Lifestyle interventions modulate cellular senescence markers in the context of Alzheimer's Disease

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M.Sc. Biomedical Sciences | **Biology of Aging** | **Rijksuniversiteit Groningen**

RESEARCH INTERNSHIP II

January 2023 – July 2023

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IDIBAPS

Funding: MCIN/AEI/10.13039/501100011033 (#PID2019-106285RB-C22)

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Abstract

The irreversible block of the cell cycle, termed cellular senescence, has recently gained interest in the aging field. Cellular senescence has been found to contribute to Alzheimer's Disease (AD) pathophysiology and the resulting loss of cognition that patients go through. Considering that lifestyle interventions such as exercise, diet or anti-inflammatory supplementation have been linked with improved symptomatology in AD patients, we assessed how senescence markers behaved under these regimens. Analysis of whole blood RNA from patients suffering from AD demonstrated a sex-specific difference in the senescence marker $p16^{INK4A}$. In the same line, 5XFAD male mice, which harbor strong AD pathological signs, also displayed more $p16^{INK4A}$ transcription and protein content. Despite a marked pathological and cognitive improvement following exercise, senescence markers remained unchanged in this AD mouse model. High-fat diet (HFD), which is described to be pro-inflammatory, increased senescence in the 5XFAD background, compared to WT mice. Complementary, anti-inflammatory perinatal treatment was also able to decrease senescence in 2–month–old mice, highlighting how inflammation and senescence are intertwined. In the present work, we propose an inflammation–based model, in which lifestyle interventions are able to decrease senescent cells by indirectly mediating senomorphic capabilities. Whether these observations will prove to be critical in the clinical practice is yet unknown, but understanding cellular senescence in the context of neuroinflammation will not only benefit AD but also age-related diseases collectively.

Introduction

Alzheimer's Disease, Aging and Cellular Senescence

Patients suffering from dementia are expected to increase from 57,4 million cases to 152,8 million by 2050, an effect largely attributed to population aging (Hou et al., 2019; The Lancet, 2022). As the most frequent cause of dementia, Alzheimer's Disease (AD) is an age-related disease defined as a synaptic dysfunction disorder characterized by progressive neurodegeneration (Knopman et al., 2021). Common early symptoms of AD include initial loss of episodic memory and reasoning (amnestic cognitive impairment), loss of capacity to make decisions, time and space disorientation and subtle language alterations, among others (Brier et al., 2016). Although anti-amyloid immunotherapies have recently gained lots of media coverage, as in the case of donanemab and lecanemab, the unavailability of an advanced-stage disease-modifying treatment highlights how pivotal it is to find preventive and curative interventions (Planche & Villain, 2023; Rafii & Aisen, 2023).

Familial AD is characterized by a progressive accumulation of extracellular β-amyloid (Aβ) plaques induced by mutations in the APP, PSEN1 or PSEN2 genes. Amyloid pathology elicits tau pathology, with consequent accumulation of intracellular neurofibrillary tangles of hyperphosphorylated tau (*p*-tau); ultimately promoting neurite degeneration, microglia activation and lysosomal dysfunction (Knopman et al., 2021). Nonetheless, since the majority of cases are sporadic, and the heterogeneity of the disease cannot be recapitulated solely through the inherent aggregation of Aβ and *p*-tau, contrary views are challenging the amyloid cascade hypothesis (Herrup, 2015; Huang et al., 2019). In this line, Geroscience, the study of the molecular mechanisms behind aging, poses that non-familial AD stems from the manifestation of a plethora of detrimental processes commonly seen in age-related diseases (Khosla et al., 2020).

These damaging features, termed The Hallmarks of Aging, explain the loss of physiological health typically seen with age, including from drivers of damage to (ultimately decompensated) compensatory mechanisms (López-Otín et al., 2013; López-Otín et al., 2023). Indeed, AD shares most (if not all) of these Hallmarks, and new lines of thought are starting to suggest that AD might just be an inherent manifestation of the brain aging process itself, albeit not necessarily developed by all elders (Ferrer, 2022; Kosyreva et al., 2022; Kumar, 2023; Susmitha & Zhao & Huai, 2023).

Of these Hallmarks, arguably the most promising one to target in the context of AD, is cellular senescence (Saez-Atienzar & Masliah, 2020). Cellular senescence refers to a state of proliferative arrest that some cells generally undergo in order to avoid pre-cancerous cells from dividing, ultimately avoiding further damage (Campisi & d'Adda di Fagagna, 2007; Serrano et al., 1997). Complementary, cellular senescence has also been seen to participate in functions as diverse as wound healing, fibrotic resolution, limb pattering or even reprogramming (Calcinotto et al., 2019).

Despite senescent cells' inability to enter the cell cycle, they remain metabolically active. Among their main cellular features, senescent cells present an increased lysosomal content and activity, a characteristic that aligns with the high amounts of genomic and proteomic stress they harbor (He & Sharpless, 2017; Huang et al., 2022). Dimri and others identified the senescence-associated β-galactosidase (SA-βgal) lysosomal enzymatic activity as a marker that is nowadays widely exploited for staining senescent cells by supplying them with with the chromogenic substrate β-D-galactopyranoside (X-gal) (Dimri et al., 1995). Other markers of senescence label different features of this cellular state. This is the case for the increased $p16^{INK4A}$ or $p21^{WAF1/Cip1}$, loss of lamin B1, decreased 5-EdU staining and the presence of telomere-associated damaged foci (TAFs) and senescence-associated heterochromatic foci (SAHFs) (Gorgoulis et al., 2019; Hewitt et al., 2012; Serrano et al., 1997; reviewed in: González-Gualda et al., 2021). In spite of the availability of a wide number

of markers, currently there is not any specific or sensitive enough to detect senescence. Due to this, a multi-faceted assessment is required to fully characterize the presence of senescent cells (González-Gualda et al., 2021, Wang et al., 2022).

On top of these markers, senescent cells also display a prominent secretome, the senescence-associated secretory phenotype (SASP), which aids in their immune surveillance and consequent elimination (Kang et al., 2011; Sturmlechner et al., 2021; Xue et al., 2007). As organisms grow older, their surveillance goes down, and senescent cells begin to accumulate in virtually all tissues, manifesting an increasingly damaging SASP that promotes tissue dysfunction and, ultimately, aging (Huang et al., 2022; Tuttle et al., 2020). These detrimental effects extend further than in the environment where senescent cells are located, as the SASP can not only act in an autocrine fashion but also in a paracrine and even endocrine manners (Coppé et al., 2008). In this sense, evolution has prioritized their quickly-arising beneficial effects of cancer suppression with the trade-off of aging promotion: senescent cell generation is crucial, but their maintained presence in tissues is detrimental (de Keizer et al., 2017).

Because of this, accumulating evidence is placing cellular senescence at the center of many peripheral age-related pathologies such as cardiovascular diseases, type II diabetes, fibrosis or physical dysfunction (Lewis-McDougall et al., 2019; Palmer et al., 2019; Roos et al., 2016; Schafer et al., 2017; Xu et al., 2018). It has also been described that senescent cell burden in the brain affects several cell types such as neurons, the microglia, astrocytes and oligodendrocyte progenitor cells, among others (Baker & Petersen, 2018). Due to this, progressive neurological diseases have also seen a great surge in interest in the field, as is the case for AD. Bussian and colleagues first described a link between cellular senescence and neurodegeneration in a tau-dependent mouse model, which they crossed with the INK-ATTAC mouse (Bussian et al., 2018). The INK-ATTAC mouse contains a suicide transgene that allows the targeted elimination of $p16^{INK4A}$-positive cells when given a transgene-activating drug (Baker et al., 2011). In this scenario, genetically eliminating senescent cells dramatically reduced insoluble tau aggregates and improved cognition significantly (Bussian et al., 2018). Furthermore, data from Zhang et al., unraveled that Aβ induced senescence markers in cells that were associated to plaques (Zhang et al., 2019). Pharmacologically treating mice with senolytics, substances that eliminate senescent cells in a hit-and-run fashion, decreased Aβ plaques in the hippocampus and rendered mice with better memory acquisition and retention (Zhang et al., 2019; Zhu et al., 2015). Another study unraveled that senolytic treatment was effective even in a 20-month-old tau-dependent model at reducing neurons with neurofibrillary tangles, reducing ventricular volume and improving blood flow in the brain (Musi et al., 2018). All in all, pharmacologically eliminating senescent cells seems a promising approach for the treatment of AD, and treatment is effective when performed intermittently as senescent cells take long to arise (Khosla et al., 2020). Because of this, clinical trials are on their way to test the feasibility and safety of senolytic substances in human patients (NCT04063124). Indeed, first preliminary reports of senolytic treatments in humans showed that short-term senolytic treatment did not cause any serious adverse effects; and that markers of senescence significantly decreased in adipose tissue and skin, altogether with reduced specific SASP factors and macrophage counts (Hickson et al., 2019; Justice et al., 2019).

Lifestyle factors modulating cellular senescence and Alzheimer's Disease

Exercise

As senolytics make their way to clinical trials, finding lifestyle choices that promote healthy aging by targeting Hallmarks of Aging is important. For example, in the case of type II diabetes, an age-related disease, proper

diet management and exercise have been proven to be key for disease remission (Magkos et al., 2020). Complementary, for cognitive impairment prevention, multidomain interventions including not only dietary counselling and exercise but also cognitive training have seen to work in randomized control trials (reviewed in: Kivipelto et al., 2018). In fact, the beneficial effects of exercise in the context of AD are known for more than a decade. A systematic review from 2014 determined that exercise had a positive effect at slowing down cognitive decline (Farina et al., 2014). Following systematic reviews concluded that most randomized controlled trials tended to report positive effects more frequently, and called for larger and better-standardized trials (Cammisuli et al., 2018). More recent systematic reviews aimed at assessing which type of physical exercise was the most effective for AD. The results concluded that aerobic, multimodal training of mid to high intensity and resistance training were beneficial for AD patients (Ayari et al., 2023). These observations hint that lifestyle interventions might be therapeutically relevant through the targeting of several Hallmarks of Aging at once.

This is indeed the case for physical exercise, which is proven to impact Hallmarks such as inflammation, the microbiota, nutrient sensing pathways, epigenetic factors and others (Gubert & Hannan, 2021). Importantly, exercise is also tightly linked to cellular senescence. Xu and colleagues unraveled that transplantation of senescent cells is detrimental for physical function, in a dose-dependent fashion with the number of senescent cells transplanted. Short-term senolytic treatment in old transplanted mice also resulted in reduced physical dysfunction (Xu et al., 2018), and treating irradiated mice with senolytics improved their exercise impairment (Zhu et al., 2015). Moreover, physical exercise has been described to harbor senolytic activity in both mice and humans in a systematic review and metanalysis (Chen et al., 2021). In a preliminary single-arm open-label trial, an acute dose of senolytics promoted improved physical function in patients with idiopathic pulmonary fibrosis (Justice et al., 2019). Quercetin, a senolytic flavonoid, has also been described to reduce neuronal lesions of AD brains such as *p*-tau, Aβ and inflammation (reviewed in: Calderaro et al., 2022).

