

Article

Estetrol Enhances Mitochondrial Bioenergetics and Neurite Outgrowth in Cellular Models of Alzheimer's Disease

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Highlights

What are the main findings?

- Estetrol (E4) boosts mitochondrial energy production and neurite outgrowth in Alzheimer's disease cell models, showing superior efficacy to 17 β -estradiol (E2) in stabilizing metabolic function under tau-pathology conditions.
- E4 exerts its neuroprotective effects by activating estrogen receptors (ER α , ER β , and GPER1) and specifically upregulating the mitochondrial phosphate carrier gene, SLC25A23.

What are the implications of the main findings?

- E4 may offer a safer therapeutic alternative to E2 for preserving neuronal function in Alzheimer's disease, potentially reducing the thrombotic and oncologic risks associated with conventional estrogen therapy.
- The discovery that E4 directly modulates mitochondrial gene expression provides a novel mechanistic validation for its clinical development as a treatment for metabolic deficits in neurodegeneration.

Abstract

Mitochondrial dysfunction is an early driver of Alzheimer's disease (AD), and the decline in sex hormones, including 17 β -estradiol (E2), at menopause has been linked to AD risk in women. While E2 exerts potent neuroprotective and mitochondrial-regulatory effects, its clinical utility in estrogen replacement therapy (ERT) may be limited by thrombotic and oncologic risks. Estetrol (E4), a fetal estrogen with a selective safety profile, may represent a promising alternative. This study evaluated the impact of E4 on mitochondrial bioenergetics and neuronal morphology in human SH-SY5Y neuroblastoma cells, including models of AD-related amyloidopathy (amyloid precursor protein overexpression) and tauopathy (P301Ltau mutation overexpression). E4 significantly enhanced ATP levels, mitochondrial membrane potential, and oxidative respiration in all cell models, notably outperforming E2 in P301L cells. E4 also promoted significant neurite outgrowth, alleviating deficits observed

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in AD models. In addition, we demonstrated that the bioenergetic effects of E4 were mediated by the estrogen receptors ER α , ER β , and GPER1. Furthermore, E4 modulated the expression of key mitochondrial genes, specifically upregulating the phosphate carrier SLC25A23 while downregulating the complex I subunit NDUFA1. In conclusion, E4 improves mitochondrial health and supports neuronal integrity via a multi-receptor mechanism, highlighting its potential as a safe neuroprotective therapy for AD.

Keywords: estetrol (E4); estradiol (E2); mitochondria; bioenergetics; neurite outgrowth; estrogen receptors; Alzheimer's disease; amyloid- β ; tau protein

1. Introduction

Estrogens are a family of steroid hormones, which includes estrone (E1), estrinol (E3), and the predominant and highly potent form, 17 β -estradiol (E2). They are synthesized in the ovaries, adrenal glands, and, to a lesser extent, other tissues such as the brain [1]. Beyond their well-established roles in reproduction and the estrous (menstrual) cycle, estrogens perform vital functions in the central nervous system, where they exhibit powerful neuroprotective, neurotrophic, and antioxidant actions [1]. The interface between estrogen signaling and cellular energy metabolism is particularly critical for neuronal maintenance. E2 is recognized as an essential regulator of the female brain's metabolic system by directly influencing mitochondrial activity, which is crucial for maintaining neuronal survival and well-being [2]. Namely, E2 has been shown to modulate mitochondrial activity in neuronal cells by enhancing bioenergetics, including increasing mitochondrial membrane potential (MMP), adenosine triphosphate (ATP) levels, and respiration [3,4]. In terms of its mechanism of action, estrogen nuclear receptors (ER α and ER β) can also translocate directly into the mitochondria, where they can bind to estrogen response elements (EREs) in mitochondrial DNA to improve the expression of mitochondrial-encoded genes and enhance the activity of the electron transport chain (ETC) [5–8].

This close metabolic control is heavily dependent on circulating hormone levels. High levels of E2 before menopause correlate with high brain energy capacity and controlled redox balance in the female brain [2,9]. However, the transition into reproductive senescence, encompassing perimenopause and postmenopause, is marked by a sudden and drastic decline in circulating estrogen levels. This hypoestrogenic state disturbs the brain's finely controlled homeostasis, leading to a significant reduction in metabolic function. The loss of E2 is linked to the emergence of bioenergetic deficits, including glucose hypometabolism, increased oxidative stress, and a decrease in antioxidant defenses. These estrogen-related deficits have major pathological consequences. The resulting impairments may increase the vulnerability of elderly women to brain degeneration and age-related pathologies, such as Alzheimer's disease (AD) [9,10]. AD is the most prevalent cause of dementia worldwide and is characterized pathologically by the accumulation of extracellular amyloid- β (A β) plaques and intracellular neurofibrillary tangles (NFTs) composed of abnormally hyperphosphorylated tau protein. The fact that women constitute approximately two-thirds of all AD patients strongly suggests that the loss of E2's neuroprotective effects is a significant risk factor. Furthermore, numerous studies have established mitochondrial dysfunction, including reduced complex IV activity, decreased ATP production, and altered mitochondrial dynamics, as an early and pivotal event in AD pathogenesis, preceding the full manifestation of classical histopathological hallmarks [11].

The evidence strongly suggests that Estrogen Replacement Therapies (ERT) represent an attractive therapeutic tool for counteracting bioenergetic impairments associated with aging and age-related disorders. Indeed, *in vitro* and *in vivo* studies have shown that

estrogen treatment is effective in reducing bioenergetic impairments observed in models of AD [3,12,13]. Estetrol (E4) is the most recently described natural estrogen, initially identified as being produced by the human fetal liver during pregnancy. It has four hydroxyl groups (hence “E4”), distinguishing it from E2 (Supplementary Figure S1A,B). It also has a long half-life (24–32 h), high oral bioavailability, and is not metabolized into active metabolites (like E2) [14]. E4 is currently used as the estrogenic component of a combined oral contraceptive and is in late-stage clinical development for use as a Menopausal Hormone Therapy (MHT) [14]. The utilization of conventional oral estrogens for menopause, such as E2, is associated with adverse effects, including an increased risk of breast cancer and venous thromboembolism (VTE). Therefore, E4 offers a promising alternative due to its more selective pharmacological profile, suggesting a favorable benefit–risk ratio compared with other estrogens [15]. This selectivity is underpinned by E4’s low impact on the liver and hemostasis balance and its unique molecular mode of action, which involves activating nuclear ER α actions while preventing or uncoupling ER α membrane (non-genomic) activation in specific cell types, a process thought to reduce proliferative and prothrombotic risks [15].

While the modulatory effects of E2 on mitochondrial function are well-documented, particularly in the context of AD, the specific impact of E4 remains unexplored. Therefore, this study aimed to evaluate the mitochondrial effects of E4 under both physiological and AD-related pathological conditions. For this, human SH-SY5Y neuroblastoma cells, a well-established neuronal model for studying mitochondrial function and neurodegenerative mechanisms, were used as previously described [3]. The effects of E4 were evaluated in wild-type (Control) cells as well as in cells overexpressing the amyloid precursor protein (APP) or the P301L-tau mutation, representing AD-related amyloidopathy and tauopathy models, respectively. Given the inherent baseline differences between these models, the effects of E4 were assessed as relative changes compared to their respective vehicle-treated conditions. The impact of E4 was assessed on mitochondrial bioenergetics, network morphology, and neurite outgrowth, using E2 as a benchmark. Furthermore, to elucidate the underlying mechanisms, we investigated the specific ERs mediating these effects and examined whether E4 influences mitochondrial-related gene expression. By characterizing the mitochondrial profile of E4, this work aims to provide essential data for the development of novel estrogen-based neuroprotective therapies.

2. Materials and Methods

2.1. Chemicals and Reagents

Estetrol powder was provided by the company Estetra SRL, a wholly owned subsidiary of Gedeon Richter Plc (Liège, Belgium). Dihydrorhodamine 123 (DHR, #D23806), CellTracker™ Blue CMAC (#C2110), and hygromycin B (#10687010) were all purchased from Thermo Fisher Scientific (Waltham, MA, USA). PBS (#MS019N1008) was purchased from Dominique Dutscher (Bernolsheim, France). All the following chemicals and reagents were purchased from Gibco (Waltham, MA, USA): B-27 Supplement (50X, #17.504.001), Gibco™ HBSS with calcium and magnesium without phenol red (#14.065.049), GlutaMax (#35.050.087), and Neurobasal™ Medium (#21.103–049). Accutase (#AT-104) was obtained from Innovative Cell Technology (San Diego, CA, USA). Collagen I, Rat Tail (#354236), and Fetal bovine serum (FBS, #35–079-CV) were purchased from Corning (Corning, NY, USA). Vectashield mounting medium (#H-1000) was obtained from Vector Labs (Newark, CA, USA). Blastidicin (#Ant-bl-1) was from InvivoGen (Toulouse, France). Seahorse XF 100 mM Pyruvate Solution (#103578-100), Seahorse XF 1 M Glucose Solution (#103577-100), and Seahorse XF DMEM, pH 7.4 (#103575-100), were purchased from Agilent Technologies (Basel, Switzerland). Penicillin/Streptomycin (#4-01F00-5) and Horse Serum (HS, #2-05F00-1) were obtained from Bioconcept (Allschwil, Switzerland). Dulbecco’s modified Eagle’s medium (DMEM) without phenol red (#D1145), 3-(4,5-Dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT, #M2128), Antimycin A, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, #C2920), DMSO (#276,855), Oligomycin (#O4876), Rotenone (#45656), Tetramethyl rhodamine methyl ester (TMRM, #T5428), 4',6-Diamidin-2-Phenylindole (DAPI, #10236276001), Triton X-100 (#T8787), Bovine Serum Albumin (BSA, #A9647), 17 β -estradiol (#E8875), and paraformaldehyde (PFA, #P6148) were all obtained from Sigma Aldrich (St. Louis, MO, USA). MPP (#HY-103454), PHTPP (#HY-103456), Fulvestrant (#HY-103636) and G-15 (#HY-103449) were obtained from MedChemExpress (Monmouth Junction, NJ, USA). MitoBrilliant™ 646 (#7700) was purchased from Tocris Bio-Techne AG (Zug, Switzerland). The anti- β -III tubulin antibody (mouse) was purchased from R&D Systems (#MAB1195, Bio-Techne GmbH, Zug, Switzerland), and the anti-mouse Alexa Fluor 568-conjugated secondary was purchased from Abcam (#175473, Abcam, Cambridge, UK). The commercial assay ATPlite 1-step Luminescence Assay (#6016739) was acquired from PerkinElmer (Waltham, MA, USA) and the MycoAlert® PLUS Mycoplasma Detection Kit (#LT07-701) was obtained from Lonza (Basel, Switzerland).

