PLOS ONE

# WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid β1-42 effects on astrocytes in primary culture.

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | PONE-D-14-56621R2 |
| **Article Type:** | Research Article |
| **Full Title:** | WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid β1-42 effects on astrocytes in primary culture. |
| **Short Title:** | WIN 55 prevents Aβ1-42 effects in cultured astrocytes |
| **Corresponding Author:** | Soraya L Valles<br>University of Valencia<br>VALENCIA, SPAIN |
| **Keywords:** | Amyloid β1-42, cannabinoids, inflammation, oxidative stress, cell death. |
| **Abstract:** | Alzheimer´s disease (AD), a neurodegenerative illness involving synaptic dysfunction with extracellular accumulation of Aβ1-42 toxic peptide, glial activation, inflammatory response and oxidative stress, can lead to neuronal death. Endogenous cannabinoid system is implicated in physiological and physiopathological events in central nervous system (CNS), and changes in this system are related to many human diseases, including AD. However, studies on the effects of cannabinoids on astrocytes functions are scarce. In primary cultured astrocytes we studied cellular viability using MTT assay. Inflammatory and oxidative stress mediators were determined by ELISA and Western-blot techniques both in the presence and absence of Aβ1-42 peptide. Effects of WIN 55,212-2 (a synthetic cannabinoid) on cell viability, inflammatory mediators and oxidative stress were also determined. Aβ1-42 diminished astrocytes viability, increased TNF-α and IL-1β levels and p-65, COX-2 and iNOS protein expression while decreased PPAR-γ and antioxidant enzyme Cu/Zn SOD. WIN 55,212-2 pretreatment prevents all effects elicited by Aβ1-42. Furthermore, cannabinoid WIN 55,212-2 also increased cell viability and PPAR-γ expression in control astrocytes. In conclusion cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes. Moreover, WIN 55,212-2 increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by Aβ1-42 in cultured astrocytes. Further studies would be needed to assess the possible beneficial effects of cannabinoids in Alzheimer's disease patients. |
| **Order of Authors:** | Diana Aguirre-Rueda |
| | Sol Guerra-Ojeda |
| | Martin Aldasoro |
| | Antonio Iradi |
| | Elena Obrador |
| | Mª Dolores Mauricio |
| | Jose Mª Vila |
| | Patricia Marchio |
| | Soraya L Valles |
| **Opposed Reviewers:** | |
| **Response to Reviewers:** | Here we indicate the response to reviewer and editor.<br>Reviewer #1: I appreciate the efforts taken by the authors to adequately address the previous concerns with respect to this manuscript.<br><br>Following are the two minor issues that I have.<br><br>1. In the figure legend for Figure 7. modify the last line to match with the conclusion from this study (delete the part that WIN 55,212-2 prevents oxidative stress, since no |

| | |
|---|---|
| | direct measure of oxidative stress was done).<br>Modifications in Figure 7 have been done to match with the conclusion.<br><br>2. In revised manuscript, authors show that 10 μM Aβ1-42 increases MnSOD protein expression. In the discussion section (page 14), can the authors further speculate regarding the conclusion of this finding with respect to mitochondria and oxidative stress during Alzhemer's disease, since mitochondria are the major organelles producing superoxide radicals leading to oxidative stress. To adequately answer this, apart from astrocytes, which are the focus of this study, what is known regarding mitochondria and oxidative stress in Alzhemier's disease in other cells like neuronal cells? And does more MnSOD mean more H2O2 production, that might be a mediator of the oxidative stress ?<br>A paragraph about all suggestions indicated by the referee has been added in discussion. |
| **Additional Information:** | |
| **Question** | **Response** |
| **Financial Disclosure**<br><br>Please describe all sources of funding that have supported your work. A complete funding statement should do the following:<br><br>Include **grant numbers and the URLs** of any funder's website. Use the full name, not acronyms, of funding institutions, and use initials to identify authors who received the funding.<br>**Describe the role** of any sponsors or funders in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. If they had no role in any of the above, include this sentence at the end of your statement: "*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*"<br><br>If the study was **unfunded**, provide a statement that clearly indicates this, for example: "*The author(s) received no specific funding for this work.*"<br><br>* typeset | Generalitat Valenciana AP-073/09 |
| **Competing Interests**<br><br>You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests. | The authors have declared that no competing interests exist. |

| | |
|---|---|
| Do any authors of this manuscript have competing interests (as described in the PLOS Policy on Declaration and Evaluation of Competing Interests)?<br><br>**If yes**, please provide details about any and all competing interests in the box below. Your response should begin with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests:*<br><br>**If no** authors have any competing interests to declare, please enter this statement in the box: "*The authors have declared that no competing interests exist.*"<br><br>* typeset | |
| **Ethics Statement**<br><br>You must provide an ethics statement if your study involved human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues. All information entered here should **also be included in the Methods section** of your manuscript. Please write "N/A" if your study does not require an ethics statement.<br><br>**Human Subject Research (involved human participants and/or tissue)**<br><br>All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written or oral, should also have been obtained from the participants. If no consent was given, the reason must be explained (e.g. the data were analyzed anonymously) and reported. The form of consent (written/oral), or reason for lack of consent, should be indicated in the Methods section of your manuscript.<br><br>Please enter the name of the IRB or Ethics Committee that approved this study | All animal work have been conducted according to relevant national and international guidelines. The relevant guidelines followed and the committee that approved the study belongs to the University of Valencia. Ethics committee specifically approved this study. |

| | |
|---|---|
| in the space below. Include the approval number and/or a statement indicating approval of this research.<br><br>**Animal Research (involved vertebrate animals, embryos or tissues)**<br><br>All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement.<br><br>If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied.<br><br>Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained.<br><br>**Field Permit**<br><br>Please indicate the name of the institution or the relevant body that granted permission. | |
| **Data Availability**<br><br>PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found.<br><br>Your answers to the following constitute your statement about data availability and | Yes - all data are fully available without restriction |

| | |
|---|---|
| will be included with the article in the event of publication. **Please note that simply stating 'data available on request from the author' is not acceptable.** ***If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.***<br><br>Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction? | |
| Please describe where your data may be found, writing in full sentences. **Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted.** If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details.<br><br>If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, "All relevant data are within the paper and its Supporting Information files."<br>If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, "All XXX files are available from the XXX database (accession number(s) XXX, XXX)." If this information will only be available after acceptance, please indicate this by ticking the box below.<br>If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example:<br><br>"Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data."<br><br>"Data are from the XXX study whose authors may be contacted at XXX."<br><br>* typeset | All relevant data are within the paper and its Supporting Information files. |
| Additional data availability information: | |

February, 2015

Chief Editor
Plos One

**Dear Editor**

Please find enclosed the manuscript **WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid $\beta_{1-42}$ effects on astrocytes in primary culture** by Aguirre-Rueda et al., which is intended for publication in Plos One after we correct again reviewer 1 suggestions.

Hoping the paper is now found suitable for publication in your Journal.

Yours sincerely,

Soraya L. Vallés, Ph.D.
Department of Physiology
Faculty of Medicine
University of Valencia
Blasco Ibañez, 15
46010-Valencia, Spain
Phone: 34963983813 Fax: 34963864642
e-mail: lilian.valles@uv.es

# WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid $\beta_{1-42}$ effects on astrocytes in primary culture.

**Diana Aguirre-Rueda[1], Sol Guerra-Ojeda[1], Martin Aldasoro[1], Antonio Iradi[1], Elena Obrador[1], Mª Dolores Mauricio[1], Jose Mª Vila[1], Patricia Marchio[1] and Soraya L. Valles[1*]**

**[1]Department of Physiology. School of Medicine, University of Valencia. Spain.**

* Corresponding Author:

E-mail: lilian.valles@uv.es. (SLV)

**Abbreviations:** Alzheimer's disease (AD); amyloid beta$_{40-1}$ ($A\beta_{40-1}$); amyloid beta$_{1-42}$ ($A\beta_{1-42}$); peroxisome proliferator activated receptor gamma (PPAR-γ); WIN 55,212-2 (WIN); Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn superoxide dismutase (Mn SOD), Central Nervous System (CNS).

**Keywords:** Amyloid $\beta_{1-42}$, cannabinoids, inflammation, oxidative stress, cell death.

# Abstract

Alzheimer´s disease (AD), a neurodegenerative illness involving synaptic dysfunction with extracellular accumulation of $A\beta_{1-42}$ toxic peptide, glial activation, inflammatory response and oxidative stress, can lead to neuronal death. Endogenous cannabinoid system is implicated in physiological and physiopathological events in central nervous system (CNS), and changes in this system are related to many human diseases, including AD. However, studies on the effects of cannabinoids on astrocytes functions are scarce. In primary cultured astrocytes we studied cellular viability using MTT assay. Inflammatory and oxidative stress mediators were determined by ELISA and Western-blot techniques both in the presence and absence of $A\beta_{1-42}$ peptide. Effects of WIN 55,212-2 (a synthetic cannabinoid) on cell viability, inflammatory mediators and oxidative stress were also determined. $A\beta_{1-42}$ diminished astrocytes viability, increased TNF-α and IL-1β levels and p-65, COX-2 and iNOS protein expression while decreased PPAR-γ and antioxidant enzyme Cu/Zn SOD. WIN 55,212-2 pretreatment prevents all effects elicited by $A\beta_{1-42}$. Furthermore, cannabinoid WIN 55,212-2 also increased cell viability and PPAR-γ expression in control astrocytes. In conclusion cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes. Moreover, WIN 55,212-2 increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by $A\beta_{1-42}$ in cultured astrocytes. Further studies would be needed to assess the possible beneficial effects of cannabinoids in Alzheimer's disease patients.

# Introduction

AD is a common neurodegenerative disease implicated in the aging process, affecting nearly 50% of people over 75 [1,2]. It involves neurofibrillary degeneration, extracellular accumulation of beta-amyloid peptide (Aβ) and synaptic dysfunction, resulting in neural cell death in the hippocampus and cerebral cortex, and in activation of glial cells [3,4]. Aβ can interact with different cellular components producing $Ca^{2+}$ deregulation, oxidative stress and inflammation [5,6].

Astrocytes are specialized neural cells serving as a structural and metabolic support and trophic help to the brain [7]. Astrocytes also release cytokines and chemokines involved both in protective and toxic roles in neuroinflammatory processes [8]. However, released cytokines in neuroinflammation may induce deleterious effects on the viability and functionality of astrocytes [9]. Furthermore, in pathological situations such as hypoxia, cytokines induce activation of vascular endothelial cells thereby modulating inflammatory responses [10]. In AD, astrocytes are found around senile plaques producing phagocytosis, and cleaning up toxic compounds such as Aβ [11]. Moreover, when stimulated with compounds such as genistein or estradiol, astrocytes block the release of pro-inflammatory mediators and induce the synthesis of anti-inflammatory proteins [12].

Endocannabinoids have been implicated in various physiopathological events in different organs, including the peripheral and CNS [13], and changes in the endocannabinoid system have been related to many human diseases, such as metabolic syndrome [14], neurodegeneration [15], inflammatory diseases [16], psychiatric disorders [17] and cancer [18]. The endocannabinoid signaling system is composed of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) interacting with CB1 and CB2 cannabinoid receptors. Receptor signaling may involve mechanisms such as adenylyl

cyclase blockade or activation of mitogen-activated protein kinases or ceramide signaling [13].

Different authors have proposed cannabinoids as preventive treatment in AD [19] due to their anti-inflammatory and neuroprotective properties [16]. In this sense, cannabinoids prevented microglial activation and cognitive impairment in Aβ-treated rats [19]. In mice exposed to Aβ, cannabinoids also suppress neuroinflammation by inhibiting iNOS expression and interleukin-1β generation [20]. However, the effects of cannabinoids on astrocytes functions have been poorly investigated. Therefore, we investigated the role of WIN 55,212-2 (WIN) as a neuroprotective agent against lesions induced by $A\beta_{1-42}$ on cultured astrocytes.

# Material and Methods

## Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gibco Invitrogen Corporation, Barcelona, Spain). The oligomers Aβ (40-1 and 1-42), were prepared following manufacture instructions (Sigma-Aldrich biotechnology). Briefly, the peptides were dissolved in $H_2O$, and, for assembly the oligomers, preparations were heated for 24 h at 37ºC. WIN and 3-(4,5-dimethyl-2-thiazolyl)-2,5-dipheniyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β and TNF-α from Pierce Biotechnology, Inc. (Rockford, USA). Western Blot Chemiluminescent Detection System (ECL) was from Amersham (Amersham Biosciences, Barcelona, Spain). Monoclonal anti-peroxisome proliferator-activated receptor antibody (PPAR-γ) (1:250) and polyclonal anti-cyclooxigenase-2 antibody (COX-2) (1:250) from Sigma Aldrich (Madrid, Spain). Monoclonal p65 antibody (p65) (1:250) from Santa Cruz Biotechnology (Madrid, Spain). Polyclonal anti-Cu/Zn superoxide dismutase antibody (Cu/Zn SOD) (1:250) from Assay Designs (Madrid, Spain). Monoclonal anti-tubuline (1:1000) from Cell Signaling (Beverly, MA, USA). All other reagents are analytical or culture grade purity.

## Primary culture of cortical astrocytes

All animals were handled according to the recommendations of the Bioethics Committee of the School of Medicine of the University of Valencia, Spain. Ethics committee specifically approved this study. Cortical astrocytes were isolated from rat fetuses of 21 days gestation. Fetuses were obtained by cesarean section and decapitated. Cerebral cortices were removed and triturated 10–15 times through a Pasteur pipette

with 10 ml DMEM. The cell suspension was filtered through nylon mesh with a pore size of 90 μm and re-suspended in DMEM containing 20% fetal bovine serum (FBS), supplemented with L-glutamine (1%), HEPES (10 mM), fungizone (1%), and antibiotics (1%). Cells were plated on T75 culture flask and maintained in a humidified atmosphere of 5% CO2/95% air at 37°C during 15 days. After 4 days of culture, the FBS was maintained at 20% and after 1 week of culture, the FBS content was reduced to 10%, and the medium was changed twice a week. The purity of astrocytes was assessed by immunofluorescence using anti-glial fibrillary acidic protein (astrocyte marker; Sigma-Aldrich), anti-CD-68 (microglial marker; Serotec), anti-myelin basic protein (olygodendroglial marker; Sigma-Aldrich) and anti-microtubule-associated protein 2 (neuronal marker; Sigma-Aldrich). The astrocyte cultures were found to be at least 99% glial fibrillary acidic protein positive. No cells were found to express CD-68, myelin basic protein, or microtubule-associated protein-2.