These results indicate that senescent cells are detrimental for physical health and that exercise is effective at preventing physical dysfunction through their seemingly targeted elimination. Due to this, it is reasonable to think that the benefits of exercise in the context of AD are in part mediated through the ability of exercise to ablate senescent cells.

Diet

Aside from exercise, an appropriate nutritional intervention can also be decisive in delaying or improving AD-derived neurodegeneration. Initial implications on the association between different diets and the risk of developing AD are now solidified (Yusufov et al., 2016). A recent systematic review published that several diets have been proven to be protective for the risk of developing mild-to-moderate AD, such as the Mediterranean or ketogenic diets (Lou et al., 2023). Supplementation has also seen a great surge in interest in the field, as several nutrients display neuroprotective effects on cognitive function (reviewed in Stefaniak et al., 2022).

On the other hand, other diets and nutritional interventions have been demonstrated to worsen AD pathology, such as the western diet and high-fat diets (HFD) (Amelianchik et al., 2022). For example, data from our group and others indicates that the 5XFAD mouse model of accelerated familial AD, increases its Aβ plaque deposition and reduces their cognitive functions after being HFD-fed (Lin et al., 2016; Reilly et al., 2020; Sarroca et al., 2021). Nonetheless, detrimental effects of a HFD have also been seen to be independent of Aβ or tau in other mouse models, like the 3xTgAD mouse (Knight et al., 2014). All in all, HFD not only worsens AD pathology and cognitive impairment but also affects Aβ load and inflammation in different mouse models (Gannon et al., 2022).

Interestingly, diets are also considered pivotal when aiming at maintaining optimal states of the Hallmarks of Aging, as is the case for gut dysbiosis and the Mediterranean diet (Gundogdu & Nalbantoglu, 2023). Other Hallmarks, like cellular senescence, have also been seen to worsen after HFD treatment. Vascular senescence and dysfunction were first identified to worsen after HFD in an Akt-mediated fashion (Wang et al., 2009). Moreover, HFD increases SA-βgal- and $p16^{INK4A}$-positive cells, as well as SASP factors. In fact, higher concentration of some HFD-induced SASP cytokines correlated with higher anxiety markers (Hou et al., 2022; Ogrodnik et al., 2019). Taken together, these results suggest that those at risk of AD can benefit from a proper nutritional plan; and that cellular senescence load, an important Hallmark of Aging, is also subject to the composition of the diet.

Anti-inflammatory drug treatment and prenatal exposure

Aside from exercise and diet, supplementation with anti-inflammatory drugs constitutes a powerful strategy to fight off the deleterious and inflammatory effects of the SASP. Senomorphic drugs, in comparison to senolytics, do not eliminate senescent cells but they dampen the detrimental paracrine and endocrine consequences of the SASP (Huang et al., 2022; Schmitt et al., 2022). Though a plethora of substances are now being studied for their senomorphic activities, several natural compounds, like resveratrol (RV), have also been described to harbor anti-SASP activity (Liu et al., 2018; Menicacci et al., 2017). Interestingly, RV harbors a biphasic dose response, as it promotes senescence in cancer contexts in high concentrations (from 25 to 50 μM) but rescues senescence in concentrations as low as 10 μM (Harris & Korolchuk, 2023; Mamun et al., 2022). Nonetheless*, in vitro* and *in vivo* evidence of anti-senescence activity has been described (Han & Kim, 2023). Indeed, RV is an anti-oxidant and anti-inflammatory compound that is accepted to harbor anti-aging benefits (Cosín-Tomàs et al., 2019; Li et al., 2018; Pyo et al., 2020).

Deciding when to start supplementation is pivotal. In this regard, the prenatal window constitutes a highly sensitive moment that can influence a plethora of age-related diseases including neurological conditions (Short & Baram, 2019). For example, prenatal malnutrition, as described during the Dutch famine during World War II, resulted in older-appearing brain MRI scans (Franke et al., 2018). In a recent systematic review, the authors described that both living in a non-ideal environment and experiencing prenatal famine exposure are associated to higher risk of developing dementia (Wiegersma et al., 2023). Complementary, telomere homeostasis during the intrauterine period of development has also been described to be crucial for processes including aging and cellular senescence (Entringer et al., 2012; Lin & Epel, 2022). All in all, the intrauterine period constitutes a remarkably sensitive window that can affect considerably the health outcome of the newborn.

Though unknown to harbor senolytic or senomorphic activity (Zhang et al., 2021), the anti-inflammatory compound TPPU (N-[1-(1-oxopropyl)-4-piperidinyl]-N'-[4-(trifluoromethoxy)phenyl] urea), that acts by inhibiting the soluble hydrolase enzyme (sEH), was seen to be neuroprotective when administered prenatally in the 5XFAD mouse model of AD (Bartra et al., 2022). In effect, TPPU and other sEH inhibitors have been seen to aid in several age-related diseases such as diabetes or hypertension and constitutes a potential new therapy for AD (Griñán-Ferré et al., 2020; Matin et al., 2021; Minaz et al., 2018).

All things considered, anti-inflammatory molecules such as RV or TPPU are great candidates to fight off the deleterious effects of the SASP, and their prenatal administration appears as an appealing strategy to prevent age-related diseases such as AD.

Rationale

Considering the importance of cellular senescence in the pathogenesis of AD, and taking into account that AD has been proven to benefit from several lifestyle interventions, we have hereby assessed cellular senescence markers in three experimental designs in both the 5XFAD mouse model and cell culture. Complementary, analysis of human blood samples of AD patients was performed. By assessing the role of exercise, HFD and RV and TPPU treatments in modulating cellular senescence, we have validated that senescence is more prevalent in the 5XFAD mouse model. Damaging lifestyle interventions inflict a greater senescence signature in 5XFAD mice; and prenatal anti-inflammatory treatment appears to decrease senescence load even in newborns. All in all, fully understanding how cellular senescence is modulated in different lifestyle interventions can shed new light in the prevention and management of AD.

Materials and Methods

Experimental cell culture, freezing and harvesting

All cell lines were maintained at 37 °C in a humidified incubator with 5% CO_2 (Water-Jacketed, US Autoflow Automatic CO_2 Incubator; NuAire, Inc.) throughout all procedures. Adherent cells were cultured according to specific protocols. Cells were seeded and grown until reaching not more than 80% confluence. For subculture, cells were incubated with a Trypsin-EDTA solution and observed under an Olympus CK40 inverted microscope until the cell layer was detached. Cells were counted manually with a Neubauer chamber and seeded with fresh media. For long-term storage, cell lines were resuspended in heat-inactivated fetal bovine serum (FBS) and 10% DMSO was slowly added to the cryovials and gently mixed. Vials were frozen at a slow pace to -80ºC. After 24 h, cells were stored in a liquid nitrogen tank.

HMC3 cells (CRL-3304™, © ATCC) were cultured in T25 flasks (Nunc™, Thermo Fisher Scientific) in Eagle's Minimum Essential Medium 30-2003™ (© ATCC) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) and gentamycin 50 µg/mL (Gibco BRL, #15750-037). For experimental procedures, cells were seeded, in complete medium, at a $0.4 \cdot 10^5$ cells/mL concentration in 96- or 24-well plates (Nunc™, Thermo-Fisher Scientific).

SH-SY5Y cells (CRL-2266™, © ATCC) were cultured in T75 flasks (Nunc™, ThermoFisher Scientific) in MEM/HAM's-F12 (1:1, v/v) medium, supplemented with non-essential amino acids, L-glutamine 1 mM, gentamycin 50 µg/ml and 10% heat inactivated FBS. For experiments, cells were seeded, in complete medium, at $1 \cdot 10^5$ cells/mL in 96- or 24-well plates (Nunc™, ThermoFisher Scientific).

SH-SY5Y differentiated cells, cells were seeded, in complete medium, at $5 \cdot 10^4$ cells/ml in 96- or 24-well plates (Nunc™, ThermoFisher Scientific). After 24 h, media was replaced for fresh medium containing MEM/HAM's-F12 (1:1, v/v) medium, supplemented with non-essential amino acids, L-glutamine 1 mM, gentamycin 50 µg/ml, retinoic acid (RA) 10 µM and B27 1X. After 72h, media was replaced with MEM/HAM's-F12 (1:1, v/v) medium, supplemented with non-essential amino acids, L-glutamine 1 mM, gentamycin 50 µg/ml, RA 10 µM, B27 1X and BDNF 50 ng/mL.

The Mycoplasma *Gel Detection* kit Biotools S.A. (ref.: 90.021) was used to test if cell cultures were mycoplasma-free, according to the manufacturer's protocol.

Palmitic acid, resveratrol and TPPU treatments

HMC3 and SH-SY5Y were treated after 24 h, except differentiated SH-SY5Y cells. Cell culture media were replaced for the corresponding fresh complete media, with either TPPU or RV at 10 or 50 µM, and 10 or 25 µM, respectively. After 1 h, PA 0.2 or 0.4 mM, was added to the chosen wells. Cells were left to incubate overnight. For PA preparation, PA was conjugated with BSA following both Listenberger et al. (2021) and Phitthayaphong et al. (2021) method description. In summary, 10.26 mg of PA (Sigma-Aldrich, Co.) were weighted and dissolved in 1 mL of NaOH 0.01 M. Having obtained a 40 mM stock, it was heated for 30 min at 80ºC. For each cell line, 1 % free fatty acid-free BSA of the corresponding media was prepared, filtered through a 0.2 µm filter, and afterwards heated in a bath at 37ºC. Finally, 40 mM PA was dissolved in the prepared media to obtain a 0.4 mM stock solution. All steps were also performed with NaOH 0.01 M to use as control.

Western Blotting

Tissue lysis (cortex and hippocampus) was performed with RIPA (radioimmunoprecipitation assay) buffer in the presence of phosphatase and protease inhibitors. Pestle homogenization and sonication of samples were

carried out in aims of disrupting cell membranes. Consequently, Bradford Assays were conducted to achieve equal loading and comparable protein samples.

Gels were mounted in Bio-Rad®'s casket system, ranging from 12 to 15% acrylamide concentrations. The prepared samples and ladder (ref.: #161-0374) were loaded and run at 100V until clear band separation. Protein transfer was carried out onto methanol-activated PVDF 0,45 µm membranes. The membranes were washed in 1X Tris-Buffered Saline 0.5% Tween® 20 Detergent (TBST) and blocked in 5% milk prior to overnight primary antibody incubation at 4ºC (Suppl. Table 1). The following day, the membranes were incubated with the corresponding Amersham™ ECL™ anti-rabbit or anti-mouse IgG antibody (1:2000), in between consequent TBST washes.