2.2. Cell Culture

Human SH-SY5Y neuroblastoma cells were cultured in a growth medium composed of DMEM, 1% penicillin/streptomycin, and 1% GlutaMax and supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5% heat-inactivated horse serum at 37 °C in 5% CO₂. The cells were cultured in 10 cm² dishes, split twice a week, and plated when they reached around 80% confluence. Amyloid precursor protein (APP)-overexpressing SH-SY5Y cells (APP cells) were used as a cellular model of AD-related amyloidopathy. Cells were stably transfected with DNA constructs harboring the entire coding region of human wild-type APP (APP-695) as described previously [16]. Stably transfected cell clones were maintained in a growth medium containing hygromycin (300 μ g/mL) and subjected to steady selection pressure. We have previously shown that these cells secrete around 150 pg/mL amyloid- β (A β)₁₋₄₀, while control cells secrete approximately 50 pg/mL A β ₁₋₄₀ [17]. P301Ltau-overexpressing SH-SY5Y cells (P301L cells) were generated by lentiviral gene transfer in the group of Jürgen Götz (Queensland Brain Institute, Brisbane, Australia) [18] and used as a cellular model of AD-related tauopathy. Stably transfected cell clones were maintained in the growth medium containing 4.5 μ g/mL blasticidin, kept under steady selection pressure, and used as described previously [19]. The untransfected SH-SY5Y cells (#CRL-2266, ATCC, Manassas, VA, USA) were used as Control cells in this study. All cell lines were regularly tested for Mycoplasma contamination using the MycoAlert® PLUS Mycoplasma Detection Kit. All experiments were performed with SH-SY5Y between passages 9 and 15.

2.3. Treatment Paradigm

Stock solutions of estetrol (E4) and estradiol (E2) were prepared in DMSO at 10 mM. Cells were plated in each assay-specific plate, and when they reached 80% confluency (24 h later), they were treated as described below. To limit cell growth and optimize mitochondrial respiration, the treatment medium contained only 5% FBS and was supplemented with 3 mM pyruvate. E4 treatment concentrations and duration were selected based on preliminary screening experiments (see Section 3). Cells were treated for 48 h, with medium replaced after 24 h. 17 β -estradiol (E2) was used at a 0.1 μ M concentration, as previously [3,4]. DMSO alone (0.001%) was used as the vehicle control condition. For the experiments with estrogen receptor (ER) antagonists (MPP, PHTPP, fulvestrant, and G-15), cells were first treated with 0.1 μ M of ER antagonist for 1 h and then treated with E2 or E4 for 48 h. Each assay was repeated at least 3–5 times.

2.4. Cell Viability Assay

Cell viability was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. Cells were plated in 96-well plates one day before treatment at a density of 1.5×10^4 cells/well, with at least 5 replicates per condition. After 48 h of treatment with E2 and E4, the cells were incubated with 5 mg/mL MTT in DMEM (10 μ L/well) for 2 h. MTT is converted into a violet formazan derivative through mitochondrial enzymatic activity. At the end of the reaction, the medium was removed, and 200 μ L of DMSO was added to each well to dissolve the formazan crystals. The MTT absorbance was measured at 550 nm using the Cytation 3 Cell Imaging Multi-Mode Plate Reader (BioTek, Agilent Technologies, Basel, Switzerland).

2.5. ATP Assay

Total ATP levels were determined using the bioluminescence assay ATPlite 1 step (Perkin Elmer), according to the manufacturer's instructions and as previously described [20]. Briefly, cells were plated in at least 5 replicates in white 96-well cell culture plates at a density of 1.5×10^4 cells/well and treated within 24 h after seeding. After 48 h of treatment with E2 and E4, wells for the ATP standard curve were prepared, and ATP substrate solution was added to every well. After 2 min of incubation at room temperature, the luminescence was measured using the Cytation 3 Cell Imaging Multi-mode Plate Reader (BioTek, Agilent Technologies, Basel, Switzerland). The method measures the formation of light from luciferin and ATP catalyzed by the enzyme luciferase. The detected luminescence was linearly correlated with the ATP concentration.

2.6. Determination of Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) was measured using the fluorescent dye TMRM [20]. Cells were plated in at least 5 replicates in black 96-well cell culture plates at a density of 1.5×10^4 cells/well and treated within 24 h after seeding. After 48 h of treatment with E2 and E4, cells were loaded with the dye at a final concentration of 0.4 μ M for 30 min at room temperature and kept in the dark. After washing twice with 200 μ L HBSS, the fluorescence signal was detected at 531 nm (excitation)/595 nm (emission) using the Cytation 3 Cell Imaging Multi-Mode Plate Reader (BioTek, Agilent Technologies, Basel, Switzerland). The dye's fluorescence intensity depends on the MMP.

2.7. Determination of Reactive Oxygen Species (ROS) Levels

The fluorescent dye dihydroethidium (DHE) was used to evaluate levels of superoxide anion radicals [20]. Cells were plated in at least 5 replicates into black 96-well cell culture plates at a density of 1.5×10^4 cells/well and treated within 24 h after seeding. After treatment with E2 and E4, cells were incubated with 10 μ M DHE for 30 min at room temperature in the dark on an orbital shaker. After three washes with HBSS, red fluorescent products were detected at 531 nm (excitation)/595 nm (emission). The fluorescence intensity was proportional to the levels of superoxide anion radicals. Fluorescence was measured using the Cytation 3 Cell Imaging Multi-mode Plate Reader (BioTek, Agilent Technologies, Basel, Switzerland).

2.8. Profiling Bioenergetic Phenotype

The Seahorse XF HS Mini Analyzer (Agilent Technologies) was used to investigate key mitochondrial respiration and glycolysis parameters. The device allows simultaneous real-time measurement of the oxygen consumption rate (OCR), reflecting mitochondrial respiration, and the extracellular acidification rate (ECAR), reflecting cellular glycolysis [20]. Cells were seeded into a Seahorse XFp Cell Culture Miniplate (Agilent Technologies) at a density of 1.5×10^4 cells per well and treated within 24 h after seeding. After 48 h of

treatment, the XF Mito Stress Test protocol was performed. For the measurements, the assay medium consisted of Seahorse XF DMEM, pH 7.4 (Agilent Technologies), supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM L-glutamine. The OCR and ECAR were recorded simultaneously, first under basal conditions, followed by the sequential injection of oligomycin (1.5 μ M), FCCP (0.5 μ M), and a combination of antimycin A (0.5 μ M) and rotenone (0.5 μ M). The collected data were analyzed on the Agilent Seahorse Analytics website, which automatically computed the following bioenergetic parameters: basal respiration, ATP production-coupled respiration, spare respiratory capacity, maximal respiration, proton leak, non-mitochondrial respiration, and basal glycolysis.

2.9. Data Normalization

ATP, MMP, ROS, and Seahorse data were normalized based on the living cell area assessed using the CellTracker Blue (CTB) dye [21]. Cells were loaded with CTB at a final concentration of 5 μ M and incubated for 30 min at 37 °C in the dark. After one wash with HBSS, the fluorescence signal was detected at 353 nm (excitation) and 466 nm (emission) using the Cytation 3 Cell Imaging Multi-mode Plate Reader (Agilent). Since the ATP measurement requires cell lysis, the CTB loading and fluorescence measurements were conducted before the ATP assay. For the other experiments, CTB loading was performed after each experimental protocol. The CellTracker Blue fluorescence intensity was used to normalize the data obtained from the different assays.

2.10. Morphometry Analysis of Mitochondrial Network

Analysis of mitochondrial network morphology was performed using fluorescence microscopy. Cells were plated in 12-well plates on collagen-coated coverslips at a density of 3.5×10^4 cells per well and treated within 24 h after seeding. After treatment, cells were incubated with 5 μ M of CellTracker Blue (CTB, ex: 353 nm–em:466 nm), to visualize the cell area, and 1 μ M of MitoBrilliant (MB, ex: 650 nm–em: 675 nm) for 60 min in the incubator at 37 °C and 5% CO₂. Subsequently, the cells were washed with PBS and fixed by immersion in a 4% PFA solution for 15 min at room temperature. After three final washes with PBS, the coverslips were mounted on slides using mounting medium (Vectashield H-1000), sealed with nail polish, and stored at 4 °C in the dark until use.

Microscopy images were acquired using a Nikon Eclipse Ti2 inverted fluorescence microscope with a 100× Immersion oil objective. Images were acquired using the fluorescent channel “DAPI” for CTB and “Cy5” for MB. Samples were scanned along the Z-axis at 21 positions, enabling optimal visualization of mitochondria at various depths. Once obtained, the images underwent a deconvolution process using the Huygens® software (v25.10) and the “Good’s Roughness Maximum Likelihood estimation” algorithm with 20 iterations and a quality factor of 0.00002.

Morphometric analysis of the mitochondrial network was conducted in a blinded manner using an automated image processing and morphometry macro in the ImageJ/FIJI® (v1.54p) image analysis and processing software as previously described [19,22]. In brief, deconvolved images underwent background subtraction (rolling ball radius = 50 pixels), and the uneven mitochondrial labeling was enhanced by local contrast improvement using contrast-limited adaptive histogram equalization (CLAHE). The “Tubeness” filter was applied to segment mitochondria. After setting an automated threshold (“Otsu”), the “Analyze Particles” plugin was used to determine the area and perimeter of individual mitochondria, and the “Skeletonize” function was used to measure mitochondrial length. The average metrics obtained included the mitochondrial length, reported in pixels after reducing mitochondria to single-pixel-wide shapes; the aspect ratio, defined as the ratio of the major and minor axes, independent of area and perimeter and reflecting the degree of

mitochondrial fusion and branching; and the form factor, defined as the inverse of circularity and reflecting particle shape complexity.

2.11. Determination of Neurite Outgrowth

SH-SY5Y neuroblastoma cells were seeded into laminin-coated black 96-well cell culture plates with transparent bottoms at a density of 5×10^3 cells/well. Differentiation was induced one day after plating by switching the growing medium to a differentiation medium composed of neurobasal medium, 1% penicillin/streptomycin, and 1% GlutaMax, supplemented with 2% B27 and 10 μ M retinoic acid (RA). After three days of differentiation, cells were treated with vehicle (DMSO), E2, or E4. Nerve growth factor (NGF) at 50 ng/mL served as a positive control. After 48 h of treatment, cells were fixed with 4% PFA for 10 min at room temperature and washed twice with PBS. They were then permeabilized with 0.15% Triton X-100 for 15 min at room temperature, followed by two washes with PBS. Cells were then incubated with a blocking solution containing 2% BSA for 1 h at room temperature. Neurites were labeled with an anti- β III-tubulin antibody (1:1000, overnight incubation at 4 °C) and an Alexa Fluor 568-conjugated secondary antibody (1:500, 1 h incubation at room temperature). Nuclei were stained with DAPI (1/1000) for 10 min at room temperature, followed by one wash with PBS.

Automated image acquisition was performed using the Cytation 5 Cell Imaging multi-mode reader (Biotek, Agilent Technologies) with a 20 $\times$ objective. Neurite outgrowth analysis was performed using the Gen5 Image Prime software (v3.17) and the Neurite Outgrowth Module (Biotek, Agilent Technologies). The following parameters were extracted from the software: average neurite length, average neurite count, and total neurite area.

2.12. RT² Profiler™ PCR Arrays

Cells were plated in 6-well plates at a density of 3×10^5 cells/well. The next day, cells were treated with vehicles, E2, or E4 for 48 h. After treatment, RNA lysates were collected to assess the effects of E4 and E2 on the expression of mitochondria-related genes using the RT² Profiler™ PCR Array Human Mitochondria (PAHS-087Z) and RT² Profiler™ PCR Array Human Mitochondrial Energy Metabolism Pathway Plus (PAHS-008Y) from Qiagen (see complete gene list in Supplementary Tables S1 and S2). In total, the expression of 164 genes was assessed. Total RNA was extracted from cell lysates using the RNeasy Mini Kit (QIAGEN), and RNA concentration was evaluated using the Take3™ Microvolume Plate in combination with the Cytation 5 Cell Imaging Multi-Mode Reader (Biotek, Agilent Technologies). cDNA was generated using the RT² First Strand Kit (Qiagen) according to the manufacturer's instructions. Next, cDNA was added to RT² Profiler Green Mastermix and loaded into 384-well PCR plates provided in the kit, which were pre-coated with assay-specific primers. Quantitative real-time PCR was performed using the CFX Opus 384 Real-Time PCR System from Bio-Rad (Cressier, Switzerland).