## Cell treatments

Ten days after seeding, WIN (10 μM) was added to culture flasks. Twenty-four hours later, 10 μM $A\beta_{1-42}$ (toxic peptide) or $A\beta_{40-1}$ (control peptide) (Sigma-Aldrich) were added to the flasks. $A\beta_{1-42}$ concentration used in our study is in the range of toxic concentrations of the peptide [21,22]. Before incubation, the peptides were diluted in 100 μM of phosphate-buffered saline (PBS) and incubated for 24 h at 37º C. Assays were performed 24 h after peptide addition.

## MTT assay

Cell viability was determined by MTT assay. The MTT assay is a well-established, widely used and easily reproducible method for the assessment of cell viability and cytotoxicity [23,24]. Astrocytes were plated in 96-well culture plate and incubated with

WIN during 24h. Subsequently, $A\beta_{40-1}$ (control) and $A\beta_{1-42}$ peptides were added to wells for another 24h. After cell treatments, the medium was removed and cells were incubated with red free medium and MTT solution [0.5 mg/ml, prepared in phosphate buffer saline (PBS) solution] for 4 h at 37ºC. Finally, the medium was removed and formazan particles were dissolved in dimethyl sulfoxide (DMSO). Cell viability, defined as the relative amount of MTT reduction, was determined by spectrophotometry at 570 nm.

## Cytokine determination, IL-1 and TNFα

Astrocytes were seeded as previously published [12]. At the time of assay, the red phenol medium was removed and replaced by PBS containing 1 mg/ml bovine serum albumin (BSA), either in the presence or absence of $A\beta_{1-42}$ (10 μM). IL-1β and TNF-α concentration (pg/ml) were ascertained using ELISA kits (Pierce Biotechnology, Inc.).

## Western blot analysis

Cultured cells were treated with lysis buffer and mechanically degraded to release the proteins. Protein concentration was determined using modified Lowry method [25]. Loading buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 0.5% (v/v) 2-mercaptoethanol, 1% bromophenolblue and 19% glycerol) was added to protein sample and heated for 5 min at 95ºC. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes in a humid environment using a transfer buffer (25mM Tris, 190mM glycine, 20% methanol). Membranes were blocked with 5% milk in TBS (0.05% Tween-20) and incubated with primary antibodies overnight at 4ºC. Membranes were washed 3 times with wash buffer TBS-T (TBS, 0.2% Tween-20) and incubated with a secondary anti-rabbit IgG or anti-mouse IgG (Cell Signalling Technologies Danvers, MA) antibody conjugated to the enzyme horseradish peroxidase (HRP) for 1 h. Membranes were washed three times and proteins were detected using the ECL method as specified by the

manufacturer. Autoradiography signals were assessed using digital image system ImageQuant LAS 4000 (GE Healthcare).

## Statistical analyses

Values are expressed as mean±S.D. Differences between groups were assessed by one-way analysis of variance (ANOVA). Statistical significance was accepted at $P \leq 0.05$. Data sets in which *F* was significant were examined by a modified *t*-test.

# Results

## Protective role of WIN on cell viability

The role of WIN on cell viability was studied using MTT conversion assay. Figure 1A shows that incubation with WIN at different concentrations induced a significant increase in cell viability at 10 μM. Consequently, that concentration was used in future experiments. Astrocytes previously incubated with 10 μM $A\beta_{1-42}$ for 24 h significantly decreased cell viability compared to control cells (Figure 1B). Furthermore, pretreating astrocytes during 48 h with WIN (10 μM) prevented the decrease in cell viability induced by $A\beta_{1-42}$ (WIN + Aβ), conversely WIN (1, 2, 5μM) did not have any effect (Figure 1B).

## WIN prevents IL-1β and TNF-α increase elicited by $A\beta_{1-42}$

Cultured astrocytes were incubated with 10 μM $A\beta_{1-42}$ and proinflammatory mediators TNF-α and IL-1β were detected by ELISA. $A\beta_{1-42}$ increased 4.5-fold IL-1β release (480.4±150.3 pg/ml) compared with control (103.9±82.9 pg/ml) (Figure 2A) and 2.4 fold TNF-α release (605.3±103.4 pg/ml *vs* 210.5±85.3 pg/ml in control group) (Figure 2B). Furthermore, WIN pre-treatment (10 μM) prevented the increase in pro-inflammatory mediators induced by $A\beta_{1-42}$ (Figure 2 A and B).

## Effect of $A\beta_{1-42}$ and WIN on p65 protein expression

NF-κB, the pro-inflammatory transcription factor, is formed by different subunits. We measured p65 protein expression by western-blot. Incubation with $A\beta_{1-42}$ increased p65 protein expression compared with control astrocytes (Figure 3), which was prevented by WIN pretreatment. ($p<0.05$ compared with $A\beta_{1-42}$ treated astrocytes)

## WIN prevents COX-2 and iNOS protein increase induced by $A\beta_{1-42}$ peptide

Incubation with $A\beta_{1-42}$ significantly increased inflammatory proteins COX-2 (Figure 4A) and iNOS (Figure 4B) expressions compared to control. Furthermore, pretreating astrocytes with WIN prevented the effects produced by $A\beta_{1-42}$.

## Effect of $A\beta_{1-42}$ and WIN on PPAR-γ protein expression

Pro-inflammatory gene expression is downregulated by PPARs family [26]. We found that pretreatment with WIN (10 μM) increased PPAR-γ expression compared to control cells (Figure 5). Incubation with $A\beta_{1-42}$ significantly decreased PPAR-γ expression that was prevented by WIN pretreatment.

## Effect of $A\beta_{1-42}$ and WIN on Cu/Zn SOD and Mn SOD protein expression.

Superoxide dismutase (SOD) is a key antioxidant enzyme. In our study, incubation with $A\beta_{1-42}$ decreased Cu/Zn SOD expression in astrocytes in primary culture which was prevented by WIN pretreatment, evidencing that WIN could play a neuro-protective role against oxidative stress induced by $A\beta_{1-42}$ peptide (Figure 6A). On the other hand, our results indicated that Mn SOD protein expression is increased in presence of $A\beta_{1-42}$.

Pretreatment with WIN did not prevent Mn SOD increase induced by $A\beta_{1-42}$ (Figure 6B).

# Discussion

Oxidative stress and inflammation are the main mechanisms in the progression of various neurodegenerative diseases, including AD [27-30]. In our study, we determined different markers involved in inflammation and oxidative stress induced by the $A\beta_{1-42}$ peptide in primary cultures of astrocytes, with the aim to assess the antioxidant and anti-inflammatory effects of cannabinoid WIN. We found that WIN significantly increased astrocytes viability compared to control cells. Furthermore, WIN prevented the decrease in astrocytes viability induced by $A\beta_{1-42}$.

It has been shown that cannabinoids preserve neurons from Aβ exposure by activating MAP kinase cascade [31] and by anti-oxidative and anti-apoptotic effects [32]. Moreover, some studies demonstrated that cannabinoids protect glial cells from death [33,34]. Nevertheless, in cancer, where cells are highly proliferative and undifferentiated, treatment with cannabinoids can block cell proliferation in a dose dependent manner [35-38], demonstrating that the effects of cannabinoids on cell viability are probably dependent on cell type [39] and developmental stage [40].

Expression of CB1 [41] and CB2 [42] receptors in rat culture astrocytes have been published and also dual activation of both cannabinoid receptors by WIN 55,212-2 (the mixed non-selective CB1/CB2 agonist) in rat cortical astrocytes have been detected [41] On the other hand, WIN confers its protective and anti-inflammatory effects against Aβ injury through both CB1 and CB2 receptors [43]. Given that our results there is expression of both types of cannabinoid receptors (CB1 and CB2), it is likely that the effect of WIN observed in our study is due to the interaction with both types of receptors, consistent with published results by Fakhfouri and cols [44].

We found that WIN prevented the increase of inflammatory mediators IL-1β, TNF-α, NF-κB, iNOS and COX-2, as well as the decrease of the anti-inflammatory mediator

PPAR-γ induced by $A\beta_{1-42}$ in astrocytes in primary culture. The inflammatory process is a characteristic mechanism in the development of AD, and pro-inflammatory agents are involved in the progression of cell damage [45,46,47,12]. Moreover, it is known that astrocytes participate in the inflammatory process induced by $A\beta_{1-42}$ [27,28,48]. Initially, inflammation is beneficial since it produces pro-inflammatory substances involved in tissue protection, limiting the survival and proliferation of cells exposed to toxic agents, such as $A\beta_{1-42}$ [49,50]. However, sustained inflammatory response could lead to neurotoxic damage or cell death [12,51,8]. NF-κB proteins are up-regulated in inflammation conditions such as astroglial activation induced by $A\beta_{1-42}$ oligomers [52]. In this regard, we found an increase in NF-kB/p-65 expression in astrocytes after addition of $A\beta_{1-42}$ that was prevented by WIN pretreatment. Valles and collaborators [53] found that the cytokine-receptor complex is able to bind to cytokines and other proteins of the extracellular matrix, producing inflammatory signals which could be important in pathologies such as Alzheimer's disease [53,54]. In agreement with our results, different authors have reported that cannabinoids mitigate neural cell activation in the neuroinflammatory response induced by $A\beta_{1-42}$, reducing the levels of pro-inflammatory molecules such as IL-1β, TNF-α, COX-2 and iNOS [55,56,57]. Likewise, the activation of cannabinoid receptors diminishes the release of IL-1β, IL-6 and TNF-α in microglial cells [58,59,60] as well as COX-2 and iNOS [61]. Studies conducted in rats pretreated with the Aβ peptide found that WIN prevented cognitive impairment, glial activation and neuronal loss [19,62,63], and also reduced COX-2, iNOS and TNF-α levels [63,64].

Kainu et al. [65] demonstrated for the first time the presence of mRNA and protein PPAR-γ in CNS cells. Subsequent studies have detected PPAR-γ expression in microglial and astrocytic cells [66,12]. PPAR-γ agonists protect against Aβ-induced

inflammatory and neuronal damage [67,68], thus making neurons and astrocytes potential therapeutic targets for PPAR-γ ligands [69,12]. Astrocytes also express the largest levels of PPAR-γ in the neural tissues [70,71]. As other authors [72,73,74], we found a decreased expression of PPAR-γ in astrocytes treated with $A\beta_{1-42}$. Esposito et al. [56] showed in neurons that cannabinoids may act as neuro-protective agents by PPAR-γ activation. In this study, we demonstrate an increase in this protein expression in astrocytic cells previously incubated with WIN. Furthermore, we found for the first time that WIN prevents PPAR-γ expression decrease induced by $A\beta_{1-42}$ peptide in astrocytes in primary culture. There is strong evidence to suggest that some cannabinoids can act on PPARs through either direct or indirect pathways. In order to directly act on nuclear transcriptional factors PPARs, exogenous cannabinoids need to pass through plasma membrane and be transported into nucleus which may involve certain membrane and intracellular transporters. However, we still cannot rule out that cannabinoids effects could be indirect through the binding of other cellular targets which in turn induces PPARs activation [75]. In fact, WIN attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-γ pathway [44].

Different authors have demonstrated the role of oxidative stress in AD [76-79]. The cumulative damage caused by free radicals induces alterations in the activity or expression of antioxidant enzymes like catalase or SOD. These enzymes were found to be decreased in both CNS and peripheral tissues of AD patients [80,81]. In this sense, we demonstrate that Cu/Zn SOD is decreased in astrocytes treated with $A\beta_{1-42}$. Our results are consistent with those reported by other authors, highlighting the role of oxidative stress in the development of AD [82]. New substances are under research to reduce damage caused by oxidative stress in this disease. Widely distributed in the body,

cannabinoids receptors were discovered few decades ago and are still under research [57]. Few studies address the effect of cannabinoids on oxidative stress. For instance, cannabinoids were found to prevent or antagonize oxidative stress toxicity in cortical neurons in cultures [83,84], and in lymphoblastic cells [85]. Studies with PC12 cells exposed to $A\beta_{1-42}$ peptide demonstrated that cannabinoids reduced reactive oxygen species production and membrane lipid oxidation [86,32]. Our results provide evidence that $A\beta_{1-42}$ decreases Cu/Zn SOD expression in astrocytes in primary culture, and pretreatment with WIN increases Cu/Zn SOD expression, preventing the decrease caused by $A\beta_{1-42}$. These findings indicate that cannabinoids could act as a protective agent against oxidative stress caused by $A\beta_{1-42}$. In Figure 7 the set of results are summarized. However our results indicate that $A\beta_{1-42}$ elevated Mn SOD protein expression, increasing mitochondrial biogenesis mechanism, such as we previously published [74]. Pretreatment with WIN did not prevent Mn SOD overexpression induced by $A\beta_{1-42.}$ Mn SOD plays a role in the adaptive response which protects brain cells from damage, as in the case of AD. In fact, Mn SOD preserves neurons against oxidative stress [87] and protects developing neurons from β-amyloid toxicity [88]. This enzyme catalyzes the conversion of superoxide radicals to molecular oxygen and $H_2O_2$, whereas glutathione peroxidase, peroxiredoxin reductase and catalase neutralize $H_2O_2$. Overexpression of Mn SOD induces cognitive recovery and reduces Aβ levels in AD animal models [89]. Furthermore, Mn SOD deficiency increases β-amyloid levels and amyloid plaque burden, promoting the development of behavioural disturbances [90].

Preclinical data suggest a beneficial role of some cannabinoids for treatment of different diseases. Dronabidol, an oil-based solution of Δ9-THC, is used as anti-emetic and appetite stimulant [91]. Δ9-THC also decreases agitation present in the advanced stage of AD [92]. In 2003, the FDA granted the patent for cannabinoids as antioxidants

and neuro-protectants (U.S. Department of Health and Human Services). Despite these promising preliminary results, the clinical utility of cannabinoids in AD is still to be determined [93].

# Conclusions

Taken together, our findings show that cannabinoid WIN increases cell viability and anti-inflammatory response in astrocytes in primary culture and prevents cell death induced by $A\beta_{1-42}$. Furthermore, WIN increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by $A\beta_{1-42}$ in astrocytes. In this sense, clinical studies are needed to evaluate the neuro-protective effects of cannabinoids in Alzheimer´s disease.

