

Peroxisome Proliferator-Activated Receptor (PPAR) γ and PPAR α Agonists Modulate Mitochondrial Fusion-Fission Dynamics: Relevance to Reactive Oxygen Species (ROS)-Related Neurodegenerative Disorders?

Juan M. Zolezzi¹, Carmen Silva-Alvarez², Daniela Ordenes², Juan A. Godoy^{2,3}, Francisco J. Carvajal², Manuel J. Santos³, Nivaldo C. Inestrosa^{2,3*}

1 Departamento de Biología, Facultad de Ciencias, Universidad de Tarapacá, Arica, Chile, **2** Centro de Envejecimiento y Regeneración (CARE), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile, **3** Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

Abstract

Recent studies showed that the activation of the retinoid X receptor, which dimerizes with peroxisome proliferator-activated receptors (PPARs), leads to an enhanced clearance of A β from the brain of transgenic mice model of Alzheimer's disease (AD), because an increased expression of apolipoprotein E and its main transporters. However, the effects observed must involve additional underlying mechanisms that have not been yet explored. Several studies conducted in our laboratory suggest that part of the effects observed for the PPARs agonist might involve mitochondrial function and, particularly, mitochondrial dynamics. In the present study we assessed the effects of oxidative stress challenge on mitochondrial morphology and mitochondrial dynamics-related proteins in hippocampal neurons. Using immunofluorescence, we evaluated the PPAR γ co-activator 1 α (PGC-1 α), dynamin related protein 1 (DRP1), mitochondrial fission protein 1 (FIS1), and mitochondrial length, in order to determine if PPARs agonist pre-treatment is able to protect mitochondrial population from hippocampal neurons through modulation of the mitochondrial fusion-fission events. Our results suggest that both a PPAR γ agonist (ciglitazone) and a PPAR α agonist (WY 14.643) are able to protect neurons by modulating mitochondrial fusion and fission, leading to a better response of neurons to oxidative stress, suggesting that a PPAR based therapy could act simultaneously in different cellular components. Additionally, our results suggest that PGC-1 α and mitochondrial dynamics should be further studied in future therapy research oriented to ameliorate neurodegenerative disorders, such as AD.

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* E-mail: ninestrosa@bio.puc.cl

Introduction

Alzheimer's disease (AD) is an age-associated neurodegenerative disorder characterized by progressive memory loss and cognitive impairment [1,2]. Brain atrophy and the gradual loss of neurons occur mainly in the hippocampus, frontal cortex and limbic areas. Senile plaques formation, derived from amyloid- β -peptide (A β) accumulation in the brain, is a pathologic hallmark of AD [3]. Until now, no effective treatment is available to stop or reverse this devastating disease.

Recently, Cramer et al. [4] found that treatment with an old drug, bexarotene, reverses the cognitive deficit observed in a mice model of AD. Bexarotene, a retinoid related molecule, is an anti-neoplastic drug, approved by the FDA (*U.S. Food and Drug Administration*) and the EMA (*European Medicines Agency*), for the treatment of some types of skin cancer [5,6]. Cramer et al. [4] exploited the retinoid-based properties of bexarotene to activate the retinoid receptor (RXR) [5], inducing apolipoprotein E

(ApoE), ABCA1 and ABCG1 expression, leading to enhanced A β clearance from the brain. However, it is well recognized that ApoE4 is an increased-risk related ApoE isoform, and patients, who carries this variant, could actually not obtain any benefit, or worst, exhibits an even greater risk to develop AD or any other neuropathology [1,2,3].

On the other hand, the mechanism proposed by Cramer [4] and others [7,8] fails to explain the wide range of effects observed after bexarotene treatment. Indeed, these studies offers a convincing explanation about A β clearance from the brain, but fails to explain the cognitive recovery observed, considering the neuronal loss and the general damage derived from A β accumulation. In fact, it has been established that A β alters synaptic function and neurotransmission by interacting with both, synaptic ion channels and mitochondria [9–13]. Moreover, several authors have demonstrated that A β also alters the neuronal metabolism due to induction of mitochondrial dysfunction which