In order to reveal the membranes, incubation with a solution containing luminol salt sodium (ref.: A4685, Sigma®) and p-coumaric acid (ref.: C9008, Sigma®) was done and revealed in Bio-Rad®'s ChemiDoc™ MP. Membrane exposure was set automatic to avoid unwanted oversaturation. Protein stripping was carried out when necessary, with 0,2 M NaOH for 5 to 10 min. Image Lab™ 6.0.0 was employed for band analysis and quantification.

RT-qPCR

In order to extract RNA from tissue samples (cortices and hippocampi), we employed the *mir*VANA™ miRNA isolation kit (ref.: AM1561), which separates RNA with superior purity for smaller fragments. Procedure was performed according to protocol, consisting in a mini-column system. RNase-free conditions were undertaken at all times when working with RNA, and the resulting samples were stored at -80ºC for long-term storage.

cDNA synthesis was performed from RNA samples (500 ng input amount) with Applied Biosystems™' high-capacity cDNA reverse transcription kit (ref.: 4368814), according to the manufacturer protocol. The resulting cDNA samples were then diluted 1:4 prior to loading.

Hard-shell® white 96-well PCR plates were used (ref.: #HSP9655) to load 1:10 dilutions of samples (in duplicates) in an aqueous solution consisting of 1:2 Quantimix Easy Probes (ref.: 10.601-4149) and the corresponding 1:20 Life Technologies® TaqMan probe (Suppl. Table 2). The Bio-Rad®'s C1000™ Thermal Cycler CFX96™ Real-Time System Data was used, and the resulting data was analyzed in Bio-Rad®'s CFX Manager™ 3.1 software and Microsoft Excel.

β–galactosidase staining

Cell Signaling Technology® Senescence β-galactosidase staining kit (ref: #9860) was employed. Prior to staining, cells were washed once with PBS and fixed with 1x Fixative Solution for 10-15 min. Staining solution was prepared fresh, with a final X-gal concentration of 1 mg/mL and a final pH ranging from 5.9 to 6.1. The staining solution was added to fixed cells after two PBS washes. Incubation for 24h at 37ºC in the absence of CO_2 was performed in aims of avoiding the change in pH. Immediately before microscopy assessment, cells were supplemented with 5 µM bisbenzimide (Hoechst 33342) to allow nuclei staining. The Inverted Olympus IX-70 fluorescence phase contrast microscope was employed for image acquisition.

5-EdU

To measure DNA synthesis, incorporation of 5-Ethynyl-2'-deoxyuridine (5-EdU, ref: CLK-N001, Jena Bioscience®) was assessed. Following the desired cell line treatments, cell lines were supplemented with 5 µM 5-EdU for 3h. After 5-EdU incorporation, the cells were fixed with 3,7% paraformaldehyde for 15 min, washed two times with 3% BSA and permeabilized with 0,5% Triton X-100 for 20 min. During this step, a freshly-made click-chemistry reaction mix was prepared, consisting of 4 mM $CuSO_4$, 0,06 mM sulfo-Cy3-Azide (ref: CLK-

AZ119, Jena Bioscience®) and 2 mg/mL ascorbic acid; diluted in 50 mM tris (pH: 8). After permeabilization, two more washes with 3% BSA were performed prior to supplementation of the click reaction cocktail to the cells for 30 min. Afterwards, the click solution was removed and cells washed with 3% BSA. Finally, the cells were incubated with 5 µM bisbenzimide (Hoechst 33342) in PBS for 10 min, and fluorescence imaging took place consequently in an Inverted Olympus IX-70 fluorescence phase contrast microscope.

Immunofluorescence

Optimal frozen brain sections were selected and washed three times in PBS, after which three more washings in TBS were performed. Blocking of unspecific unions was carried out with 10% goat serum in 0,2% triton X-100 in TBS for at least 1 h. Following, overnight incubation with the selected primary antibodies (varying concentrations, see Suppl. Table 1) was done in 3% goat serum in 0,2% triton X-100 in TBS. Primary antibodies were then washed three times with 0,2% triton X-100 in TBS prior to secondary antibody incubation (raised in goat). Afterwards, secondary antibodies were washed three times in 0,2% triton X-100 in TBS and consequently mounted in gelatinated slides with Fluoroshield™ with DAPI (ref: F6057, Sigma-Aldrich®). Mounted slides were kept at -20º C before capturing pictures of the brain sections. Imaging was performed with either the Eclipse E-100 Nikon fluorescence motorized microscope or the Inverted Olympus IX-70 fluorescence phase contrast microscope.

Human blood samples

Human samples of whole blood RNA were provided by Dr. Anna Antonell from the Hospital Clínic de Barcelona, as a collaborative research project, and following all the required ethical procedures. A total of 35 samples, with 17 controls (12 females, 5 males) and 18 AD patients (10 females, 8 males) were analyzed to assess senescent cell markers. Median age of all control patients was 55,4 years, while for AD patients it was 55,6 years (Student's *t*-test = 0,7506).

Mouse models and experimental designs

Mouse samples employed in the current Research Project were obtained from three prior experimental projects from Dr. Sanfeliu's lab. 5XFAD mice that went through voluntary exercise treatment were caged with or without a running wheel that they could use at free will. WT controls were included for reference but no experimental group was considered in this genetic background. Mice were sacrificed from 7.5 to 8 months of age for experimental testing (unpublished data). For the HFD section, male WT and 5XFAD mice were fed during a 16-week period from 2 to 6 months of age with either a control diet, a diet high in animal saturated fats (60% of the calories from fat) or the same high fat diet supplemented with 0.1% trans-RV (published in: Sarroca et al., 2021). For the TPPU-prenatal treatment, 2-month-old 5XFAD heterozygous or WT pups were employed. These were bred from WT females crossed with 5XFAD mice, and the mothers received 5mg/kg TPPU or vehicle during pregnancy (published in: Bartra et al., 2022). Further and more complete information such as mouse strains, commercial references or precise experimental design layouts can be retrieved from the original publications.

Data analysis

Statistical analysis of qPCR, WB and 5-EdU results were processed in GraphPad Prism 6.01. Gaussian distribution was ensured in all datasets with D'Agostino-Pearson and Shapiro-Wilk normality tests before statistical analysis; and sex differences, when needed, were checked through two-way ANOVA testing. In the absence of significant sex factor, data of male and female samples were pooled together for the final analysis and graph representation. Most of the analyses were performed with two- or one-way ANOVA, or Student's

t-tests. Significance of statistical tests is indicated in the upper left corner of each graph, and when significant, post-hoc analyses were carried out with Tukey's testing, allowing multiple comparisons between groups. Post-hoc significance is labelled with one or more asterisks across groups. Significance was indicated: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Outliers were taken out through Grubbs' test (alpha = 0.05).

Results

Whole Blood RNA analysis of human samples reveals sex differences

The assessment of blood markers in the context of AD is relevant because it can not only reflect the pathogenic degree but it may also be used for early-detection (Hansson et al., 2023). A metanalysis of omics data from brain tissues and blood from AD and non-AD patients, revealed cellular senescence as the second most enriched pathway in AD (Shokhirev & Johnson, 2022). Due to this, we reasoned that it was important to assess the transcription of *CDKN2A*, *CDKN1A* and *TP53*; which are the transcripts for $p16^{INK4A}$, $p21^{WAF1/CIP1}$ and p53 respectively. To do this, we analyzed the RNA from whole blood of control and AD. Interestingly, male and female participants were considered distinct groups when looking at their *CDKN2A* levels (two-way ANOVA, p = 0,0280), and were separated only for the analysis of this transcript.

Male participants with AD displayed statistically higher levels of *CDKN2A* than female AD patients (**Fig. 1a**). While *CDKN1A* displayed no differences between controls and AD patients, its activator and effector *TP53* surprisingly was decreased in AD (**Figs. 1b–c**). As p53 is a master antiapoptotic protein in which a multitude of signals converge, we decided to elucidate if such decrease could be consistent with a change in the double-strand break pathway. In it, while the ATM protein is activated by the γH2AX histone, which is found in sites of double strand breaks, ATR mediates replicative stress (Merighi et al., 2021). Unexpectedly, ATM levels were significantly reduced (**Fig. 1d**), yet ATR levels remained unchanged (**Suppl. Fig. 1**).

These results indicate that transcription of senescence-associated genes is not clear in the nucleated blood cells in this 55-years-old cohort. Important sex differences hint that senescence pathology might differ between male and female AD patients.

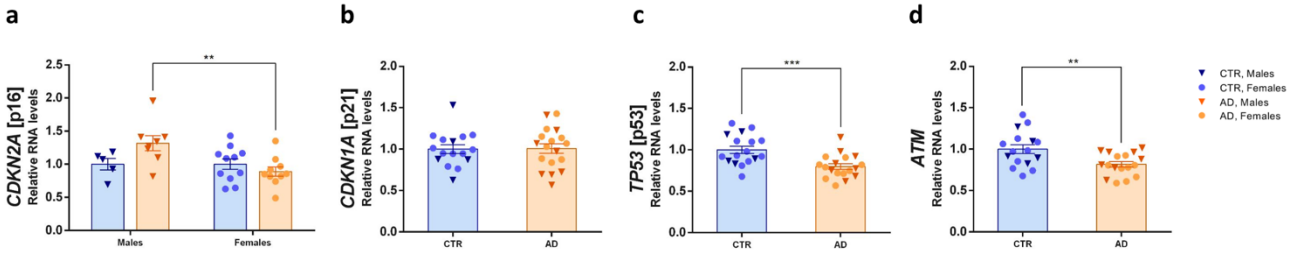

Figure 1. **qPCR analysis of RNA from human whole blood from healthy controls and AD patients. a|** *CDKN2A* transcript was analyzed with two-way ANOVA given that the sex factor was statistically significant (p = 0.0280). **b – d|** *CDKN1A*, *TP53* and *ATM* transcription was statistically indistinguishable in male and female participants (two-way ANOVA, $p \geq 0.05$), and results were analyzed with Student's unpaired *t*-tests.

Assessment of senescence and mitochondrial respiration in 5XFAD exercise mice

As aforementioned, exercise has been seen to be beneficial for AD, and at the same time harbor senolytic activity (Ayari et al., 2023, Chen et al., 2021). Because of this, we assessed the transcription of *Cdkn2a*, *Cdkn1a* and *Tp53* in the hippocampus (HC) and cortex (CTX) of 5XFAD sedentary and exercise mice. Mice started voluntary treatment at 2 months of age and were sacrificed at 8 months of age. Analysis of $p16^{INK4A}$ transcription indicated that 5XFAD mice had more *Cdkn2a* levels in the HC and CTX, as consistent with prior evidence (Wei et al., 2016). Complementary, our results show that males had considerably higher levels in the HC and CTX of *Cdkn2a* than females, but exercise did not protect them (**Figs. 2a, 2e**). On the contrary, 5XFAD females exhibited more *Cdkn1a* transcription than males (**Figs. 2b, 2f**), altogether with more *Trp53* in the HC but not in the CTX (**Figs. 2c, 2g**). Nonetheless, exercise did not seem to have a protective effect overall, except

for the non-significant decrease of *Cdkn1a*'s levels in the 5XFAD female cortex (**Fig. 2f**). Once again, we set on to assess if the *Tp53* increase was partly due to double-strand break effect and transcriptional stress. Indeed, regardless of sex, 5XFAD mice displayed higher levels of *Atm* (**Figs. 2d, 2h**). While a non-significant trend of increasing *Tp53*'s levels were seen in the cortex, the *Atm* transcript was significantly more prevalent in 5XFAD mice after exercise treatment (**Figs. 2g, 2h**). Immunoblot analysis of $p16^{INK4A}$ in the HC revealed a significant increase in the 5XFAD exercise group, suggesting that voluntary running might somewhat enfeeble these mice (**Fig. 2i, 2j**).