# Conflict of Interest Statement:

The authors declare that there are no conflicts of interest.

# References

[1] Selkoe DJ. (1977) Alzheimer's disease: genotypes, phenotypes, and treatments. *Science*. 275:630-31.

[2] Ferri CP, Prince M, Brayne C, et al. (2005) Alzheimer's Disease International. Global prevalence of dementia: a Delphi consensus study. *Lancet* 366:2112-17.

[3] Heneka MT and O'Banion MK. (2007) Inflammatory processes in Alzheimer's disease. *J Neuroimmunol* 184:69-91.

[4] Pratico D. (2008) Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows. *Ann N Y Acad Sci.* 1147:70-78.

[5] Inestrosa NC, Reyes AE, Chacón MA, et al. (2005) Human-like rodent amyloid-beta-peptide determines Alzheimer pathology in aged wild-type Octodon degu. *Neurobiol Aging* 26:1023-28.

[6] Vallés SL, Borrás C, Gambini J, et al. (2008) Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38. *Aging Cell* 7:112-8.

[7] Sofroniew MV and Vinters HV. (2010) Astrocytes: biology and pathology. *Acta Neuropathol* 119:7-35.

[8] Choi SS, Lee HJ, Lim I, et al. (2014) Human astrocytes: secretome profiles of cytokines and chemokines. *PLoS One* 1:9(4):e92325. doi:10.1371/journal.pone.0092325.

[9] van Kralingen C, Kho DT, Costa J, et al. (2013) Exposure to inflammatory cytokines IL-1β and TNFα induces compromise and death of astrocytes; implications for chronic neuroinflammation. *PLoS One* 19;8(12):e84269. doi: 10.1371/journal.pone.0084269.

[10] Zhang W, Smith C, Howlett C, et al. (2000) Inflammatory activation of human brain endothelial cells by hypoxic astrocytes in vitro is mediated by IL-1beta. *J Cereb Blood Flow Metab* 20:967-78.

[11] Li C, Zhao R, Gao K, et al. (2011) Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* 8:67-80.

[12] Valles SL, Dolz-Gaiton P, Gambini J, et al. (2010) Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression in cultured astrocytes. *Brain Res* 1312:138-44.

[13] Pacher P and Kunos G. (2013) Modulating the endocannabinoid system in human health and disease-successes and failures. *FEBS J* 280:1918-43.

[14] Kunos G and Tam J. (2011) The case for peripheral $CB_1$ receptor blockade in the treatment of visceral obesity and its cardiometabolic complications. *Br J Pharmacol* 163:1423-31.

[15] Centonze D, Finazzi-Agrò A, Bernardi G, et al. (2007) The endocannabinoid system in targeting inflammatory neurodegenerative diseases. *Trends Pharmacol Sci* 28:180-7.

[16] Klein WK. (2005) Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nat Rev Immunol* 5:400-11.

[17] Hillard CJ, Weinlander KM, Stuhr KL. (2012) Contributions of endocannabinoid signaling to psychiatric disorders in humans: genetic and biochemical evidence. *Neuroscience* 1:207-29.

[18] Guindon J and Hohmann AG. (2011) The endocannabinoid system and cancer: therapeutic implication. *Br J Pharmacol* 163:1447-63.

[19] Ramírez BG, Blázquez C, Gómez del Pulgar T, et al. (2005) Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *J Neurosci* 25:1904-13.

[20] Esposito G, Scuderi C, Savani C, et al. (2007) Cannabidiol in vivo blunts beta-amyloid induced neuroinflammation by suppressing IL-1beta and iNOS expression. *Br J Pharmacol* 151:1272-79.

[21] Blanchard BJ, Chen A, Rozeboom LM, et al. (2004) Efficient reversal of Alzheimer's disease fibril formation and elimination of neurotoxicity by a small molecule. *Proc Natl Acad Sci* 101:14326-332.

[22] McGeer PL and McGeer EG. (2013) The amyloid cascade-inflammatory hypothesis of Alzheimer disease: implications for therapy. *Acta Neuropathol.* 126:479-97.

[23] Rönicke R, Klemm A, Meinhardt J, et al. (2008) Abeta mediated diminution of MTT reduction--an artefact of single cell culture?. *PLoS One* 3:e3236.

[24] Palanca JM, Aguirre-Rueda D, Granell MV, et al. (2013) Sugammadex, a neuromuscular blockade reversal agent, causes neuronal apoptosis in primary cultures. *Int J Med Sci* 10:1278-85.

[25] Peterson GL. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 83:346-56.

[26] Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature.* 391(6662):79-82.

[27] Eikelenboom P and van Gool WA. (2004) Neuroinflammatory perspectives on the two faces of Alzheimer's disease. *J Neural Transm* 111:281-94.

[28] Zhu X, Smith MA, Perry G, et al. (2004) Mitochondrial failures in Alzheimer's disease. *Am J Alzheimers Dis Other Demen* 19:345-52.

[29] Jiménez-Jiménez FJ, Alonso-Navarro H, Ayuso-Peralta L, et al. (2006) Oxidative stress and Alzheimer's disease. *Rev Neurol* 42:419-27.

[30] Quintanilla RA, Orellana JA, von Bernhardi R. (2012) Understanding risk factors for Alzheimer's disease: interplay of neuroinflammation, connexin-based communication and oxidative stress. *Arch Med Res* 43:632-44.

[31] Milton NG. (2002) Anandamide and noladin ether prevent neurotoxicity of the human amyloid-beta peptide. *Neurosci Lett* 332:127-30.

[32] Iuvone T, Esposito G, Esposito R, et al. (2004) Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on beta-amyloid-induced toxicity in PC12 cells. *J Neurochem* 89:134-41.

[33] Marsicano G, Wotjak CT, Azad SC, et al. (2002) The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 1:530-34.

[34] Gómez del Pulgar T, de Ceballos ML, Guzmán M, et al. (2002) Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. *J. Biol. Chem* 277: 36527–33.

[35] Pourkhalili N, Ghahremani MH, Farsandaj N, et al. (2013) Evaluation of anti-invasion effect of cannabinoids on human hepatocarcinoma cells. *Toxicol Mech Methods* 23:120-6.

[36] De Petrocellis L, Ligresti A, Schiano-Moriello A, et al. (2013) Non-THC cannabinoids inhibit prostate carcinoma growth in vitro and in vivo: pro-apoptotic effects and underlying mechanisms. *Br J Pharmacol* 168:79-102.

[37] Vara D, Salazar M, Olea-Herrero N, et al. (2011) Anti-tumoral action of cannabinoids on hepatocellular carcinoma: role of AMPK-dependent activation of autophagy. *Cell Death Differ* 18:1099-111.

[38] Rocha FC, Dos Santos Júnior JG, Stefano SC, et al. (2014) Systematic review of the literature on clinical and experimental trials on the antitumor effects of cannabinoids in gliomas. *J Neurooncol* 116:11-24.

[39] Sánchez C, Galve-Roperh I, Canova C, et al. (1998) Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Lett* 436:6-10.

[40] Guzmán M, Sánchez C, Galve-Roperh I. (2002) Cannabinoids and cell fate. *Pharmacol Ther* 95:175-84.

[41] Stella N. (2004) Cannabinoid signaling in glial cells. *Glia* 48(4):267-277.

[42] Sheng WS, Hu S, min X, Cabral GA, Lokensgard JR, Peterson P. (2005) Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta.stimulated human astrocytes. *Glia* 49:211-219.

[43] Palmer SL, Thakur GA, Makriyannis A. (2002) Cannabinergic ligands. *Chem Phys Lipids* 121:3-19.

[44] Fakhfouri G, Ahmadiani A, Rahimian R, et al. (2012) WIN55212-2 attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-γ pathway. *Neuropharmacology.* 63:653-66

[45] Strohmeyer R and Rogers J. (2001) Molecular and cellular mediators of Alzheimer's disease inflammation. *J Alzheimers Dis* 3:131-57.

[46] Wyss-Coray T and Mucke L. (2002) Inflammation in neurodegenerative disease-A doubleedged sword. *Neuron* 35:419-32.

[47] Griffin WS and Mrak RE. (2002) Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease. *J Leukoc Biol* 72:233-8.

[48] McGeer PL, Rogers J, McGeer EG. (2006) Inflammation, anti-inflammatory agents and Alzheimer disease: the last 12 years. *J Alzheimers Dis* 9:271-6.

[49] Allan SM and Rothwell NJ. (2001) Cytokines and acute neurodegeneration. *Nat Rev Neurosci* 2:734-44.

[50] Allan SM and Rothwell NJ. (2003) Inflammation in central nervous system injury. *Philos Trans R Soc Lond B Biol Sci* 358:1669-77.

[51] Burkert K, Moodley K, Angel CE, et al. (2012) Detailed analysis of inflammatory and neuromodulatory cytokine secretion from human NT2 astrocytes using multiplex bead array. *Neurochem Int* 60:573-80.

[52] Carrero I, Gonzalo MR, Martin B, et al. (2012) Oligomers of β-amyloid protein (Aβ1-42) induce the activation of cyclooxygenase-2 in astrocytes via an interaction with interleukin-1β, tumour necrosis factor-α, and a nuclear factor κ-B mechanism in the rat brain. *Exp Neurol* 236:215-27.

[53] Vallés S, Tsoi C, Huang WY, et al. (1999) Recruitment of a heparan sulfate subunit to the interleukin-1 receptor complex. Regulation by fibronectin attachment. *J Biol Chem* 274:20103-9.

[54] Bondareff W. (2013) Age-related changes in brain extracellular space affect processing of amyloid-β peptides in Alzheimer's disease. *J Alzheimers Dis* 35:1-6.

[55] Sheng WS, Hu S, Min X, et al. (2005) Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta-stimulated human astrocytes. *Glia* 49:211-9.

[56] Esposito G, Scuderi C, Valenza M, et al. (2011) Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PLoS One* 6:e28668.

[57] Scuderi C, Esposito G, Blasio A, et al. (2011) Palmitoylethanolamide counteracts reactive astrogliosis induced by β-amyloid peptide. *J Cell Mol Med* 15:2664-74.

[58] Puffenbarger RA, Boothe AC, Cabral GA. (2000) Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. *Glia* 29:58-69.

[59] Facchinetti F, Del Giudice E, Furegato S, et al. (2003) Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide. *Glia* 41:161-8.

[60] Ehrhart J, Obregon D, Mori T, et al. (2005) Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. *J Neuroinflammation* 12:2-29.

[61] Castillo A, Tolón MR, Fernández-Ruiz J, et al. (2010) The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. *Neurobiol Dis* 37:434-40.

[62] Martín-Moreno AM, Reigada D, Ramírez BG, et al. (2011) Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease. *Mol Pharmacol* 79:964-73.

[63] Martín-Moreno AM, Brera B, Spuch C, et al. (2012) Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β-amyloid levels and improves cognitive performance in Tg APP 2576 mice. *J Neuroinflammation* 16:8.

[64] Esposito G, De Filipis D, Carnuccio R, et al. (2006) The marijuana component cannabidiol inhibits β-amyloid-induced tau protein hyperphosphorylation through Wnt/β-catenin pathway rescue PC12 cells. *J Mol Med* 84:253-8.

[65] Kainu T, Wikström AC, Gustafsson JA, et al. (1994) Localization of the peroxisome proliferator-activated receptor in the brain. *Neuroreport* 5:2481-5.

[66] Luna-Medina R, Cortes-Canteli M, Alonso M, et al. (2005) Regulation of inflammatory response in neural cells in vitro by thiadiazolidinones derivatives through peroxisome proliferator-activated receptor gamma activation. *J Biol Chem* 280:21453-62.

[67] Bright JJ, Kanakasabai S, Chearwae W, et al. (2008) PPAR Regulation of Inflammatory Signaling in CNS Diseases. *PPAR Res* doi: 10.1155/2008/658520.

[68] Kapadia R, Yi JH, Vemuganti R. (2008) Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-γ agonists. *Front Biosci* 13:1813–26.

[69] Landreth G, Jiang Q, Mandrekar S, et al. (2008) PPARgamma agonists as therapeutics for the treatment of Alzheimer's disease. *Neurotherapeutics* 5:481-9.

[70] Bodles AM and Barger SW. (2005) Secreted β-amyloid precursor protein activates microglia via JNK and p38-MAPK. *Neurobiol Aging* 26:9–16.

[71] Ho GJ, Drego R, Hakimian E, et al. (2005) Mechanisms of cell signaling and inflammation in Alzheimer's disease. *Curr Drug Targets Inflamm Allergy* 4:247–56.

[72] Wang HM, Zhao YX, Zhang S, et al. (2010) PPARgamma agonist curcumin reduces the amyloid-beta-stimulated inflammatory responses in primary astrocytes. *J Alzheimers Dis* 20:1189-99.

[73] Chen YC, Wu JS, Tsai HD, et al. (2012) Peroxisome proliferator-activated receptor gamma (PPAR-γ) and neurodegenerative disorders. *Mol Neurobiol* 46:114–24.

[74] Aguirre-Rueda D, Guerra-Ojeda S, Aldasoro M, et al. (2015) Astrocytes protect neurons from Aβ1-42 peptide-induced neurotoxicity increasing TFAM and PGC-1 and decreasing PPAR-γ and SIRT-1. *Int J Med Sci* 12:48-56.

[75] Sun Y, Bennett A (2007) Cannabinoids: a new group of agonists of PPARs. *PPAR Res.* 2007:23513.

[76] Wallace DC. (2000) Mitochondrial defects in cardiomyopathy and neuromuscular disease. *Am Heart J* 139:S70–S85.

[77] Lesnefsky EJ, Moghaddas S, Tandler B, et al. (2001) Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. *J Mol Cell Cardiol* 33:1065-89.

[78] Chen Q, Vazquez EJ, Moghaddas S, et al. (2003) Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* 278:36027-31.

[79] Abeti R and Duchen MR. (2012) Activation of PARP by Oxidative Stress Induced by β-Amyloid: Implications for Alzheimer's Disease. *Neurochem Res* 37:2589-96.

[80] Marcus DL, Thomas C, Rodriguez C, et al. (1998) Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. *Exp Neurol* 150:40-4.

[81] Padurariu M, Ciobica A, Hritcu L, et al. (2010) Changes of some oxidative stress markers in the serum of patients with mild cognitive impairment and Alzheimer's disease. *Neuroscience Letters* 469:6–10.