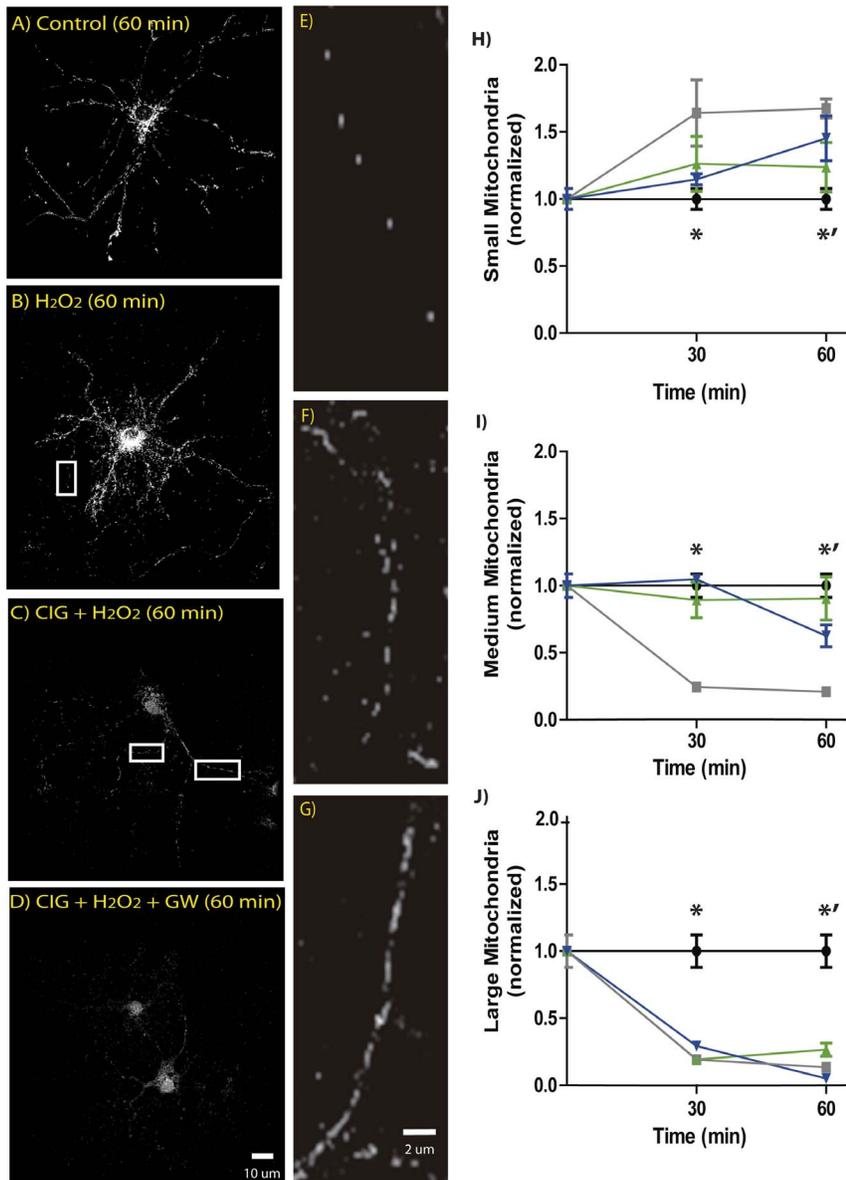


Figure 1. H₂O₂-induced mitochondrial size decrease is prevented by CIG pre-treatment in Hippocampal neurons. A, B, C and D, are representative microphotographs of mitochondrial population under different experimental conditions. E, F, G: image enlargement to show the variations in the mitochondrial morphology observed under different experimental conditions. H, I, J: Normalized mitochondrial size quantification (black: control; gray: H₂O₂; green: CIG+H₂O₂; blue: CIG+H₂O₂+ GW). H) * and *' indicate statistically significant differences between H₂O₂ and Control (*: $F 6.542$, $d.f. 3$, $C.I. 0.07389$ to 0.6266 , $t 3.856$, $p < 0.001$; *: $C.I. 0.08504$ to 0.6519 , $t 3.955$, $p < 0.001$); I) * and *' indicate statistically significant differences between H₂O₂ and Control (*: $F 17.8$, $d.f. 3$, $C.I. -0.3665$ to -0.1151 , $t 5.829$, $p < 0.001$; *: $C.I. -0.3805$ to -0.1227 , $t 5.937$, $p < 0.001$). J) * and *' indicate statistically significant differences between Control and all other experimental conditions (*: $F 18.99$, $d.f. 3$, H_2O_2 $C.I. -0.1696$ to -0.04917 , $t 5.527$, $p < 0.001$; H₂O₂+ CIG $C.I. -0.1708$ to -0.04728 , $t 5.371$, $p < 0.001$; *: H₂O₂ $C.I. -0.1787$ to -0.05512 , $t 5.757$, $p < 0.001$; H₂O₂+ CIG $C.I. -0.1595$ to -0.03905 , $t 5.015$, $p < 0.001$).

