nutrients

MDPI

Article

Effect of Xanthohumol, a Bioactive Natural Compound from Hops, on Adenosine Pathway in Rat C6 Glioma and Human SH-SY5Y Neuroblastoma Cell Lines

Adrián Tejero, David Agustín León-Navarro * and Mairena Martín

Department of Inorganic and Organic Chemistry and Biochemistry, Faculty of Chemical Sciences and Technologies, Institute of Biomedicine, IDISCAM, University of Castilla-La Mancha, Avenida Camilo José Cela 10, 13071 Ciudad Real, Spain; adrian.tejero@uclm.es (A.T.); mairena.martin@uclm.es (M.M.)
* Correspondence: davidagustin.leon@uclm.es

Abstract: Xanthohumol (Xn) is an antioxidant flavonoid mainly extracted from hops (*Humulus lupulus*), one of the main ingredients of beer. As with other bioactive compounds, their therapeutic potential against different diseases has been tested, one of which is Alzheimer's disease (AD). Adenosine is a neuromodulatory nucleoside that acts through four different G protein-coupled receptors: A_1 and A_3, which inhibit the adenylyl cyclases (AC) pathway, and A_{2A} and $A_{2B,}$ which stimulate this activity, causing either a decrease or an increase, respectively, in the release of excitatory neurotransmitters such as glutamate. This adenosinergic pathway, which is altered in AD, could be involved in the excitotoxicity process. Therefore, the aim of this work is to describe the effect of Xn on the adenosinergic pathway using cell lines. For this purpose, two different cellular models, rat glioma C6 and human neuroblastoma SH-SY5Y, were exposed to a non-cytotoxic 10 µM Xn concentration. Adenosine A_1 and A_{2A}, receptor levels, and activities related to the adenosine pathway, such as adenylate cyclase, protein kinase A, and 5′-nucleotidase, were analyzed. The adenosine A_1 receptor was significantly increased after Xn exposure, while no changes in A_{2A} receptor membrane levels or AC activity were reported. Regarding 5′-nucleotidases, modulation of their activity by Xn was noted since CD73, the extracellular membrane attached to 5′-nucleotidase, was significantly decreased in the C6 cell line. In conclusion, here we describe a novel pathway in which the bioactive flavonoid Xn could have potentially beneficial effects on AD as it increases membrane A1 receptors while modulating enzymes related to the adenosine pathway in cell cultures.

Keywords: xanthohumol; adenosine receptors; cell cultures

check for updates

Citation: Tejero, A.; León-Navarro, D.A.; Martín, M. Effect of Xanthohumol, a Bioactive Natural Compound from Hops, on Adenosine Pathway in Rat C6 Glioma and Human SH-SY5Y Neuroblastoma Cell Lines. *Nutrients* **2024**, *16*, 1792. https://doi.org/10.3390/nu16111792

Academic Editors: Cristiana Caliceti and Paola Nieri

Received: 16 April 2024
Revised: 17 May 2024
Accepted: 4 June 2024
Published: 6 June 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Numerous natural bioactive molecules, such as polyphenols, flavonoids, alkaloids, and terpenes, have been discovered and studied as potential therapeutic candidates to act against neurodegenerative diseases [1]. One such compound belonging to the group of flavonoids is xanthohumol (Xn, 2′,4′,4-trihydroxy-6′-methoxy-3′-prenylchalcone), found in the hop flower (*Humulus lupulus*), one of the main ingredients of beer, which contributes at least 30% of its polyphenol content [2]. This prenylated chalcone has been reported to have anti-inflammatory [3,4], antioxidant [5–7], and antiproliferative properties [8] on various cell lines and animal models. It has also been postulated as a potential therapeutic agent against the neurodegeneration characteristic of Alzheimer's disease (AD), where it reduces the accumulation of amyloid β-peptide and the hyperphosphorylation of the tau protein in cellular models of AD [9].

Neurodegenerative diseases comprise a type of neurological abnormality in which progressive neuronal population loss occurs in the central nervous system (CNS). This loss leads to several alterations in functions such as movement, learning, and memory. AD is the most predominant neurodegenerative disease worldwide [10]. The pathogenesis, however,

has not yet been discovered, but several studies suggest that oxidative stress [11], mitochondrial dysfunction [12], excitotoxicity [13], and neuroinflammation [14] may be involved.

Excitotoxicity is a complex process that leads to neuronal loss and is triggered by overactivation of glutamate receptors due to an increased release of excitatory transmitters such as L-glutamate and the consequent intracellular calcium enhancement. Adenosine is a very well-known neuromodulator in the nervous system, where it can modulate the release of several neurotransmitters, including L-glutamate. Adenosine acts through four different types of receptors: A_1, A_{2A}, A_{2B}, and A_3. The activation of A_1 and A_3 receptors inhibits adenylate cyclase activity and reduces the level of glutamate released into the extracellular medium, whereas the activation of A_{2A} and A_{2B} causes an increase in extracellular glutamate levels [15].

Several studies carried out using human samples have reported that in AD, the A_1 receptor is decreased in some brain regions such as the hippocampus [16], temporal cortex, and thalamus [17], while the A_{2A} receptor is increased in studies performed in animal models of AD [18–21] and in AD patients [22]. However, this increase does not translate into hyperexcitability in AD-affected regions, where A_{2A}R preserves basal transmission [19,23]. Additionally, a previous study from our group has shown that the adenosinergic network is disrupted in the brains of human post-mortem AD patients [24].

Moreover, the adenosine network is involved in several pathological processes. The extracellular ATP release can act as a stress signal. Ecto-5′-nucleotidase, or CD73, converts AMP derived from this ATP into adenosine, which activates mainly A_{2A}R, causing an overactivation of this receptor [25]. The enzymes converting adenosine into AMP, adenosine kinases [26], and equilibrative nucleoside transporters (ENT) have also been studied in the search for potential inhibitors of this network, as they can act as therapeutic agents against neurological diseases [27].

The search for possible natural bioactive compounds that modulate the functionality of adenosine receptors could, therefore, be very helpful in the prevention and identification of new therapeutic molecules for AD. In that sense, previous work carried out in our group has shown that Xn causes a modulation at the level of gene expression of adenosine receptors in SH-SY5Y cells [28]. However, until now, it has not been fully reported how it could affect the adenosinergic pathway, including levels of these receptors in the membrane, functionality, or related enzymatic activities. Due to the importance of this pathway in neurodegenerative diseases, the present study aims to characterize the effect of Xn on the adenosinergic pathway in cellular models of rat C6 glioma and human SH-SY5Y neuroblastoma.

2. Materials and Methods

2.1. Cell Culture

C6 rat glioma and human SH-SY5Y neuroblastoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were grown in a humidified atmosphere consisting of 95% air, 5% CO_2, and at a temperature of 37 °C. For C6 cell culture medium, Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 1% antibiotic–antimycotic, 10% decomplemented fetal bovine serum (FBS) (PAA, Pasching, Austria), and 50 µg/µL gentamicin (Gibco, Waltham, MA, USA) was used. For the SH-SY5Y human cell line medium, DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic was used. At confluence, cells were detached from 10 mL Petri dishes (Nunc, Roskilde, Denmark) by mechanical action in the case of C6 or trypsin (TrypLE Express, Gibco, USA) in the case of SH-SY5Y cells. The detached cells were then transferred to other dishes as required depending on the experiment carried out, which included using 10 mL dishes for membrane isolation or 96-well dishes (Nunc, Roskilde, Denmark) for viability assays.

2.2. Cell Viability Assay

For cell viability measurement, an in vitro assay kit (Cell Proliferation Kit II, XTT, Roche, Manheim, Germany) based on the reduction of tetrazolium salt (XTT) to formazan,

delivering a colorimetric signal, was used. C6 and SH-SY5Y cells were detached and counted on a TC 20™ Automated Cell Counter (Bio-Rad, Madrid, Spain) and seeded in 96-well dishes (10^4 cells/well) for their exposure to xanthohumol (Sigma-Aldrich, Madrid, Spain) prepared following manufacturer indications at different concentrations during 24 h of treatment. Xn was solubilized in DMSO and then diluted in the corresponding cell medium, depending on the cell line. Then, the cells were incubated for 2 h with the XTT solution at 37 °C. The orange formazan color produced by viable cells was studied by recording the absorbance at 475 nm and 690 nm. Each single experiment was run in sextuplicate. Photographs were made with a digital camera (Leica DFC350FX) attached to a Leica DMI6000B (Leica Microsystems, Wetzlar, Germany) microscope.

2.3. RNA Isolation and cDNA Preparation

RNA isolation was performed using the PureLinkTM RNA Mini Kit from Invitrogen by Thermo Fisher Scientific (Waltham, MA, USA) (cat. number 12183018A) according to the manufacturer's protocol. RNA purity was confirmed by measuring absorbance at 260 nm and 280 nm (the A260/A280 ratio) and making sure it was between 1.8 and 2.0. After that, retrotranscription of RNA was performed with a High-Capacity cDNA Archive Kit from Applied Biosystems (Waltham, MA, USA). RNA was maintained for 10 min at 25 °C and then incubated for 2 h at 37 °C before interrupting retrotranscription at 85 °C for 5 s.