[82] Pappolla MA, Chyan YJ, Omar RA, et al. (1998) Evidence of oxidative stress and in vivo neurotoxicity of beta-amyloid in a transgenic mouse model of Alzheimer's disease: a chronic oxidative paradigm for testing antioxidant therapies in vivo. *Am J Pathol* 152:871-7.

[83] Hampson AJ, Grimaldi M, Axelrod J, et al. (1998) Cannabidiol and (-) Δ9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci* 95:8268–73.

[84] Hampson AJ and Grimaldi M. (2001) Cannabinoid receptor activation and elevated cyclic AMP reduce glutamate neurotoxicity. *Eur J Neurosci* 13:1529–36.

[85] Chen Y and Buck J. (2000) Cannabinoids protect cells from oxidative cell death: a receptor-independent mechanism. *J. Pharmacol. Exp. Ther* 293:807–12.

[86] Pratico D, MY Lee V, Trojanowski JQ, et al. (1998) Increased F2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation in vivo. *FASEB J* 12:1777-83.

[87] Keller JN, Kindy MS, Holtsberg FW, et al. (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J Neurosci*. 18:687-97.

[88] Sompol P, Ittarat W, Tangpong J, et al. (2008) A neuronal model of Alzheimer's disease: an insight into the mechanisms of oxidative stress-mediated mitochondrial injury. *Neuroscience* 153:120-30.

[89] Dumont M, Wille E, Stack C, et al. (2009) Reduction of oxidative stress, amyloid deposition, and memory deficit by manganese superoxide dismutase overexpression in a transgenic mouse model of Alzheimer's disease. *FASEB J.* 23:2459-66.

[90] Esposito L, Raber J, Kekonius L, et al. (2006) Reduction in mitochondrial superoxide dismutase modulates Alzheimer's disease-like pathology and accelerates the onset of behavioral changes in human amyloid precursor protein transgenic mice. *J Neurosci*. 26:5167-79.

[91] Volicer L, Stelly M, Morris J, et al. (1997) Effects of dronabinol on anorexia and disturbed behavior in patients with Alzheimer's disease. *Int J Geriatr Psychiatry* 12:913-9.

[92] Walther S, Mahlberg R, Eichmann U, et al. (2006) 9-tetrahydrocannabinol for nighttime agitation in severe dementia. *Psychopharmacology* 185:524–8.

[93] Krishnan S, Cairns R, Howard R. (2009) Cannabinoids for the treatment of dementia. *Cochrane Database Syst Rev* 15(2):CD007204.

# Figure Legends

**Figure 1. Astrocytes viability.** (A) Astrocytes viability induced by WIN. Concentration-dependent viability of WIN (1, 2, 5, 10 μM) was determined by MTT assay for 24 h. Data are means ± SD for 4 independent experiments. *$p$<0.04 comparing WIN vs control cells. (B) Astrocytes viability in cells treated during 24 h with 10 μM $A\beta_{40\text{-}1}$ (control peptide, C), 10 μM $A\beta_{1\text{-}42}$ (Aβ) and WIN (1, 2, 5, 10 μM) + 10 μM $A\beta_{1\text{-}42}$ (WIN + Aβ). Data are means ± SD of 3 independent experiments. *$p$<0.05 *vs* control cells.

**Figure 2. IL-1β and TNF-α secretion.** WIN prevents the increase of IL-1β and TNF-α secretion caused by $A\beta_{1\text{-}42}$ in astrocytes. Cells were incubated with 10 μM $A\beta_{40\text{–}1}$ (control peptide, C), 10 μM $A\beta_{1\text{–}42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1\text{–}42}$ (WIN + Aβ). Cell culture supernatants were harvested, and IL-1β (panel A) and TNF-α (panel B) secretion were determined by ELISA. Values are means ± SD of replicate experiments from 4 independent astrocytes cultures. *$p$<0.05 *vs* control astrocytes. #$p$<0.05 *vs* $A\beta_{1\text{-}42}$ treated cells.

**Figure 3. p65 protein expression.** WIN 55, 212-2 prevents p65 expression induced by $A\beta_{1\text{-}42}$ in astrocytes in primary culture. p65 and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40\text{-}1}$ (control peptide, C), 10 μM $A\beta_{1\text{-}42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1\text{-}42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 5 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1\text{-}42}$.

**Figure 4. COX-2 and iNOS protein expression.** WIN prevents COX-2 and iNOS expression induced by $A\beta_{1-42}$. COX-2 (panel A), iNOS (panel B) and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40-1}$ (control peptide, C), 10 μM $A\beta_{1-42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1-42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 6 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1-42}$.

**Figure 5. PPAR-γ protein expression.** WIN induces PPAR-γ expression in astrocytes in primary culture treated with $A\beta_{1-42}$. PPAR-γ and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40-1}$ (control peptide, C), 10 μM $A\beta_{1-42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1-42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 4 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1-42}$.

**Figure 6. Cu/Zn-SOD and Mn-SOD protein expressions.** WIN prevents Cu/Zn-SOD expression decrease in astrocytes in primary culture treated with $A\beta_{1-42}$. Cu/Zn-SOD, Mn-SOD and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40-1}$ (control peptide, C), 10 μM $A\beta_{1-42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1-42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 4 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1-42}$.

**Figure 7. Preventive function of cannabinoid WIN on $A\beta_{1-42}$-induced toxic effects in astrocytes in primary culture.** Cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes and prevents inflammatory effects induced by $A\beta_{1-42}$.