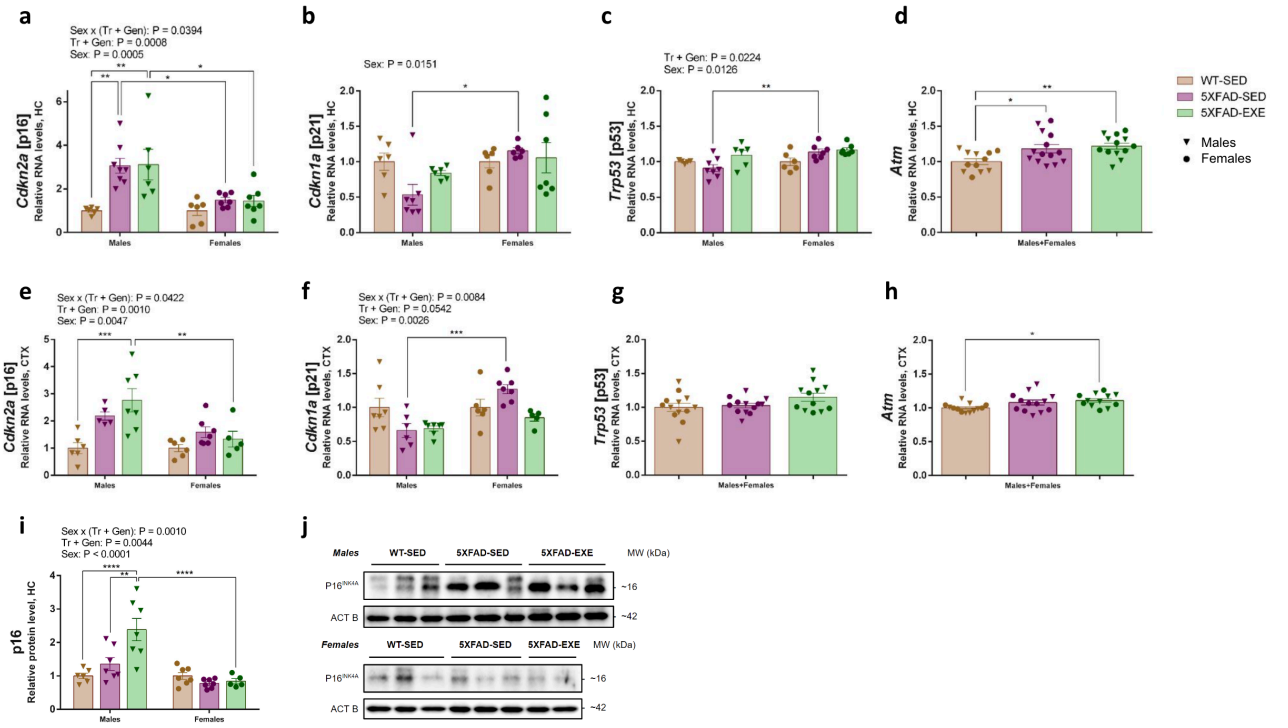

Figure 2. **qPCR and protein immunoblot analysis of WT and 5XFAD mice following a sedentary or exercise intervention. a |** *Cdkn2a* transcription in the HC. **b |** *Cdkn1a* transcription in the HC. **c |** *Trp53* transcription in the HC. **d |** *Atm* transcription in the HC, which was indistinguishable within males and females. **e |** *Cdkn2a* transcription in the CTX. **f |** *Cdkn1a* transcription in the CTX. **g |** *Trp53* transcription in the CTX, which was indistinguishable within males and females. **h |** *Atm* transcription in the CTX, which was indistinguishable within males and females. **i |** $P16^{INK4A}$ protein quantification of Western Blot membranes. **j |** Representative immunoblots of $p16^{INK4A}$ membranes.

Sex differences were analyzed through two-way ANOVAs, and, when significant, groups were separated accordingly, as indicated. ANOVA's source of variation analysis of Group (Tr + Gen) or Interaction (Sex x (Tr + Gen)) is indicated in the upper left corner of each graph, when significant. Post-hoc analysis was performed with Tukey's multiple comparisons test (specified with stars). Groups with indistinguishable differences between sexes were analyzed through one-way ANOVA, followed by post-hoc analysis when significant. Significance was indicated: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Considering the fact that exercise seemed to worsen the senescent signature of male 5XFAD mice, we decided to test their mitochondrial state. Indeed, DNA damage is tightly associated to aging and neurodegenerative disorders (Madabhushi et al., 2014; Schumacher et al., 2021); and under genomic stress, mitochondrial homeostasis suffers as the nucleus–mitochondria crosstalk becomes impaired. Because nuclear DNA encodes for most mitochondrial proteins, cells with abundant genomic stress (i.e., increased double strand breaks, increased ATM signaling) can harbor weakened mitochondria (Haynes & Ron, 2010). Complementary, dysfunctional mitochondria have been observed to induce a cellular senescence state with a specific SASP factor signature (Wiley et al., 2016).

Analysis of the Electron Transport Chain (ETC) of the mitochondria can provide a good image of mitochondrial function and mass (Visavadiya et al., 2021). Experimental data from the lab indicated that 3xTg-AD mice had an impaired ratio of ETC complexes, and that dual treatment of exercise with melatonin supplementation rescued such trend (García-Mesa et al., 2012). To assess these ratios, a representative protein of each complex is normalized with succinate dehydrogenase complex iron sulfur subunit B (SDHB), which is stable in different conditions (**Suppl. Fig. 2a**). Our data, on the contrary, do not show any impaired ratio of ECT complex proteins in male or female 5XFAD mice. However, an increase of complex V in males submitted to exercise suggests a high production of ATP derived from intense exercise engagement (**Figs. 3a-b**). On top of that, a non-significant increase in 5XFAD exercised mice of UQCRC2 (CIII) and a non-significant decrease of NDUFB8 (CI) was observed regardless of sex (**Figs. 3a, 3d–e**). Transcript analysis of Nd1, another protein in CI, indicated a significant decrease in 5XFAD's HC and CTX that was even more prominent in the exercise group regardless of sex (**Suppl. Fig. 3b, d**). On the contrary, CoxI transcript (part of CIV) was significantly increased in the CTX of 5XFAD females (**Suppl. Fig. 3a, c**).

Taken together, these results indicate that exercise alters ETC complex ratios in a targeted manner. While CV had a marked increase in non-sedentary male 5XFAD, exercise caused a mild decrease in CI:CII ratio. Considering that the complexes need to be coupled to work optimally, a CI deficiency might be detrimental.

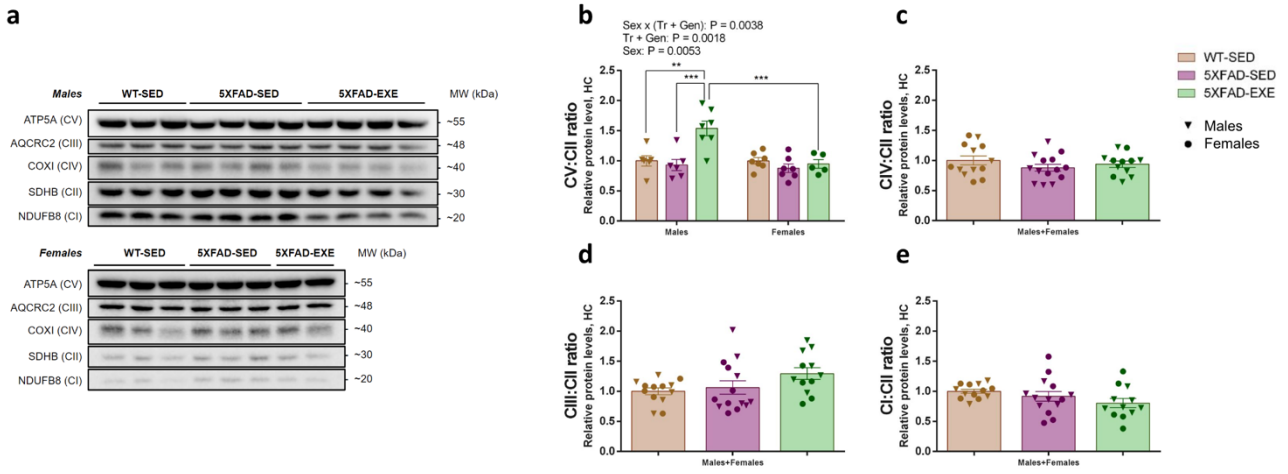

Figure 3. **Electron Transport Chain complexes immunoblot and analysis of WT and 5XFAD mice following a sedentary or exercise intervention. a |** Representative immunoblots of CV to CI membranes. **b |** ATP5A to SDHB (CV:CII) protein ratio. **c |** COXI to SDHB (CIV:CII) protein ratio. **d |** AQCRC2 to SDHB (CIII:CII) protein ratio. **e |** NDUFB8 to SDHB (CI:CII) protein ratio.
Sex differences were analyzed through two-way ANOVAs, and, when significant, groups were separated accordingly, as indicated. ANOVA's source of variation analysis of Group (Tr + Gen) or Interaction (Sex x (Tr + Gen)) is indicated in the upper left corner of each graph, when significant. Post-hoc analysis was performed with Tukey's multiple comparisons test. Groups with indistinguishable differences between sexes were analyzed through one-way ANOVA. Significance was indicated: **, $p < 0.01$; ***, $p < 0.001$.