2.4. Quantitative Real-Time RT-PCR Analysis

Gene expression was quantified by quantitative real-time RT-PCR in an ABI Prism 7500 Fast SDS, using 20 ng of cDNA for each reaction and TaqMan Universal PCR Master Mix following the manufacturer's indications (Applied Biosystems, Foster City, CA, USA). The TaqMan probes used for experiments were for A_1 (Hs00181231_m1), for A_{2A} (Hs00169123_m1, Rn00583935_m1), for AC1 (Adenylate cyclase 1) (Rn02115682_s1), and for β-actin (Hs99999903_m1, Rn00667869_m1). The reaction was conducted at 95 °C for 20 s, then followed by 40 cycles at 95 °C for 3 s and 60 °C for another 30 s. Gene expression was analyzed using the 7500 Fast System SDS 1.3.1 program. Each gene expression level was normalized to its endogenous control and relativized according to a calibrator using the following equation: RQ = 2 $-\Delta\Delta$t = 2 − ((Ct target gene − Ct β-actin) sample − (Ct target gene − Ct β-actin) calibrator). All cDNA samples were run in duplicate, and the average represents a single value for each mRNA and sample.

2.5. Plasma Membrane Isolation Procedure

For cellular plasma membrane isolation, cells were detached from plates with a ThermoScientificTM NuncTM cell scraper. The cells were then resuspended and homogenized in an isolation buffer consisting of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and protease inhibitors, specifically phenylmethylsulfonyl fluoride and bacitracin at 100 μM and 100 μg/μL, respectively, in a Dounce homogenizer. Once samples were homogenized, they were centrifuged at 1000× *g* for 5 min and 4 °C in a Beckman JA 21 centrifuge (Coulter, Madrid, Spain). After that, the supernatant was rescued to perform another centrifugation, this time for 30 min at 27,000× *g* and 4 °C. Both fractions were collected: the supernatant corresponding to the cytosol and the pellet corresponding to the plasmatic membrane. Finally, the protein concentration of both fractions was quantified following the Lowry method, using bovine serum albumin (BSA) as a standard.

2.6. Western Blot Assay

Plasmatic membrane and cytosolic fractions were separated by 10% polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) as a detergent, loading 30 μg of protein from each sample. Then, the protein content in the gel was transferred to a nitrocellulose or PVDF membrane (iBlotTM Gel Transfer Stack, nitrocellulose, or iBlotTM Gel Transfer Stack PVDF, regular size) using the iBlotTM Dry Blotting System (Invitrogen, Barcelona, Spain). After blocking the membranes with 5% skimmed milk dissolved in

water for 60 min at room temperature, they were washed three times for 10 min each with phosphate-buffered saline (PBS) at 1× concentration. Subsequently, the membranes were incubated overnight at 4 °C while shaking with the following primary antibodies: Anti-Adenosine A_1 Receptor antibody (1:1000, ab124780 from Abcam, Cambridge, UK), Anti-Adenosine A_{2A} Receptor antibody (1:1000, ab79714 from Abcam), Anti-PKA antibody (1:1000, ABIN1532391 from Antibodies online, Limerick, PA, USA), β-Actin antibody (1:5000, ab8226 from Abcam), and GAPDH antibody (1:1000 from Abcam, ab8245) used as gel loading control. Once this time elapsed, three more washes with PBS were conducted before incubating with the secondary antibody for 1 h at room temperature. These antibodies were anti-rabbit (GAR) or anti-mouse (GAM) IgG, depending on the species in which the primary antibody was produced, coupled to the horseradish peroxidase enzyme (1:5000, GARPO 172-1019, and GAMPO 170-6516 from Bio-Rad). After this incubation, membranes were washed again three times with PBS, and the respective protein bands were visualized using the ECL chemiluminescent detection kit from GE Healthcare (Madrid, Spain), incubated for three minutes with the mixture of both reagents of the kit in a 1:1 ratio, and then revealed using a G:Box camera. Also, quantification of the optical density of the bands was conducted using Gene-Tools software version number 4.03.02.0 (Syngene, Bristol, UK). The results of the densitometry were expressed as percentages of control values.

2.7. Adenylate Cyclase Activity Assay

Adenylate cyclase activity determination in C6 and SH-SY5Y plasma membranes was performed with 20 μg of protein in a final volume of 250 μL of 50 mM Tris-HCl buffer pH 7.4, 10 mM creatine phosphate, 1 mM Dithiothreitol (DTT), 1 mg/mL BSA, 5 mM $MgCl_2$, 1 mg/mL creatine kinase, and 0.1 mM of the selective phosphodiesterase inhibitor Ro 20/1724. Previously, the plasma membrane samples were incubated with the adenosine deaminase enzyme (ADA) (0.2 U/mg protein) for 30 min at 37 °C to eliminate endogenous adenosine from the samples. Subsequently, they were incubated for 6 min at 37 °C in the absence (basal activity) or presence of 10 μM forskolin to stimulate the activity and 10 μM forskolin plus CPA at 10 μM to see the inhibition through the A_1 receptor, which could also activate A_2 receptors, followed by a 200 μM ATP incubation for 10 min at 37 °C. The samples were boiled at 100 °C for 3 min in order to stop the reaction. Then, centrifugation was carried out for 4 min at 12,000× *g* and 4 °C, and the supernatant (50 μL) was used for the determination of the 3′-5′ cyclic adenosine monophosphate (cAMP) accumulation. Briefly, samples were incubated with 0.25 pmol of [^{3}H] cAMP and 6.25 μg of AMP-dependent protein kinase (PKA) in a final assay volume of 200 μL of determination buffer (50 mM Tris-HCl pH 7.4, 4 mM ethylenediaminetetraacetic acid (EDTA)) for 2–18 h at 4 °C. Standard samples were prepared in the same buffer in the concentration range of 0–16 pmol. The reaction was stopped by rapid filtration through Whatman GF/B filters, previously preincubated with 0.3% polyethyleneimine using a Filter Mate Harvester (PerkinElmer, Shelton, CT, USA) for at least 30 min. Finally, for the radioactivity measurement, scintillation liquid was added to a Micro Beta JET counter (PerkinElmer) using Betaplate Scint (PerkinElmer). Results of basal activity are presented as pmol cAMP/mg protein·min, forskolin-mediated activity is presented as percentage respect to basal activity, and CPA-mediated inhibition is presented as percentage respect to forskolin-stimulated activity.

2.8. 5′-Nucleotidase Activity Assay

Membrane or cytosolic fractions (20 μg protein) from C6 or SH-SY5Y cell lines were preincubated in a reaction medium consisting of 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 9, at 37 °C for 10 min with shaking. The reaction was started with the addition of 10 mM AMP (500 μM final concentration) and stopped by the addition of 10% trichloroacetic acid 20 min later. Once stopped, samples were kept on ice (at 4 °C) for at least 10 min. Later, samples were centrifuged at 12,000 rpm for 4 min at 4 °C. Finally, 40 μL of each sample supernatant was collected to quantify the inorganic phosphate (Pi) release, using KH_2PO_4

as the Pi standard. All experiments were performed in duplicate. 5′-nucleotidase activity is expressed as nmol Pi released/min·mg protein.

2.9. Statistical and Data Analysis

Statistical analyses were performed using an unpaired two-tailed Student's *t*-test using GraphPad Prism 8. The results are expressed as the mean ± standard error of the mean (SEM). Differences between mean values were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of Xn on C6 and SH-SY5Y Cell Lines Viability

First, a study of the cell viability of C6 and SH-SY5Y cells in the presence of different concentrations of Xn was conducted in order to establish a proper concentration that has no severe toxicity to these cell lines. Previous results of our group showed no significant effect of 10 μM Xn in these cell lines; therefore, 5 and 15 μM Xn were tested [28]. Results presented in Figure 1 show a significant decrease in cell viability when both C6 and SH-SY5Y cells were exposed to 15 μM of Xn (C6: 100.0 ± 3.9 vs. 65.8 ± 3.1%, * $p < 0.05$; SH-SY5Y: 100.0 ± 4.7 vs. 77.8 ± 5.1%, * $p < 0.05$. Panels Figure 1B and Figure 1C, respectively). However, no significant changes in viability were observed when exposing cells to 5 or 10 μM of Xn. The changes in the number of cells were consistent with the results of the viability tests, as we can see in panels Figure 1D and Figure 1E for C6 and SH-SY5Y cell lines, respectively.

