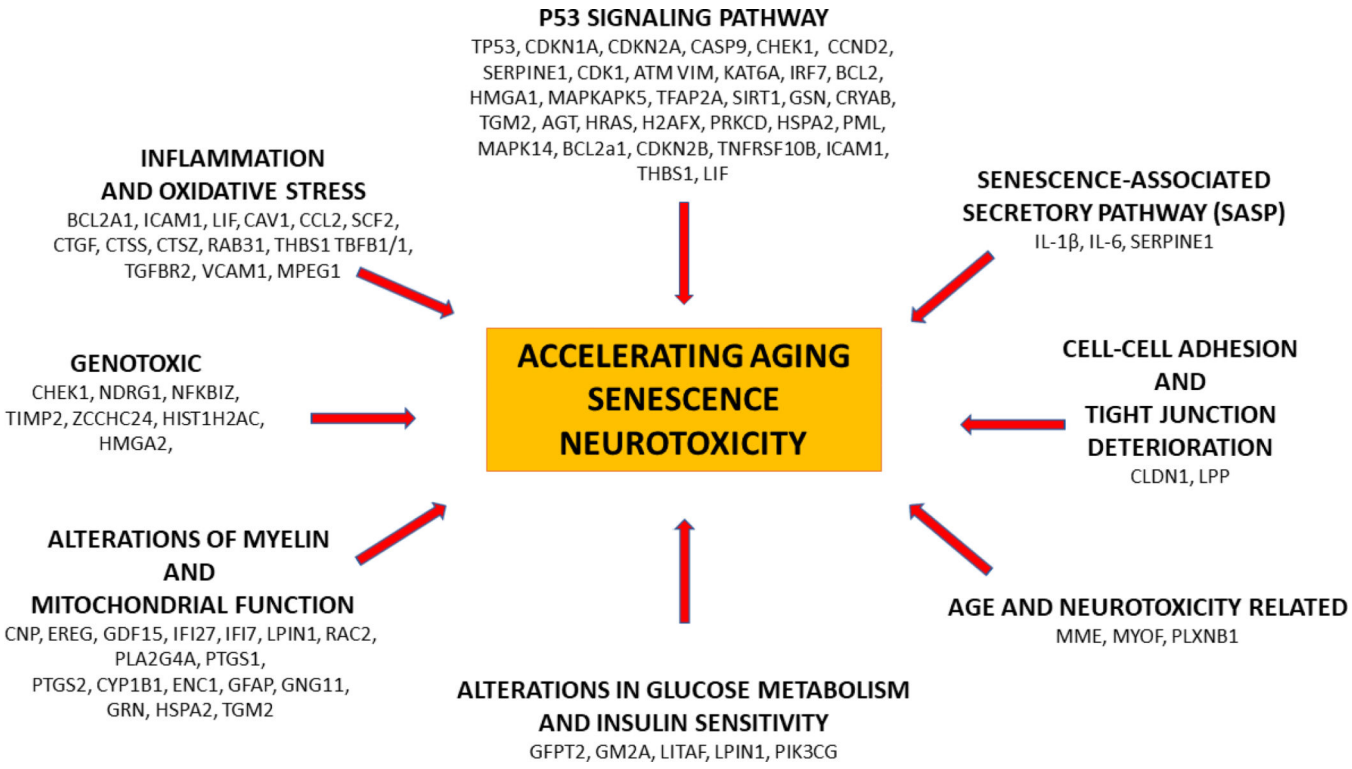

Fig 4. Influence of Candesartan on genes associated with hallmarks of senescence.

The Fig represents principal hallmarks of senescence and associated genes upregulated by glutamate in our database, normalized by Candesartan and enriched in selected datasets (Figs 1 and 2, Online Resource 3). Many genes associate and influence each other.

Table 1.

Genesets names, accession IDs, and links for the 19 genesets analyzed in this manuscript.

GENESET NAME	ACCESSION ID	LINK
GO_AGING	GO:0007568	http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=GO_AGING&keywo
GO_CELL_AGING	GO:0007569	http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=GO_CELL_AGING&keywords=GO_CELL_AGING
GO_CELLULAR_SENESCENCE	GO:0090398	http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=GO_CELLULAR_SENESCENCE&keywords=GO_CELLULAR_SENESCENCE
KYNG_WERNER_SYNDROM_AND_NORMAL_AGING_UP_		http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=KYNG_WERNER_SYNDROM_AND_NORMAL_AGING_UP&keywords=KYNG
LU_AGING_BRAIN_UP		http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=LU_AGING_BRAIN_UP&keywords=LU_AGING_BRAIN_UP
DEMAGALHAES_AGING_UP		http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=DEMAGALHAES_AGING_UP&keywords=DEMAGALHAES_AGING_UP
FRIDMAN_SENESCENCE_UP		http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=FRIDMAN_SENESCENCE_UP&keywords=FRIDMAN_SENESCENCE_UP
TANG_SENESCENCE_TP53_TARGETS_UP		http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=TANG_SENESCENCE_TP53_TARGETS_UP&keywords=TANG_SENESCENCE_T
RODWELL_AGING_KIDNEY_UP		http://software.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=RODWELL_AGING_KIDNEY_UP&keywords=RODWELL_AGING_KIDNEY_UP
LEE_AGING_MUSCLE_UP		http://software.broadinstitute.org/gsea/msigdb/cards/LEE_AGING_MUSCLE_UP
KAYO_AGING_MUSCLE_UP		http://software.broadinstitute.org/gsea/msigdb/cards/KAYO_AGING_MUSCLE_UP.html
LEE_AGING_NEOCORTEX_UP		https://www.ncbi.nlm.nih.gov/pubmed/10888876
LEE_AGING_CEREBELLUM_UP		https://www.ncbi.nlm.nih.gov/pubmed/10888876
Reiter E_GSE8150_NEOCORTEX_AGING UP	GSE8150	https://www.ncbi.nlm.nih.gov/pubmed/17316780
GSE60652_IMR90_RAS_SENESCENCE_RB-KNOCKDOWN_DOWN	GSE60652	https://www.ncbi.nlm.nih.gov/pubmed/26009982
GSE28464_IMR90CELLS_RAS_SENESCENCE_UP	GSE28464	https://www.ncbi.nlm.nih.gov/pubmed/21512002
GSE11882_aging	GSE11882	https://www.ncbi.nlm.nih.gov/pubmed/18832152
GSE11697_HippoCA1_Aging	GSE11697	https://www.ncbi.nlm.nih.gov/pubmed/20427664
GSE17757_SFG_Aging	GSE17757	https://www.ncbi.nlm.nih.gov/pubmed/20647238