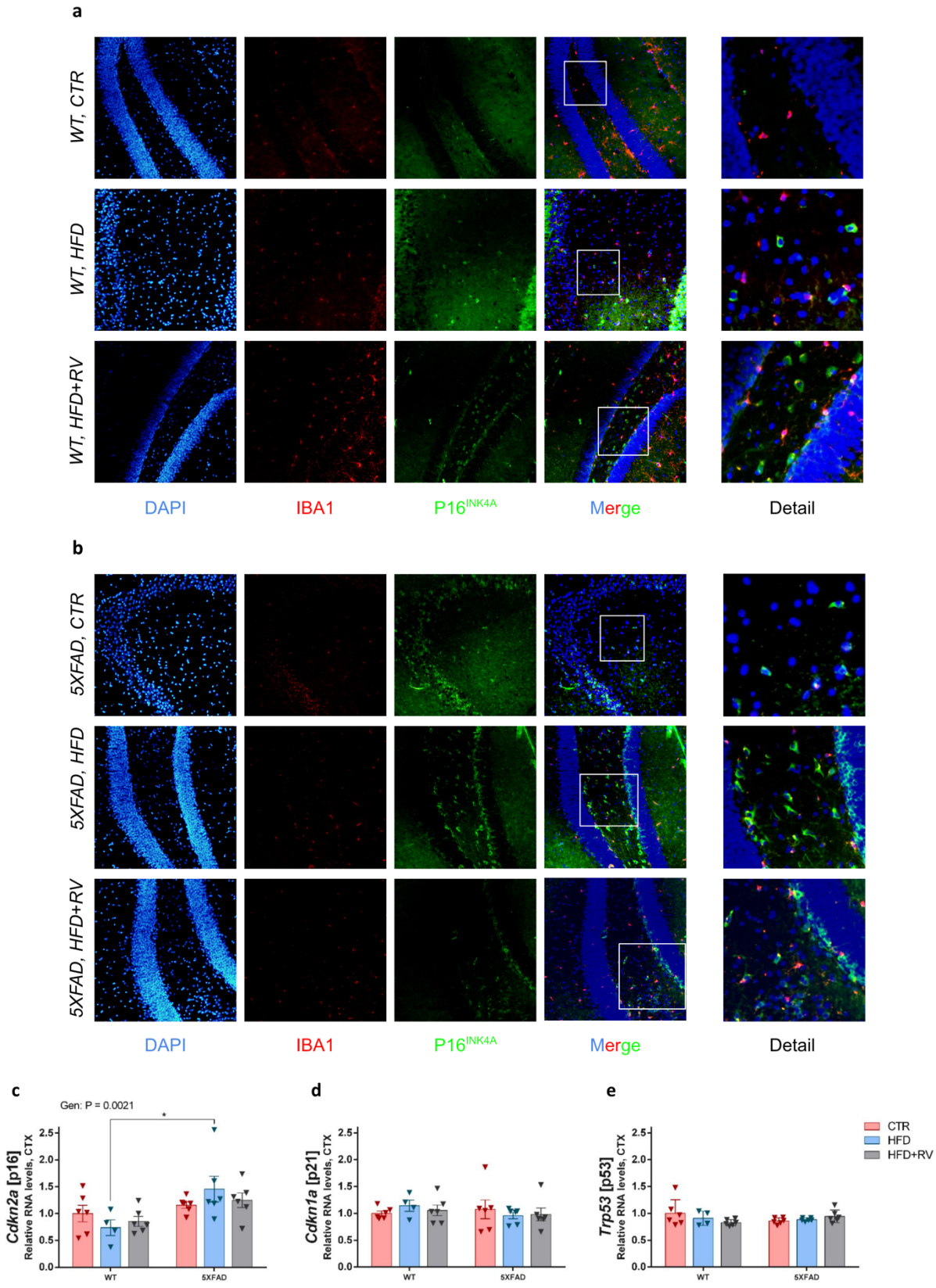

Figure 4. **Immunofluorescence labeling (HC) and qPCR analysis (CTX) of WT and 5XFAD male mouse following standard diet, HFD or HFD+RV. a |** Representative view of WT mice dentate gyrus, labeled for IBA1 and $P16^{INK4A}$, white square indicates the area represented in the Detail section. **b |** Representative view of 5XFAD mice dentate gyrus, labeled for IBA1 and $P16^{INK4A}$, white square indicates the area represented in the Detail section. **c |** *Cdkn2a* transcription in the CTX. **d |** *Cdkn1a* transcription in the CTX. **e |** *Trp53* transcription in the CTX.

Two-way ANOVA's source of variation analysis of Genotype (Gen) is indicated in the upper left corner of each graph, when significant. Post-hoc analysis was performed with Tukey's multiple comparisons test. Significance was indicated: *, $p < 0.05$.

HFD and PA treatment in 5XFAD mice and cell culture, with anti-inflammatory supplementation

Considering that a HFD has been extensively described to worsen AD pathology and induce cellular senescence (Bracko et al., 2020; Mazzei et al., 2021; Ogrodnik et al., 2019), we decided to assess several markers of male 6-month-old 5XFAD mice. Moreover, as a HFD is highly inflammatory, and RV is considered a senomorphic compound, we tested whether RV could also assist in maintaining senescent cell counts low. Immunostaining of $p16^{INK4A}$ in the dentate gyrus, revealed that WT mice under a HFD had an increased number of $p16^{INK4A}$-immunoreactive cells, which were not positive for Iba1, a marker of microglia (**Fig. 4a**). On the contrary, 5XFAD control mice already displayed $p16^{INK4A}$-positive cells, which were markedly increased in HFD-fed mice (**Fig. 4b**). RV, nonetheless, did not appear to induce a general effect in decreasing the number of $p16^{INK4A}$-positive cells. Though unlabeled for NeuN, possible neurons tended to accumulate in the subgranular zone of the dentate gyrus in 5XFAD-HFD mice, with or without RV. The identification of the cells entering in a senescence and a complete stereological cell count will allow to confirm a senolytic effect of RV in specific cell types in these mice.

These results are compatible in the transcript analysis, as HFD produced a two-fold increase of the $p16^{INK4A}$ transcript *Cdkn2a* in 5XFAD mice, but not of *Cdkn1a* or *Trp53* (**Fig. 4c-d**). Interestingly, RV induced a decreasing *Cdkn2a* transcript trend in 5XFAD mice. (**Fig. 4c**).

Palmitic acid (PA) is one of the main fatty acids comprised in a HFD (Yustisia et al., 2022). Because of this, we decided to treat with PA the differentiated and non-differentiated SH-SY5Y neuroblastoma cell line as well as HMC3 microglia. Prior evidence suggested that PA diminished cell viability in the SH-SY5Y neuroblastoma and the HMC3 microglia cell lines (Hsiao et al., 2014; Phitthayaphong et al., 2021). In order to test if an anti-inflammatory treatment would avoid the entry into senescence, cell lines were incubated with varying concentrations of RV or TPPU. Despite multiple SA-βgal stainings, no apparent differences could be observed across treatments (**Suppl. Fig. 4**). Nonetheless, we proceeded with 5-EdU staining, an analogue of thymidine that can be employed to detect cycling cells when coupled with a click-chemistry reaction (Buck et al., 2008). Here, RV had the biggest effect promoting cells into the cell cycle, but results were only significant in the absence of PA (**Fig. 5a–d**). Unexpectedly, PA did not alter the ability of cells to cycle in any case. SH-SY5Y differentiated cells, as they are not expected to divide, did not show any differences (**Suppl. Fig. 5a**). TPPU, on the contrary, did not appear to promote cell proliferation (**Suppl. Fig. 5b-d**). These results show that further experimental work should be done to safely conclude that PA promotes cellular senescence in these conditions, and that anti-inflammatory treatment rescues this increase of senescence.

RNA analysis of TPPU perinatally–treated 5XFAD mice

Either 2-month-old WT or 5XFAD pups were screened for senescence cell cycle regulators *Cdkn2a*, *Cdkn1a* and *Trp53*. The mothers of these were administered TPPU or vehicle prenatally and postnatally until weanling of the pups, which conferred neuroprotection in the treated group (Bartra et al., 2022). Whether TPPU is a senolytic substance is unknown (Zhang et al., 2021), but due to its anti-inflammatory properties we argued that its preventive administration could affect the SASP's ability to promote and expand senescence to other cells (Kumari & Jat, 2021). Remarkably, TPPU-treated female mice gave birth to pups with significantly less *Cdkn2a*, *Cdkn1a* and *Trp53* in the HC at 2 months of age, and this effect was stronger in the 5XFAD genetic background (**Figs. 6a–c**). On the contrary, no differences were seen in the CTX for *Cdkn1a* and *Trp53* (**Suppl. Fig. 6**). Complementary, 5XFAD mice did not appear to have more senescence markers at 2 months of age, and they are not described to have more *Cdkn2a* and *Cdkn1a* transcription at this timepoint (Wei et al., 2016). These results indicate, for the first time, that modulation of senescence before birth is possible and that it might confer long-lasting effects.