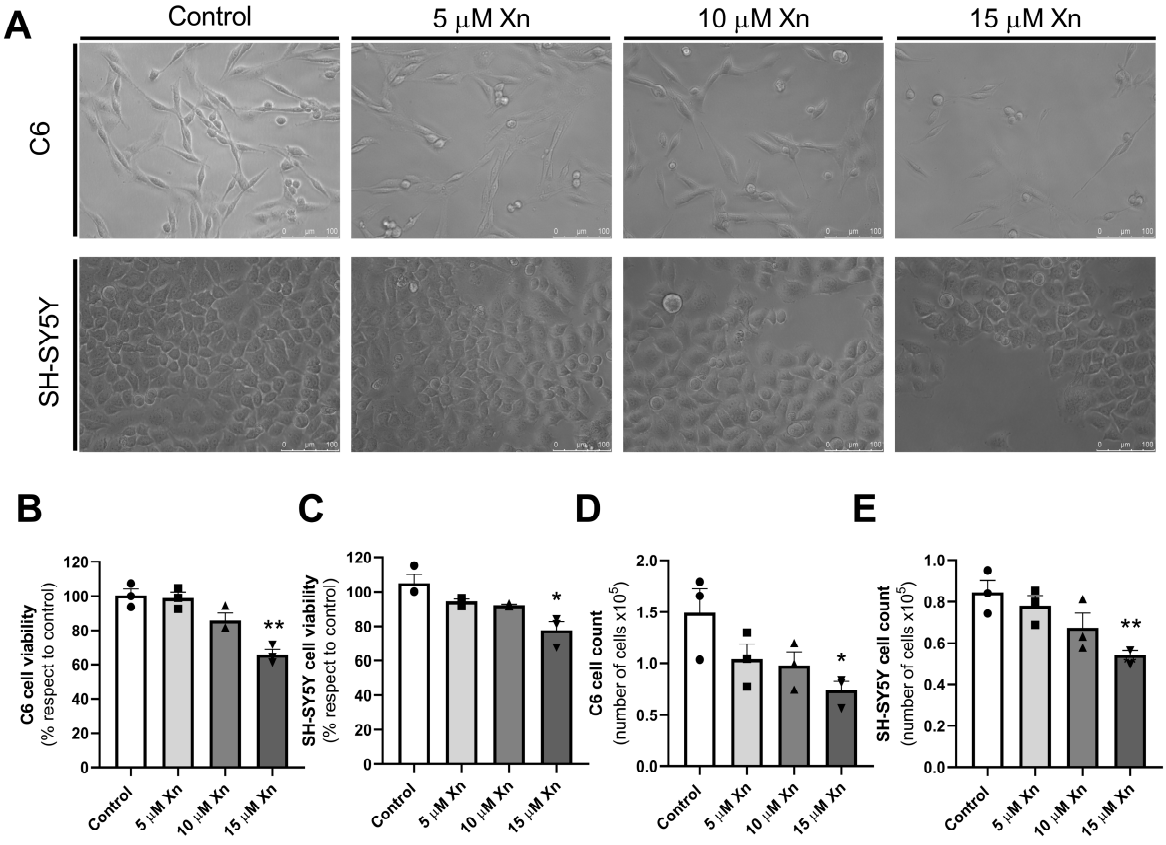


Figure 1. Effect of Xn on viability and cell number of C6 (panel (**A**) top row and panel (**B**) and (**D**), respectively) and SH-SY5Y (panel (**A**) bottom row and panel (**C**) and (**E**), respectively) cell lines. Cells were treated with different concentrations of Xn, and cell viability was assayed. Histograms showing data, expressed as percentages with respect to control values (**B**,**C**) or the number of cells $\times 10^5$ (**D**,**E**), correspond to the mean ± SEM of three different experiments carried out in sextuplicate. Individual data are shown in circles, squares and triangles. * $p < 0.05$ and ** $p < 0.01$ are significantly different from the control values according to the Student's *t*-test.

3.2. Effect of Xn on Adenosine Receptor Gene Expression

Once viability assays were conducted and after bibliography research to choose a concentration of Xn that had reported beneficial effects in other cell lines such as PC12 (0.1–10 μM), cementoblasts (10 nM–10 μM), or HT29 (0.2–8 μM) [7,29,30], a 10 μM concentration of Xn was chosen for subsequent experiments. As adenosine receptors have been widely studied in neurodegenerative pathologies, we wanted to investigate if this flavonoid was able to modulate the expression of these receptors in C6 and SH-SY5Y cell lines. Thus, RT-qPCR assays were performed. Also, adenylate cyclase 1 enzyme isoform expression was tested.

Results presented in Figure 2 show an increase in A_{2A} receptor gene expression (1.2 ± 0.1 vs. 3.0 ± 0.4 relative units, $p < 0.05$) in SH-SY5Y (panel D), while no changes were observed in the A_1 receptor measured in this cell line (panel B). On the other hand, $A_{2A}R$ expression in the C6 cell line was significantly reduced (0.9 ± 0.3 vs. 0.5 ± 0.1 relative units, $p < 0.05$) by Xn treatment (panel C), whereas AC1 was not significantly modulated (0.95 ± 0.27 vs. 0.49 ± 0.13 relative units, $p = 0.1711$, panel A). However, under our experimental conditions, we were unable to amplify the A_1R and AC1 genes for C6 and SH-SY5Y cells, respectively.

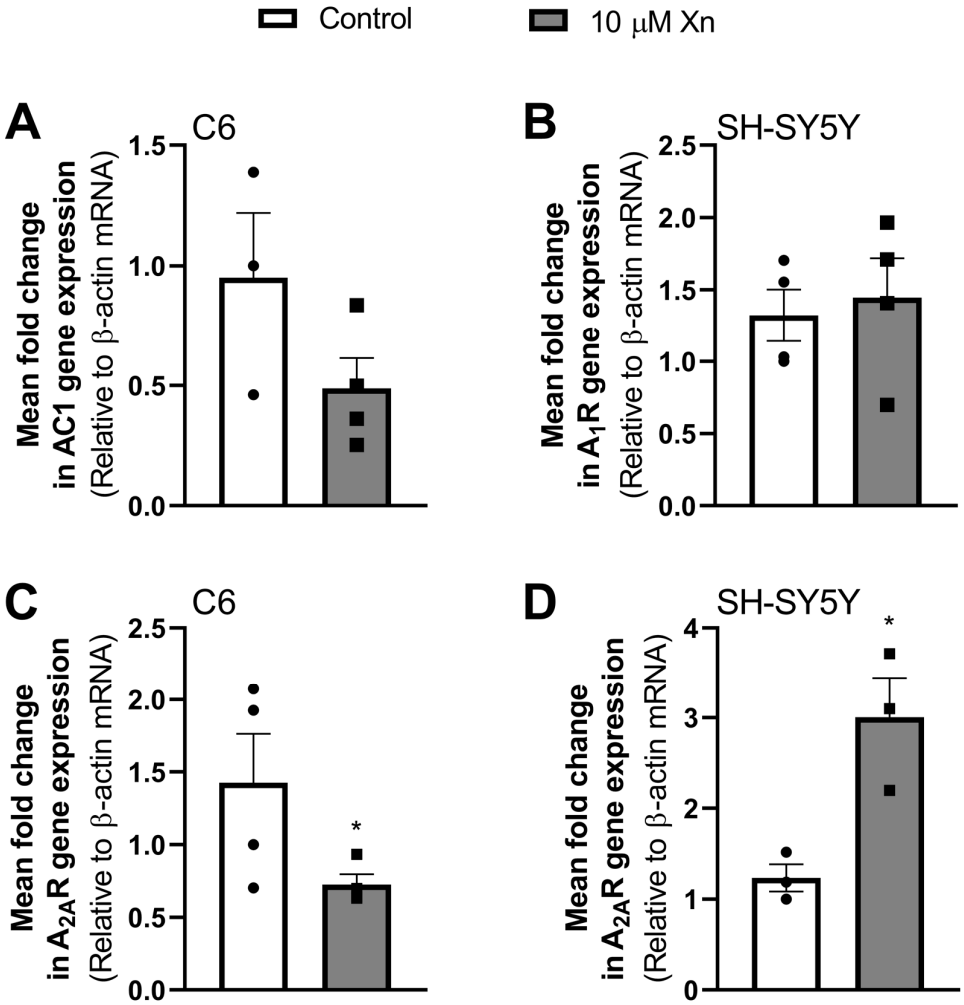


Figure 2. Gene expression of A_1 (panel (**B**)) and A_{2A} (panels (**C**,**D**)) adenosine receptors and AC1 enzyme isoform (panel (**A**)) in C6 and SH-SY5Y cell lines. The expression of the β-actin gene was used as an endogenous control for each sample. Histograms show data corresponding to the mean ± SEM of three–four different experiments performed in duplicate. Individual data are shown in circles and squares. * $p < 0.05$ is significantly different according to the Student's *t*-test.

3.3. Effect of Xn on Adenosine Receptors Levels in Plasma Membrane

The effect of Xn on adenosine receptor levels in plasma membranes from both cell lines was analyzed by Western blotting. As shown in Figure 3 (panel A), adenosine A_1 receptor levels were significantly increased in the C6 cell line (79.7 ± 15.7 vs. 169.3 ± 8.4% from control values, $p < 0.01$), while in SH-SY5Y (panel B), it was also observed a tendency to increase, although not significant (100.8 ± 16.5 vs. 159.3 ± 18.3% from control values, $p = 0.06$). On the other hand, adenosine A_{2A} receptor levels were not significantly affected by the 10 μM Xn treatment, neither in C6 (61.58 ± 22.00 vs. 84.04 ± 31.08% from control values, $p = 0.5768$) nor in SH-SY5Y (120.73 ± 25.41 vs. 219.83 ± 45.58% from control values, $p = 0.1063$) cell lines (panels Figure 3C,D, respectively), although a tendency to increase has been shown for SH-SY5Y cells.