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contributes to oxidative damage because an increased production of mitochondrial reactive oxygen species (ROS) [14–16]. In fact, *in vitro* experiments have confirmed that one of the main neurotoxic mechanisms of A β aggregates is through oxidative stress [3,10,17,18]. Recently, mitochondrial dynamics, which considers fusion-fission events, has been described as a critical mechanism related with mitochondrial and cellular fate after critical insults, mainly because successive fusion-fission cycles allows to eliminate dysfunctional organelles and to repair the

mitochondrial DNA that could ended damaged after a toxic challenge [19].

Since a decade ago, we have studied Nuclear Receptors (NR), particularly the peroxisome proliferator activated-receptors (PPARs) [20–24]. PPARs, a type II NR, are characterized by dimer formation with the RXR [25]. Activation of RXR:PPAR requires removal of transcriptional co-repressors and addition of co-activators, such as peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α) [25,26]. Moreover, PGC-1 α has been described as highly expressed in the brain [26] and, recently, it has

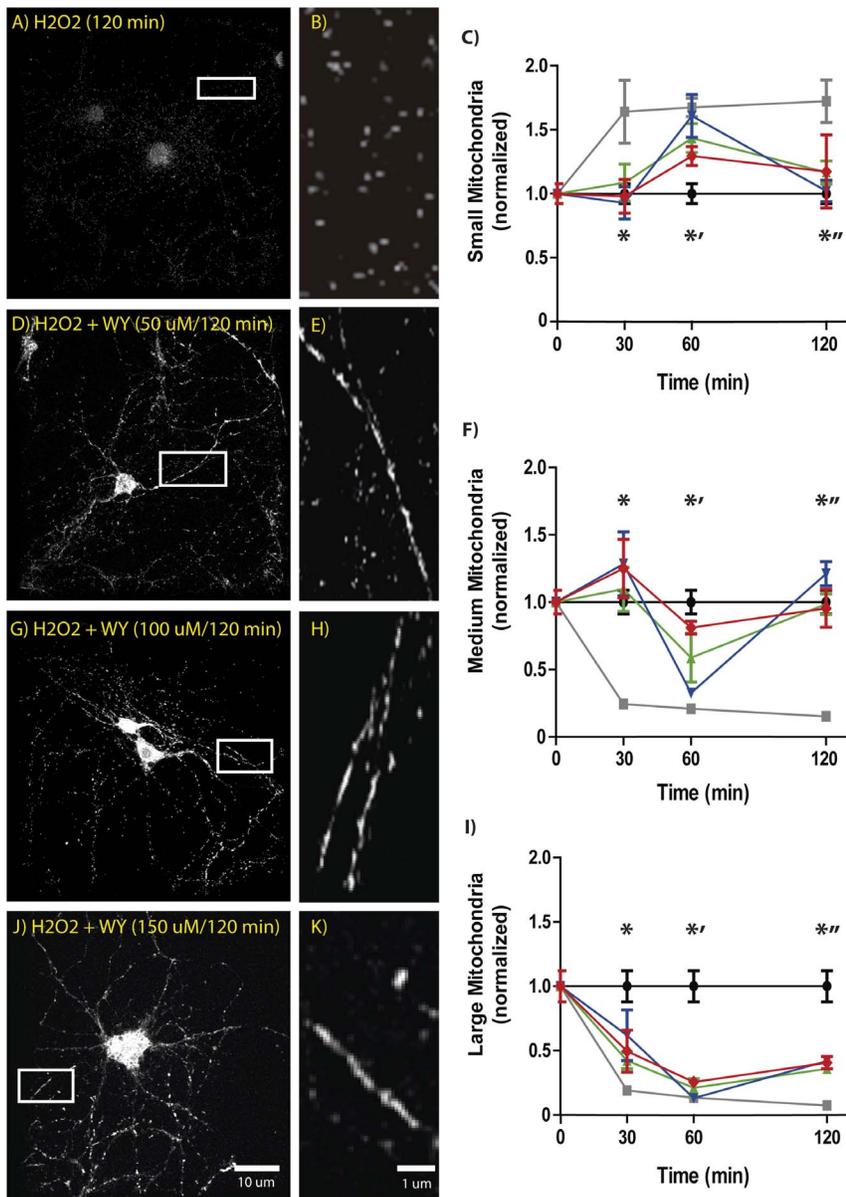


Figure 2. H₂O₂-induced mitochondrial size decrease is prevented by WY pre-treatment in hippocampal neurons. A, D, G, J microphotographs of mitochondrial population under different experimental conditions. B, E, H, K: image enlargement to show variations in mitochondrial morphology observed under different experimental conditions. C, F, I: Normalized mitochondrial size quantification (black: control; gray: H₂O₂; green: 50 μM WY+H₂O₂; blue: 100 μM WY+H₂O₂; red: 150 μM WY+H₂O₂). C) * indicates statistically significant differences between H₂O₂ and Control ($F_{9.275, d.f. 4, C.I. 0.06947 \text{ to } 0.631, t 4.078, p < 0.001$); *' indicates statistically significant differences between H₂O₂ and Control ($F_{9.275, d.f. 4, C.I. 0.08051 \text{ to } 0.6564, t 4.183, p < 0.001$), interestingly at 60 min only 100 μM WY shows significant differences respect Control ($F_{9.275, d.f. 4, C.I. -0.0009977 \text{ to } 0.6653, t 3.259, p < 0.01$); *'' indicates significant differences between H₂O₂ and all other treatments ($F_{9.275, d.f. 4, C.I. 0.1069 \text{ to } 0.6828, t 4.482, p < 0.001$; 50 μM WY C.I. $-0.617 \text{ to } 0.01, t 3.165, p < 0.01$; 100 μM WY C.I. $-0.7046 \text{ to } -0.06217, t 3.902, p < 0.001$; 150 μM WY C.I. $-0.6306 \text{ to } 0-0303, t 2.969, p < 0.05$). F) * *' and *'' indicate significant differences between H₂O₂ and Control ($F_{21.3, d.f. 4, C.I. -0.3851 \text{ to } -0.09656, t 5.457, p < 0.001$; *' C.I. $-0.3995 \text{ to } -0.1036, t 5.558, p < 0.001$; *'' C.I. $-0.4178 \text{ to } -0.1218, t 5.961, p < 0.001$). No significant differences were observed between Control and WY concentrations, except for 100 μM WY at 60 min ($F_{21.3, d.f. 4, C.I. -0.386 \text{ to } -0.04365, t 4.102, p < 0.001$). I) At each single time point significant differences were registered between Control and all other experimental conditions; however, at 120 min was possible to observe a different tendency for WY pre-treated curves and that of H₂O₂. doi:10.1371/journal.pone.0064019.g002

been demonstrated that AD brain exhibits decreased expression of PGC-1 α [27]. Further investigations have led to consider PGC-1 α as a critical transcriptional co-activator linked to cellular metabolism and mitochondrial biogenesis, and which abnormal expression could further account for the altered neuronal metabolism and diminished mitochondrial density often reported

in several AD models [27,28]. Recent research carried out in our laboratory has demonstrated that WY 14,643, a PPAR α agonist, improves significantly the cognitive abilities and reduces neuronal damage in a mouse model of AD [24]. These findings have prompted us to suggest that part of the effects observed after WY