Figure 1
Click here to download high resolution image

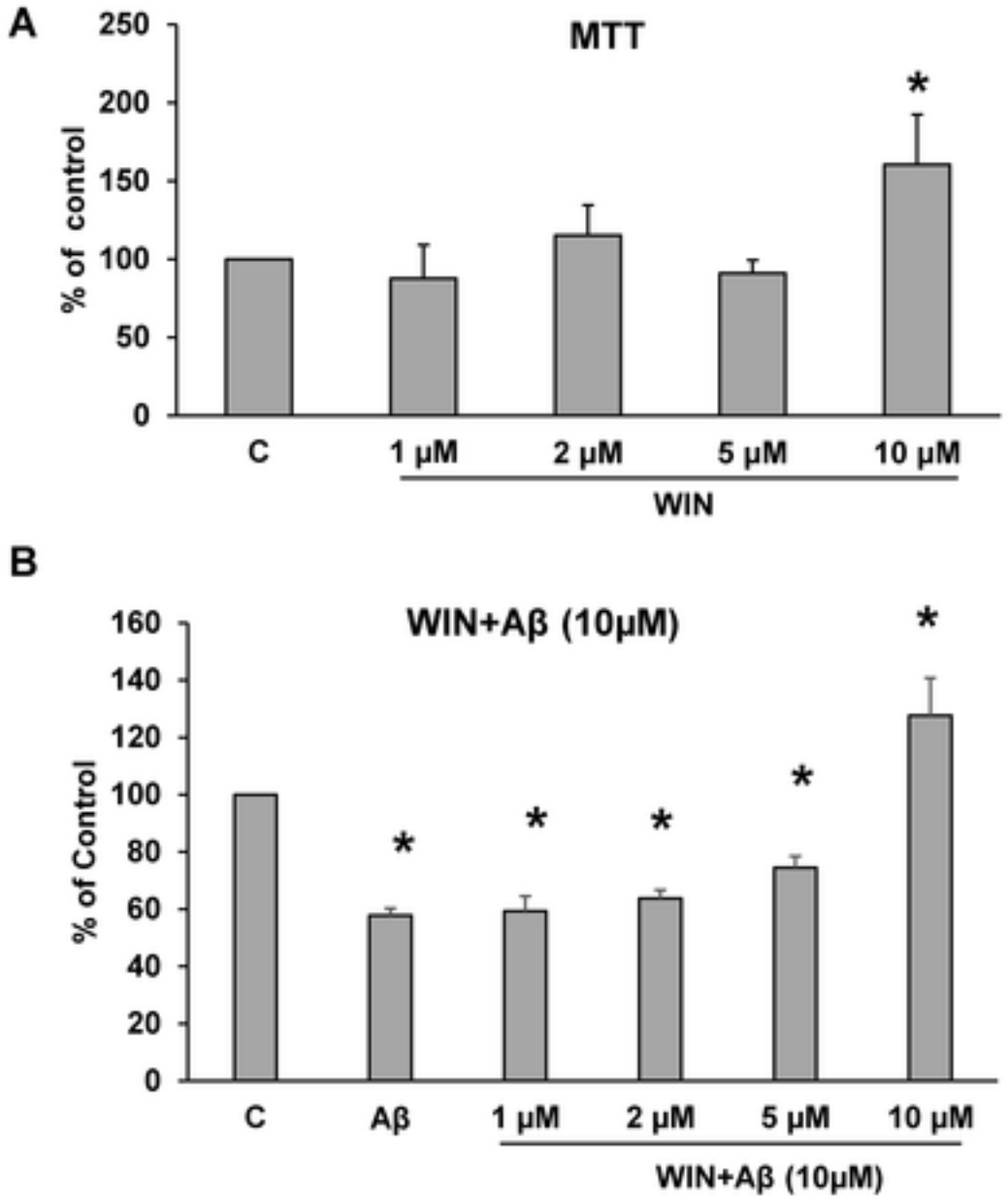


Figure 2
Click here to download high resolution image

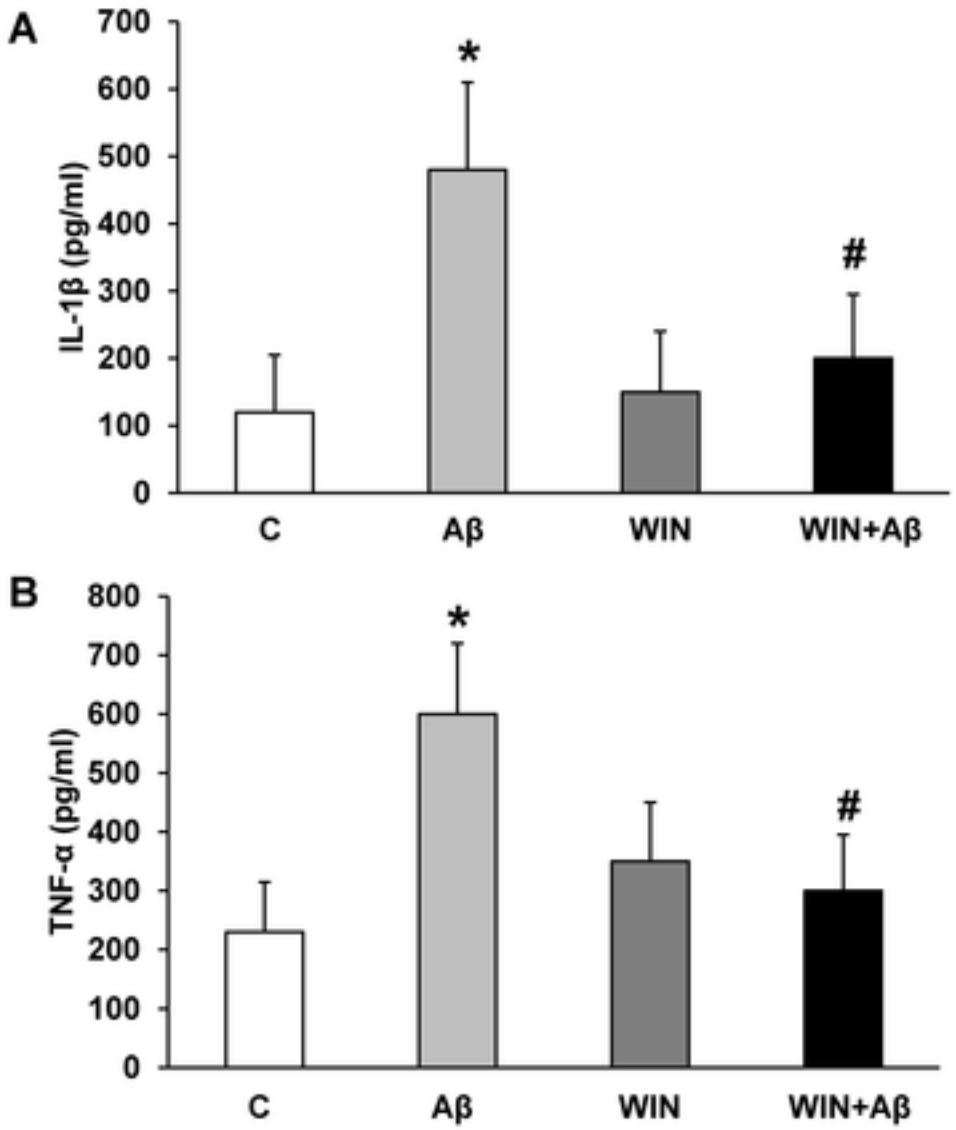


Figure 3
Click here to download high resolution image

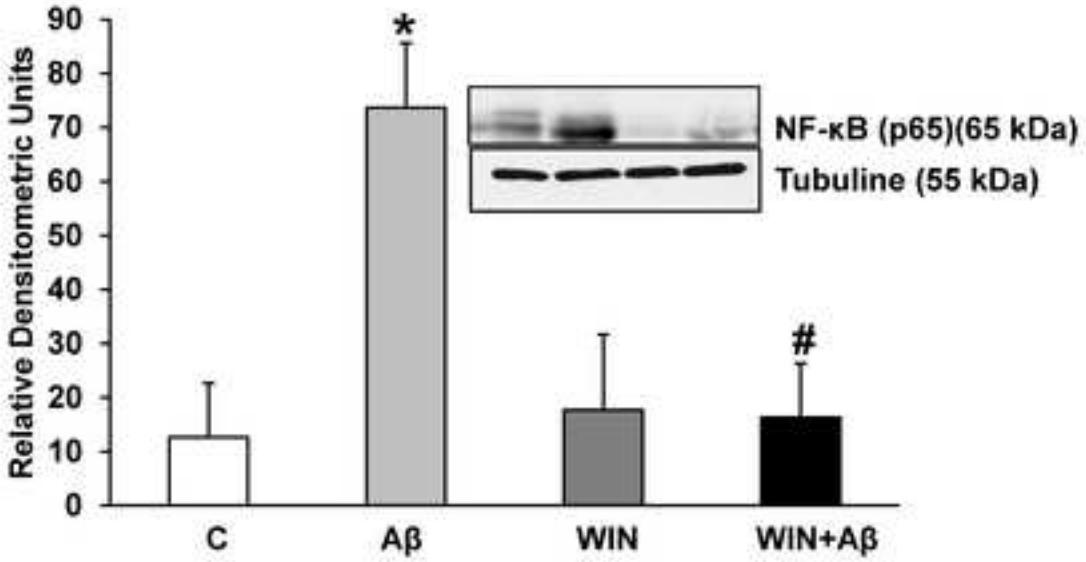


Figure 4
Click here to download high resolution image

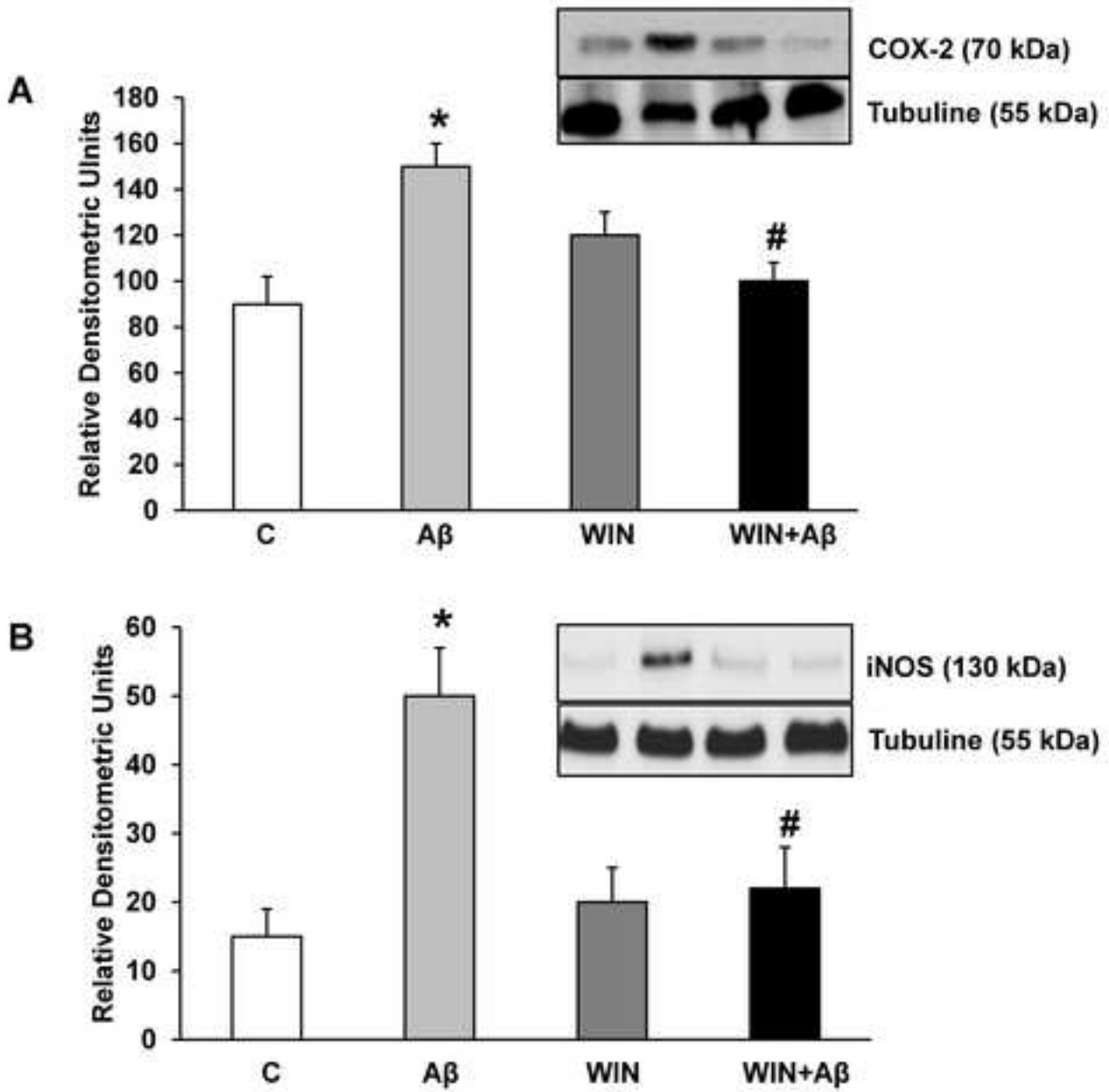


Figure 5
Click here to download high resolution image

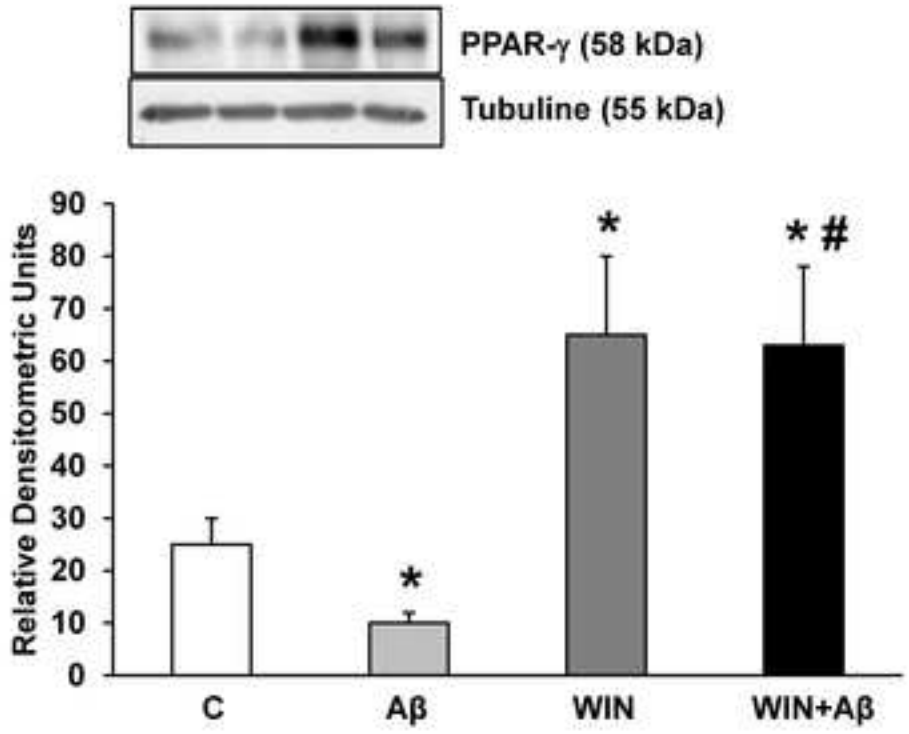


Figure 6
Click here to download high resolution image

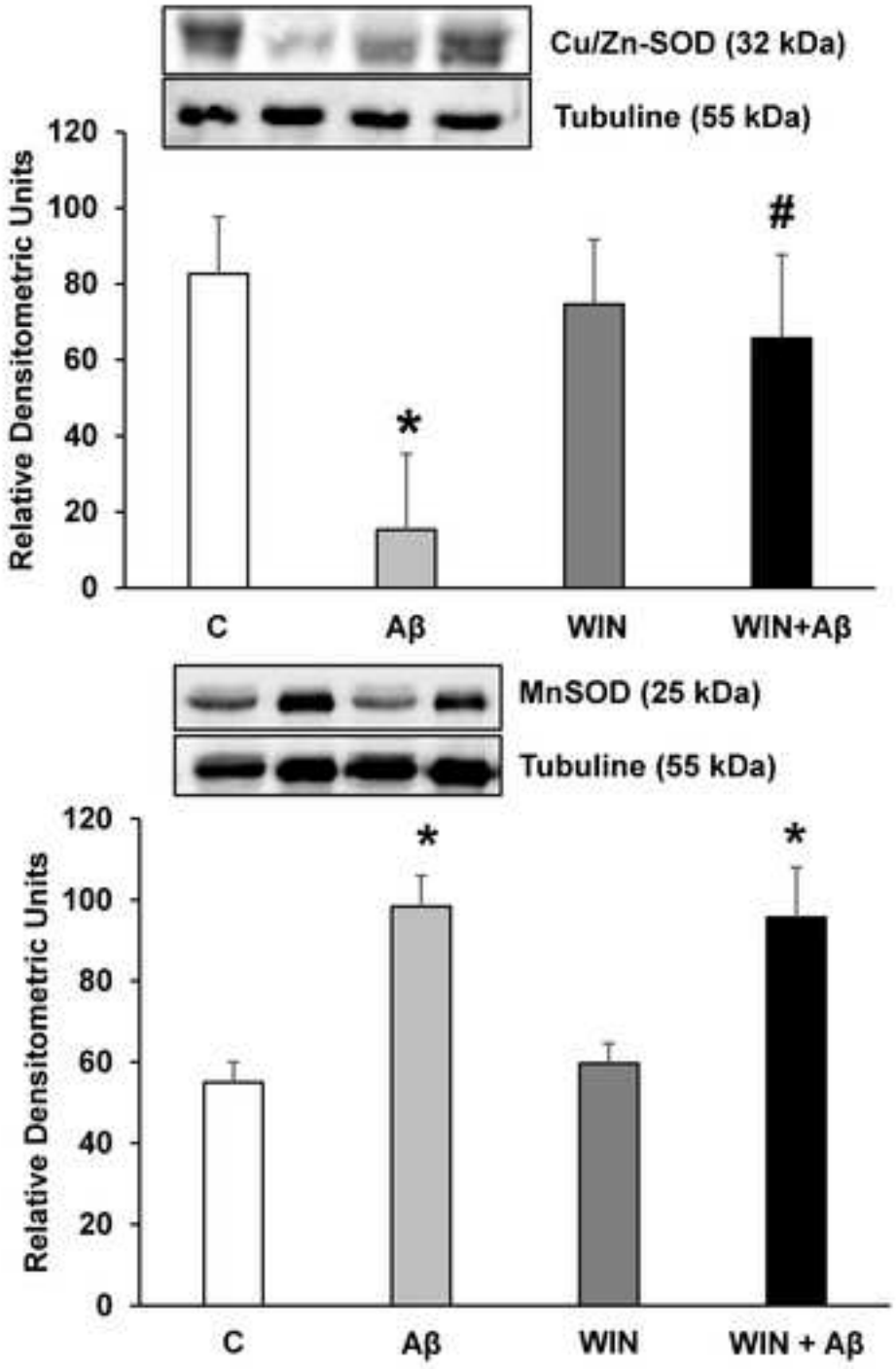
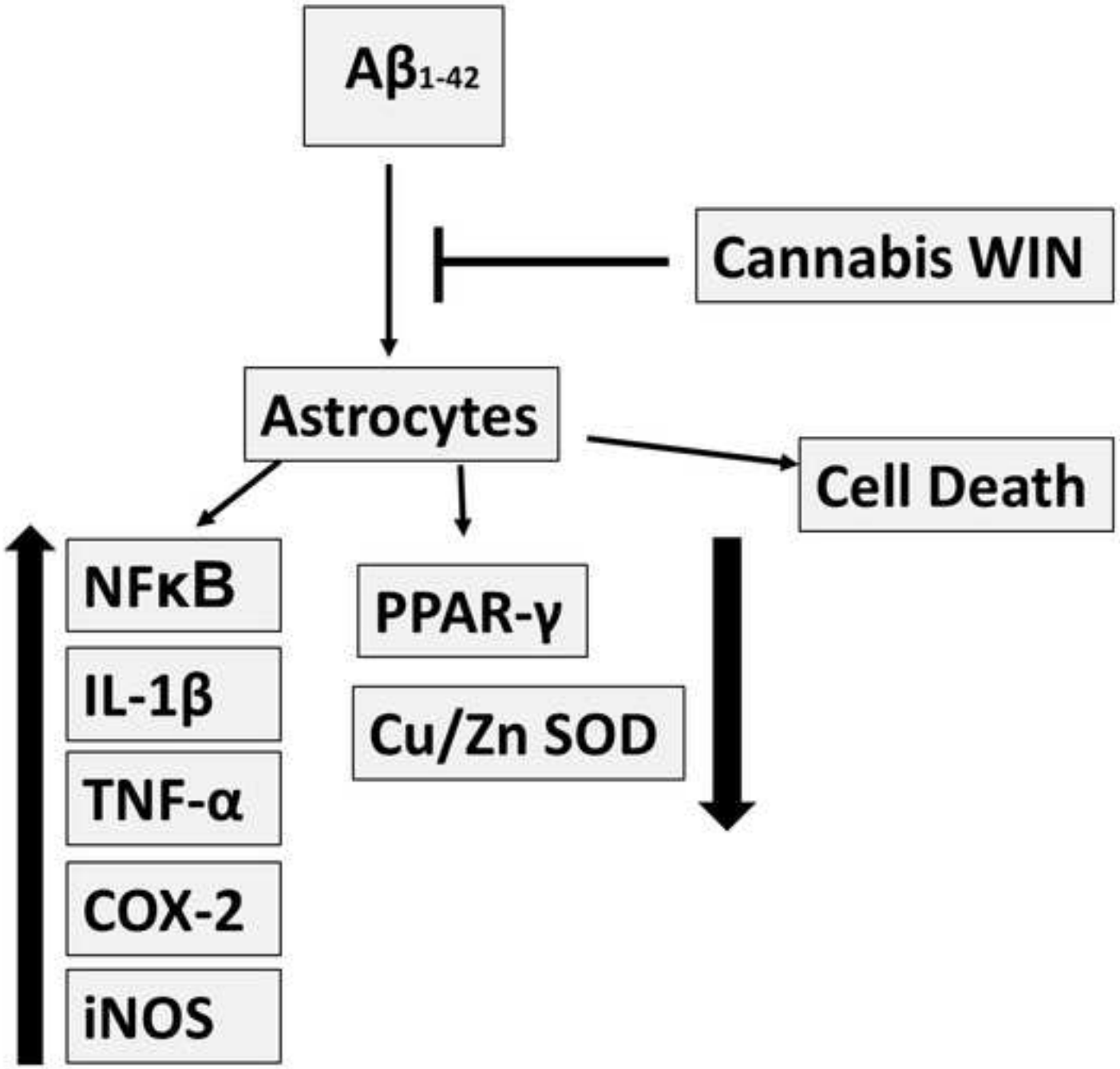


Figure 7
Click here to download high resolution image



# WIN 55,212-2, agonist of cannabinoid receptors, prevents Amyloid $\beta_{1-42}$ effects on astrocytes in primary culture.