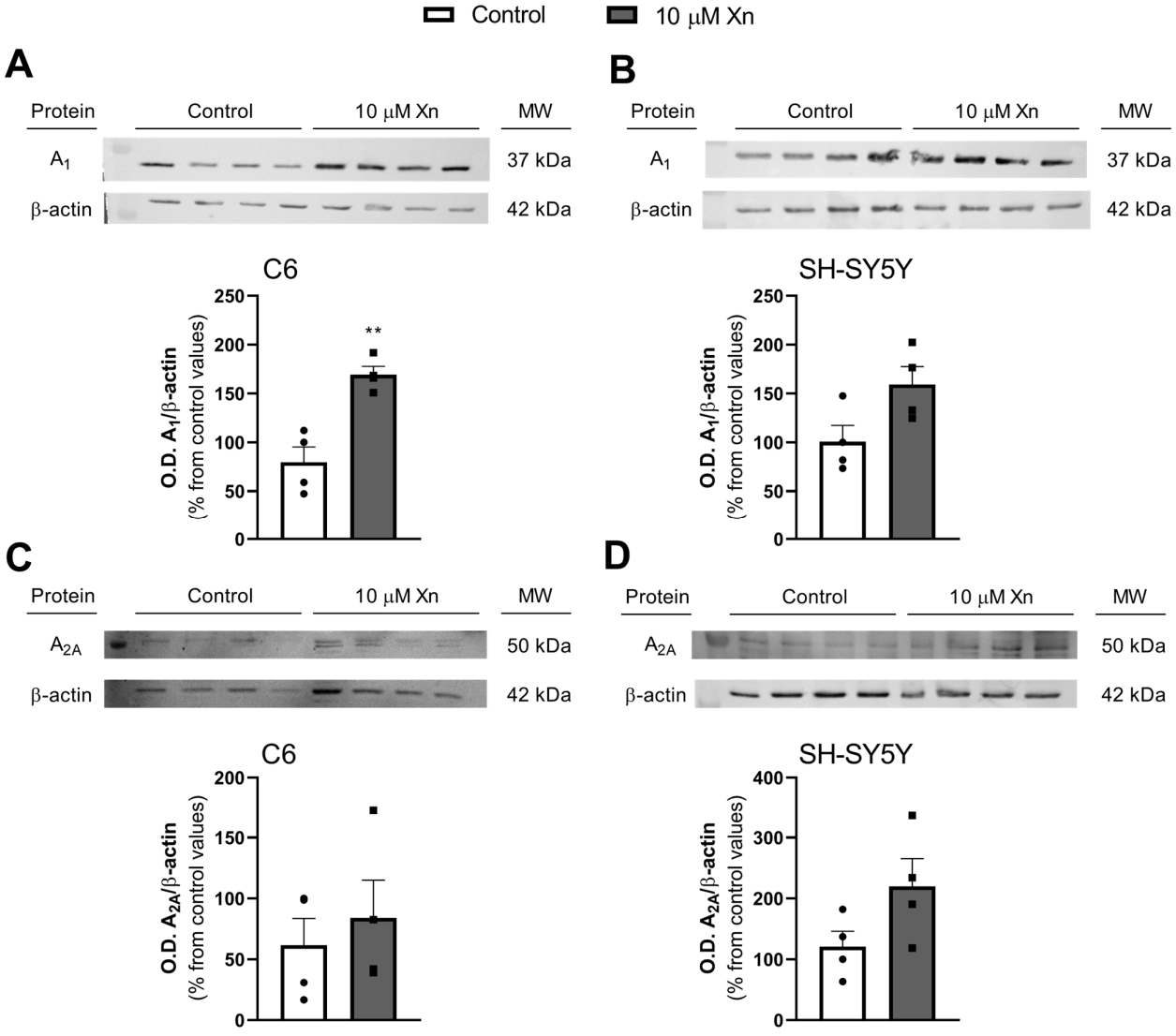


Figure 3. Adenosine A_1 and A_{2A} receptor levels in plasma membranes from C6 and SH-SY5Y after Xn exposure. Western blot analysis was performed with plasma membranes obtained after Xn treatment for 24 h for A_1R (panel (**A**) and (**B**) for C6 and SH-SY5Y cell lines, respectively) and A_{2A}R (panel (**C**,**D**) for C6 and SH-SY5Y cell lines, respectively). Graphs representing data, expressed as % from control values, correspond to the mean ± SEM of four experiments carried out with different plasmatic membrane isolations normalized using β-actin as the control loading. Individual data are shown in circles and squares.** $p < 0.01$ is significantly different from the control according to the Student's *t*-test.

3.4. Effect of Xn on Adenylate Cyclase Activity and PKA Levels

Next, adenylate cyclase activity was measured to determine whether the increase in adenosine A_1 receptor level could also affect its signaling pathway. The results are presented in Figure 4. Interestingly, no effect due to Xn treatment was observed in C6 respecting basal activity (panel Figure 4A) or forskolin-stimulated activity (panel Figure 4B). The functionality through the A_1 receptor after stimulating with forskolin and inhibiting with the A_1 receptor agonist, CPA, also remained unaltered (panel Figure 4C). The same results were obtained for the human neuroblastoma SH-SY5Y cell line (panels Figure 4D, Figure 4E, and Figure 4F, respectively).

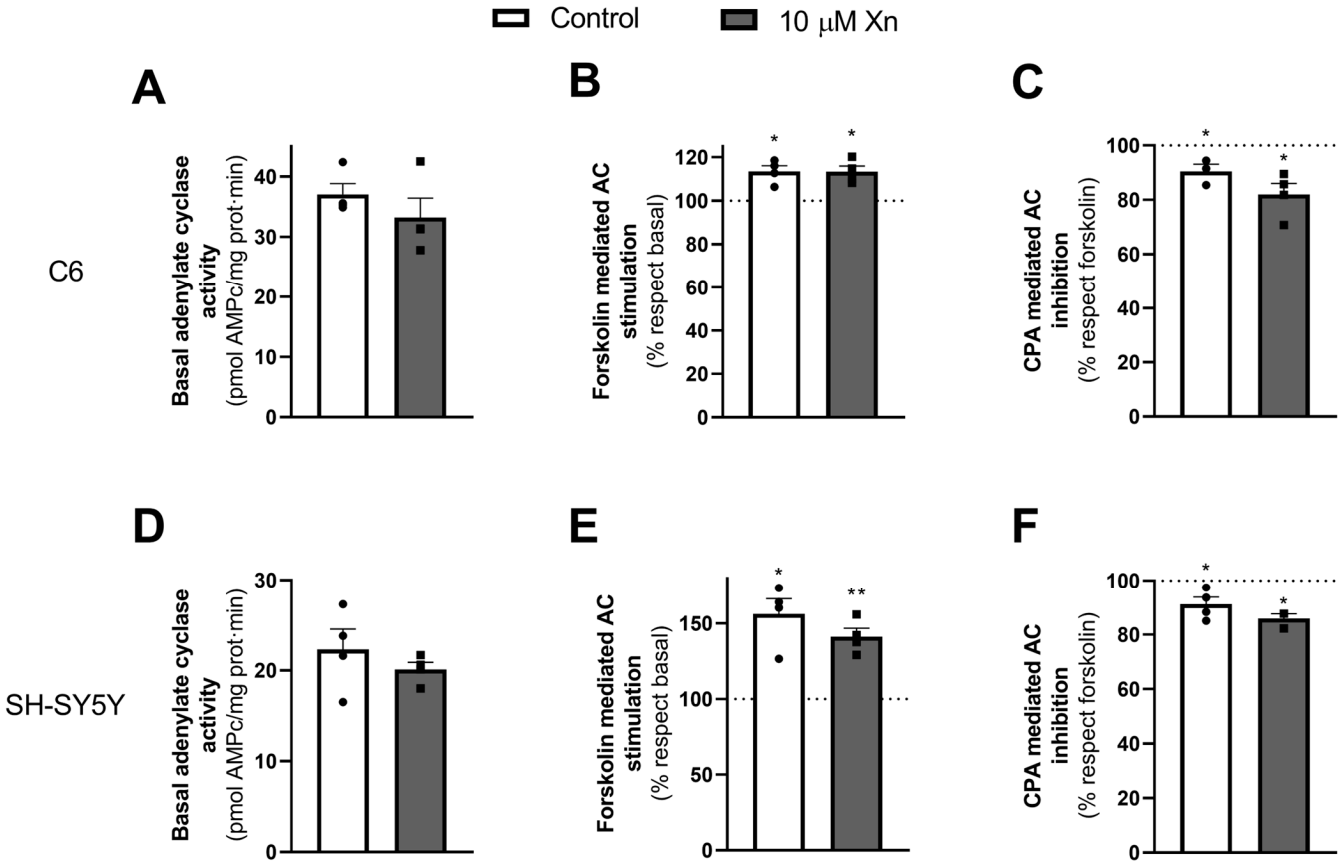


Figure 4. Adenylate cyclase activity in plasmatic membrane fractions from C6 and SH-SY5Y cells. Adenylate activity was analyzed in plasma membranes after Xn treatment for 24 h. Histograms show data representing basal (panels (**A**,**D**)), forskolin-stimulated (panels (**B**–**E**)), and CPA inhibited over forskolin-stimulated activity (panels (**C**,**F**)) in C6 and SH-SY5Y cell lines, respectively. Data are expressed as pmol cAMP/mg protein·min for basal activity, as a percentage of basal values for forskolin-stimulated, and as % from forskolin-stimulated for CPA-inhibited activity, and they correspond to the mean ± SEM of three–four experiments performed using different plasma membrane isolations. Individual data are shown in circles and squares. Dashed line in B, E correspond to basal value, set to 100 %; dashed line in C, F correspond to forskolin-stimulated value, set to 100 % * $p < 0.05$ and ** $p < 0.01$ are significantly different from basal (panels (**B**,**E**)) or forskolin-stimulated activity (panels (**C**,**F**)) according to the Student's *t*-test.