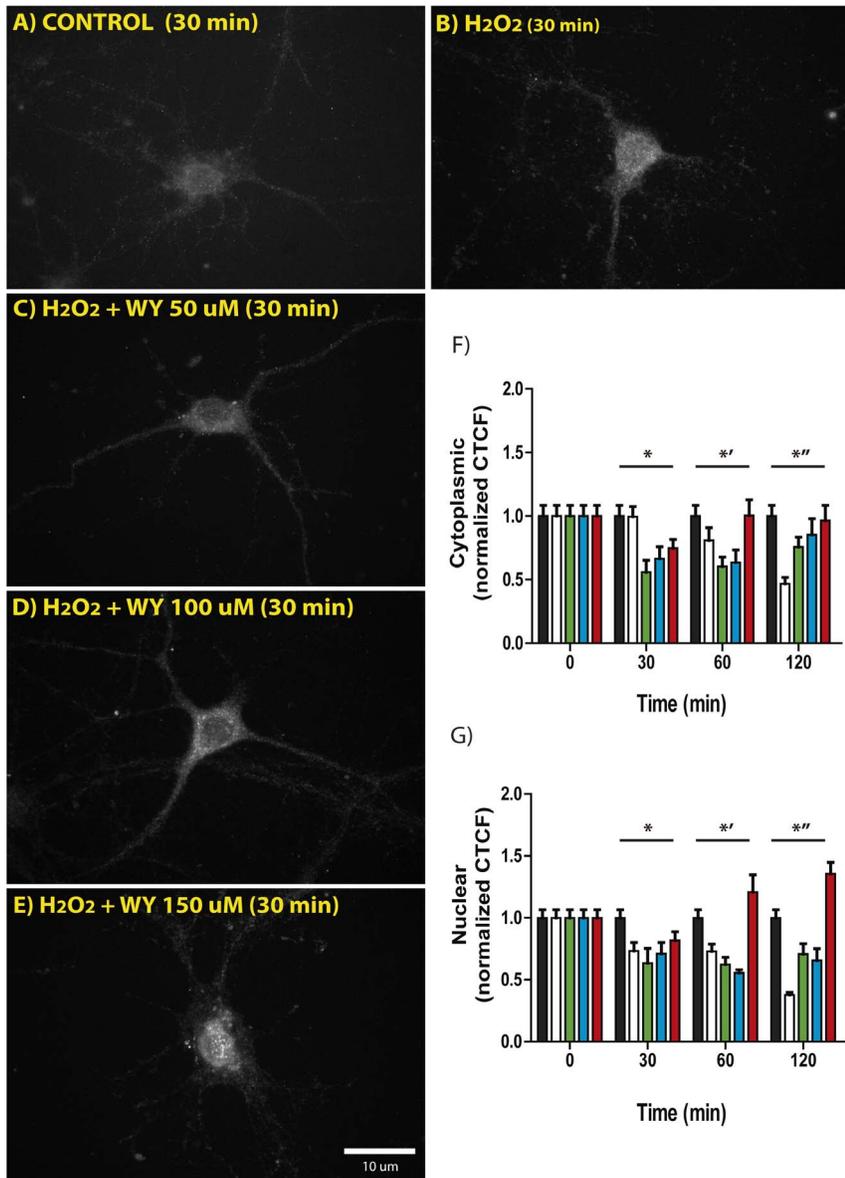


Figure 3. H₂O₂-induced PGC-1 α signal intensity loss is prevented by WY pre-treatment in hippocampal neurons. A–E representative microphotographs of PGC-1 α immunodetection in hippocampal neurons. F (cytoplasmic) and G (nuclear), normalized PGC-1 α signal intensity quantification by means of the corrected total cell fluorescence (CTCF) (black: control; white: H₂O₂; green: 50 μ M WY+H₂O₂; blue: 100 μ M WY+H₂O₂; red: 150 μ M WY+H₂O₂). F) * and ** indicates statistically significant differences between Control and 50 μ M and 100 μ M WY (F 5.131, *d.f.* 4, 50 μ M WY *C.I.* -0.08866 to -0.007103 , t 2.545, $p < 0.05$, and *C.I.* -0.07239 to -0.000643 , t 3.041, $p < 0.05$; 100 μ M WY *C.I.* -0.6401 to -0.001898 , t 2.816, $p < 0.05$, and *C.I.* -0.05095 to 0.02356 , t 2.580, $p < 0.05$). *** indicates statistically significant differences between H₂O₂ and Control (F 5.131, *d.f.* 4, *C.I.* -0.07803 to -0.02051 , t 5.118, $p < 0.001$). G) At 30 and 60 min (* and **) only 150 μ M WY did not show statistically significant differences respect Control situation. Interestingly, at 120 min (***) also 150 μ M WY exhibits significant differences regarding Control, showing an increment of the PGC-1 α signal intensity (F 5.131, *d.f.* 4, *C.I.* 0.002515 to 0.09452 , t 3.448, $p < 0.01$). doi:10.1371/journal.pone.0064019.g003

treatment might be due to an underlying mechanism which involves a PGC-1 α -related response.