For PCR data analysis, Cq values were extracted from the Bio-Rad CFX Maestro Software 2.3 using a threshold of 400 across all experiments. The data were then exported and analyzed in Excel. Gene expression was normalized using the housekeeping genes (HKG: Actb, Gapdh, and Rplp0) as follows: $\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{mean HKG}}$. Then, differential expression was calculated relative to the control (vehicle-treated) group: $\Delta\Delta Cq = \Delta Cq_{\text{treated}} - \text{mean}(\Delta Cq_{\text{Veh}})$. Fold changes were computed using the $2^{-\Delta\Delta Cq}$ method. Fold changes were transformed to log₂ scale for statistical analysis and visualization ($\log_2(\text{Fold Change}) = -\Delta\Delta Cq$) was performed in GraphPad Prism (v10.4.2). Unpaired two-tailed *t*-tests were performed to compare treated groups (e.g., E4, E2) vs. vehicle-treated controls. A False Discovery Rate (FDR) correction was applied using the two-stage step-up method of Benjamini, Krieger, and Yekutieli, with a desired FDR (Q) of 10%. Genes were considered significantly regulated when the adjusted q-value (FDR) < 0.10. Significantly regulated genes were

subsequently reanalyzed using multiple *t*-tests with the Set threshold for *p*-value approach, applying the Holm–Sidak method with $\alpha = 0.05$. Significantly regulated genes (adjusted *q*-value (FDR) < 0.10) were used for functional enrichment analysis with the g:Profiler tool (<https://biit.cs.ut.ee/gprofiler/gost> (accessed on 24 September 2025)). The Gene Ontology sources GO:BP (Biological Process), GO:MF (Molecular Function), and GO:CC (Cellular Component) were queried. Enriched terms were identified based on adjusted *p*-values provided by g:Profiler and visualized using GraphPad Prism.

Promoter sequences (2000 bp upstream of the transcription start site) for human genes of interest (NDUFA1 and SLC25A23) were retrieved from the Ensembl Genome Browser (<https://www.ensembl.org/index.html> (accessed on 5 December 2025)) using the GRCh38 assembly. Transcription factor binding motifs were obtained in MEME format from the JASPAR 2024 CORE vertebrate non-redundant database (<https://jaspar.elixir.no/> (accessed on 5 December 2025)). Motif discovery was performed using the FIMO (Find Individual Motif Occurrences) tool from the MEME Suite (v5.5.0, <https://meme-suite.org/meme/tools/fimo> (accessed on 5 December 2025)). Each promoter sequence was scanned against selected motifs (e.g., ESR1, SP1, ERR α/β) using a *p*-value threshold of 1×10^{-4} , and hits with *q*-values < 0.05 were considered significant. Motif matches were interpreted in the context of known regulatory interactions relevant to estrogen signaling and mitochondrial gene regulation.

2.13. Statistical Analysis and Manuscript Preparation

Statistical analysis and graph creation were performed using GraphPad Prism 10 software (v10.4.2). Outliers were identified using the ‘Identify Outliers’ function in GraphPad Prism (ROUT method, $Q = 1\%$) and excluded from subsequent analyses. In each independent experiment, conditions were performed in technical replicates (a minimum of three per condition), and the average of these technical replicates was used as a single data point for statistical analysis. Three to five independent experiments were performed for each test (biological replicates). Comparisons between multiple groups were performed using the Kruskal–Wallis test to identify significant differences between treatment groups and the control condition (vehicle, DMSO). Results were considered statistically significant if *p*-values were < 0.05.

During the preparation of this manuscript, the authors used Google’s large language model [Gemini, Advanced version] to refine the language and grammar of the text and to assist with literature searches to identify relevant publications. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

3. Results

3.1. Selection of E4 Concentrations

First, we aimed to determine the E4 concentration that was not toxic to cells. Cell viability was assessed in Control SH-SY5Y cells after 24 h, 48 h, and 72 h of treatment with 0.01 μM , 0.1 μM , 1 μM , 10 μM , and 50 μM (Supplementary Figure S1C–E). Based on prior studies showing that E2 increases mitochondrial bioenergetics in the same cellular model, E2 at 0.1 μM was used for comparison [3,4]. DMSO alone (0.001%) was used as the vehicle control condition (Veh). We did not observe any cell toxicity at concentrations below 10 μM at any of the time points investigated. Only the 50 μM E4 concentration showed decreased cell viability, which could be attributed to the high DMSO concentration in this preparation (0.05%). Lower concentrations of E4 induced an increase in cell viability, reaching approximately a 10% increase with 0.01 μM , 0.1 μM , and 1 μM E4 after 48 h of treatment. To see if the selected E4 concentrations influenced cell bioenergetics, ATP levels were measured after 24 h, 48 h, and 72 h of treatment (Supplementary Figure S1F–H). A significant increase in ATP levels was observed after 48 h of treatment with 0.1 μM , 1 μM , and 10 μM (+5%, +4.3%, +2.3%, respectively). Again, the 50 μM E4 treatment was associated with a decrease in ATP

levels, suggesting that it was linked to reduced cell viability. In line with our prior studies [3,4], treatment with E2 at 0.1 μM significantly improved cell viability at 24 h and 72 h and increased ATP levels by about 5% after 24 h, 48 h, and 72 h of treatment.

Based on the ATP data, we selected the 0.1 μM and 1 μM concentrations of E4 and a 48 h treatment duration for our subsequent bioenergetic investigations. These concentrations are physiologically relevant, within the range of fetal E4 levels reported during pregnancy [15], and in line with our previous studies with E2 [3,4].

3.2. E4 Enhances Cell Bioenergetics in Control, APP, and P301L SH-SY5Y Cells

Based on our preliminary screening, Control, APP, and P301L SH-SY5Y cells were treated with 0.1 μM and 1 μM E4 or 0.1 μM E2 for 48 h. In control cells, we confirmed that E4 induced a 9% and 11% increase in cell viability compared with the vehicle (Veh) at 0.1 μM and 1 μM , respectively (Figure 1A). E2 had no significant effect on cell viability. Both E4 concentrations used induced a 10% rise in ATP levels, similar to E2 treatment, compared with the Veh (Figure 1B). Next, we investigated the effects of E4 on the mitochondrial membrane potential (MMP), an indicator of the proton motive force required for ATP synthesis, to assess whether the increase in ATP levels was linked to mitochondrial activity. We observed that E4 at 0.1 μM and 1 μM , as well as E2, increased MMP by 23–25% compared to the Veh-treated group (Figure 1C). Because an increase in mitochondrial bioenergetic activity is often coupled with increased oxidative phosphorylation byproducts, such as superoxide anion radicals, we next investigated the effects of E4 treatments on superoxide anion levels. However, we did not observe any difference in total superoxide anion levels between Veh-treated cells and cells treated with E4 or E2 (Figure 1D).

We have previously shown that APP- and P301L-overexpressing SH-SY5Y cells present mitochondrial bioenergetic dysfunction [3]. In the present study, we observed that these cells exhibit a significant decrease in cell viability and ATP levels and an increase in superoxide anion levels when compared with control cells (Supplementary Figure S2A–C), confirming the APP- and P301L-related bioenergetic impairments. We then tested the effects of E4 on APP and P301L cell bioenergetics (Figure 1E–L). In APP cells, a 48 h treatment with E4 at 0.1 μM significantly increased cell viability by 9% compared with the Veh-treated group (Figure 1E). E4 at 1 μM and E2 had no significant effect. Nevertheless, E4 (both concentrations) increased ATP levels by 6% compared with Veh (Figure 1F), while the effect of E2 was not significant. Regarding the MMP, a 21% and 32% increase was observed after E4 treatment at 0.1 μM and 1 μM , respectively (Figure 1G). Surprisingly, E2 induced a 12% decrease in MMP compared with Veh, although this difference was not statistically significant. This suggests that E2 has a rather uncoupling effect on the mitochondrial respiratory chain. E4 at 1 μM and E2 also induced a 7% and 14% decrease in superoxide anion levels, respectively (Figure 1H). Since APP cells present higher superoxide anion levels compared with control cells (Supplementary Figure S2C), these data suggest that E4 and E2 may have antioxidant effects in this cell line.

In P301L cells, 48 h treatment with E4 at 0.1 μM , 1 μM , as well as E2, induced a modest but significant increase in cell viability (+5%) compared with the Veh-treated group (Figure 1I). Only E4 at 0.1 μM and 1 μM significantly increased ATP levels by 6% compared with Veh (Figure 1J). E4 at 1 μM and E2 also induced a 38% and 50% increase in MMP, respectively (Figure 1K). Of note, while E2 treatment significantly increased MMP, it did not translate into a net increase in total cellular ATP levels, suggesting that ATP synthesis may be limited in this specific context. In parallel, E4 at 1 μM significantly decreased the superoxide anion levels by 5% compared with Veh (Figure 1L). Consistent with the APP cells, these data suggest that E4 may have antioxidant effects in P301L cells.