Moreover, protein kinase A (PKA) enzyme levels, which are involved in the downstream adenosine pathway through adenylate cyclase, were measured by Western blot. Regarding this enzyme, no changes were observed in any cell lines, as can be observed in Figure 5.

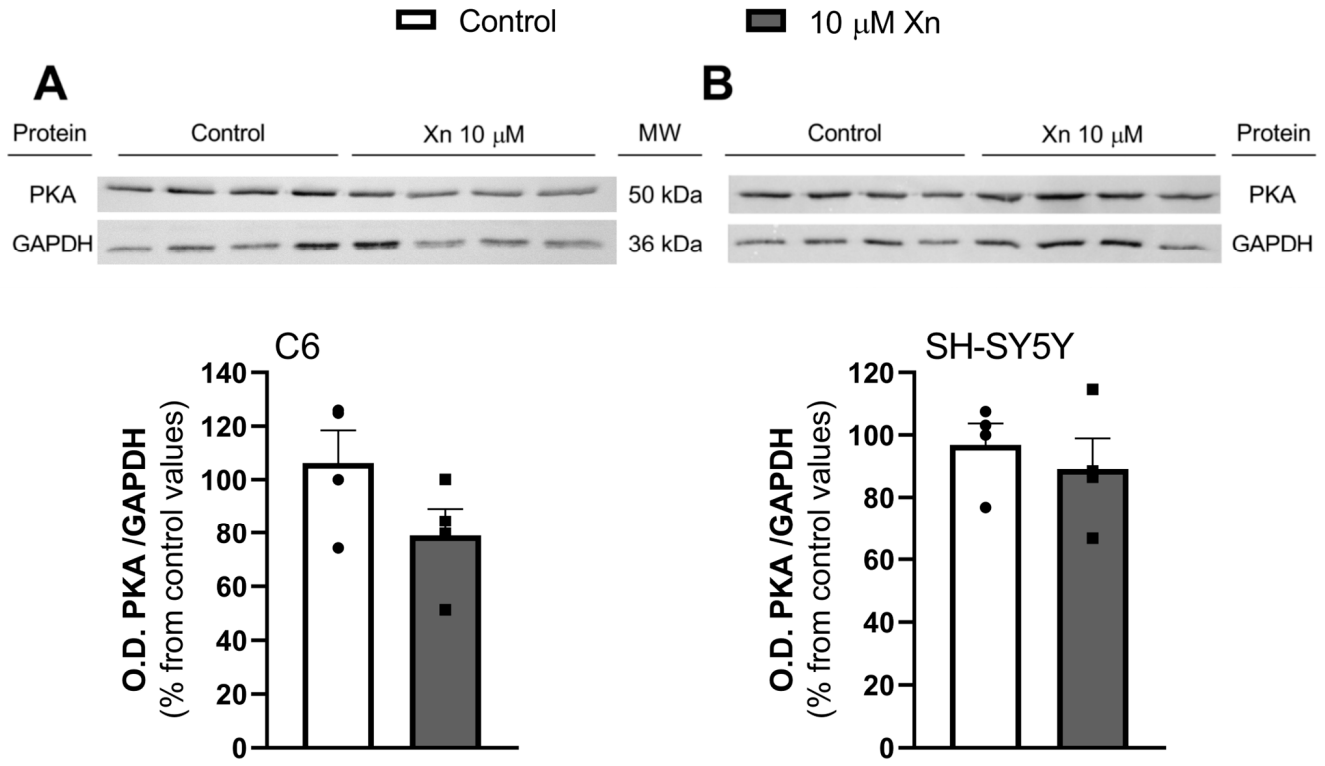


Figure 5. PKA levels in C6 (panel (**A**)) and SH-SY5Y (panel (**B**)) cells. Western blots were performed in cytosol fractions from cells after 10 μM Xn treatment for 24 h. Histograms show that results, expressed as % of control values, correspond to the mean ± SEM of four experiments performed using different cytosol fractions and normalized using GAPDH as the control loading. Individual data are shown in circles and squares. No differences were found according to the statistical analysis.

3.5. Effect of Xn in 5′-Nucleotidase Activity (CD73)

After assessing adenosine receptors and functionality, other enzymatic activity related to this nucleoside was studied, such as 5′-nucleotidase activity. This enzyme is responsible for degrading mainly AMP into adenosine, and it can be found attached to the plasma membrane (ecto-5′-nucleotidase, or CD73) or in the cytosolic fraction. Xn exposure at 10 μM for 24 h caused a significant decrease in CD73 activity (42.7 ± 1.15 vs. 23.6 ± 4.0 nmol Pi/mg protein·min, $p < 0.05$), while the cytosolic activity of the enzyme remains unaltered by the treatment in C6 glioma cells, as can be observed in Figures 6A and 6C, respectively. As for SH-SY5Y cells, no change was observed in CD73 activity, while an increase in cytosolic activity was noted (4.5 ± 0.7 vs. 10.6 ± 1.9 nmol Pi/mg protein·min, $p < 0.05$), as shown in Figures 6B and 6D, respectively.

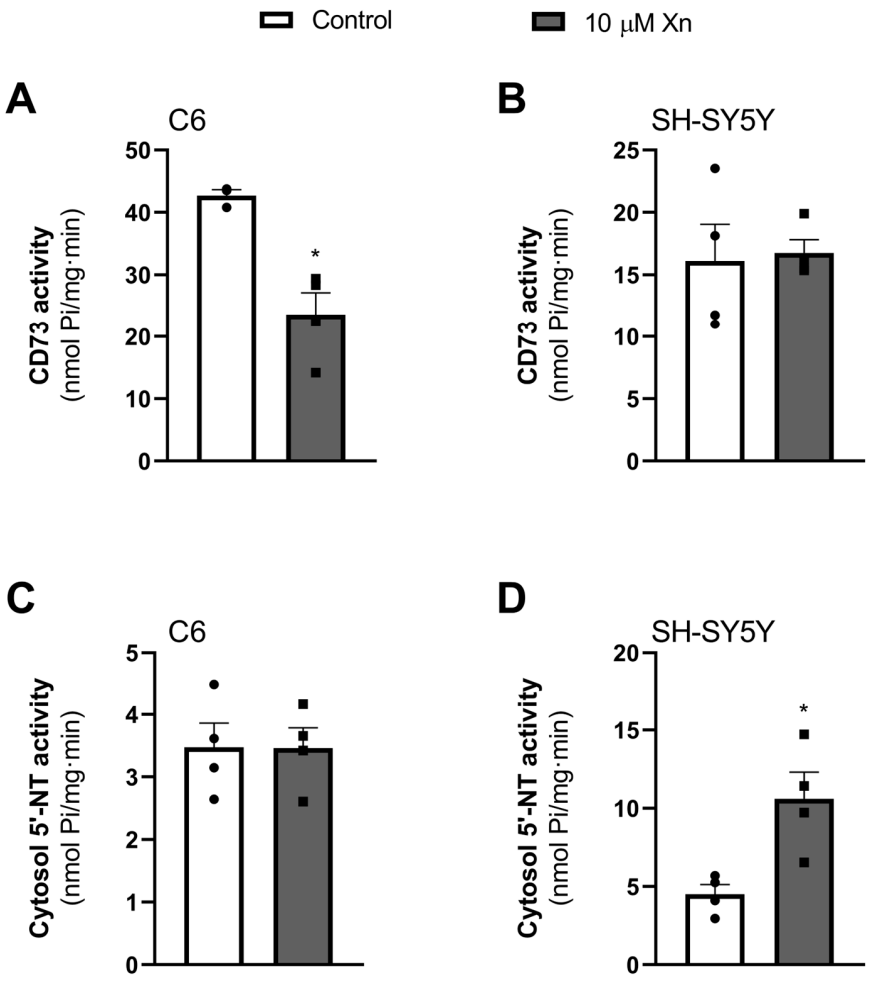


Figure 6. 5′-Nucleotidase enzymatic activity in the plasmatic membrane (CD73) and cytosol fractions from C6 (panels (**A**,**C**)) and SH-SY5Y (panels (**B**,**D**)) cell lines, respectively, after 10 μM Xn exposure for 24 h. Results are expressed as the mean ± SEM of four different experiments performed in duplicate. Individual data are showin in circles and squares. * $p < 0.05$ is significantly different according to the Student's *t*-test.