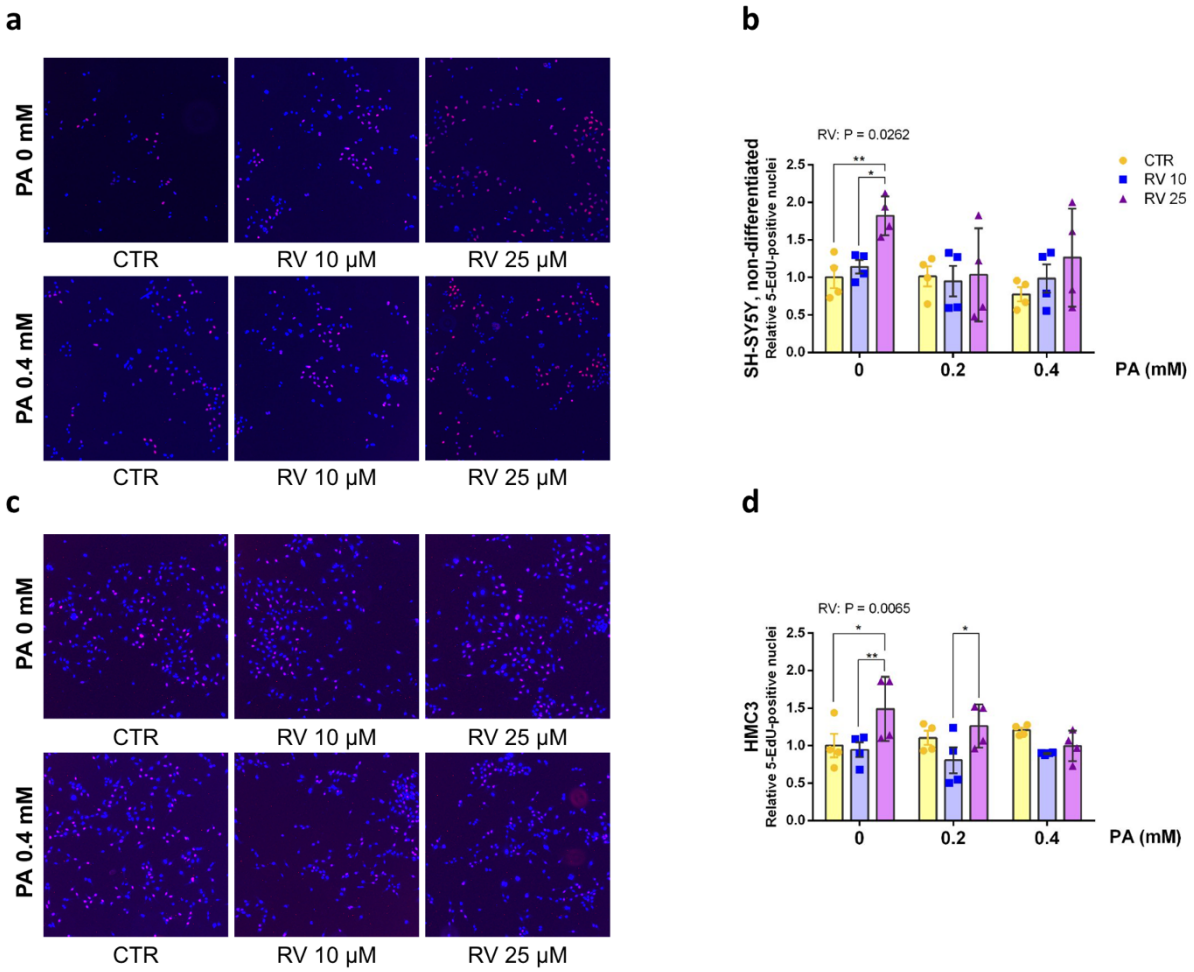

Figure 5. **5-EdU labeling of cultured SH-SY5Y and HMC3 cells. a |** Representative merge images of SH-SY5Y cells under the specified conditions. The blue channel indicates the nuclei, whereas the red channel indicates 5-EdU incorporation. **b |** Quantification of the images taken for each condition, N=4. **c |** Representative merge images of HMC3 cells under the specified conditions. The blue channel indicates the nuclei, whereas the red channel indicates 5-EdU incorporation. **d |** Quantification of the images taken for each condition, N=4. Two-way ANOVA's source of variation analysis of Treatment (RV) is indicated in the upper left corner of each graph, when significant. Post-hoc analysis was performed with Tukey's multiple comparisons test. Significance was indicated: *, $p < 0.05$; **, $p < 0.01$.

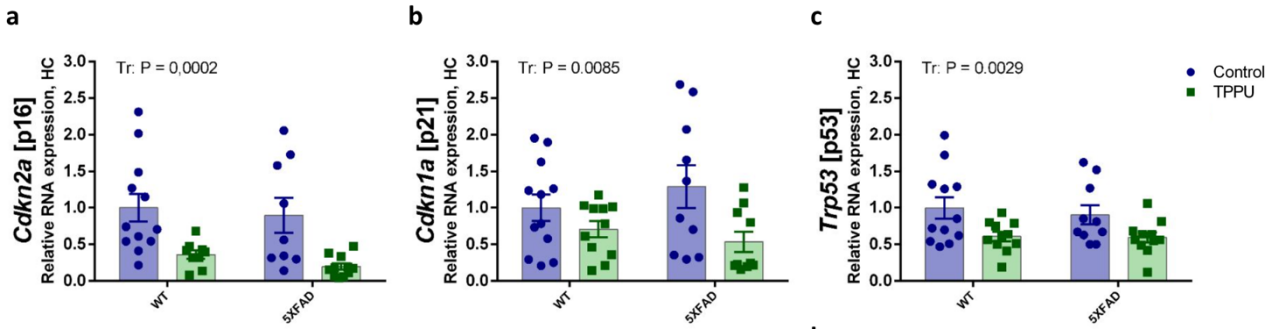

Figure 6. **qPCR analysis of selected genes in 5XFAD mice prenatally treated with TPPU or vehicle. a |** *Cdkn2a* transcription in the HC. **b |** *Cdkn1a* transcription in the HC. **c |** *Trp53* transcription in the HC.
Two-way ANOVA's source of variation analysis of TPPU treatment (Tr) is indicated in the upper left corner of each graph.

Discussion

Cellular senescence is a powerful anti-cancer response that ultimately contributes to aging and age-related diseases when loss of immune surveillance takes place, which promotes their accumulation (Gonçalves et al., 2021; Kale et al., 2020; Song et al., 2020). Specifically, senescent cells accumulate in the aging brain and in sites of damage, such as around Aβ plaques (Zhang et al., 2019). Although detrimental in the long-term for the pathogenesis of neurological diseases like AD, senescent cell genesis is considered to be a potent mechanism to avoid pre-cancerous cells from cycling (Wang et al., 2022). These findings are compatible with the observed inverse relationship seen between AD and cancer (Lanni et al., 2020). Nonetheless, in the context of AD, senescence markers are not only found in the brain. Assessment of whole blood from healthy and AD participants revealed significant sex differences in the AD group. Despite females seemingly having a greater likelihood of senescence onset (Hägg & Jylhävä, 2021; Ng & Hazrati, 2022), in the current work we validated a higher count of senescent markers in both male human and male 5XFAD mice. Understanding the onset of cellular senescence in the context of sex can be crucial since senescence is known to be implicated in multiple diseases with described sex differences. Moreover, as senolytic substances make their way into clinical trials, proper doses and treatment plans should consider different senescence signatures across sexes.

Another important aspect to consider is the disentanglement of apoptosis and cellular senescence. ATM, a marker of DNA damage, was seen to be decreased in our analysis of whole blood human samples. These data are compatible with observations that ATM transcription is reduced in neurons in the frontal cortex or the hippocampus but increased in the superior temporal cortex (Tse et al., 2017). Nevertheless, an RNA-seq of AD patient's blood found ATR to be significantly downregulated, but no markers of senescence were (Shigemizu et al., 2020). Contrarily, *Atm* was significantly increased in 5XFAD mice, and its transcription was further aggravated by exercise. In this scenario, understanding what part of the cell cycle is being repressed by DNA damage-induced apoptosis or cellular senescence is necessary. In the same line, the amount of exercise that yields the most beneficial effects needs to be assessed. While exercise is broadly considered to block neuroinflammation (Wang et al., 2023), AD is a highly debilitating condition, that, at later stages, could even be additionally affected by exercise. Despite high-intensity training being beneficial for AD patients (Ayari et al., 2023), our results suggest that 5XFAD mice, which harbor severe amyloid pathology, might not benefit from exercise. Not only did treatment not reduce senescence markers, but physical voluntary exercise even increased p16 in the male CTX. In this scenario, exercise mimetic drugs could be an interesting approach to overcome this problem (Gubert & Hannan, 2021). To untangle this unexpected result, it would have been highly informative to have a WT experimental group to assess if the same regimen of exercise decreases senescence markers in a non-AD genetic background.

Mitochondria dysfunction, as seen by the loss of mitochondrial structure and integrity, is a feature that mediates the pathogenesis of AD; and depletion of the mtDNA to nDNA ratio can be used to infer mitochondrial state (Wang et al., 2020). Our analysis of the composition of the ETC did not reveal 5XFAD to have an impaired ratio of mitochondrial complexes, but we did observe an exercise-mediated improvement in the complex V in males. This finding does not align with our lab's prior observations linking the 3xTg-AD mouse having a general decreased complex ratio (García-Mesa et al., 2012). In fact, RNA transcription of the complex IV's COXI protein (*CoxI*) tended to increase in females, while *Nd1*'s transcription had an opposing trend in both the HC and CTX. In this regard, it is important to establish which control fits better in the experimental design: while complex ratios are normalized against the most stable complex, qPCR analysis is normalized with nuclear controls. Exercise alone was sufficient to revert the mtDNA:nDNA reduction observed in 3xTg-AD mice (García-Mesa et al., 2012), but our data suggests that exercise aggravates the loss of this ratio

in 5XFAD mice, as assessed through the ratio of *Nd1* transcription. All in all, finding reliable markers and standardization of these measurements is important. What is more, more faithful measurements of mitochondria dysfunction like oxygen consumption rate should be performed (reviewed in: Connolly et al., 2017).

Another important research matter that should be discussed is whether RV harbors senolytic or senomorphic activities. This question is important because it determines the endpoint that is to be assessed. When working with a senomorphic compound, senescence markers should remain unchanged, but SASP factors would be expected to reflect a significant decrease. In our experimental model, 5XFAD mice following a HFD were supplemented with 0,1% RV in food, corresponding to 120 mg/kg body weight. Despite a trend to reduction, RV did not show clear senolytic activity. Nonetheless, senolysis could well be cell type specific. In our work, senescent $p16^{INK4A}$-positive cells identified resulted to be Iba1-negative, indicating the need for a $p16^{INK4A}$ and NeuN or GFAP costaining. Future work in the lab will fully characterize the nature of these $p16^{INK4A}$-positive cells.

Experimental cell culture with RV and PA in SH-SY5Y and HMC3 cells showed increased DNA replication in the absence of PA, an indirect measure of reduced senescence. On the contrary, SA-βgal staining were not indicative of an altered senescence burden in cultured plates. This can be due to two possible main reasons. The first one is that under our experimental settings no differences in senescence are present, which goes against published evidence of PA promoting senescence in these cell types. The second, most plausible, refers to the complexity of the set-up for each cell line and conditions, altogether with the necessity of including validated and clear positive and negative senescence-inducing treatments. Due to the limited time of the project, this was not possible.

Conversely, TPPU, which is not known to have senolytic activity but is an anti-inflammatory drug, strongly decreased senescence markers when administered prenatally. Unlike the HFD-RV design, prenatally-treated mothers with TPPU could have had the ability to avoid expansion of SASP factors (not assessed), ultimately avoiding the onset of new senescent cells.

In summary, the lifestyle interventions hereby characterized share a common feature that is also pivotal for both senescence and AD: inflammation. Indeed, aged organisms present what is known as “inflammaging”, defined as a state of chronic inflammation in the absence of an inflammatory stimulus (Franceschi et al., 2000). Inflammaging is associated to an increase in the cellular senescence load (and thus its noxious SASP), and is being studied as a target of healthy-aging therapeutics (Dugan et al., 2023). Lifestyle interventions have also been seen to target inflammaging. Exercise has been linked with a decreased inflammatory state and reduced immunosenescence, while diets like the Mediterranean Diet are also known to be anti-inflammatory (Teissier et al., 2022; Wang et al., 2023). Because our results do not clearly recapitulate that exercise or RV reduced senescent cell burden, we hypothesize that the beneficial effects seen in prior publications from the group come from the improvement of the inflammatory state (Bartra et al., 2022; Sarroca et al., 2021). Complementary to this, breaking the SASP–senescence loop in the brain can directly have a beneficial effect on neuroinflammation (**Fig. 7**). In this line, we propose that exercise, RV and TPPU might not be intrinsically senolytic, but that they mediate their effects on senescence depletion through senomorphic means. By avoiding the spread of SASP factors locally and at long distances (i.e., the blood), these interventions can aid senescence immune surveillance and favor the immune system’s task of keeping new senescent cells at bay. In fact, senescence accumulation takes place when senescence surveillance fails and the immune system is no longer able to recognize and eliminate them. In this regard, elucidating senescence immunoevasive mechanisms in the context of lifestyle interventions is also necessary.