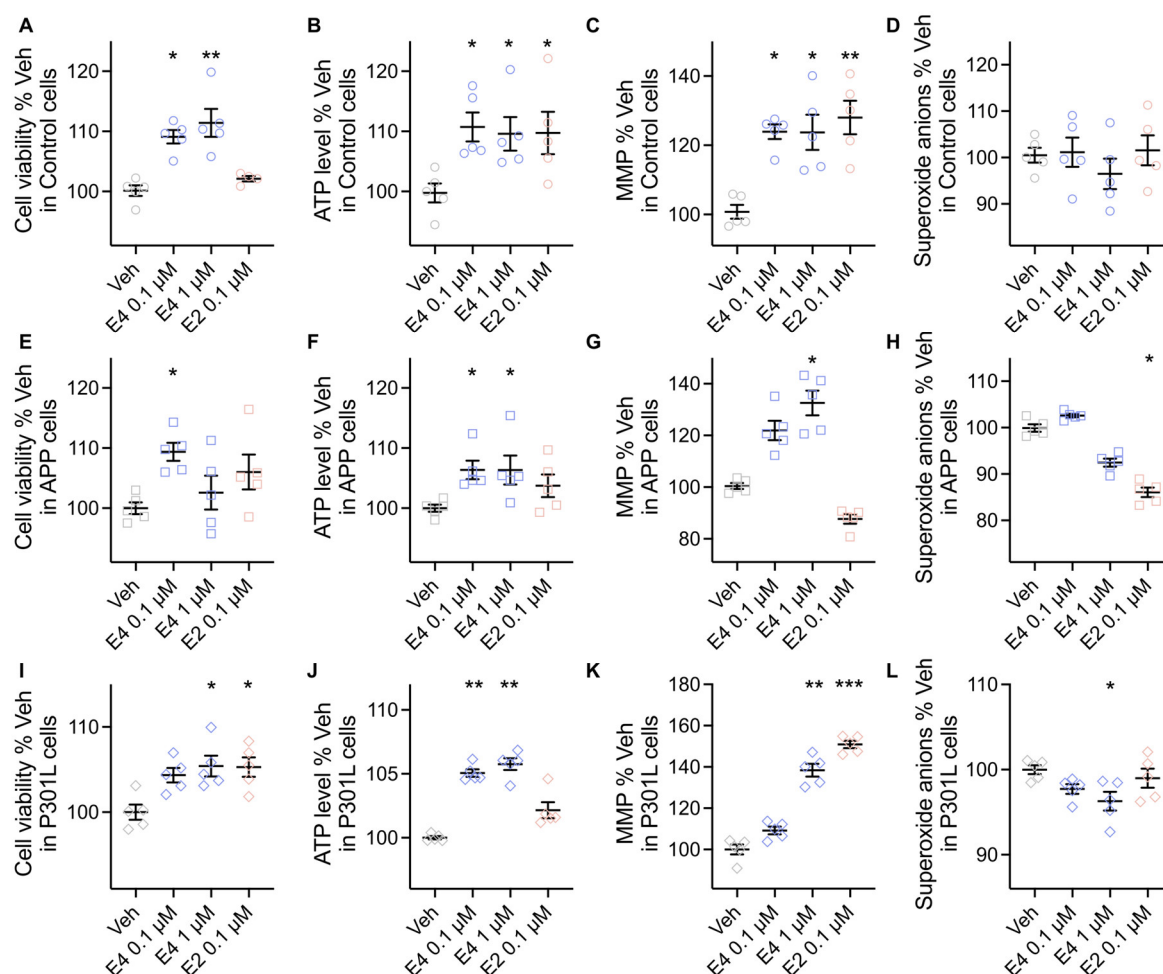


Figure 1. Effects of estetrol (E4) on cell bioenergetics in the Control, APP and P301L cells. Cells were treated with vehicle alone (Veh = DMSO), E4 0.1 μ M, E4 1 μ M, or E2 0.1 μ M for 48 h. Bioenergetic parameters were then assessed in Control (A–D), APP (E–H), and P301L (I–L) cells. Namely, cell viability (A,E,I), ATP levels (B,F,J), mitochondrial membrane potential (MMP, C,G,K), and superoxide anion levels (D,H,L) were measured. All data were normalized to CellTracker Blue fluorescence intensity, corresponding to the area of living cells. Data are presented as mean and SEM of 3–5 independent experiments and expressed as a percentage of the vehicle (Veh) condition. In addition, the mean of each independent experiment (biological replicates) is presented as circles (for Control cells), squares (for APP cells), or diamonds (for P301L cells). Total number of technical replicates: $n = 13$ –61 (A); $n = 46$ –58 (B); $n = 41$ –61 (C); $n = 33$ –47 (D); $n = 24$ –32 (E); $n = 72$ –109 (F); $n = 70$ –95 (G); $n = 48$ –62 (H); $n = 34$ –45 (I); $n = 32$ –45 (J); $n = 35$ –45 (K); $n = 32$ –46 (L). One-way ANOVA and Kruskal–Wallis multiple comparison test versus the Veh condition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ATP: adenosine triphosphate, Veh: vehicle, E2: estradiol, E4: estetrol.

As a next step, we investigated the impact of E4 at 1 μ M, the optimal concentration based on the initial bioenergetic assessment (Figure 1), on mitochondrial respiration and cellular glycolysis using the Seahorse XF Analyzer (Figure 2). In Control cells, a 48 h treatment with 1 μ M E4 significantly increased the basal respiration (+11%), basal glycolysis (+16%), mitochondrial respiration coupled with ATP production (+17%), and maximal respiration (+17%) compared to the Veh group (Figure 2B). E2 induced a 7% increase in basal respiration, a 21% increase in basal glycolysis, and a 16% increase in maximal respiration. These data suggest that the increases in ATP levels and MMP previously observed after treatment with E4 and E2 (Figure 1B,C) is linked to enhanced mitochondrial respiration and glycolysis. Indeed, by drawing the energy map with the basal oxygen consumption rate

(OCR, basal respiration) on the ordinate and the basal extracellular acidification rate (ECAR, basal glycolysis) on the abscissa, we observed that cells shifted to a metabolically more active state after treatment with E2 and E4, with an increase in both basal respiration and glycolysis compared to Veh-treated cells (Figure 2C).

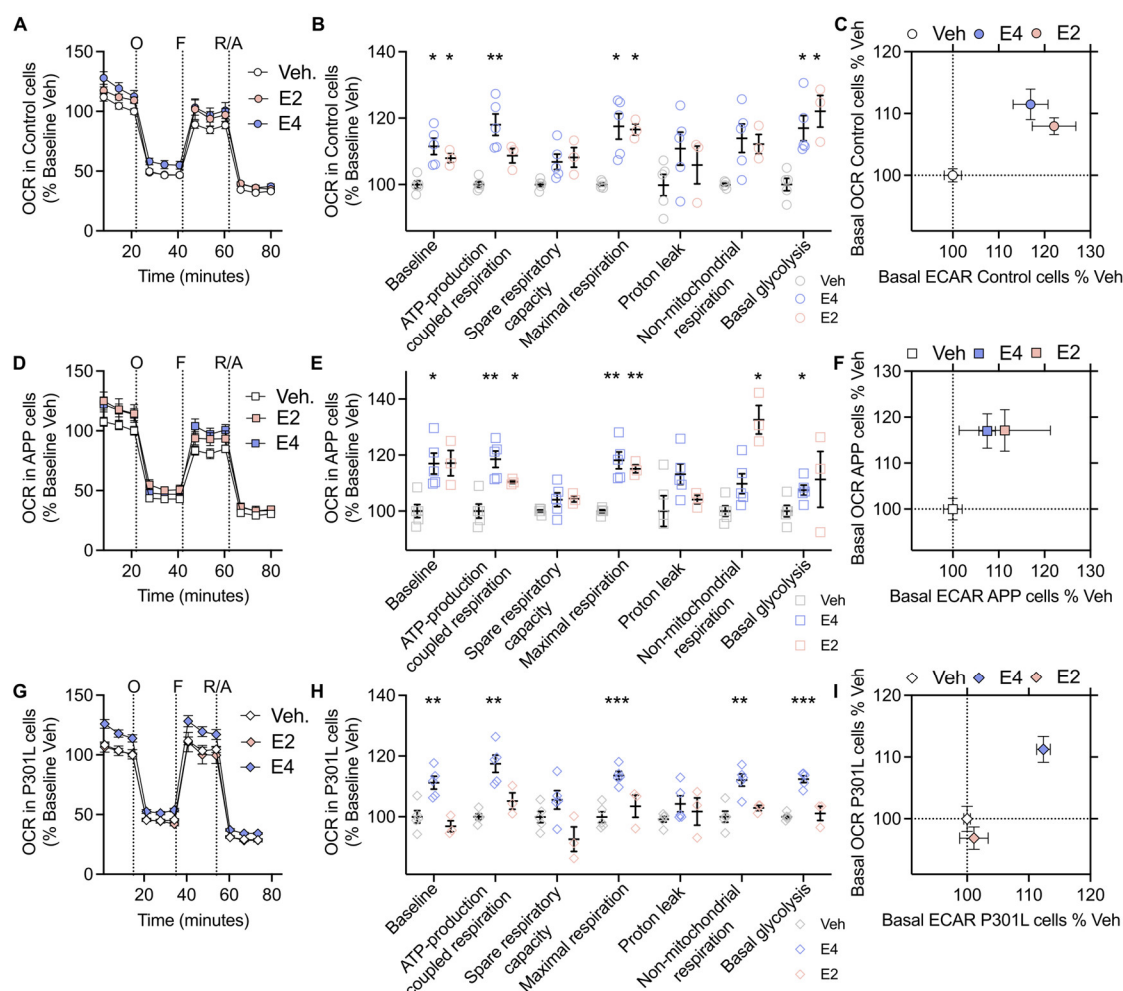


Figure 2. Effects of estretol (E4) on mitochondrial respiration parameters in the Control, APP and P301L cells. Cells were treated with vehicle alone (Veh = DMSO), E4 at 1 μ M, or E2 at 0.1 μ M for 48 h. (A,D,G) Oxygen consumption rate (OCR) was measured after 48 h of treatment in Control (A), APP (D), and P301L (G) cells using the Seahorse XF HS Mini Analyzer (Agilent). Sequential injection of mitochondrial inhibitors, namely oligomycin (O), FCCP (F), and rotenone/antimycin A (R/A), is indicated (see details in the Section 2). Data are presented as mean $\pm$ SEM as a percentage of the baseline (last point before oligomycin injection) of the vehicle (Veh) condition. (B,E,H) Values corresponding to different respiratory parameters and basal glycolysis in Control (B), APP (E) and P301L (H) cells are presented as mean $\pm$ SEM of 3–5 independent experiments, expressed as a percentage of the vehicle (Veh) condition. In addition, the mean of each independent experiment (biological replicates) is presented as circles (for Control cells), squares (for APP cells), or diamonds (for P301L cells). (C,F,I) Energy maps depict the basal OCR versus basal extracellular acidification rate (ECAR) in Control (C), APP (F) and P301L (I) cells after 48 h of treatment. Values represent mean $\pm$ SEM of ECAR on the abscissa versus the mean of the OCR on the ordinate, expressed as a percentage of the Veh-treated group. Total number of technical replicates: $n = 9$ –24 (B); $n = 4$ –18 (E); and $n = 6$ –24 (H). All data were normalized to CellTracker Blue fluorescence intensity, corresponding to the area of living cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA versus Veh. Veh: vehicle, E2: estradiol, E4: estretol.

In APP cells, E4 significantly increased basal respiration (+16%), mitochondrial respiration coupled with ATP production (+18%), maximal respiration (+18%), and basal glycolysis (+7%) (Figure 2E). E2 significantly increased mitochondrial respiration coupled with ATP production (+10%), maximal respiration (+15%) and non-mitochondrial respiration (+32%) (Figure 2E). Interestingly, while E2 effectively reduced superoxide anion levels in APP cells (Figure 1H), it concomitantly decreased MMP (Figure 1G) and increased non-mitochondrial respiration (Figure 2E). This suggests that E2 may drive a compensatory oxygen-consuming detoxification mechanism that scavenges ROS but may also affect mitochondrial bioenergetics in APP cells. In contrast, E4 increased mitochondrial respiration, ATP levels, and MMP and decreased ROS in APP cells without necessitating elevated non-mitochondrial oxygen consumption (Figures 1F–H and 2E). The bioenergetic map revealed that both E4 and E2 enhanced basal OCR and basal ECAR (Figure 2F).

In P301L cells, only E4 significantly increased basal respiration (+11%), ATP-production coupled respiration (+17%), maximal respiration (+13%), non-mitochondrial respiration (+12%) and basal glycolysis (+12%) compared with Veh (Figure 2H). In addition, E4 increased both basal OCR and basal ECAR, while E2 showed no significant effect (Figure 2I). These data suggest that E4 stimulates a global upregulation of cellular bioenergetics, simultaneously enhancing mitochondrial oxidative phosphorylation (OxPhos) and glycolytic flux to restore metabolic flexibility. While E2 treatment elicited a significant hyperpolarization of the MMP (Figure 1K), this was not paralleled by an increase in ATP production or respiratory flux in P301L cells. This suggests that E2 effectively increases the MMP but may fail to engage the machinery required for ATP synthesis.

Taken together, these findings demonstrate that E4 consistently enhances key bioenergetic parameters, including cell viability, ATP production, MMP, and oxidative metabolism, across Control, APP, and P301L SH-SY5Y cells. While both E4 and E2 positively modulate mitochondrial function in control and APP cells, E4 shows superior efficacy in the P301L model, significantly improving mitochondrial respiration and glycolysis, whereas E2 exerts less effect than E4. This suggests that E4 may provide broader and more potent support for cellular energy metabolism, particularly in neurodegenerative contexts such as tauopathy.