4. Discussion

Beer is one of the most consumed beverages worldwide, and 80% of its characteristic bitterness comes from the addition of hops during the brewing process [31]. Its polyphenolic compounds content ranges from 74 to 256 mg/L, derived from hops (30%) and malt (70–80%) [32], and Xn is its major prenylated flavonoid, being beer the main source of this molecule [33].

Despite the high prevalence of Alzheimer's disease in the human population, research conducted to date has yet to discover an effective drug capable of halting the progression of the disease. Consequently, efforts have been made to develop new cellular models that could serve as potential predictive platforms for the development of novel drugs. As a result, various cell lines with neuronal properties have been extensively utilized to replicate the pathological processes of Alzheimer's disease. Among these cell lines are PC12, as well as those utilized in this study, SH-SY5Y and C6, derived from rat glioma [34]. However, a more precise study of its action over viability in these cell lines was needed to prove if this molecule by itself could have a potential positive impact on neurodegeneration without affecting viability. It has shown protective effects against the toxicity of several molecules, such as methylglyoxal in osteoblastic cells, at which Xn by itself at 1 μM caused a significant decrease in this cell line viability [35], or in cementoblasts, which started to decrease their viability at 4 μM Xn [29]. It also caused a significant diminution of viability

in PC12 neurons like rat pheochromocytoma cells at concentrations greater than 2 μM [7]. Thus, in our case, both cell lines performed equally well in terms of viability when adding Xn for a 24 h treatment. Therefore, a 10 μM concentration was employed for the subsequent experiments in C6 and SH-SY5Y cell lines in order to avoid this cytotoxicity and to ensure the usage of a concentration of this flavonoid, which has reported effects in other cell lines.

Adenosine is a neuromodulator nucleoside that acts through four different receptors, with A_1R and $A_{2A}R$ being the main ones responsible for the adenosine function in the brain [36]. A_1R has been known for its neuroprotective effect [37], acting pre-synaptically, decreasing the calcium influx, and, in turn, glutamate release, among other functions [38]. In fact, some studies carried out in the post-mortem brains of AD patients have observed that adenosine A_1 receptors are decreased, even though their expression remains unaltered [39], promoting an environment of excess glutamate release that ends up in an excitotoxicity process [13]. Other works propose that simultaneously boosting A_1R activity and blocking $A_{2A}R$ may accomplish maximal neuroprotection, preventing brain damage [15]. In fact, other polyphenolic bioactive compounds such as resveratrol have shown these effects in in vitro [40] and in vivo studies [41], and caffeine has also been proposed as neuroprotective mainly due to its $A_{2A}R$ antagonism [18,42].

Regarding the presence of adenosine receptors in the plasma membrane, A_1 levels were significantly increased, while no changes in A_{2A} levels were found. Thus, an increase in A_1R membrane levels could indicate a possible neuroprotective effect of Xn. However, different results were found between cell lines in adenosine receptor gene expression. Our data show a clear modulation of A_{2A} expression by Xn, while no modulation in A_1 gene expression in the SH-SY5Y cell line was observed. As reported, these results are not paralleled by changes in the corresponding presence of these receptors in the plasma membrane. The discrepancies between gene expression and their respective protein levels have been previously reported in different cell lines, such as HeLa [40], and tissues by our group [39,41,43]. A possible explanation for these results could be that Xn exposure time is not enough to observe an effect on the translation of the A_{2A} receptor. Also, for the A_1 receptor, the stability of its mRNA could have been modulated by the Xn treatment. Despite this, the different effects of this flavonoid on A_{2A} expression should be considered since it acts differently depending on the cell type or perhaps the host species of these cells.

Once we studied the modulation of receptor expression and their membrane levels, AC activity at different conditions was measured to probe if Xn was affecting the functionality of adenosine receptors through this pathway. AC activity is crucial for the Ca^{2+} and cAMP second messenger balance inside the cell [44], and dysregulation of this activity or PKA activity may translate into structural and functional abnormalities [45]. Therefore, their modulation has been considered a promising target for drugs with neuroregenerative activity for the treatment of dementia [46]. Also, some studies remark that AC activity is diminished in neurodegenerative diseases in some brain areas [47]; hence, a possible modulation of this activity by Xn must be studied. Interestingly, no effect was observed in adenylate cyclase basal activity after Xn treatment nor in its activity through the A_1 adenosine receptor in any of the cell lines tested under our conditions. Additionally, PKA levels were not affected.

Regarding 5′-nucleotidase activity, a different modulation occurred when treating cells for 24 h with Xn in C6 or SH-SY5Y cell lines, decreasing the activity of the ecto-5′-nucleotidase or CD73 in the rat model, the major enzyme responsible for the extracellular AMP degradation into adenosine [48], while increasing its activity in the cytosolic fraction of the SH-SY5Y human cellular model. The role of ecto-5′-nucleotidases in pathologies has been widely described as they participate in a large variety of processes, such as extracellular availability of nucleosides [49], synaptogenesis [50], and cell proliferation [51], and it has been described as decreased in some early stages of AD [24]. According to that, this enzyme activity has been targeted in several neurodegenerative diseases, as $A_{2A}R$ is mainly activated by adenosine coming from CD73 ecto-5′-nucleotidase activity [25,52]. Even a physical interaction through coimmunoprecipitation between this enzyme and the

receptor has been described [53]. It has been described how the adenosine produced by the catabolism of ATP directly activates the A_{2A} receptor, causing synaptic disruption and memory dysfunction in a mouse model of AD [23], proposing CD73 activity as a novel target for the modulation of A2AR function. Other works reported the importance of this ecto-5′-nucleotidase in the regulation of A_{2A} function in animal models of Parkinson's disease [25], brain damage produced by convulsive activity, which leads to an extracellular ATP increase and, therefore, affects the CD73-A_{2A}R pathway [54], and even the damage caused by repeated stress on adult rats [55]. In addition, this enzyme is important in embryonic brain development, taking part in the migration of cortical projection neurons [56]. These studies comment on the role of this enzyme in multiple neurological processes and propose it as a potential target for several diseases. Thus, a decrease in this activity could moderate A_{2A} function and become another crucial factor contributing to the potential neuroprotective effect of Xn. Concerning the decrease in cytosolic 5′-nucleotidase activity observed in SH-SY5Y cells following Xn exposure, this effect may also contribute to a protective effect against Alzheimer's disease since a previous study conducted by our group has also revealed a significant reduction in cytosolic 5′-nucleotidase activity in the frontal cortex of human patients with Alzheimer's [24].

In addition, due to the antiproliferative and antioxidant effects provided by Xn in different cell lines, it could also be a promising antitumoral molecule since gliomas are the most frequent malignant tumors of the CNS in adults, and currently, there are no strategies to achieve their successful eradication [57].

5. Conclusions

In summary, here we describe a novel route in which Xn could act in preventing neurodegeneration as well as its reported prevention of oxidative stress, both of which are common signs of dementia, proposing this molecule as a potential neuroprotective agent through the adenosine pathway modulation in different cell lines.

Author Contributions: Conceptualization, D.A.L.-N. and M.M.; formal analysis, A.T., D.A.L.-N. and M.M.; funding acquisition, M.M.; investigation, A.T.; supervision, D.A.L.-N. and M.M.; writing—original draft preparation, A.T.; writing—review and editing, D.A.L.-N. and M.M. All authors have read and agreed to the published version of the manuscript.

Funding: Grant PID2022-140602NB-100 by MICIN and by FEDER ("A Way of Making Europe"), Grant 2022-GRIN-34201 by UCLM cofinanced with FEDER, and Grant SBPLY/23/180225/000156 by JCCM. A.T. is a recipient of a predoctoral Manuel Oya 2022 fellowship from Fundación Cerveza y Salud (CR-2023-103).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author due to privacy.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References

1. Phukan, B.C.; Roy, R.; Gahatraj, I.; Bhattacharya, P.; Borah, A. Therapeutic Considerations of Bioactive Compounds in Alzheimer's Disease and Parkinson's Disease: Dissecting the Molecular Pathways. *Phytother. Res.* **2023**, *37*, 5657–5699. [CrossRef] [PubMed]
2. Nardini, M. An Overview of Bioactive Phenolic Molecules and Antioxidant Properties of Beer: Emerging Trends. *Molecules* **2023**, *28*, 3221. [CrossRef] [PubMed]
3. Fernández-García, C.; Rancan, L.; Paredes, S.D.; Montero, C.; de la Fuente, M.; Vara, E.; Tresguerres, J.A.F. Xanthohumol Exerts Protective Effects in Liver Alterations Associated with Aging. *Eur. J. Nutr.* **2019**, *58*, 653–663. [CrossRef] [PubMed]
4. Wang, Q.; Chen, T.; Shuqing, Z.; Yu, L.; Chen, S.; Lu, H.; Zhu, H.; Min, X.; Li, X.; Liu, L. Xanthohumol Relieves Arthritis Pain in Mice by Suppressing Mitochondrial-Mediated Inflammation. *Mol. Pain* **2023**, *19*, 17448069231204051. [CrossRef] [PubMed]