**Diana Aguirre-Rueda[1], Sol Guerra-Ojeda[1], Martin Aldasoro[1], Antonio Iradi[1], Elena Obrador[1], Mª Dolores Mauricio[1], Jose Mª Vila[1], Patricia Marchio[1] and Soraya L. Valles[1*]**

**[1]Department of Physiology. School of Medicine, University of Valencia. Spain.**

* Corresponding Author:

E-mail: lilian.valles@uv.es. (SLV)

**Abbreviations:** Alzheimer's disease (AD); amyloid beta$_{40-1}$ ($A\beta_{40-1}$); amyloid beta$_{1-42}$ ($A\beta_{1-42}$); peroxisome proliferator activated receptor gamma (PPAR-γ); WIN 55,212-2 (WIN); Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn superoxide dismutase (Mn SOD), Central Nervous System (CNS).

**Keywords:** Amyloid $\beta_{1-42}$, cannabinoids, inflammation, oxidative stress, cell death.

# Abstract

Alzheimer´s disease (AD), a neurodegenerative illness involving synaptic dysfunction with extracellular accumulation of $A\beta_{1-42}$ toxic peptide, glial activation, inflammatory response and oxidative stress, can lead to neuronal death. Endogenous cannabinoid system is implicated in physiological and physiopathological events in central nervous system (CNS), and changes in this system are related to many human diseases, including AD. However, studies on the effects of cannabinoids on astrocytes functions are scarce. In primary cultured astrocytes we studied cellular viability using MTT assay. Inflammatory and oxidative stress mediators were determined by ELISA and Western-blot techniques both in the presence and absence of $A\beta_{1-42}$ peptide. Effects of WIN 55,212-2 (a synthetic cannabinoid) on cell viability, inflammatory mediators and oxidative stress were also determined. $A\beta_{1-42}$ diminished astrocytes viability, increased TNF-α and IL-1β levels and p-65, COX-2 and iNOS protein expression while decreased PPAR-γ and antioxidant enzyme Cu/Zn SOD. WIN 55,212-2 pretreatment prevents all effects elicited by $A\beta_{1-42}$. Furthermore, cannabinoid WIN 55,212-2 also increased cell viability and PPAR-γ expression in control astrocytes. In conclusion cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes. Moreover, WIN 55,212-2 increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by $A\beta_{1-42}$ in cultured astrocytes. Further studies would be needed to assess the possible beneficial effects of cannabinoids in Alzheimer's disease patients.

# Introduction

AD is a common neurodegenerative disease implicated in the aging process, affecting nearly 50% of people over 75 [1,2]. It involves neurofibrillary degeneration, extracellular accumulation of beta-amyloid peptide (Aβ) and synaptic dysfunction, resulting in neural cell death in the hippocampus and cerebral cortex, and in activation of glial cells [3,4]. Aβ can interact with different cellular components producing $Ca^{2+}$ deregulation, oxidative stress and inflammation [5,6].

Astrocytes are specialized neural cells serving as a structural and metabolic support and trophic help to the brain [7]. Astrocytes also release cytokines and chemokines involved both in protective and toxic roles in neuroinflammatory processes [8]. However, released cytokines in neuroinflammation may induce deleterious effects on the viability and functionality of astrocytes [9]. Furthermore, in pathological situations such as hypoxia, cytokines induce activation of vascular endothelial cells thereby modulating inflammatory responses [10]. In AD, astrocytes are found around senile plaques producing phagocytosis, and cleaning up toxic compounds such as Aβ [11]. Moreover, when stimulated with compounds such as genistein or estradiol, astrocytes block the release of pro-inflammatory mediators and induce the synthesis of anti-inflammatory proteins [12].

Endocannabinoids have been implicated in various physiopathological events in different organs, including the peripheral and CNS [13], and changes in the endocannabinoid system have been related to many human diseases, such as metabolic syndrome [14], neurodegeneration [15], inflammatory diseases [16], psychiatric disorders [17] and cancer [18]. The endocannabinoid signaling system is composed of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) interacting with CB1 and CB2 cannabinoid receptors. Receptor signaling may involve mechanisms such as adenylyl

cyclase blockade or activation of mitogen-activated protein kinases or ceramide signaling [13].

Different authors have proposed cannabinoids as preventive treatment in AD [19] due to their anti-inflammatory and neuroprotective properties [16]. In this sense, cannabinoids prevented microglial activation and cognitive impairment in Aβ-treated rats [19]. In mice exposed to Aβ, cannabinoids also suppress neuroinflammation by inhibiting iNOS expression and interleukin-1β generation [20]. However, the effects of cannabinoids on astrocytes functions have been poorly investigated. Therefore, we investigated the role of WIN 55,212-2 (WIN) as a neuroprotective agent against lesions induced by $A\beta_{1-42}$ on cultured astrocytes.

# Material and Methods

## Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Gibco Invitrogen Corporation, Barcelona, Spain). The oligomers Aβ (40-1 and 1-42), were prepared following manufacture instructions (Sigma-Aldrich biotechnology). Briefly, the peptides were dissolved in $H_2O$, and, for assembly the oligomers, preparations were heated for 24 h at 37ºC. WIN and 3-(4,5-dimethyl-2-thiazolyl)-2,5-dipheniyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β and TNF-α from Pierce Biotechnology, Inc. (Rockford, USA). Western Blot Chemiluminescent Detection System (ECL) was from Amersham (Amersham Biosciences, Barcelona, Spain). Monoclonal anti-peroxisome proliferator-activated receptor antibody (PPAR-γ) (1:250) and polyclonal anti-cyclooxigenase-2 antibody (COX-2) (1:250) from Sigma Aldrich (Madrid, Spain). Monoclonal p65 antibody (p65) (1:250) from Santa Cruz Biotechnology (Madrid, Spain). Polyclonal anti-Cu/Zn superoxide dismutase antibody (Cu/Zn SOD) (1:250) from Assay Designs (Madrid, Spain). Monoclonal anti-tubuline (1:1000) from Cell Signaling (Beverly, MA, USA). All other reagents are analytical or culture grade purity.

## Primary culture of cortical astrocytes

All animals were handled according to the recommendations of the Bioethics Committee of the School of Medicine of the University of Valencia, Spain. Ethics committee specifically approved this study. Cortical astrocytes were isolated from rat fetuses of 21 days gestation. Fetuses were obtained by cesarean section and decapitated. Cerebral cortices were removed and triturated 10–15 times through a Pasteur pipette

with 10 ml DMEM. The cell suspension was filtered through nylon mesh with a pore size of 90 μm and re-suspended in DMEM containing 20% fetal bovine serum (FBS), supplemented with L-glutamine (1%), HEPES (10 mM), fungizone (1%), and antibiotics (1%). Cells were plated on T75 culture flask and maintained in a humidified atmosphere of 5% CO2/95% air at 37°C during 15 days. After 4 days of culture, the FBS was maintained at 20% and after 1 week of culture, the FBS content was reduced to 10%, and the medium was changed twice a week. The purity of astrocytes was assessed by immunofluorescence using anti-glial fibrillary acidic protein (astrocyte marker; Sigma-Aldrich), anti-CD-68 (microglial marker; Serotec), anti-myelin basic protein (olygodendroglial marker; Sigma-Aldrich) and anti-microtubule-associated protein 2 (neuronal marker; Sigma-Aldrich). The astrocyte cultures were found to be at least 99% glial fibrillary acidic protein positive. No cells were found to express CD-68, myelin basic protein, or microtubule-associated protein-2.

## Cell treatments

Ten days after seeding, WIN (10 μM) was added to culture flasks. Twenty-four hours later, 10 μM $A\beta_{1-42}$ (toxic peptide) or $A\beta_{40-1}$ (control peptide) (Sigma-Aldrich) were added to the flasks. $A\beta_{1-42}$ concentration used in our study is in the range of toxic concentrations of the peptide [21,22]. Before incubation, the peptides were diluted in 100 μM of phosphate-buffered saline (PBS) and incubated for 24 h at 37º C. Assays were performed 24 h after peptide addition.

## MTT assay

Cell viability was determined by MTT assay. The MTT assay is a well-established, widely used and easily reproducible method for the assessment of cell viability and cytotoxicity [23,24]. Astrocytes were plated in 96-well culture plate and incubated with

WIN during 24h. Subsequently, $A\beta_{40-1}$ (control) and $A\beta_{1-42}$ peptides were added to wells for another 24h. After cell treatments, the medium was removed and cells were incubated with red free medium and MTT solution [0.5 mg/ml, prepared in phosphate buffer saline (PBS) solution] for 4 h at 37ºC. Finally, the medium was removed and formazan particles were dissolved in dimethyl sulfoxide (DMSO). Cell viability, defined as the relative amount of MTT reduction, was determined by spectrophotometry at 570 nm.

## Cytokine determination, IL-1 and TNFα

Astrocytes were seeded as previously published [12]. At the time of assay, the red phenol medium was removed and replaced by PBS containing 1 mg/ml bovine serum albumin (BSA), either in the presence or absence of $A\beta_{1-42}$ (10 μM). IL-1β and TNF-α concentration (pg/ml) were ascertained using ELISA kits (Pierce Biotechnology, Inc.).

## Western blot analysis

Cultured cells were treated with lysis buffer and mechanically degraded to release the proteins. Protein concentration was determined using modified Lowry method [25]. Loading buffer (0.125 M Tris-HCl, pH 6.8, 2% SDS, 0.5% (v/v) 2-mercaptoethanol, 1% bromophenolblue and 19% glycerol) was added to protein sample and heated for 5 min at 95ºC. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes in a humid environment using a transfer buffer (25mM Tris, 190mM glycine, 20% methanol). Membranes were blocked with 5% milk in TBS (0.05% Tween-20) and incubated with primary antibodies overnight at 4ºC. Membranes were washed 3 times with wash buffer TBS-T (TBS, 0.2% Tween-20) and incubated with a secondary anti-rabbit IgG or anti-mouse IgG (Cell Signalling Technologies Danvers, MA) antibody conjugated to the enzyme horseradish peroxidase (HRP) for 1 h. Membranes were washed three times and proteins were detected using the ECL method as specified by the

manufacturer. Autoradiography signals were assessed using digital image system ImageQuant LAS 4000 (GE Healthcare).

## Statistical analyses

Values are expressed as mean±S.D. Differences between groups were assessed by one-way analysis of variance (ANOVA). Statistical significance was accepted at $P \leq 0.05$. Data sets in which *F* was significant were examined by a modified *t*-test.

# Results

## Protective role of WIN on cell viability

The role of WIN on cell viability was studied using MTT conversion assay. Figure 1A shows that incubation with WIN at different concentrations induced a significant increase in cell viability at 10 μM. Consequently, that concentration was used in future experiments. Astrocytes previously incubated with 10 μM $A\beta_{1-42}$ for 24 h significantly decreased cell viability compared to control cells (Figure 1B). Furthermore, pretreating astrocytes during 48 h with WIN (10 μM) prevented the decrease in cell viability induced by $A\beta_{1-42}$ (WIN + Aβ), conversely WIN (1, 2, 5μM) did not have any effect (Figure 1B).

## WIN prevents IL-1β and TNF-α increase elicited by $A\beta_{1-42}$

Cultured astrocytes were incubated with 10 μM $A\beta_{1-42}$ and proinflammatory mediators TNF-α and IL-1β were detected by ELISA. $A\beta_{1-42}$ increased 4.5-fold IL-1β release (480.4±150.3 pg/ml) compared with control (103.9±82.9 pg/ml) (Figure 2A) and 2.4 fold TNF-α release (605.3±103.4 pg/ml *vs* 210.5±85.3 pg/ml in control group) (Figure 2B). Furthermore, WIN pre-treatment (10 μM) prevented the increase in pro-inflammatory mediators induced by $A\beta_{1-42}$ (Figure 2 A and B).

## Effect of $A\beta_{1-42}$ and WIN on p65 protein expression

NF-κB, the pro-inflammatory transcription factor, is formed by different subunits. We measured p65 protein expression by western-blot. Incubation with $A\beta_{1-42}$ increased p65 protein expression compared with control astrocytes (Figure 3), which was prevented by WIN pretreatment. ($p<0.05$ compared with $A\beta_{1-42}$ treated astrocytes)

## WIN prevents COX-2 and iNOS protein increase induced by $A\beta_{1-42}$ peptide

Incubation with $A\beta_{1-42}$ significantly increased inflammatory proteins COX-2 (Figure 4A) and iNOS (Figure 4B) expressions compared to control. Furthermore, pretreating astrocytes with WIN prevented the effects produced by $A\beta_{1-42}$.

## Effect of $A\beta_{1-42}$ and WIN on PPAR-γ protein expression

Pro-inflammatory gene expression is downregulated by PPARs family [26]. We found that pretreatment with WIN (10 μM) increased PPAR-γ expression compared to control cells (Figure 5). Incubation with $A\beta_{1-42}$ significantly decreased PPAR-γ expression that was prevented by WIN pretreatment.

## Effect of $A\beta_{1-42}$ and WIN on Cu/Zn SOD and Mn SOD protein expression.

Superoxide dismutase (SOD) is a key antioxidant enzyme. In our study, incubation with $A\beta_{1-42}$ decreased Cu/Zn SOD expression in astrocytes in primary culture which was prevented by WIN pretreatment, evidencing that WIN could play a neuro-protective role against oxidative stress induced by $A\beta_{1-42}$ peptide (Figure 6A). On the other hand, our results indicated that Mn SOD protein expression is increased in presence of $A\beta_{1-42}$.

Pretreatment with WIN did not prevent Mn SOD increase induced by $A\beta_{1-42}$ (Figure 6B).

# Discussion

Oxidative stress and inflammation are the main mechanisms in the progression of various neurodegenerative diseases, including AD [27-30]. In our study, we determined different markers involved in inflammation and oxidative stress induced by the $A\beta_{1-42}$ peptide in primary cultures of astrocytes, with the aim to assess the antioxidant and anti-inflammatory effects of cannabinoid WIN. We found that WIN significantly increased astrocytes viability compared to control cells. Furthermore, WIN prevented the decrease in astrocytes viability induced by $A\beta_{1-42}$.

It has been shown that cannabinoids preserve neurons from Aβ exposure by activating MAP kinase cascade [31] and by anti-oxidative and anti-apoptotic effects [32]. Moreover, some studies demonstrated that cannabinoids protect glial cells from death [33,34]. Nevertheless, in cancer, where cells are highly proliferative and undifferentiated, treatment with cannabinoids can block cell proliferation in a dose dependent manner [35-38], demonstrating that the effects of cannabinoids on cell viability are probably dependent on cell type [39] and developmental stage [40].