3.3. E4 Effects on Mitochondrial Network Morphology

Improved mitochondrial bioenergetics is often coupled with changes in mitochondrial network morphology [23]. APP and P301Ltau overexpression have previously been shown to alter mitochondrial network morphology. Indeed, APP overexpression induced mitochondrial fragmentation, whereas P301Ltau overexpression triggered mitochondrial network elongation in vitro and in vivo [19,24–26]. In the present study, we did not observe a significant difference in mitochondrial length or branching (aspect ratio or shape complexity) between Control and APP cells (Figure 3A, Supplementary Figure S2D). However, P301L cells presented a significant decrease in mitochondrial branching ($p = 0.02$, t -test versus Control cells) and an increase in mitochondria length ($p < 0.001$, t -test versus Control cells), indicating an elongation of mitochondria, in line with previous reports [19] (Supplementary Figure S2D).

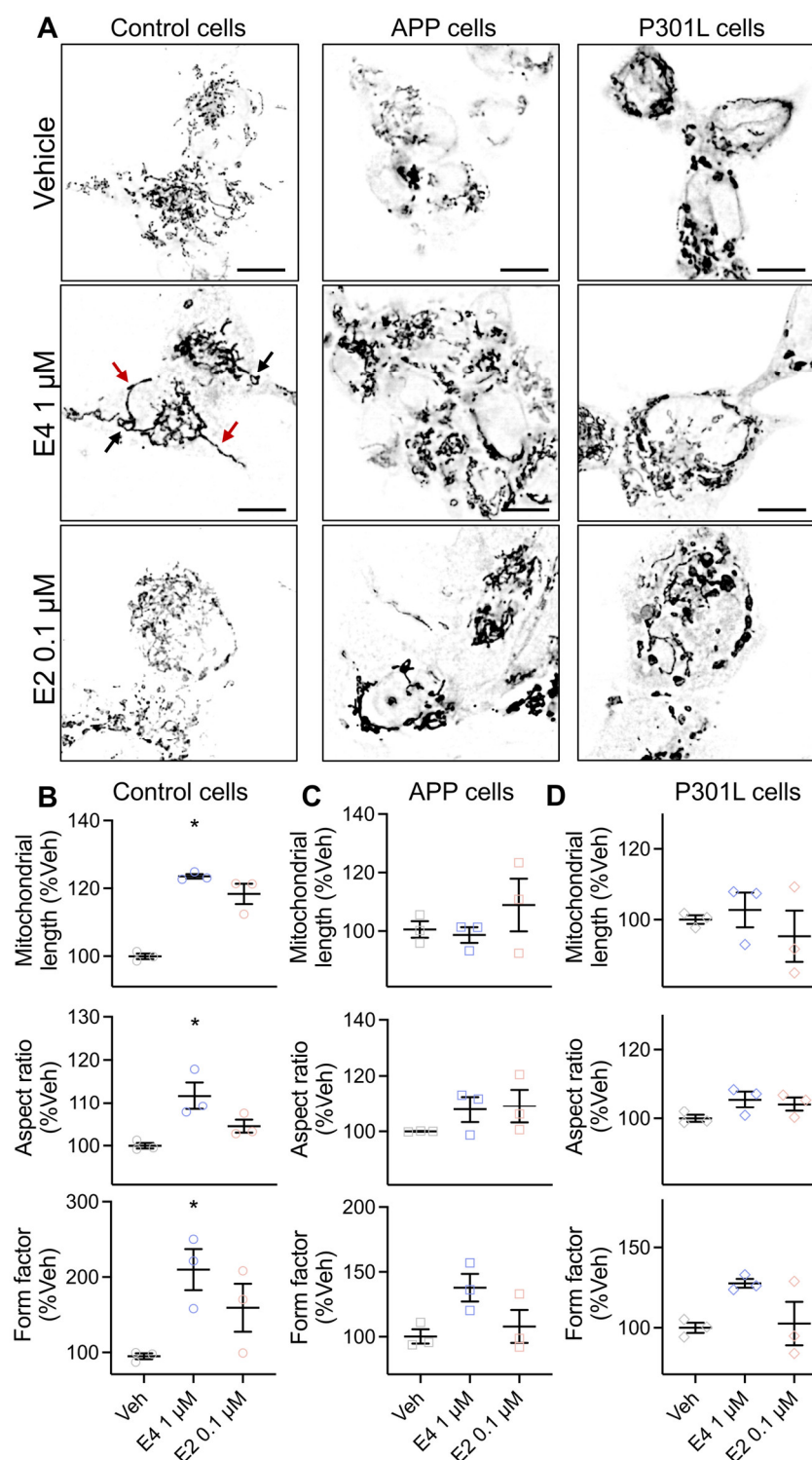


Figure 3. Effects of estretol (E4) on mitochondrial network shape in Control, APP and P301L cells. Cells were treated with vehicle alone (Veh = DMSO), E4 1 μ M, or E2 0.1 μ M for 48 h. Then, cells were stained with the mitochondrial dye MitoBrilliant-647 to visualize mitochondria and perform morphometric analysis using Fiji (see details in the Section 2). **(A)** Representative images of MitoBrilliant-647-stained mitochondria (inverted gray LUT, false color) from Control (left panels), APP (middle panels), and P301L (right panels) cells. In the image corresponding to the E4-treated group of the Control cells (left panel, middle image), red arrows indicate elongated mitochondria, and black arrows indicate branched or non-linear mitochondrial shapes. **(B–D)** Morphometric analysis of the mitochondrial network, including mitochondrial length (top), aspect ratio (middle), and form factor (bottom) in Control **(B)**, APP **(C)**, and P301L **(D)** cells after 48 h of treatment with Veh, E2, or E4. Data are presented as the

mean and SEM of three independent experiments, expressed as a percentage of the vehicle (Veh) condition. In addition, the mean of each independent experiment (biological replicates) is presented as circles (for Control cells), squares (for APP cells), or diamonds (for P301L cells). Total number of cells analyzed per condition: $n = 48\text{--}89$ (B); $n = 55\text{--}126$ (C); and $n = 70\text{--}86$ (D). Please see Supplementary Figure S3 for the uncropped images. * $p < 0.05$, one-way ANOVA + Kruskal–Wallis multiple comparison test versus Veh. Veh: vehicle, E2: estradiol, E4: estetrol.

Morphometric analysis of the mitochondrial network was performed after treatment with vehicle, E4, or E2 and included the following parameters: mitochondrial length; aspect ratio, an indicator of mitochondrial fusion/fission dynamics, with a higher aspect ratio indicating more branched and fused mitochondria; and form factor, an indicator of mitochondrial shape complexity, with a higher form factor indicating more complex, branched, or elongated shapes, while a lower value indicates a spherical or fragmented shape. E4 significantly increased mitochondrial length (+23%), aspect ratio (+11%), and form factor (+109%) in Control cells (Figure 3A,B, Supplementary Figure S3A), while E2 showed no significant effect. In APP and P301L cells, none of the treatments significantly modulated mitochondrial shape (Figure 3C,D, Supplementary Figure S3B,C).

Interestingly, although E4 promoted mitochondrial elongation in Control cells, it did not affect mitochondrial morphology in APP or P301L models. However, despite this lack of structural modulation, E4 successfully increased bioenergetic parameters in APP and P301L cells (Figures 1 and 2). This dissociation between structure and function suggests that E4-mediated neuroprotection does not rely on restoring the macroscopic mitochondrial network but rather on optimizing OxPhos efficiency under pathological conditions.

3.4. E4 Increases Neurite Outgrowth in Control, APP, and P301L SH-SY5Y Cells

Our data demonstrate that E4 improved ATP production in Control, APP, and P301L cells after 48 h of treatment. As ATP synthesis is essential for neuronal functions, including the building of neuronal networks via neurite outgrowth, a morphological analysis was performed to assess neurite outgrowth parameters in Control, APP, and P301L cells after two days of treatment with E4 1 μM , E2 0.1 μM (for comparison), and NGF 50 ng/mL (positive control). Namely, the average neurite length, the average neurite count, and the neurite area were assessed after treatment. Of note, compared with Control cells, APP and P301L cells exhibited shorter and fewer neurites (Figure 4A,E,I, Supplementary Figure S2E,F).

In Control cells, we observed a 30% increase in neurite length in E4-treated cells (Figure 4B), paralleled by a 6% increase in neurite count (Figure 4C) and a 29% increase in neurite area (Figure 4D), compared with the Veh-treated group. Although E2 induced a 20% increase in neurite length, it did not reach statistical significance and showed no effect on the other parameters (Figure 4B–D). In comparison, the positive control NGF induced a 42% increase in neurite length, a 9% increase in neurite count, and a 45% increase in neurite area.

In APP cells, E4 induced a significant increase in neurite length, neurite count, and neurite area by 16%, 9%, and 25%, respectively, compared with the vehicle (Figure 4E–G). E2 also increased the neurite length (+16%) and neurite count (+9%), but not the neurite area. NGF was again the most potent in APP cells, increasing neurite length, neurite count, and neurite area by 19%, 10%, and 49%, respectively.