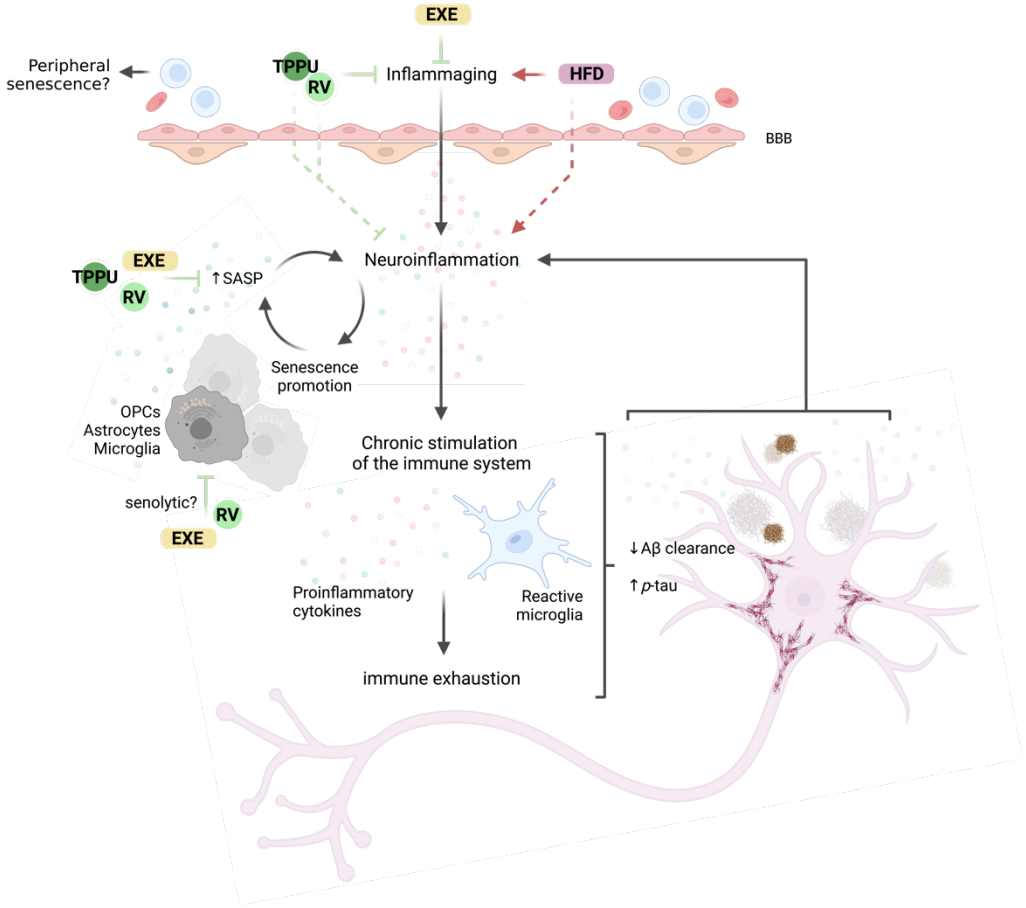

Figure 7. **Proposed interplay between lifestyle factors, cellular senescence and neuroinflammation.** Cellular senescence in the brain constitutes a self-feeding cycle that, when out of balance, promotes more senescence through the actions of the SASP. The SASP contributes directly to increasing neuroinflammation, which ultimately promotes a state of chronic stimulation of the immune system and its subsequent exhaustion. In these high inflammatory conditions, hyperphosphorylation of intracellular tau takes place, altogether with a reduced ability to clear extracellular Aβ plaques. This environment worsens the neuroinflammatory status. We propose that lifestyle interventions are able to improve the senescence signature in the brain through modulation of the noxious SASP and by enhancing inflammaging in peripheral tissues. Indeed, cytokines and different inflammatory factors have the ability to reach distant tissues through the blood. In this regard, exercise and RV or TPPU supplementation promote a healthier inflammaging signature. By avoiding the spread of senescence–inducing SASP components, they appear to harbor a senolytic effect, thereby reducing senescent markers in the AD brain.

The present work highlights how necessary the assessment of senescence markers is in lifestyle interventions within the context of AD. Since cellular senescence plays a significant role in both aging and AD pathogenesis, understanding its dynamics and impact on the different sexes will be crucial for developing effective senolytic and senomorphic strategies. As research delves deeper into the complexities of cellular senescence and its intricate relationship with inflammation, its full assessment will be key for the understanding of not only AD but also age-related diseases as a whole.

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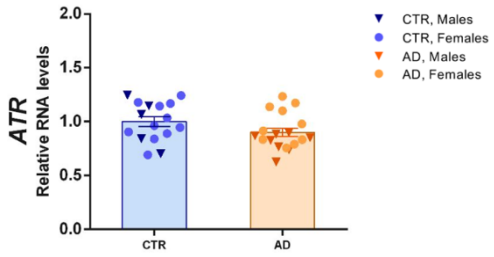
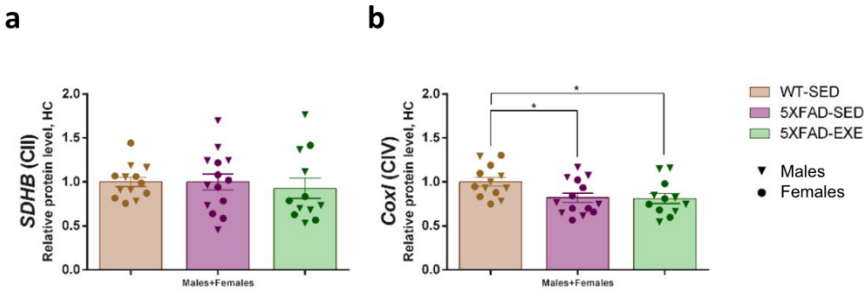
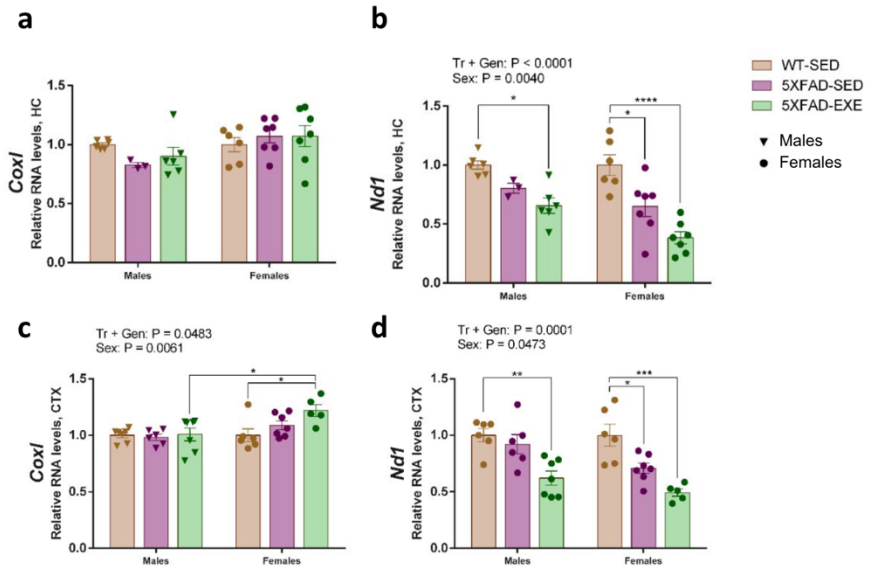
Supplementary figures



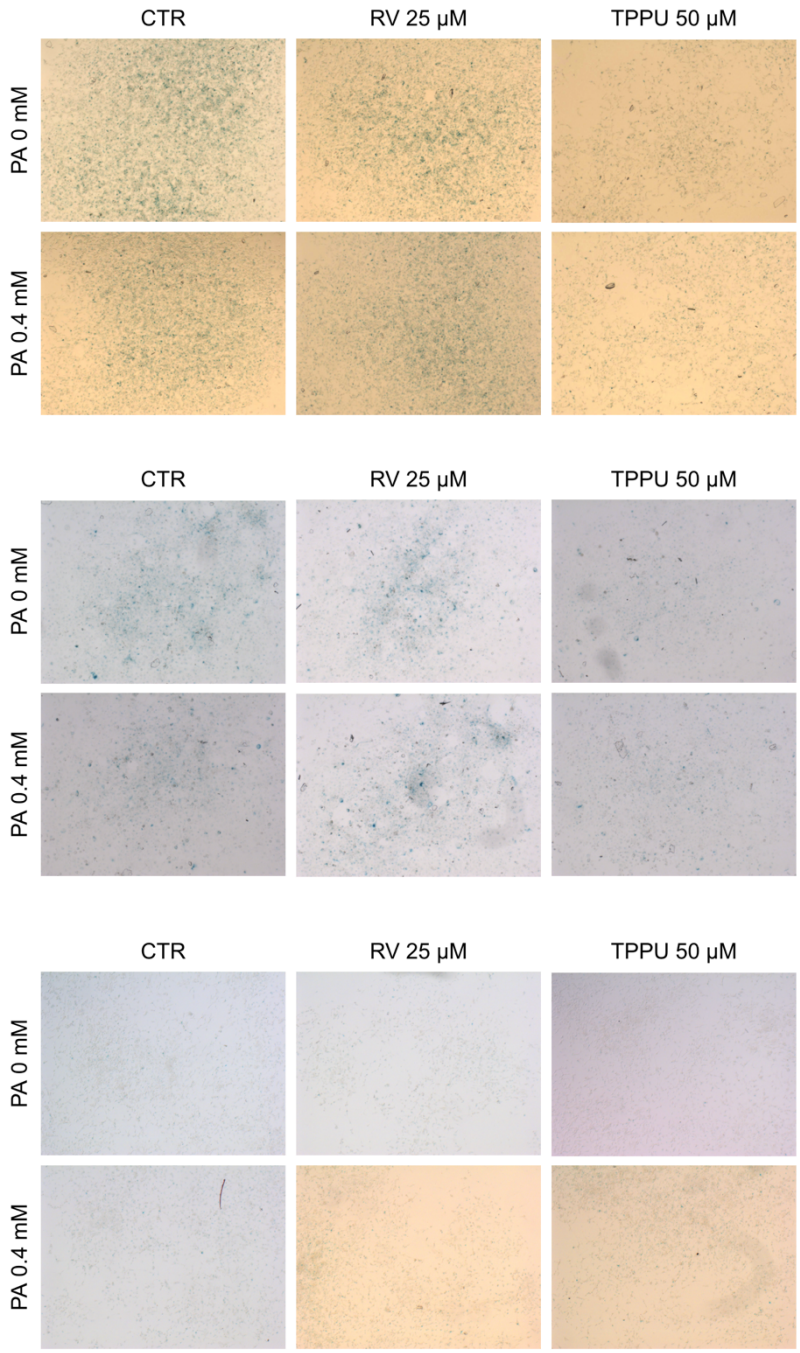
Supplementary Figure 1. **qPCR analysis of RNA from human whole blood from healthy controls and AD patients.** *ATR* transcript was analyzed with Student's unpaired *t*-test (non-significant) given that the sex factor was not statistically significant.