Expression of CB1 [41] and CB2 [42] receptors in rat culture astrocytes have been published and also dual activation of both cannabinoid receptors by WIN 55,212-2 (the mixed non-selective CB1/CB2 agonist) in rat cortical astrocytes have been detected [41] On the other hand, WIN confers its protective and anti-inflammatory effects against Aβ injury through both CB1 and CB2 receptors [43]. Given that our results there is expression of both types of cannabinoid receptors (CB1 and CB2), it is likely that the effect of WIN observed in our study is due to the interaction with both types of receptors, consistent with published results by Fakhfouri and cols [44].

We found that WIN prevented the increase of inflammatory mediators IL-1β, TNF-α, NF-κB, iNOS and COX-2, as well as the decrease of the anti-inflammatory mediator

PPAR-γ induced by $A\beta_{1-42}$ in astrocytes in primary culture. The inflammatory process is a characteristic mechanism in the development of AD, and pro-inflammatory agents are involved in the progression of cell damage [45,46,47,12]. Moreover, it is known that astrocytes participate in the inflammatory process induced by $A\beta_{1-42}$ [27,28,48]. Initially, inflammation is beneficial since it produces pro-inflammatory substances involved in tissue protection, limiting the survival and proliferation of cells exposed to toxic agents, such as $A\beta_{1-42}$ [49,50]. However, sustained inflammatory response could lead to neurotoxic damage or cell death [12,51,8]. NF-κB proteins are up-regulated in inflammation conditions such as astroglial activation induced by $A\beta_{1-42}$ oligomers [52]. In this regard, we found an increase in NF-kB/p-65 expression in astrocytes after addition of $A\beta_{1-42}$ that was prevented by WIN pretreatment. Valles and collaborators [53] found that the cytokine-receptor complex is able to bind to cytokines and other proteins of the extracellular matrix, producing inflammatory signals which could be important in pathologies such as Alzheimer's disease [53,54]. In agreement with our results, different authors have reported that cannabinoids mitigate neural cell activation in the neuroinflammatory response induced by $A\beta_{1-42}$, reducing the levels of pro-inflammatory molecules such as IL-1β, TNF-α, COX-2 and iNOS [55,56,57]. Likewise, the activation of cannabinoid receptors diminishes the release of IL-1β, IL-6 and TNF-α in microglial cells [58,59,60] as well as COX-2 and iNOS [61]. Studies conducted in rats pretreated with the Aβ peptide found that WIN prevented cognitive impairment, glial activation and neuronal loss [19,62,63], and also reduced COX-2, iNOS and TNF-α levels [63,64].

Kainu et al. [65] demonstrated for the first time the presence of mRNA and protein PPAR-γ in CNS cells. Subsequent studies have detected PPAR-γ expression in microglial and astrocytic cells [66,12]. PPAR-γ agonists protect against Aβ-induced

inflammatory and neuronal damage [67,68], thus making neurons and astrocytes potential therapeutic targets for PPAR-γ ligands [69,12]. Astrocytes also express the largest levels of PPAR-γ in the neural tissues [70,71]. As other authors [72,73,74], we found a decreased expression of PPAR-γ in astrocytes treated with $A\beta_{1-42}$. Esposito et al. [56] showed in neurons that cannabinoids may act as neuro-protective agents by PPAR-γ activation. In this study, we demonstrate an increase in this protein expression in astrocytic cells previously incubated with WIN. Furthermore, we found for the first time that WIN prevents PPAR-γ expression decrease induced by $A\beta_{1-42}$ peptide in astrocytes in primary culture. There is strong evidence to suggest that some cannabinoids can act on PPARs through either direct or indirect pathways. In order to directly act on nuclear transcriptional factors PPARs, exogenous cannabinoids need to pass through plasma membrane and be transported into nucleus which may involve certain membrane and intracellular transporters. However, we still cannot rule out that cannabinoids effects could be indirect through the binding of other cellular targets which in turn induces PPARs activation [75]. In fact, WIN attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-γ pathway [44].

Different authors have demonstrated the role of oxidative stress in AD [76-79]. The cumulative damage caused by free radicals induces alterations in the activity or expression of antioxidant enzymes like catalase or SOD. These enzymes were found to be decreased in both CNS and peripheral tissues of AD patients [80,81]. In this sense, we demonstrate that Cu/Zn SOD is decreased in astrocytes treated with $A\beta_{1-42}$. Our results are consistent with those reported by other authors, highlighting the role of oxidative stress in the development of AD [82]. New substances are under research to reduce damage caused by oxidative stress in this disease. Widely distributed in the body,

cannabinoids receptors were discovered few decades ago and are still under research [57]. Few studies address the effect of cannabinoids on oxidative stress. For instance, cannabinoids were found to prevent or antagonize oxidative stress toxicity in cortical neurons in cultures [83,84], and in lymphoblastic cells [85]. Studies with PC12 cells exposed to $A\beta_{1-42}$ peptide demonstrated that cannabinoids reduced reactive oxygen species production and membrane lipid oxidation [86,32]. Our results provide evidence that $A\beta_{1-42}$ decreases Cu/Zn SOD expression in astrocytes in primary culture, and pretreatment with WIN increases Cu/Zn SOD expression, preventing the decrease caused by $A\beta_{1-42}$. These findings indicate that cannabinoids could act as a protective agent against oxidative stress caused by $A\beta_{1-42}$. In Figure 7 the set of results are summarized. However our results indicate that $A\beta_{1-42}$ elevated Mn SOD protein expression, increasing mitochondrial biogenesis mechanism, such as we previously published [74]. Pretreatment with WIN did not prevent Mn SOD overexpression induced by $A\beta_{1-42.}$ Mn SOD plays a role in the adaptive response which protects brain cells from damage, as in the case of AD. In fact, Mn SOD preserves neurons against oxidative stress [87] and protects developing neurons from β-amyloid toxicity [88]. This enzyme catalyzes the conversion of superoxide radicals to molecular oxygen and $H_2O_2$, whereas glutathione peroxidase, peroxiredoxin reductase and catalase neutralize $H_2O_2$. Overexpression of Mn SOD induces cognitive recovery and reduces Aβ levels in AD animal models [89]. Furthermore, Mn SOD deficiency increases β-amyloid levels and amyloid plaque burden, promoting the development of behavioural disturbances [90].

Preclinical data suggest a beneficial role of some cannabinoids for treatment of different diseases. Dronabidol, an oil-based solution of Δ9-THC, is used as anti-emetic and appetite stimulant [91]. Δ9-THC also decreases agitation present in the advanced stage of AD [92]. In 2003, the FDA granted the patent for cannabinoids as antioxidants

and neuro-protectants (U.S. Department of Health and Human Services). Despite these promising preliminary results, the clinical utility of cannabinoids in AD is still to be determined [93].

# Conclusions

Taken together, our findings show that cannabinoid WIN increases cell viability and anti-inflammatory response in astrocytes in primary culture and prevents cell death induced by $A\beta_{1-42}$. Furthermore, WIN increases expression of anti-oxidant Cu/Zn SOD and is able to prevent inflammation induced by $A\beta_{1-42}$ in astrocytes. In this sense, clinical studies are needed to evaluate the neuro-protective effects of cannabinoids in Alzheimer´s disease.

# Conflict of Interest Statement:

The authors declare that there are no conflicts of interest.

# References

[1] Selkoe DJ. (1977) Alzheimer's disease: genotypes, phenotypes, and treatments. *Science*. 275:630-31.

[2] Ferri CP, Prince M, Brayne C, et al. (2005) Alzheimer's Disease International. Global prevalence of dementia: a Delphi consensus study. *Lancet* 366:2112-17.

[3] Heneka MT and O'Banion MK. (2007) Inflammatory processes in Alzheimer's disease. *J Neuroimmunol* 184:69-91.

[4] Pratico D. (2008) Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows. *Ann N Y Acad Sci.* 1147:70-78.

[5] Inestrosa NC, Reyes AE, Chacón MA, et al. (2005) Human-like rodent amyloid-beta-peptide determines Alzheimer pathology in aged wild-type Octodon degu. *Neurobiol Aging* 26:1023-28.

[6] Vallés SL, Borrás C, Gambini J, et al. (2008) Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38. *Aging Cell* 7:112-8.

[7] Sofroniew MV and Vinters HV. (2010) Astrocytes: biology and pathology. *Acta Neuropathol* 119:7-35.

[8] Choi SS, Lee HJ, Lim I, et al. (2014) Human astrocytes: secretome profiles of cytokines and chemokines. *PLoS One* 1:9(4):e92325. doi:10.1371/journal.pone.0092325.

[9] van Kralingen C, Kho DT, Costa J, et al. (2013) Exposure to inflammatory cytokines IL-1β and TNFα induces compromise and death of astrocytes; implications for chronic neuroinflammation. *PLoS One* 19;8(12):e84269. doi: 10.1371/journal.pone.0084269.

[10] Zhang W, Smith C, Howlett C, et al. (2000) Inflammatory activation of human brain endothelial cells by hypoxic astrocytes in vitro is mediated by IL-1beta. *J Cereb Blood Flow Metab* 20:967-78.

[11] Li C, Zhao R, Gao K, et al. (2011) Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* 8:67-80.

[12] Valles SL, Dolz-Gaiton P, Gambini J, et al. (2010) Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression in cultured astrocytes. *Brain Res* 1312:138-44.

[13] Pacher P and Kunos G. (2013) Modulating the endocannabinoid system in human health and disease-successes and failures. *FEBS J* 280:1918-43.

[14] Kunos G and Tam J. (2011) The case for peripheral $CB_1$ receptor blockade in the treatment of visceral obesity and its cardiometabolic complications. *Br J Pharmacol* 163:1423-31.

[15] Centonze D, Finazzi-Agrò A, Bernardi G, et al. (2007) The endocannabinoid system in targeting inflammatory neurodegenerative diseases. *Trends Pharmacol Sci* 28:180-7.

[16] Klein WK. (2005) Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nat Rev Immunol* 5:400-11.

[17] Hillard CJ, Weinlander KM, Stuhr KL. (2012) Contributions of endocannabinoid signaling to psychiatric disorders in humans: genetic and biochemical evidence. *Neuroscience* 1:207-29.

[18] Guindon J and Hohmann AG. (2011) The endocannabinoid system and cancer: therapeutic implication. *Br J Pharmacol* 163:1447-63.

[19] Ramírez BG, Blázquez C, Gómez del Pulgar T, et al. (2005) Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *J Neurosci* 25:1904-13.

[20] Esposito G, Scuderi C, Savani C, et al. (2007) Cannabidiol in vivo blunts beta-amyloid induced neuroinflammation by suppressing IL-1beta and iNOS expression. *Br J Pharmacol* 151:1272-79.

[21] Blanchard BJ, Chen A, Rozeboom LM, et al. (2004) Efficient reversal of Alzheimer's disease fibril formation and elimination of neurotoxicity by a small molecule. *Proc Natl Acad Sci* 101:14326-332.

[22] McGeer PL and McGeer EG. (2013) The amyloid cascade-inflammatory hypothesis of Alzheimer disease: implications for therapy. *Acta Neuropathol.* 126:479-97.

[23] Rönicke R, Klemm A, Meinhardt J, et al. (2008) Abeta mediated diminution of MTT reduction--an artefact of single cell culture?. *PLoS One* 3:e3236.

[24] Palanca JM, Aguirre-Rueda D, Granell MV, et al. (2013) Sugammadex, a neuromuscular blockade reversal agent, causes neuronal apoptosis in primary cultures. *Int J Med Sci* 10:1278-85.

[25] Peterson GL. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 83:346-56.

[26] Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature.* 391(6662):79-82.

[27] Eikelenboom P and van Gool WA. (2004) Neuroinflammatory perspectives on the two faces of Alzheimer's disease. *J Neural Transm* 111:281-94.

[28] Zhu X, Smith MA, Perry G, et al. (2004) Mitochondrial failures in Alzheimer's disease. *Am J Alzheimers Dis Other Demen* 19:345-52.

[29] Jiménez-Jiménez FJ, Alonso-Navarro H, Ayuso-Peralta L, et al. (2006) Oxidative stress and Alzheimer's disease. *Rev Neurol* 42:419-27.

[30] Quintanilla RA, Orellana JA, von Bernhardi R. (2012) Understanding risk factors for Alzheimer's disease: interplay of neuroinflammation, connexin-based communication and oxidative stress. *Arch Med Res* 43:632-44.

[31] Milton NG. (2002) Anandamide and noladin ether prevent neurotoxicity of the human amyloid-beta peptide. *Neurosci Lett* 332:127-30.

[32] Iuvone T, Esposito G, Esposito R, et al. (2004) Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on beta-amyloid-induced toxicity in PC12 cells. *J Neurochem* 89:134-41.

[33] Marsicano G, Wotjak CT, Azad SC, et al. (2002) The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 1:530-34.

[34] Gómez del Pulgar T, de Ceballos ML, Guzmán M, et al. (2002) Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. *J. Biol. Chem* 277: 36527–33.

[35] Pourkhalili N, Ghahremani MH, Farsandaj N, et al. (2013) Evaluation of anti-invasion effect of cannabinoids on human hepatocarcinoma cells. *Toxicol Mech Methods* 23:120-6.

[36] De Petrocellis L, Ligresti A, Schiano-Moriello A, et al. (2013) Non-THC cannabinoids inhibit prostate carcinoma growth in vitro and in vivo: pro-apoptotic effects and underlying mechanisms. *Br J Pharmacol* 168:79-102.

[37] Vara D, Salazar M, Olea-Herrero N, et al. (2011) Anti-tumoral action of cannabinoids on hepatocellular carcinoma: role of AMPK-dependent activation of autophagy. *Cell Death Differ* 18:1099-111.

[38] Rocha FC, Dos Santos Júnior JG, Stefano SC, et al. (2014) Systematic review of the literature on clinical and experimental trials on the antitumor effects of cannabinoids in gliomas. *J Neurooncol* 116:11-24.

[39] Sánchez C, Galve-Roperh I, Canova C, et al. (1998) Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells. *FEBS Lett* 436:6-10.

[40] Guzmán M, Sánchez C, Galve-Roperh I. (2002) Cannabinoids and cell fate. *Pharmacol Ther* 95:175-84.