In the present study we have evaluated the impact of oxidative stress challenge and PPAR-agonists treatment on mitochondrial morphology and on the PGC-1 α -related mitochondrial fusion-fission events on hippocampal neurons. The scope of this research is not only to complement the actual knowledge regarding PPARs-mechanisms of action, but also to propose mitochondrial dynamics as an additional target for future oxidative stress-related neurodegenerative disorders therapies.

Materials and Methods

Ethics Statement

Sprague-Dawley rats used in these experiments were housed at the Faculty of Biological Sciences of the P. Universidad Catolica de Chile and handled according to guidelines outlined and approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Biological Sciences of the P. Catholic University of Chile; and in accordance with the main directions from the Science and Technology National Commission (CON-

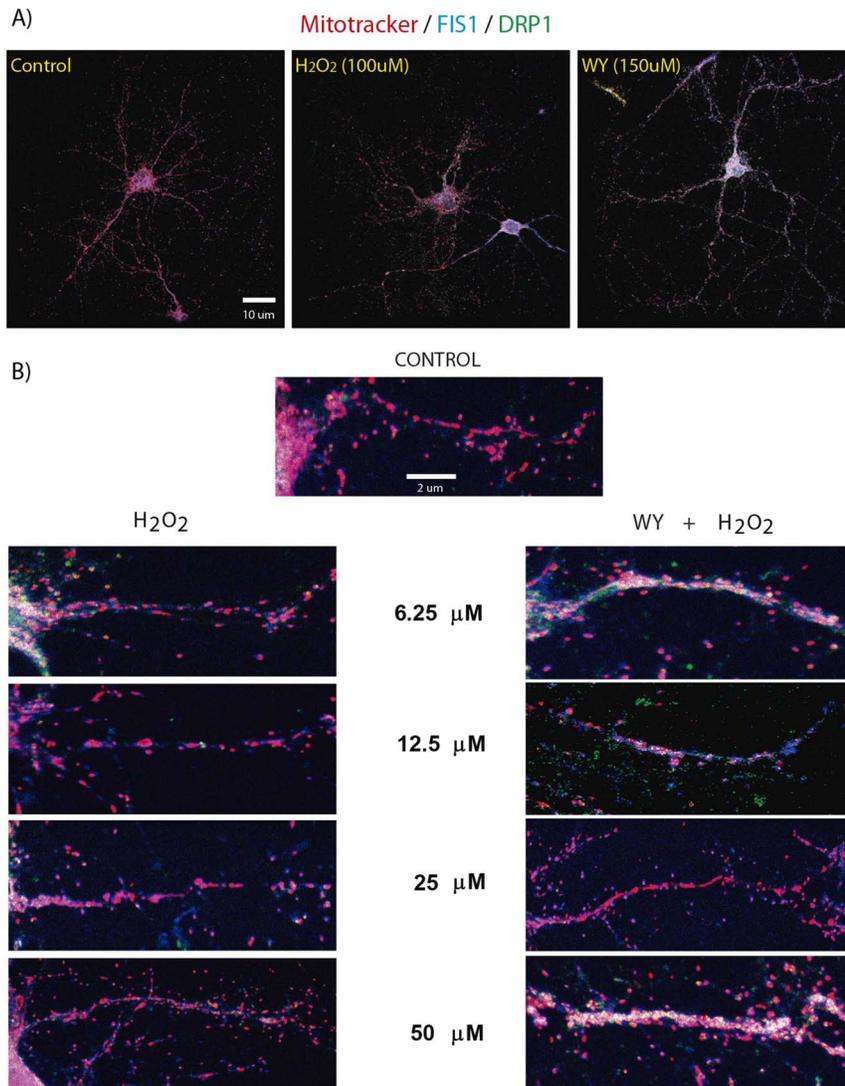


Figure 4. WY pre-treatment induces mitochondrial morphology changes in hippocampal neurons after H₂O₂ challenge. A) representative microphotographs of hippocampal neurons showing color merge under specific experimental conditions. B) mitochondrial morphology registered after H₂O₂ challenge and with WY pre-treatment. doi:10.1371/journal.pone.0064019.g004

ICYT) about experimental animal research. Animals were euthanized by anesthesia overdose. In our case, the IACUC did not require approval of specific protocols, because housing and handling procedures, as well as euthanasia, are well standardized protocols conceived and constantly monitored by IACUC itself. No experimental procedures were performed on the rats prior to euthanasia.