5. Carvalho, D.O.; Oliveira, R.; Johansson, B.; Guido, L.F. Dose-Dependent Protective and Inductive Effects of Xanthohumol on Oxidative DNA Damage in Saccharomyces Cerevisiae. *Food Technol. Biotechnol.* **2016**, *54*, 60–69. [CrossRef] [PubMed]
6. Wongchum, N.; Dechakhamphu, A. Xanthohumol Prolongs Lifespan and Decreases Stress-Induced Mortality in Drosophila Melanogaster. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2021**, *244*, 108994. [CrossRef] [PubMed]
7. Yao, J.; Zhang, B.; Ge, C.; Peng, S.; Fang, J. Xanthohumol, a Polyphenol Chalcone Present in Hops, Activating Nrf2 Enzymes to Confer Protection against Oxidative Damage in PC12 Cells. *J. Agric. Food Chem.* **2015**, *63*, 1521–1531. [CrossRef] [PubMed]
8. Girisa, S.; Saikia, Q.; Bordoloi, D.; Banik, K.; Monisha, J.; Daimary, U.D.; Verma, E.; Ahn, K.S.; Kunnumakkara, A.B. Xanthohumol from Hop: Hope for Cancer Prevention and Treatment. *IUBMB Life* **2021**, *73*, 1016–1044. [CrossRef] [PubMed]
9. Huang, X.; Wang, J.; Chen, X.; Liu, P.; Wang, S.; Song, F.; Zhang, Z.; Zhu, F.; Huang, X.; Liu, J.; et al. The Prenylflavonoid Xanthohumol Reduces Alzheimer-Like Changes and Modulates Multiple Pathogenic Molecular Pathways in the Neuro2a/APPswe Cell Model of AD. *Front. Pharmacol.* **2018**, *9*, 199. [CrossRef]
10. Dugger, B.N.; Dickson, D.W. Pathology of Neurodegenerative Diseases. *Cold Spring Harb. Perspect. Biol.* **2017**, *9*, a028035. [CrossRef]
11. Bai, R.; Guo, J.; Ye, X.-Y.; Xie, Y.; Xie, T. Oxidative Stress: The Core Pathogenesis and Mechanism of Alzheimer's Disease. *Ageing Res. Rev.* **2022**, *77*, 101619. [CrossRef]
12. Dhapola, R.; Sarma, P.; Medhi, B.; Prakash, A.; Reddy, D.H. Recent Advances in Molecular Pathways and Therapeutic Implications Targeting Mitochondrial Dysfunction for Alzheimer's Disease. *Mol. Neurobiol.* **2022**, *59*, 535–555. [CrossRef] [PubMed]
13. Ong, W.-Y.; Tanaka, K.; Dawe, G.S.; Ittner, L.M.; Farooqui, A.A. Slow Excitotoxicity in Alzheimer's Disease. *J. Alzheimers Dis.* **2013**, *35*, 643–668. [CrossRef] [PubMed]
14. Mangalmurti, A.; Lukens, J.R. How Neurons Die in Alzheimer's Disease: Implications for Neuroinflammation. *Curr. Opin. Neurobiol.* **2022**, *75*, 102575. [CrossRef] [PubMed]
15. Cunha, R.A. How Does Adenosine Control Neuronal Dysfunction and Neurodegeneration? *Journal of Neurochemistry* **2016**, *139*, 1019–1055. [CrossRef] [PubMed]
16. Kalaria, R.N.; Sromek, S.; Wilcox, B.J.; Unnerstall, J.R. Hippocampal Adenosine A1 Receptors Are Decreased in Alzheimer's Disease. *Neurosci. Lett.* **1990**, *118*, 257–260. [CrossRef] [PubMed]
17. Fukumitsu, N.; Ishii, K.; Kimura, Y.; Oda, K.; Hashimoto, M.; Suzuki, M.; Ishiwata, K. Adenosine A(1) Receptors Using 8-Dicyclopropylmethyl-1-[(11)C]Methyl-3-Propylxanthine PET in Alzheimer's Disease. *Ann. Nucl. Med.* **2008**, *22*, 841–847. [CrossRef] [PubMed]
18. Espinosa, J.; Rocha, A.; Nunes, F.; Costa, M.S.; Schein, V.; Kazlauckas, V.; Kalinine, E.; Souza, D.O.; Cunha, R.A.; Porciúncula, L.O. Caffeine Consumption Prevents Memory Impairment, Neuronal Damage, and Adenosine A2A Receptors Upregulation in the Hippocampus of a Rat Model of Sporadic Dementia. *J. Alzheimers Dis.* **2013**, *34*, 509–518. [CrossRef] [PubMed]
19. Viana da Silva, S.; Haberl, M.G.; Zhang, P.; Bethge, P.; Lemos, C.; Gonçalves, N.; Gorlewicz, A.; Malezieux, M.; Gonçalves, F.Q.; Grosjean, N.; et al. Early Synaptic Deficits in the APP/PS1 Mouse Model of Alzheimer's Disease Involve Neuronal Adenosine A2A Receptors. *Nat. Commun.* **2016**, *7*, 11915. [CrossRef]
20. Silva, A.C.; Lemos, C.; Gonçalves, F.Q.; Pliássova, A.V.; Machado, N.J.; Silva, H.B.; Canas, P.M.; Cunha, R.A.; Lopes, J.P.; Agostinho, P. Blockade of Adenosine A2A Receptors Recovers Early Deficits of Memory and Plasticity in the Triple Transgenic Mouse Model of Alzheimer's Disease. *Neurobiol. Dis.* **2018**, *117*, 72–81. [CrossRef]
21. Lopes, C.R.; Silva, A.C.; Silva, H.B.; Canas, P.M.; Agostinho, P.; Cunha, R.A.; Lopes, J.P. Adenosine A2A Receptor Up-Regulation Pre-Dates Deficits of Synaptic Plasticity and of Memory in Mice Exposed to Aβ1–42 to Model Early Alzheimer's Disease. *Biomolecules* **2023**, *13*, 1173. [CrossRef] [PubMed]
22. Temido-Ferreira, M.; Ferreira, D.G.; Batalha, V.L.; Marques-Morgado, I.; Coelho, J.E.; Pereira, P.; Gomes, R.; Pinto, A.; Carvalho, S.; Canas, P.M.; et al. Age-Related Shift in LTD Is Dependent on Neuronal Adenosine A2A Receptors Interplay with mGluR5 and NMDA Receptors. *Mol. Psychiatry* **2020**, *25*, 1876–1900. [CrossRef] [PubMed]
23. Gonçalves, F.Q.; Lopes, J.P.; Silva, H.B.; Lemos, C.; Silva, A.C.; Gonçalves, N.; Tomé, Â.R.; Ferreira, S.G.; Canas, P.M.; Rial, D.; et al. Synaptic and Memory Dysfunction in a β-Amyloid Model of Early Alzheimer's Disease Depends on Increased Formation of ATP-Derived Extracellular Adenosine. *Neurobiol. Dis.* **2019**, *132*, 104570. [CrossRef] [PubMed]
24. Alonso-Andrés, P.; Albasanz, J.L.; Ferrer, I.; Martín, M. Purine-Related Metabolites and Their Converting Enzymes Are Altered in Frontal, Parietal and Temporal Cortex at Early Stages of Alzheimer's Disease Pathology. *Brain Pathol.* **2018**, *28*, 933–946. [CrossRef] [PubMed]
25. Carmo, M.; Gonçalves, F.Q.; Canas, P.M.; Oses, J.-P.; Fernandes, F.D.; Duarte, F.V.; Palmeira, C.M.; Tomé, A.R.; Agostinho, P.; Andrade, G.M.; et al. Enhanced ATP Release and CD73-Mediated Adenosine Formation Sustain Adenosine A2A Receptor over-Activation in a Rat Model of Parkinson's Disease. *Br. J. Pharmacol.* **2019**, *176*, 3666–3680. [CrossRef] [PubMed]
26. Murugan, M.; Fedele, D.; Millner, D.; Alharfoush, E.; Vegunta, G.; Boison, D. Adenosine Kinase: An Epigenetic Modulator in Development and Disease. *Neurochem. Int.* **2021**, *147*, 105054. [CrossRef] [PubMed]
27. Boison, D. Modulators of Nucleoside Metabolism in the Therapy of Brain Diseases. *Curr. Top Med. Chem.* **2011**, *11*, 1068–1086. [CrossRef]
28. Alonso, P.; Albasanz, J.L.; Martín, M. Modulation of Adenosine Receptors by Hops and Xanthohumol in Cell Cultures. *ACS Chem. Neurosci.* **2021**, *12*, 2373–2384. [CrossRef]