Table 2:

Representative IPA upstream regulator's z-score and p-value for aging up-regulated genes and CGC Glutamate-up/Candesartan-down regulated genes.

The table includes the z-scores and p-values for GSE11882_Aging_UP and for CGC_Glutamate-UP_Candesartan-Down.

IPA UP-stream regulators	z-score for GSE11882_ Aging_UP	p-value for GSE11882_Aging_ UP	z-score for CGC_ glutamate-UP_ Candesartan-Down	p-value for CGC_glutamate-UP_Candesartan-Down
resveratrol	−1.618	1.49E-06	2.411	2.97E-20
TNF	7.042	3.26E-49	−9.366	7.93E-71
IL6	5.686	2.02E-22	−6.103	3.12E-36
Insulin	1.879	4.78E-07	−1.198	7.21E-08
SIRT1	−2.697	5.43E-06	2.004	1.89E-09
FOXO1	3.896	1.08E-07	−4.695	2.89E-17
FOXO3	2.856	1.65E-08	−4.011	5.92E-19
TP53	4.253	5.97E-24	−4.793	4.07E-26
NFkB (complex)	6.52	2.72E-14	−7.381	8.12E-47
IGF1	4.264	8.11E-10	−5.135	4.00E-28
TGFB1	7.112	7.65E-29	−8.078	1.70E-60
IL1B	6.36	2.58E-31	−8.866	1.32E-53
PI3K (complex)	3.979	1.49E-06	−4.606	8.16E-18
P38 MAPK	3.876	2.19E-12	−5.777	6.24E-31
SMAD3	3.883	6.84E-08	−4.574	1.59E-15
SMAD4	2.318	4.54E-09	−2.852	3.20E-18
Mek	−0.156	8.47E-05	−3.35	6.10E-23
HRAS	1.824	4.56E-24	−2.673	8.01E-33
ERK1/2	3.268	1.89E-08	−4.736	3.74E-23
PTEN	−1.579	6.83E-13	2.116	7.38E-16
TSC2	−4.303	4.49E-13	3.949	3.35E-16
MAPK14	3.33	2.76E-06	−4.292	1.40E-20
JUN	4.161	2.16E-11	−3.877	2.14E-35
FOS	1.686	8.25E-09	−3.339	2.46E-28

Table 3.

p53-associated genes and dataset enrichment correlation.

The table includes genes associated to the p53 pathway that are enriched in analyzed datasets, including gene symbols, corresponding encoding protein and dataset enrichment correlation

Gene symbol	Encoded protein	Dataset enrichment correlation
TP53	tumor protein p53	FRIDMAN_SENESCENCE_UP GO_AGING GO_CELL_AGING GO_CELLULAR_SENESCENCE
CDKN1A	cyclin dependent kinase inhibitor 1A	FRIDMAN_SENESCENCE_UP GO_AGING GO_CELL_AGING GO_CELLULAR_SENESCENCE GSE28464_IMR90CELLS_RAS_SENESCENCE_UP KAYO_AGING_MUSCLE_UP TANG_SENESCENCE_TP53_TARGETS_UP
CDKN2A	cyclin dependent kinase inhibitor 2A	FRIDMAN_SENESCENCE_UP GO_AGING GO_CELL_AGING GO_CELLULAR_SENESCENCE GSE28464_IMR90CELLS_RAS_SENESCENCE_UP
CASP9	caspase 9	GO_AGING GSE40349_IMR90_RAS_SENECENCE_UP Reiter E_GSE8150_NEOCORTEX_AGING UP
CHEK1	checkpoint kinase 1	GO_AGING GO_CELL_AGING KYNG_WERNER_SYNDROM_AND_NORMAL_AGING_UP
CCND2	cyclin D2	GSE40349_IMR90_RAS_SENECENCE_UP RODWELL_AGING_KIDNEY_UP TANG_SENESCENCE_TP53_TARGETS_UP
SERPINE1	serpin family E member 1	FRIDMAN_SENESCENCE_UP GO_AGING GO_CELL_AGING KAYO_AGING_MUSCLE_UP
CDK1	cyclin dependent kinase 1	GO_AGING GO_CELL_AGING KYNG_WERNER_SYNDROM_AND_NORMAL_AGING_UP
ATM	ATM serine/threonine kinase	GO_AGING GO_CELL_AGING GSE28464_IMR90CELLS_RAS_SENESCENCE_UP