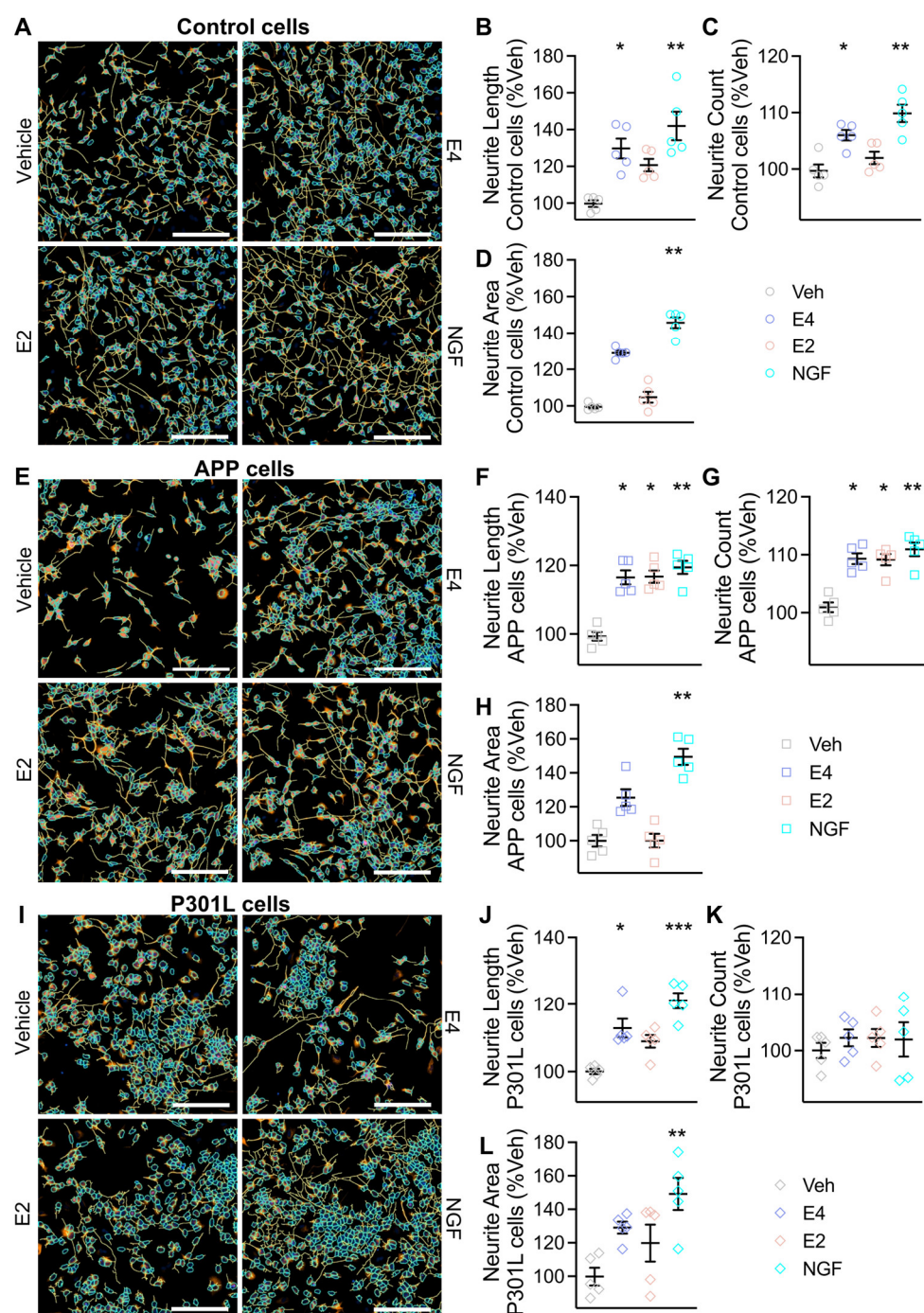


Figure 4. Effects of estetrol (E4) on neurite outgrowth in Control, APP, and P301L cells. After three days of differentiation in neurobasal medium, 2% B27 and 10 μ M retinoic acid, cells were treated with vehicle alone (Veh = DMSO), E4 1 μ M, E2 0.1 μ M or NGF (positive control) 50 ng/mL for 48 h. (**A,E,I**) Representative images showing neurite outgrowth in Control (**A**), APP (**E**), and P301L (**I**) cells after treatment. Cells were fixed with 4% PFA and stained with β III-tubulin (red color) and DAPI (nuclei in blue) to visualize the soma and neurites. All images were captured at 20 $\times$ magnification on the Agilent BioTek Cytation 5 instrument, and image analysis was performed with the Gen5 software Neurite Outgrowth module. A mask was generated to detect the soma (light blue mask) and neurites (yellow mask) (see details in the Section 2). (**B,F,J**) Average Neurite Length, determined by the Neurite Outgrowth module, in Control (**B**), APP (**F**), and P301L cells (**J**) after 48 h of treatment. (**C,G,K**) Average Neurite Count, determined by the Neurite Outgrowth module, in Control (**C**), APP (**G**), and P301L cells (**K**) after 48 h of treatment. (**D,H,L**) Total neurite area, determined by the Neurite Outgrowth module, in Control (**D**), APP (**H**), and P301L cells (**L**) after 48 h of treatment. Data are presented as the

mean and SEM of five independent experiments and expressed as a percentage of the vehicle (Veh) condition. In addition, the mean of each independent experiment (biological replicates) is presented as circles (for Control cells), squares (for APP cells), or diamonds (for P301L cells). Total number of images analyzed per condition: $n = 66\text{--}132$ (B–D), $n = 64\text{--}124$ (F–H), and $n = 52\text{--}117$ (J–L). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA + Kruskal–Wallis multiple comparison test versus Veh. Veh: vehicle, E2: estradiol, E4: estetrol, NGF: nerve growth factor.

In P301L cells, E4 significantly increased neurite length (+12%) and area (+29%) but did not affect neurite count compared with the vehicle group (Figure 4I–L). Similarly, NGF induced a 21% increase in neurite length and a 49% increase in neurite area but did not affect neurite count in P301L cells, indicating a limited capacity of tau-mutant cells to generate additional neurites. E2 did not significantly change any of the neurite outgrowth parameters in P301L cells.

Taken together, our data demonstrate that E4 significantly promotes neurite outgrowth in both healthy and AD-pathology cells, mimicking the profile of the positive control (NGF), albeit with slightly lower potency. In contrast, E2 failed to reach significance in Control cells and showed no effect in the P301L model. In addition, in P301L cells, where the capacity to initiate new neurites appears blocked (no change in count even with NGF), E4 successfully induced the elongation of existing neurites, proving it can stabilize the cytoskeleton even in the presence of tau pathology.

3.5. E4 Modulates Cell Bioenergetics via Estrogen Receptor Activation.

To determine through which main pathway (genomic or non-genomic) E4 modulates mitochondrial bioenergetics, selective antagonists of different estrogen receptors (ERs) were used, including Fulvestrant (an antagonist of estrogen receptors ER α and ER β), methyl-piperidino-pyrazole (MPP, a selective antagonist of ER α), PHTPP (a selective antagonist of ER β), and G-15 (an antagonist of the G protein-coupled estrogen receptor GPER1). Cells were first pretreated with ER antagonists for 1 h and then treated with E2 and E4 for 48 hr. ATP levels were then used as a readout to assess whether ER inhibition abolished E4's effect on mitochondrial bioenergetic activity.

In the Control cells, E2 and E4 significantly increased ATP levels (Figure 5A), confirming previously obtained results (Figure 1B). None of the ER antagonists affected ATP levels in the Control cells per se (Figure 5A, gray circles). However, all ER antagonists abolished the effects of E2 and E4 on ATP levels.

The same experiments were performed in APP cells. Treatment with ER antagonists alone did not alter ATP levels (Figure 5B, gray squares). All antagonists reduced or abolished E4's effects on ATP levels. MPP, Fulvestrant, and G-15 also reduced the effects of E2, but not PHTPP, suggesting that E2 does not act via ER β in APP cells.

Finally, in P301L cells, none of the ER antagonists affected ATP levels per se (Figure 5C). All antagonists decreased or abolished the effects of E2 and E4 on ATP levels, with varying efficacy. In particular, Fulvestrant and G-15 reduced ATP levels by 7% and 8%, respectively, in the E4-treated cells.

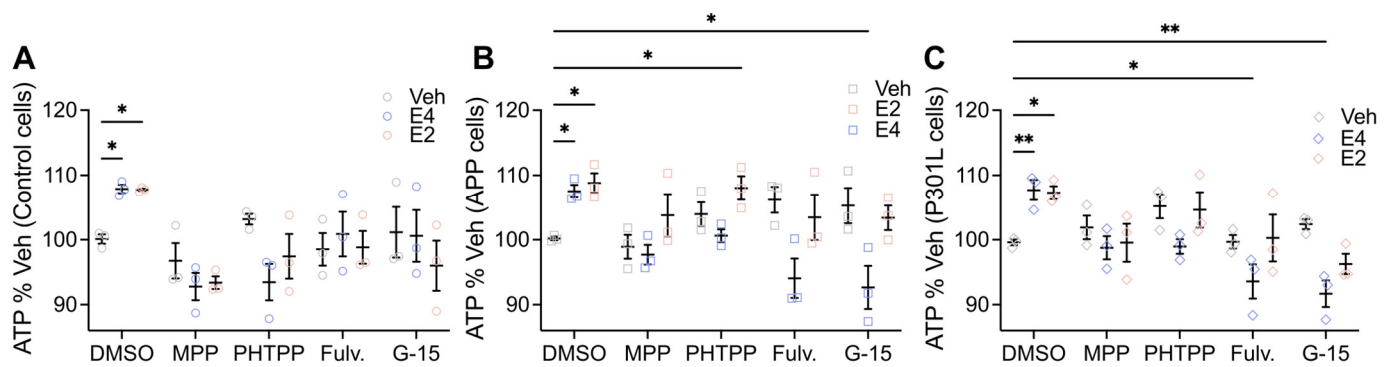


Figure 5. Impact of estrogen receptor antagonists on the E2- and E4-mediated increase in ATP levels in Control, APP and P301L cells. Cells were first pre-treated with 0.1 μ M MPP (ER α antagonist), 0.1 μ M PHTPP (ER β antagonist), 0.1 μ M fulvestrant (ER antagonist), or 0.1 μ M G-15 (GPER1 antagonist) for 1 h and then treated with vehicle alone (Veh = DMSO), E2 0.1 μ M, and E4 1 μ M for 48 h. Data represent the ATP levels as mean $\pm$ SEM of three independent experiments, expressed as a percentage of the vehicle (Veh) condition. In addition, the mean of each independent experiment (biological replicates) is presented as circles (for Control cells), squares (for APP cells), or diamonds (for P301L cells). Total number of technical replicates per condition: $n = 11$ –36 (A); $n = 11$ –36 (B); and $n = 12$ –32 (C). All the data were normalized on the CellTracker Blue fluorescence intensity, which corresponds to the area of living cells. * $p < 0.05$, ** $p < 0.01$, two-way ANOVA versus Veh/DMSO. MPP: Methyl-piperidino-pyrazole, E2: estradiol, E4: estetrol.

These findings indicate that E4 modulates mitochondrial bioenergetics via estrogen receptor-dependent mechanisms. In all three cell models, Control, APP, and P301L, pharmacological inhibition of ER α , ER β , or GPER1 abolished or significantly reduced the E4-induced increase in ATP production. This suggests that E4 engages both classical nuclear estrogen receptors (ER α and ER β) and the membrane-associated GPER1 to exert its bioenergetic effects. The consistent involvement of multiple ER pathways across models supports the idea that E4 acts through a broad, receptor-mediated mechanism to modulate mitochondrial function.

3.6. E4 Modulates Mitochondria-Related Gene Expression

To assess the effects of E4 on mitochondrial gene expression, RT² Profiler PCR arrays targeting 164 mitochondrial-related genes were used in Control, APP, and P301L cell lines. Only one gene, SLC25A21, could not be detected and is likely not expressed in SH-SY5Y cells. The complete list of genes is available in Supplementary Tables S1 and S2.

Specifically, between 4 and 16 genes were differentially expressed in each condition, with variation in the number and direction of regulation depending on the genetic background (Control, APP, or P301L). Venn diagram analyses showed that, while E4 influenced gene expression in a cell line-specific manner, subsets of regulated genes were shared across multiple models (Supplementary Figure S4A). Volcano plot analysis identified a subset of genes that were significantly up- or downregulated based on an FDR-adjusted q-value threshold of <0.10 (Figure 6A–C; Supplementary Table S3). Namely, E4 significantly influenced the expression of 10 mitochondria-related genes in Control cells, 4 in APP cells, and 16 in P301L cells. Functional enrichment analysis using g: Profiler revealed overrepresentation of pathways involved in OxPhos, respiratory chain complexes, electron transport, mitochondrial membrane composition, and transmembrane transport across the three cell lines (Figure 6D–F), indicating that E4 significantly impacts mitochondrial bioenergetics and structure in these models. Graphs of selected genes confirmed robust changes in expression following treatment in Control (Figure 6G), APP (Figure 6H), and P301L (Figure 6I) cells. Notably, NDUFA1 (NADH dehydrogenase ubiquinone A1 subcomplex) and SLC25A23

(Solute carrier family 25, mitochondrial/phosphate carrier, member 23) were commonly down-regulated and up-regulated, respectively, by E4 in the three cell lines.