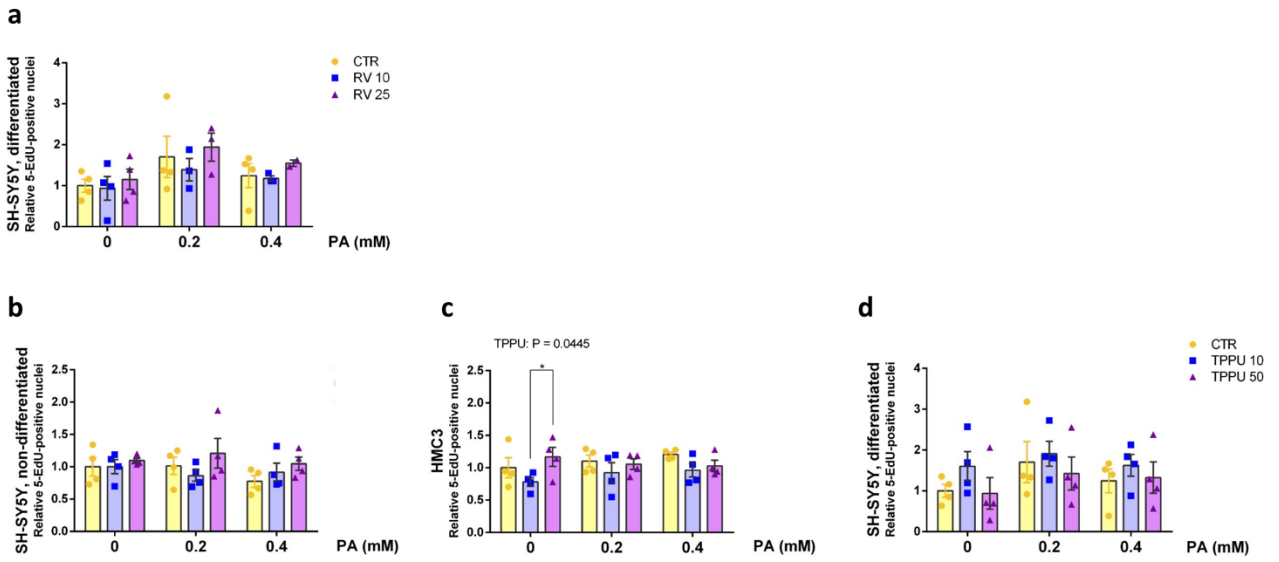
Supplementary Figure 2. **Electron Transport Chain complexes immunoblot analysis of WT and 5XFAD mice following a sedentary or exercise intervention. a |** SDHB protein content, normalized to GAPDH. **b |** COXI protein content, normalized to GAPDH.
Sex differences were analyzed through two-way ANOVAs, and were not significant (not indicated). One way ANOVA was performed to assess differences between groups. Post-hoc analysis was performed. Significance was indicated: *, $p < 0.05$.



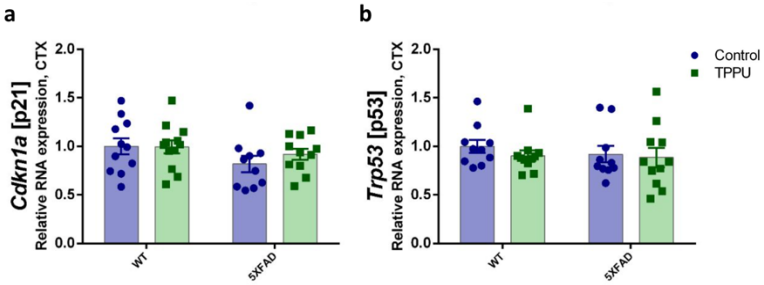
Supplementary Figure 3. **qPCR analysis of WT and 5XFAD mice following a sedentary or exercise intervention. a |** *Cox1* transcription in the HC. **b |** *Nd1* transcription in the HC. **c |** *Cox1* transcription in the CTX. **d |** *Nd1* transcription in the CTX.
Sex differences were analyzed through two-way ANOVAs, and, when significant, groups were separated accordingly. ANOVA's source of variation analysis of Group (Tr + Gen) is indicated in the upper left corner of each graph, when significant. Post-hoc analysis was performed with Tukey's multiple comparisons test. Significance was indicated: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



Supplementary Figure 4. **Senescence-Associated beta-galactosidase stainings, under PA, RV and TPPU treatments. TOP |** SH-SY5Y non-differentiated cell line. **MIDDLE |** HMC3 microglia cell line. **BOTTOM |** SH-SY5Y differentiated cell line.
Color differences only reflect whether blank background was performed, and the backgrounds were slightly edited to correct differences in microscope light instability, due to microscope–coupled camera issues. Since no clear differences were seen, definite pictures were not taken. Other treatment concentrations were performed (not shown); such as 0.2 mM PA, 10 µM RV, 10 µM TPPU and the combination of these.



Supplementary Figure 5. **5-EdU analysis of cultured SH-SY5Y and HMC3 cells. a |** Quantification of the images taken for each condition (PA & RV) in differentiated SH-SY5Y cells, N=4. **b |** Quantification of the images taken for each condition (PA & TPPU) in non-differentiated SH-SY5Y cells, N=4. **b |** Quantification of the images taken for each condition (PA & TPPU) in HMC3 cells, N=4. **b |** Quantification of the images taken for each condition (PA & TPPU) in differentiated SH-SY5Y cells, N=4.
Two-way ANOVA's source of variation analysis of Treatment (TPPU) is indicated in the upper left corner of each graph, when significant. Post-hoc analysis was performed with Tukey's multiple comparisons test. Significance was indicated: *, $p < 0.05$.
Microscopy images of the quantification are not shown for simplicity matters.



Supplementary Figure 6. **qPCR analysis of selected genes in 5XFAD mice CTX, prenatally treated with TPPU or vehicle. a |** *Cdkn1a* transcription in the CTX. **b |** *Trp53* transcription in the CTX.
Two-way ANOVA's source of variation analysis of Treatment (Tr) revealed no significant differences.

Supplementary tables

Antibody (clone)	Raised in	Dilution (WB)	Dilution (IF)	Supplier	Catalog #
Actin	Rabbit	1:500	-	Sigma-Aldrich®	A5060
GAPDH (1D4)	Mouse	1:1000	-	Invitrogen®	MA1-16757
β-tubulin (TUB 2.1)	Mouse	1:1000	-	Sigma-Aldrich®	T4026
OXPHOS	Mouse	1:1000	-	Abcam®	ab110413
OXPHOS	Mouse	1:250	-	MitoSciences®	MS604
p53 (1C12)	Mouse	1:1000	-	Cell Signaling Technology®	2524
$p21^{Waf1/Cip1}$ (F-5)	Mouse	1:200	-	Santa Cruz Biotechnology, INC.	sc-6246
$p21^{Waf1/Cip1}$ (187)*	Mouse	1:200	-	Santa Cruz Biotechnology, INC.	sc-817
$p16^{INK4a}$ (1E12E10)	Mouse	1:1000	1:200	Invitrogen®	MA5-17142
NeuN*	Rabbit	-	1:750	GeneTex, INC.	GTX132974
Iba1	Rabbit	-	1:500	GeneTex, INC.	GTX100042
Iba1*	Rabbit	-	1:500	FUJIFILM Wako Chemicals©	019-19741
H2A.XS139ph (GT2311)*	Mouse	-	1:500	GeneTex, INC.	GTX628789
GFAP*	Rabbit	-	1:500	Agilent Dako®	Z0334
GFAP (G-A-5)*	Mouse	-	1:500	Sigma-Aldrich®	G3893

Supplementary Table 1. Primary antibody list used for Western Blotting and/or Immunofluorescence. Antibodies labeled with an asterisk (*) do not appear in any of the figures included in the present report but have been added to the list since they provide a true picture of the totality of the antibodies used.

TaqMan gene	Species	Supplier	Reference
Actb	*M. musculus*	Life Technologies™	Mm02619580_g1
Atm	*M. musculus*	Life Technologies™	Mm01177457_m1
ATM	*H. sapiens*	Life Technologies™	Hs01112355_g1
ATMIN	*H. sapiens*	Life Technologies™	Hs00796220_s1
ATR	*H. sapiens*	Life Technologies™	Hs00992123_m1
ATRIP	*H. sapiens*	Life Technologies™	Hs04335019_s1
B2M	*H. sapiens*	Life Technologies™	Hs00984230_m1
Cdkn1a	*M. musculus*	Life Technologies™	Mm00432448_m1
CDKN1A	*H. sapiens*	Life Technologies™	Hs00355782_m1
Cdkn2a	*M. musculus*	Life Technologies™	Mm00494449_m1
CDKN2A	*H. sapiens*	Life Technologies™	Hs00923894_m1
Cox1	*M. musculus*	Life Technologies™	Mm04225243_g1
Nd1	*M. musculus*	Life Technologies™	Mm04225274_s1
PGK1	*H. sapiens*	Life Technologies™	Hs00943178_g1
Tbp	*M. musculus*	Life Technologies™	Mm00446971_m1
TP53	*H. sapiens*	Life Technologies™	Hs01034249_m1
Trp53	*M. musculus*	Life Technologies™	Mm01731287_m1

Supplementary Table 2. List of probes used for RT-qPCR.

Acknowledgments

The current Research Project came at one of the hardest times in my academic career. Having taken mental health for granted all my life, I went through several experiences that would make me conclude that research was over for me. Because of this, I had to gather strengths to finish my very last M.Sc. project at home, Catalonia. The reason behind these acknowledgments is to perpetuate my gratitude towards the people that helped me build myself up.

Thank you, Clara. For your care, your sensitivity, your understanding. Thank you for teaching me how to trust myself; thank you for teaching me that I am better when I take care of myself. Your supervision has not only shaped the person I am today, but it has already shaped the future supervisor I will become. I can only be grateful to have had such an exceptional scientist by my side. I can only hope that our scientific careers will meet again.

Thank you, Coral. For being the first Principal Investigator I've had to *always* prioritize humanity over workload. Know that your lab has been the first one where I've actually enjoyed. It's really heartbreaking to have to say goodbye to such a fun and healthy workplace. You made us all feel like we were family. Thank you for your trust in me.

Thank you, Kiki. For teaching me that things are not that serious. With you I've been able to let go when things were not going the way I wanted them to go. Thank you for creating a space where I could talk about my frustration, my insecurities, my fears; I hope you felt this way too. It really wouldn't have been the same without you.

Thank you, Arnau. For always being there for me. Despite not having been physically present in the lab, know that you were always present. I'm not sure I could do anything without you by my side. Thank you.