[41] Stella N. (2004) Cannabinoid signaling in glial cells. *Glia* 48(4):267-277.

[42] Sheng WS, Hu S, min X, Cabral GA, Lokensgard JR, Peterson P. (2005) Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta.stimulated human astrocytes. *Glia* 49:211-219.

[43] Palmer SL, Thakur GA, Makriyannis A. (2002) Cannabinergic ligands. *Chem Phys Lipids* 121:3-19.

[44] Fakhfouri G, Ahmadiani A, Rahimian R, et al. (2012) WIN55212-2 attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-γ pathway. *Neuropharmacology.* 63:653-66

[45] Strohmeyer R and Rogers J. (2001) Molecular and cellular mediators of Alzheimer's disease inflammation. *J Alzheimers Dis* 3:131-57.

[46] Wyss-Coray T and Mucke L. (2002) Inflammation in neurodegenerative disease-A doubleedged sword. *Neuron* 35:419-32.

[47] Griffin WS and Mrak RE. (2002) Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease. *J Leukoc Biol* 72:233-8.

[48] McGeer PL, Rogers J, McGeer EG. (2006) Inflammation, anti-inflammatory agents and Alzheimer disease: the last 12 years. *J Alzheimers Dis* 9:271-6.

[49] Allan SM and Rothwell NJ. (2001) Cytokines and acute neurodegeneration. *Nat Rev Neurosci* 2:734-44.

[50] Allan SM and Rothwell NJ. (2003) Inflammation in central nervous system injury. *Philos Trans R Soc Lond B Biol Sci* 358:1669-77.

[51] Burkert K, Moodley K, Angel CE, et al. (2012) Detailed analysis of inflammatory and neuromodulatory cytokine secretion from human NT2 astrocytes using multiplex bead array. *Neurochem Int* 60:573-80.

[52] Carrero I, Gonzalo MR, Martin B, et al. (2012) Oligomers of β-amyloid protein (Aβ1-42) induce the activation of cyclooxygenase-2 in astrocytes via an interaction with interleukin-1β, tumour necrosis factor-α, and a nuclear factor κ-B mechanism in the rat brain. *Exp Neurol* 236:215-27.

[53] Vallés S, Tsoi C, Huang WY, et al. (1999) Recruitment of a heparan sulfate subunit to the interleukin-1 receptor complex. Regulation by fibronectin attachment. *J Biol Chem* 274:20103-9.

[54] Bondareff W. (2013) Age-related changes in brain extracellular space affect processing of amyloid-β peptides in Alzheimer's disease. *J Alzheimers Dis* 35:1-6.

[55] Sheng WS, Hu S, Min X, et al. (2005) Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta-stimulated human astrocytes. *Glia* 49:211-9.

[56] Esposito G, Scuderi C, Valenza M, et al. (2011) Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PLoS One* 6:e28668.

[57] Scuderi C, Esposito G, Blasio A, et al. (2011) Palmitoylethanolamide counteracts reactive astrogliosis induced by β-amyloid peptide. *J Cell Mol Med* 15:2664-74.

[58] Puffenbarger RA, Boothe AC, Cabral GA. (2000) Cannabinoids inhibit LPS-inducible cytokine mRNA expression in rat microglial cells. *Glia* 29:58-69.

[59] Facchinetti F, Del Giudice E, Furegato S, et al. (2003) Cannabinoids ablate release of TNFalpha in rat microglial cells stimulated with lypopolysaccharide. *Glia* 41:161-8.

[60] Ehrhart J, Obregon D, Mori T, et al. (2005) Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. *J Neuroinflammation* 12:2-29.

[61] Castillo A, Tolón MR, Fernández-Ruiz J, et al. (2010) The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. *Neurobiol Dis* 37:434-40.

[62] Martín-Moreno AM, Reigada D, Ramírez BG, et al. (2011) Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease. *Mol Pharmacol* 79:964-73.

[63] Martín-Moreno AM, Brera B, Spuch C, et al. (2012) Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β-amyloid levels and improves cognitive performance in Tg APP 2576 mice. *J Neuroinflammation* 16:8.

[64] Esposito G, De Filipis D, Carnuccio R, et al. (2006) The marijuana component cannabidiol inhibits β-amyloid-induced tau protein hyperphosphorylation through Wnt/β-catenin pathway rescue PC12 cells. *J Mol Med* 84:253-8.

[65] Kainu T, Wikström AC, Gustafsson JA, et al. (1994) Localization of the peroxisome proliferator-activated receptor in the brain. *Neuroreport* 5:2481-5.

[66] Luna-Medina R, Cortes-Canteli M, Alonso M, et al. (2005) Regulation of inflammatory response in neural cells in vitro by thiadiazolidinones derivatives through peroxisome proliferator-activated receptor gamma activation. *J Biol Chem* 280:21453-62.

[67] Bright JJ, Kanakasabai S, Chearwae W, et al. (2008) PPAR Regulation of Inflammatory Signaling in CNS Diseases. *PPAR Res* doi: 10.1155/2008/658520.

[68] Kapadia R, Yi JH, Vemuganti R. (2008) Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-γ agonists. *Front Biosci* 13:1813–26.

[69] Landreth G, Jiang Q, Mandrekar S, et al. (2008) PPARgamma agonists as therapeutics for the treatment of Alzheimer's disease. *Neurotherapeutics* 5:481-9.

[70] Bodles AM and Barger SW. (2005) Secreted β-amyloid precursor protein activates microglia via JNK and p38-MAPK. *Neurobiol Aging* 26:9–16.

[71] Ho GJ, Drego R, Hakimian E, et al. (2005) Mechanisms of cell signaling and inflammation in Alzheimer's disease. *Curr Drug Targets Inflamm Allergy* 4:247–56.

[72] Wang HM, Zhao YX, Zhang S, et al. (2010) PPARgamma agonist curcumin reduces the amyloid-beta-stimulated inflammatory responses in primary astrocytes. *J Alzheimers Dis* 20:1189-99.

[73] Chen YC, Wu JS, Tsai HD, et al. (2012) Peroxisome proliferator-activated receptor gamma (PPAR-γ) and neurodegenerative disorders. *Mol Neurobiol* 46:114–24.

[74] Aguirre-Rueda D, Guerra-Ojeda S, Aldasoro M, et al. (2015) Astrocytes protect neurons from Aβ1-42 peptide-induced neurotoxicity increasing TFAM and PGC-1 and decreasing PPAR-γ and SIRT-1. *Int J Med Sci* 12:48-56.

[75] Sun Y, Bennett A (2007) Cannabinoids: a new group of agonists of PPARs. *PPAR Res.* 2007:23513.

[76] Wallace DC. (2000) Mitochondrial defects in cardiomyopathy and neuromuscular disease. *Am Heart J* 139:S70–S85.

[77] Lesnefsky EJ, Moghaddas S, Tandler B, et al. (2001) Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. *J Mol Cell Cardiol* 33:1065-89.

[78] Chen Q, Vazquez EJ, Moghaddas S, et al. (2003) Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* 278:36027-31.

[79] Abeti R and Duchen MR. (2012) Activation of PARP by Oxidative Stress Induced by β-Amyloid: Implications for Alzheimer's Disease. *Neurochem Res* 37:2589-96.

[80] Marcus DL, Thomas C, Rodriguez C, et al. (1998) Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. *Exp Neurol* 150:40-4.

[81] Padurariu M, Ciobica A, Hritcu L, et al. (2010) Changes of some oxidative stress markers in the serum of patients with mild cognitive impairment and Alzheimer's disease. *Neuroscience Letters* 469:6–10.

[82] Pappolla MA, Chyan YJ, Omar RA, et al. (1998) Evidence of oxidative stress and in vivo neurotoxicity of beta-amyloid in a transgenic mouse model of Alzheimer's disease: a chronic oxidative paradigm for testing antioxidant therapies in vivo. *Am J Pathol* 152:871-7.

[83] Hampson AJ, Grimaldi M, Axelrod J, et al. (1998) Cannabidiol and (-) Δ9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci* 95:8268–73.

[84] Hampson AJ and Grimaldi M. (2001) Cannabinoid receptor activation and elevated cyclic AMP reduce glutamate neurotoxicity. *Eur J Neurosci* 13:1529–36.

[85] Chen Y and Buck J. (2000) Cannabinoids protect cells from oxidative cell death: a receptor-independent mechanism. *J. Pharmacol. Exp. Ther* 293:807–12.

[86] Pratico D, MY Lee V, Trojanowski JQ, et al. (1998) Increased F2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation in vivo. *FASEB J* 12:1777-83.

[87] Keller JN, Kindy MS, Holtsberg FW, et al. (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J Neurosci*. 18:687-97.

[88] Sompol P, Ittarat W, Tangpong J, et al. (2008) A neuronal model of Alzheimer's disease: an insight into the mechanisms of oxidative stress-mediated mitochondrial injury. *Neuroscience* 153:120-30.

[89] Dumont M, Wille E, Stack C, et al. (2009) Reduction of oxidative stress, amyloid deposition, and memory deficit by manganese superoxide dismutase overexpression in a transgenic mouse model of Alzheimer's disease. *FASEB J.* 23:2459-66.

[90] Esposito L, Raber J, Kekonius L, et al. (2006) Reduction in mitochondrial superoxide dismutase modulates Alzheimer's disease-like pathology and accelerates the onset of behavioral changes in human amyloid precursor protein transgenic mice. *J Neurosci*. 26:5167-79.

[91] Volicer L, Stelly M, Morris J, et al. (1997) Effects of dronabinol on anorexia and disturbed behavior in patients with Alzheimer's disease. *Int J Geriatr Psychiatry* 12:913-9.

[92] Walther S, Mahlberg R, Eichmann U, et al. (2006) 9-tetrahydrocannabinol for nighttime agitation in severe dementia. *Psychopharmacology* 185:524–8.

[93] Krishnan S, Cairns R, Howard R. (2009) Cannabinoids for the treatment of dementia. *Cochrane Database Syst Rev* 15(2):CD007204.

# Figure Legends

**Figure 1. Astrocytes viability.** (A) Astrocytes viability induced by WIN. Concentration-dependent viability of WIN (1, 2, 5, 10 μM) was determined by MTT assay for 24 h. Data are means ± SD for 4 independent experiments. *$p$<0.04 comparing WIN vs control cells. (B) Astrocytes viability in cells treated during 24 h with 10 μM $A\beta_{40\text{-}1}$ (control peptide, C), 10 μM $A\beta_{1\text{-}42}$ (Aβ) and WIN (1, 2, 5, 10 μM) + 10 μM $A\beta_{1\text{-}42}$ (WIN + Aβ). Data are means ± SD of 3 independent experiments. *$p$<0.05 *vs* control cells.

**Figure 2. IL-1β and TNF-α secretion.** WIN prevents the increase of IL-1β and TNF-α secretion caused by $A\beta_{1\text{-}42}$ in astrocytes. Cells were incubated with 10 μM $A\beta_{40\text{–}1}$ (control peptide, C), 10 μM $A\beta_{1\text{–}42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1\text{–}42}$ (WIN + Aβ). Cell culture supernatants were harvested, and IL-1β (panel A) and TNF-α (panel B) secretion were determined by ELISA. Values are means ± SD of replicate experiments from 4 independent astrocytes cultures. *$p$<0.05 *vs* control astrocytes. #$p$<0.05 *vs* $A\beta_{1\text{-}42}$ treated cells.

**Figure 3. p65 protein expression.** WIN 55, 212-2 prevents p65 expression induced by $A\beta_{1\text{-}42}$ in astrocytes in primary culture. p65 and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40\text{-}1}$ (control peptide, C), 10 μM $A\beta_{1\text{-}42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1\text{-}42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 5 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1\text{-}42}$.

**Figure 4. COX-2 and iNOS protein expression.** WIN prevents COX-2 and iNOS expression induced by $A\beta_{1-42}$. COX-2 (panel A), iNOS (panel B) and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40-1}$ (control peptide, C), 10 μM $A\beta_{1-42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1-42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 6 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1-42}$.

**Figure 5. PPAR-γ protein expression.** WIN induces PPAR-γ expression in astrocytes in primary culture treated with $A\beta_{1-42}$. PPAR-γ and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40-1}$ (control peptide, C), 10 μM $A\beta_{1-42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1-42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 4 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1-42}$.

**Figure 6. Cu/Zn-SOD and Mn-SOD protein expressions.** WIN prevents Cu/Zn-SOD expression decrease in astrocytes in primary culture treated with $A\beta_{1-42}$. Cu/Zn-SOD, Mn-SOD and α-tubulin expressions were determined by Western-blot in astrocytes treated for 24 h with 10 μM $A\beta_{40-1}$ (control peptide, C), 10 μM $A\beta_{1-42}$ (Aβ), 10 μM WIN + 10 μM control peptide (WIN) and 10 μM WIN + 10 μM $A\beta_{1-42}$ (WIN + Aβ). A representative immunoblot of each protein is shown and tubulin was used as control amount of protein. Data are means ± SD of 4 independent experiments. *$p$<0.05 *vs* control cells. #$p$<0.05 *vs* $A\beta_{1-42}$.

**Figure 7. Preventive function of cannabinoid WIN on $A\beta_{1-42}$-induced toxic effects in astrocytes in primary culture.** Cannabinoid WIN 55,212-2 increases cell viability and anti-inflammatory response in cultured astrocytes and prevents inflammatory effects induced by $A\beta_{1-42}$.

Reviewer #1: I appreciate the efforts taken by the authors to adequately address the previous concerns with respect to this manuscript.

Following are the two minor issues that I have.

1. In the figure legend for Figure 7. modify the last line to match with the conclusion from this study (delete the part that WIN 55,212-2 prevents oxidative stress, since no direct measure of oxidative stress was done).

Modifications in Figure 7 have been done to match with the conclusion.

2. In revised manuscript, authors show that 10 µM Aβ1-42 increases MnSOD protein expression. In the discussion section (page 14), can the authors further speculate regarding the conclusion of this finding with respect to mitochondria and oxidative stress during Alzhemer's disease, since mitochondria are the major organelles producing superoxide radicals leading to oxidative stress. To adequately answer this, apart from astrocytes, which are the focus of this study, what is known regarding mitochondria and oxidative stress in Alzhemier's disease in other cells like neuronal cells? And does more MnSOD mean more H2O2 production, that might be a mediator of the oxidative stress ?

A paragraph about all suggestions indicated by the referee has been added in discussion.