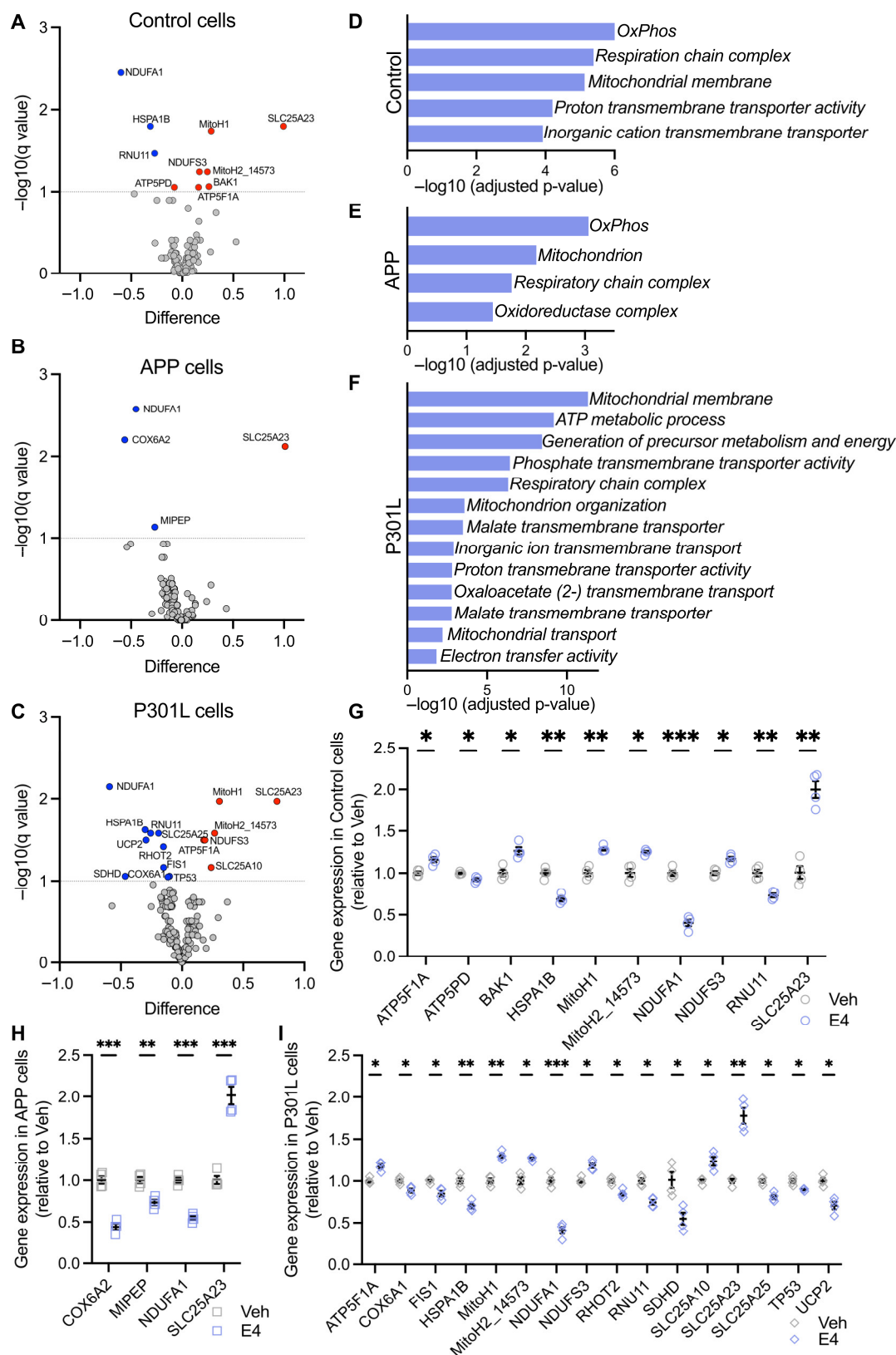


Figure 6. Effects of E4 on mitochondrial gene expression and associated pathways in Control, APP, and P301L cells. (A–C) Volcano plots showing significantly regulated genes in Control (A), APP (B), and P301L (C) cells following treatment with E4 compared with vehicle (Veh). The X-axis represents the $\log_2$ fold change (difference), and the Y-axis represents $-\log_{10}$ (adjusted q-value). Genes with FDR-

adjusted $q < 0.10$ are considered significantly regulated. Selected upregulated (red) and downregulated (blue) genes are highlighted. (D–F) Functional enrichment analysis of significantly regulated genes in Control (A), APP (B), and P301L (C) cells following E4 treatment using g:Profiler. The top enriched Gene Ontology terms are shown (GO:BP, GO:MF, GO:CC), plotted by $-\log_{10}$ (adjusted p -value). (G–I) Expression levels of selected significantly regulated genes after E4 relative to the vehicle in Control (G), APP (H), and P301L (I) cells. Data are presented as mean $\pm$ SEM of four biological replicates per group. In addition, each biological replicate is represented as a circle (Control cells), a square (APP cells), or a diamond (P301L cells). Significance was assessed using multiple unpaired t -tests and Holm–Šidák post hoc testing ($\alpha = 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Of note, genes involved in mitochondrial fusion/fission (DNM1L: dynamin 1-like protein; FIS1: fission 1; MFN1/2: mitofusin 1/2; OPA1: optic atrophy 1) were not significantly affected by E4 in Control and APP cells (Supplementary Table S3). In P301L cells, only FIS1 was slightly downregulated by E4 (Figure 6I), but this had no consequence on mitochondrial shape (Figure 3A,D). This suggests that the increased mitochondrial elongation observed in the Control cells after E4 treatment is not due to changes in the expression of fusion/fission genes but possibly to post-translational modifications or rapid signaling cascades that regulate the assembly and activity of the fission/fusion machinery at the mitochondrial membrane site. Similarly, the decrease in superoxide anion levels observed in APP and P301L cells after E4 treatment (Figure 1H,I) was not paralleled by increased expression of SOD1/2 (superoxide dismutase 1/2, Supplementary Table S3). This suggests that E4's antioxidant effects may be mediated by another mechanism, such as the increased SOD activity.

The same experiments were performed after E2 treatment for comparison (Supplementary Figure S4). Again, E2 influenced gene expression in a cell line-specific manner, but subsets of regulated genes were shared across the cell lines (Supplementary Figure S4A). Functional enrichment analysis revealed that E2 enriches processes related to mitochondrial membrane and transmembrane transporter activity (Supplementary Figure S4B–D). Graphs of selected genes illustrate expression patterns of genes identified as significant by the FDR method (Supplementary Figure S4E–G). Notably, two genes, MitoH2_12106 and MitoH2_14573, were up-regulated by E2 in the Control and P301L cells, and down-regulated in APP cells. Interestingly, we observed that E2 treatment significantly downregulated key genes of the electron transport chain and mitochondrial membrane integrity (Supplementary Figure S4F), including Complex I subunits (NDUFA1, A6, B6, S4, and S7) and complex IV (COX6A1 and A2). This may provide a molecular explanation for the observed decrease in MMP induced by E2 in APP cells (Figure 1G).

Thus, E4 selectively modulates mitochondria-related gene expression across Control, APP, and P301L SH-SY5Y cells, with the strongest effects observed in the P301L model. E4-regulated genes are involved in key mitochondrial pathways, including OxPhos and membrane transport. Notably, NDUFA1 and SLC25A23 were consistently affected in all cell models, highlighting E4's potential to modulate mitochondrial function in neurodegenerative contexts.

4. Discussion

The present study provides the first comprehensive characterization of the effects of E4 on mitochondrial bioenergetics and neuronal morphology in cellular models of AD. While the neuroprotective and mitochondrial effects of E2 are well-documented [27,28], the potential of E4, a fetal estrogen with a distinct safety profile, has remained largely unexplored in the context of neurodegeneration. Our findings demonstrate that E4 acts as a potent modulator of mitochondrial bioenergetics, significantly increasing ATP levels, MMP, and respiratory capacity in human SH-SY5Y cells (Figure 7). Notably, E4 exerted these beneficial effects not only under physiological conditions (Control cells) but also in cells modeling AD-

related amyloidopathy (APP cells) and tauopathy (P301L cells), displaying similar or even (in some parameters) superior efficacy compared with E2. Furthermore, we identified that these effects are mediated by broad engagement of estrogen receptors (ERs) and are accompanied by modulation of specific mitochondrial gene networks (Figure 7).

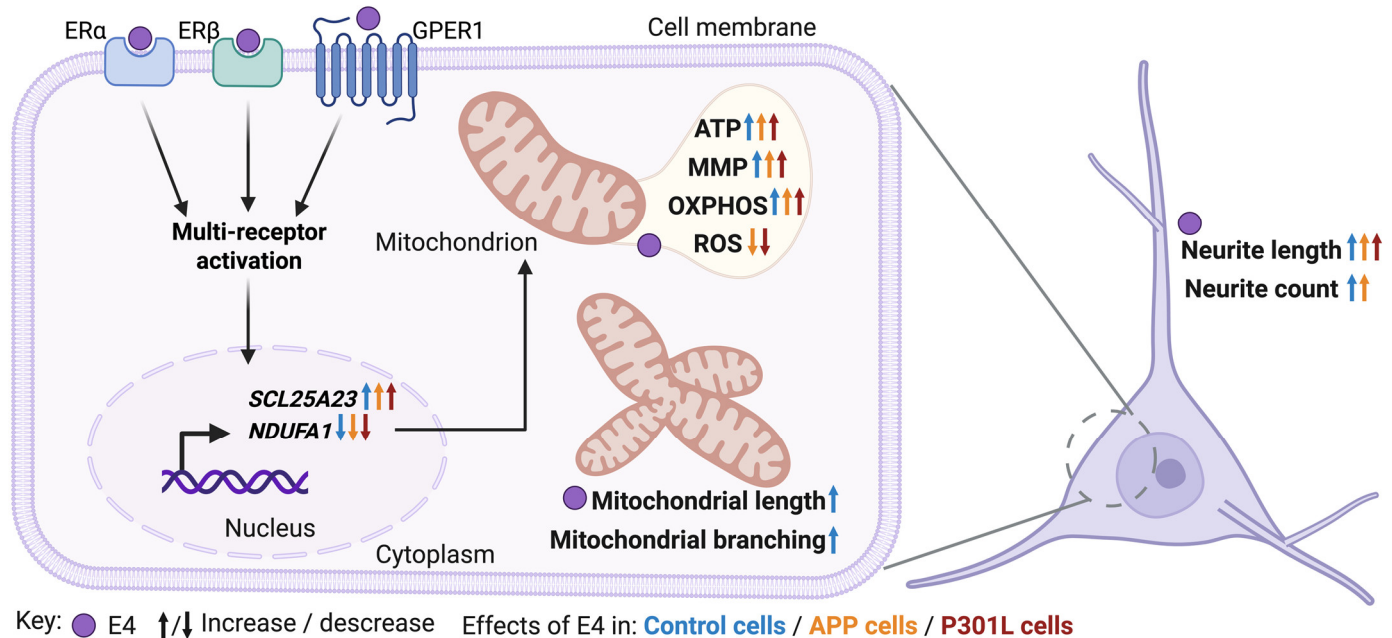


Figure 7. Overview of the effects of E4 on mitochondria and neurite outgrowth in healthy and AD-related conditions. E4 activates a specific multi-receptor network involving ER α , ER β , and GPER1. This activation modulates mitochondrial gene expression, notably upregulating the phosphate carrier SLC25A23 and downregulating NDUFA1. These genomic changes may contribute to the enhancement of mitochondrial bioenergetics and the promotion of mitochondrial fusion. Because neurite extension is an energy-demanding process, the E4-induced energy boost may also be linked to the increase in neurite outgrowth observed in our study. Effects of E4 in the specific cell lines are indicated by blue arrows for the Control cells, orange arrows for the APP cells, and red arrows for the P301L cells. ATP: adenosine triphosphate, MMP: mitochondrial membrane potential, OXPHOS: oxidative phosphorylation, ROS: reactive oxygen species, ER α /ER β : estrogen receptor α / β , GPER1: G-protein coupled estrogen receptor 1.