29. Niederau, C.; Bhargava, S.; Schneider-Kramman, R.; Jankowski, J.; Craveiro, R.B.; Wolf, M. Xanthohumol Exerts Anti-Inflammatory Effects in an in Vitro Model of Mechanically Stimulated Cementoblasts. *Sci. Rep.* **2022**, *12*, 14970. [CrossRef]
30. Torrens-Mas, M.; Alorda-Clara, M.; Martínez-Vigara, M.; Roca, P.; Sastre-Serra, J.; Oliver, J.; Pons, D.G. Xanthohumol Reduces Inflammation and Cell Metabolism in HT29 Primary Colon Cancer Cells. *Int. J. Food Sci. Nutr.* **2022**, *73*, 471–479. [CrossRef]
31. Oladokun, O.; Tarrega, A.; James, S.; Smart, K.; Hort, J.; Cook, D. The Impact of Hop Bitter Acid and Polyphenol Profiles on the Perceived Bitterness of Beer. *Food Chem.* **2016**, *205*, 212–220. [CrossRef] [PubMed]
32. Vazquez-Cervantes, G.I.; Ortega, D.R.; Blanco Ayala, T.; Pérez de la Cruz, V.; Esquivel, D.F.G.; Salazar, A.; Pineda, B. Redox and Anti-Inflammatory Properties from Hop Components in Beer-Related to Neuroprotection. *Nutrients* **2021**, *13*, 2000. [CrossRef] [PubMed]
33. Stevens, J.F.; Page, J.E. Xanthohumol and Related Prenylflavonoids from Hops and Beer: To Your Good Health! *Phytochemistry* **2004**, *65*, 1317–1330. [CrossRef] [PubMed]
34. Xie, D.; Deng, T.; Zhai, Z.; Sun, T.; Xu, Y. The Cellular Model for Alzheimer's Disease Research: PC12 Cells. *Front. Mol. Neurosci.* **2022**, *15*, 1016559. [CrossRef] [PubMed]
35. Suh, K.S.; Chon, S.; Choi, E.M. Cytoprotective Effects of Xanthohumol against Methylglyoxal-Induced Cytotoxicity in MC3T3-E1 Osteoblastic Cells. *J. Appl. Toxicol.* **2018**, *38*, 180–192. [CrossRef] [PubMed]
36. Fredholm, B.B.; Chen, J.-F.; Cunha, R.A.; Svenningsson, P.; Vaugeois, J.-M. Adenosine and Brain Function. *Int. Rev. Neurobiol.* **2005**, *63*, 191–270. [CrossRef] [PubMed]
37. Stockwell, J.; Jakova, E.; Cayabyab, F.S. Adenosine A1 and A2A Receptors in the Brain: Current Research and Their Role in Neurodegeneration. *Molecules* **2017**, *22*, 676. [CrossRef] [PubMed]
38. Ambrósio, A.F.; Malva, J.O.; Carvalho, A.P.; Carvalho, C.M. Inhibition of N-,P/Q- and Other Types of Ca^{2+} Channels in Rat Hippocampal Nerve Terminals by the Adenosine A1 Receptor. *Eur. J. Pharmacol.* **1997**, *340*, 301–310. [CrossRef]
39. Albasanz, J.L.; Perez, S.; Barrachina, M.; Ferrer, I.; Martín, M. Up-Regulation of Adenosine Receptors in the Frontal Cortex in Alzheimer's Disease. *Brain Pathol.* **2008**, *18*, 211–219. [CrossRef]
40. Muñoz-López, S.; Sánchez-Melgar, A.; Martín, M.; Albasanz, J.L. Resveratrol Enhances A1 and Hinders A2A Adenosine Receptors Signaling in Both HeLa and SH-SY5Y Cells: Potential Mechanism of Its Antitumoral Action. *Front. Endocrinol.* **2022**, *13*, 1007801. [CrossRef]
41. Sánchez-Melgar, A.; Albasanz, J.L.; Palomera-Ávalos, V.; Pallàs, M.; Martín, M. Resveratrol Modulates and Reverses the Age-Related Effect on Adenosine-Mediated Signalling in SAMP8 Mice. *Mol. Neurobiol.* **2019**, *56*, 2881–2895. [CrossRef] [PubMed]
42. Rivera-Oliver, M.; Díaz-Ríos, M. Using Caffeine and Other Adenosine Receptor Antagonists and Agonists as Therapeutic Tools against Neurodegenerative Diseases: A Review. *Life Sci.* **2014**, *101*, 1–9. [CrossRef] [PubMed]
43. Castillo, C.A.; Albasanz, J.L.; León, D.; Jordán, J.; Pallàs, M.; Camins, A.; Martín, M. Age-Related Expression of Adenosine Receptors in Brain from the Senescence-Accelerated Mouse. *Exp. Gerontol.* **2009**, *44*, 453–461. [CrossRef] [PubMed]
44. Lezmy, J.; Arancibia-Cárcamo, I.L.; Quintela-López, T.; Sherman, D.L.; Brophy, P.J.; Attwell, D. Astrocyte Ca2+-Evoked ATP Release Regulates Myelinated Axon Excitability and Conduction Speed. *Science* **2021**, *374*, eabh2858. [CrossRef] [PubMed]
45. Sobolczyk, M.; Boczek, T. Astrocytic Calcium and cAMP in Neurodegenerative Diseases. *Front. Cell Neurosci.* **2022**, *16*, 889939. [CrossRef] [PubMed]
46. Zyuz'kov, G.N.; Miroshnichenko, L.A.; Kotlovskaya, L.Y.; Chaikovskii, A.V. The Role of cAMP-Dependent Intracellular Signaling Pathways in the Regulation of the Functions of Neural Stem Cells and Neuroglial Cells in Amyloid-β-Induced Neurodegeneration. *Bull. Exp. Biol. Med.* **2023**, *175*, 437–441. [CrossRef] [PubMed]
47. Schnecko, A.; Witte, K.; Bohl, J.; Ohm, T.; Lemmer, B. Adenylyl Cyclase Activity in Alzheimer's Disease Brain: Stimulatory and Inhibitory Signal Transduction Pathways Are Differently Affected. *Brain Res.* **1994**, *644*, 291–296. [CrossRef] [PubMed]
48. Zimmermann, H.; Zebisch, M.; Sträter, N. Cellular Function and Molecular Structure of Ecto-Nucleotidases. *Purinergic Signal* **2012**, *8*, 437–502. [CrossRef] [PubMed]
49. Grković, I.; Drakulić, D.; Martinović, J.; Mitrović, N. Role of Ectonucleotidases in Synapse Formation During Brain Development: Physiological and Pathological Implications. *Curr. Neuropharmacol.* **2019**, *17*, 84–98. [CrossRef]
50. Sebastião, A.M.; Ribeiro, J.A. Neuromodulation and Metamodulation by Adenosine: Impact and Subtleties upon Synaptic Plasticity Regulation. *Brain Res.* **2015**, *1621*, 102–113. [CrossRef]
51. Coppi, E.; Cellai, L.; Maraula, G.; Dettori, I.; Melani, A.; Pugliese, A.M.; Pedata, F. Role of Adenosine in Oligodendrocyte Precursor Maturation. *Front. Cell Neurosci.* **2015**, *9*, 155. [CrossRef] [PubMed]
52. Meng, F.; Guo, Z.; Hu, Y.; Mai, W.; Zhang, Z.; Zhang, B.; Ge, Q.; Lou, H.; Guo, F.; Chen, J.; et al. CD73-Derived Adenosine Controls Inflammation and Neurodegeneration by Modulating Dopamine Signalling. *Brain* **2019**, *142*, 700–718. [CrossRef] [PubMed]
53. Augusto, E.; Matos, M.; Sévigny, J.; El-Tayeb, A.; Bynoe, M.S.; Müller, C.E.; Cunha, R.A.; Chen, J.-F. Ecto-5′- Nucleotidase (CD73)-Mediated Formation of Adenosine Is Critical for the Striatal Adenosine A2A Receptor Functions. *J. Neurosci.* **2013**, *33*, 11390–11399. [CrossRef]
54. Augusto, E.; Gonçalves, F.Q.; Real, J.E.; Silva, H.B.; Pochmann, D.; Silva, T.S.; Matos, M.; Gonçalves, N.; Tomé, Â.R.; Chen, J.-F.; et al. Increased ATP Release and CD73-Mediated Adenosine A2A Receptor Activation Mediate Convulsion-Associated Neuronal Damage and Hippocampal Dysfunction. *Neurobiol. Dis.* **2021**, *157*, 105441. [CrossRef] [PubMed]

55. Dias, L.; Pochmann, D.; Lemos, C.; Silva, H.B.; Real, J.I.; Gonçalves, F.Q.; Rial, D.; Gonçalves, N.; Simões, A.P.; Ferreira, S.G.; et al. Increased Synaptic ATP Release and CD73-Mediated Formation of Extracellular Adenosine in the Control of Behavioral and Electrophysiological Modifications Caused by Chronic Stress. *ACS Chem. Neurosci.* **2023**, *14*, 1299–1309. [CrossRef] [PubMed]
56. Alçada-Morais, S.; Gonçalves, N.; Moreno-Juan, V.; Andres, B.; Ferreira, S.; Marques, J.M.; Magalhães, J.; Rocha, J.M.M.; Xu, X.; Partidário, M.; et al. Adenosine A2A Receptors Contribute to the Radial Migration of Cortical Projection Neurons through the Regulation of Neuronal Polarization and Axon Formation. *Cereb. Cortex* **2021**, *31*, 5652–5663. [CrossRef]
57. Colopi, A.; Fuda, S.; Santi, S.; Onorato, A.; Cesarini, V.; Salvati, M.; Balistreri, C.R.; Dolci, S.; Guida, E. Impact of Age and Gender on Glioblastoma Onset, Progression, and Management. *Mech. Ageing Dev.* **2023**, *211*, 111801. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.