Mitochondrial dysfunction is an early and consistent feature of AD, characterized by deficits in OxPhos and ATP production that precede plaque and tangle formation [11]. E2 has been shown to improve mitochondrial respiration in several in vitro and in vivo AD models (reviewed in [2,27]). We previously demonstrated that the sex steroid hormones, including E2 and E1 at 100 nM, improved ATP levels, MMP, and mitochondrial respiration parameters in Control SH-SY5Y cells [4] and in APP- or P301L-overexpressing SH-SY5Y cells [3]. In the present study, we demonstrated that E4 improves mitochondrial bioenergetics in both APP and P301L cells. While these findings provide novel mechanistic insights, we acknowledge that they were obtained in human neuroblastoma-derived SH-SY5Y cell lines. Although these cells do not fully recapitulate the electrophysiological complexity of primary neurons, they are a well-characterized model for AD-related mitochondrial dysfunction [3,17,19,20]. Importantly, we previously demonstrated that neuroactive steroids elicit comparable bioenergetic responses, specifically regarding ATP production and oxygen consumption rates, in SH-SY5Y cells and primary murine cortical neurons [4]. This conserved metabolic response strongly supports the validity of the SH-SY5Y model for investigating steroid-mediated mitochondrial regulation. Notably, our study was designed to

assess the modulatory capacity of E4 relative to the vehicle-treated baseline in each cell model rather than to quantify a direct restoration of absolute bioenergetic values to healthy control levels. Thus, while we do not claim a complete “rescue” to a physiological baseline, our data conclusively demonstrate that E4 robustly boosts mitochondrial function in the presence of AD-related pathological proteins. Moreover, although the magnitude of bioenergetic upregulation by E4 may appear moderate relative to acute pharmacological stimulants, it is consistent with our previous findings on sex steroid modulation of neuronal bioenergetics [3]. Even a 10–15% sustained increase in bioenergetic efficiency significantly increased neurite outgrowth. E4 also induced a metabolic shift toward a more active state by enhancing both mitochondrial respiration (OCR) and glycolysis (ECAR), supporting the metabolic flexibility essential for neuronal function. Notably, in the P301L tauopathy model, E4, but not E2, significantly improved basal and maximal respiration. This suggests E4 offers a unique therapeutic advantage against tau-mediated mitochondrial toxicity. These findings align with our previous report that estrogens preferentially alleviate tau- over APP-related bioenergetic impairments [3], while highlighting E4’s superior efficacy in this context. Furthermore, E4 reduced superoxide levels, demonstrating antioxidant properties likely linked to its phenolic structure [15]. Because SOD1 and SOD2 gene expression remained unchanged in our study, E4 might also enhance antioxidant defenses via activation of SOD, similar to the activity-boosting effects of other estrogens in this model [4].

Regarding mitochondrial morphology, E4 promoted mitochondrial elongation and branching in Control cells, a phenotype linked to efficient ATP production [23], and maintained network integrity in the AD models, although no significant morphological changes were observed. Notably, E2 did not impact mitochondrial dynamics in our study. Because neither hormone altered the expression of key fusion/fission genes, the morphogenic effects of E4 likely arise from distinct post-translational modifications rather than from the transcriptional regulation previously reported for E2 [29,30].

The E4-mediated bioenergetic boost observed in our study likely drove structural recovery, as E4 significantly enhanced energy-demanding neurite outgrowth across all cell lines. Notably, while E2 only increased neurite outgrowth in APP cells, E4’s broader efficacy mirrored the neurotrophic effects of NGF in Control cells and in both AD models. Although E2 has been shown to stimulate neuritogenesis via ER-mediated kinase cascades (e.g., PI3K/Akt, MAPK/ERK) and WNT signaling [2,31–33], our data suggest that E4 engages these broad ER-dependent pathways to promote cytoskeletal reorganization and maintain neuronal connectivity. Future investigations are needed to explore the spatial and functional dynamics of this rescue. Specifically, assessing mitochondrial trafficking within neurites and evaluating synaptic connectivity (e.g., via synaptophysin markers and electrophysiological recordings in human iPSC-derived models) will be crucial to understanding how E4-mediated metabolic recovery translates into functional neuronal networking.

E4 has been shown to act as a nuclear ER α agonist in the liver [34] and as a GPER1 ligand in breast cancer cells [35], eliciting anti-migratory and anti-invasive effects. In our neuronal models, E4 effects were completely abolished by antagonists of ER α , ER β , and GPER1. This indicates that E4 requires the concurrent engagement of this broad receptor network, likely to drive rapid, non-genomic signaling pathways vital for mitochondrial calcium handling and function [36,37]. Interestingly, E2’s effects were similarly abolished by all antagonists, except by the ER β inhibitor (PHTPP) in APP cells. This highlights a key pharmacological distinction: while APP overexpression may alter ER expression ratios, allowing E2 to bypass ER β blockade via redundant ER α /GPER1 signaling, E4 lacks this redundancy and relies strictly on the cooperative integrity of the entire estrogen receptor network for its neuroprotective efficacy.

Mechanistically, E4 distinctly regulated specific mitochondrial genes compared to E2. The phosphate/calcium carrier SLC25A23 was consistently upregulated by E4 across all cell

lines (and by E2 in Control and APP cells). Because inorganic phosphate import is rate limiting for ATP synthesis, SLC25A23 upregulation provides a strong mechanistic link to the observed bioenergetic boost. Conversely, the Complex I subunit NDUFA1 was downregulated by E4, likely reflecting compensatory remodeling of the electron transport chain. Promoter analysis (Supplementary Table S4) revealed that both genes lack strong canonical estrogen response elements (EREs) but contain multiple high-confidence SP1 sites, suggesting non-classical regulation via ER-SP1 tethering. Crucially, the SLC25A23 promoter also harbors a highly significant $ERR\alpha/ERR\beta$ motif, which NDUFA1 lacks. This SP1-only configuration in NDUFA1 may explain its repression, whereas SLC25A23 undergoes direct transcriptional activation via estrogen-related receptors. Indeed, previous genomic data validate SLC25A23 as a direct physical target of $ERR\alpha$ in mitochondrial metabolism [38]. Together, these findings indicate that E4 enhances ATP transport by engaging conserved ERR-mediated machinery and non-canonical transcription factor crosstalk, rather than through direct ER α binding.

The search for safe therapies to delay AD onset is urgent. While E2 is neuroprotective, its clinical use is hindered by oncologic and thrombotic risks. Furthermore, the efficacy of estrogen replacement therapy (ERT) is tied to a “critical period” or “window of opportunity” for therapeutic intervention [10]. Research indicates that ERT may be primarily beneficial if initiated early in endocrine aging, specifically during perimenopause and early postmenopause, before irreversible cellular damage accumulates. Intervening during this crucial window may prevent cognitive decline and decrease dementia risk. This hypothesis plausibly explains the failure of large-scale trials such as the Women’s Health Initiative (WHI), which enrolled older postmenopausal women (mean age > 63), thereby missing the neuroprotective window [39]. Beyond the timing of intervention, ERT safety and efficacy depend significantly on formulation, posology, and route of administration (e.g., transdermal vs. oral). Oral administration of E2 or ethinylestradiol (EE) is often associated with hepatic first-pass effects, increasing the synthesis of coagulation factors and sex hormone-binding globulin (SHBG), thereby heightening the risk of venous thromboembolism (VTE) [14]. In contrast, transdermal E2 formulations are associated with superior cognitive outcomes, likely by maintaining stable physiological hormone levels and avoiding hepatic metabolism [40]. Crucially, these benefits are maximized when therapy is initiated within five years of menopause, further supporting the critical window hypothesis for neuroprotection [40]. Despite estrogen’s neuroprotective potential, using ERT to prevent dementia remains heavily debated due to conflicting clinical evidence. For instance, recent Women’s Health Initiative (WHI) data do not support ERT for preventing chronic diseases like dementia [41]. Conversely, retrospective analyses indicate that longer ERT duration and specific formulations significantly reduce AD risk [42]. This discrepancy highlights the critical need for precision medicine approaches tailored to patient age, formulation, and treatment timing.

In this context, E4 presents a compelling alternative. Clinical data indicate that E4 minimally affects liver metabolism, SHBG levels, and hemostasis compared with EE-containing products, thereby suggesting a lower thrombotic risk, and displays potentially reduced breast proliferation risk [15]. By coupling these safety advantages with the robust neuroprotection demonstrated here, E4 emerges as a highly promising candidate for long-term preventive strategies in menopausal women. Ultimately, our data provide the first preclinical evidence that E4 improves mitochondrial health and neuronal integrity in the context of AD pathology, potentially offering a safer “window of opportunity” intervention than current standard-of-care estrogens.

5. Conclusions

This study establishes E4 as a potent modulator of neuronal bioenergetics. By enhancing mitochondrial respiration, mitigating oxidative stress, and promoting neurite

outgrowth via a multi-receptor mechanism, E4 demonstrates significant neuroprotective potential (Figure 7). These findings serve as a mechanistic proof of concept requiring further validation in more physiologically relevant systems, such as primary neurons, human iPSC-derived neurons, and in vivo AD models. Additionally, while we demonstrated a robust rescue of the bioenergetic phenotype, the direct impact of E4 on A β load and tau aggregation dynamics remains to be elucidated. Collectively, these results support the continued clinical investigation of E4 as a promising therapeutic candidate for the prevention or treatment of neurological conditions driven by mitochondrial dysfunction, including AD.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells15050452/s1>, Supplementary Figures S1–S4; Supplementary Tables S1–S4. Figure S1: Chemical structure of Estradiol and Estetrol, and preliminary ATP and MTT assays; Figure S2: Impairments in APP- and P301L-overexpressing SH-SY5Y cells; Figure S3: Effects of estetrol (E4) on mitochondrial network morphology in Control, APP, and P301L cells; Figure S4: Effects of E2 on mitochondrial gene expression and associated pathways in Control, APP, and P301L cells; Table S1: Human Mitochondrial Energy Metabolism Pathway Plus; Table S2: Human Mitochondria; Table S3: PCR arrays analysis; Table S4: Promoter analysis.

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Abbreviations

The following abbreviations are used in this manuscript:

AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
ATP	Adenosine triphosphate
CTB	CellTracker Blue
DMSO	Dimethyl sulfoxide
E2	17 β -estradiol
E4	Estetrol
ECAR	Extracellular Acidification Rate
ER α	Estrogen Receptor α
ER β	Estrogen Receptor β

ERE	Estrogen Responsive Element
ERT	Estrogen Replacement Therapy
ETC	Electron Transport Chain
GPER1	G Protein-coupled Estrogen Receptor 1
MMP	Mitochondrial Membrane Potential
NDUFA1	NADH dehydrogenase ubiquinone 1 α subcomplex
OCR	Oxygen Consumption Rate
P301L	P301Ltau mutation
ROS	Reactive Oxygen Species
SLC25A23	Solute carrier family 25, mitochondrial/phosphate carrier, member 23

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