Hippocampal Neuronal Cultures

Mixed 18 days embryonic Sprague-Dawley rat hippocampi were dissected, and primary hippocampal cultures were prepared as described previously [29]. Hippocampal cells were seeded in polylysine-coated wells and cultured in Neurobasal medium (Invitrogen Corp., Carlsbad, CA), supplemented with B27 (Invitrogen). 11 DIV cultured hippocampal neurons were used for several experiments. 2 μ M 1- β -D-arabinofuranosylcytosine (Ara-C, Cat. N^oC-6645, Sigma-Aldrich, St. Louis, MO) was used to reduce the glial content in the culture.

Ciglitazone (PPAR γ agonist)-H₂O₂ Challenge Assay

Hippocampal neurons (11 DIV) were pre-treated for 2 h with 10 μ M ciglitazone (CIG) (Sigma Aldrich) prior to 30 and 60 min of 25 μ M H₂O₂ challenge. Additionally, 5 μ M GW9662 (PPAR γ inhibitor) (Sigma Aldrich) was used as control. At the end of experimental time, neurons were washed and labeled with 50 nM Mitotracker Red (Molecular Probes, Eugene, OR) for 30 min. at 37°C. Cells were then fixed by paraformaldehyde-sucrose 4% fixative and permeabilized with PBS-Triton X 0.01%. Immunofluorescence images were captured with a Zeiss LSM 5 Pascal confocal microscope. Mitochondrial length was assessed using Image J software by Feret's diameter analysis. To allow comparison, mitochondrial length was classified into three different categories ranging from 0.21 to 0.54 μ m (small), 0.55 to 1.59 μ m (medium); and 1.60 to 10 μ m (large). A minimum of 10 microphotographs were recorded for each treatment (per replicate) and scored.

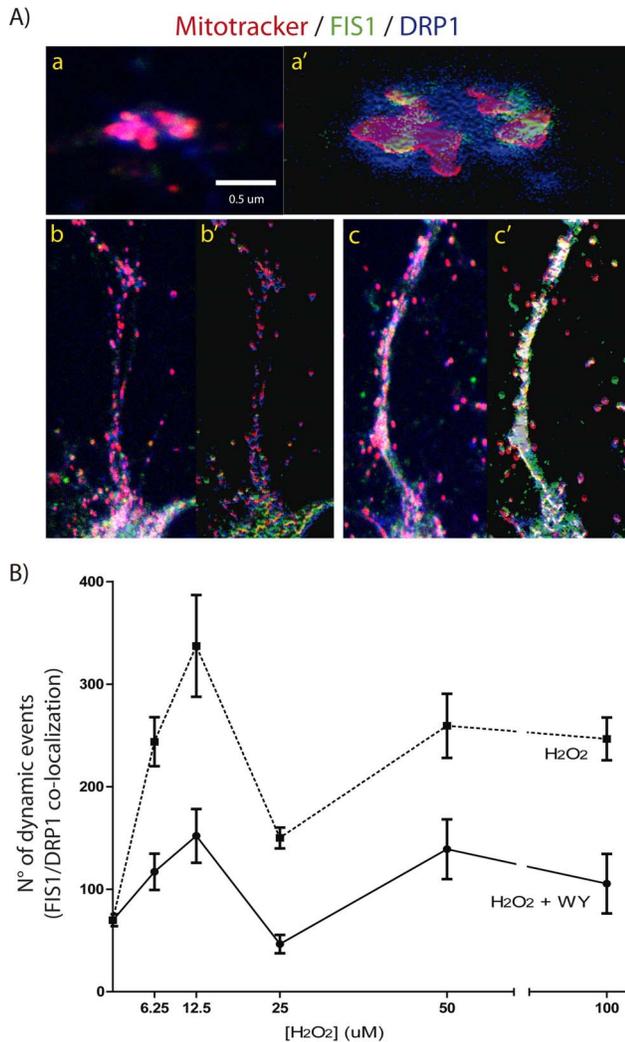


Figure 5. WY pre-treatment increases FIS1/DRP1 co-localization events in hippocampal neurons after H₂O₂ challenge. A) microphotograph close up showing localization of FIS1 (green) and DRP1 (blue) on a fissioned mitochondria (a); and on a neuronal projection under H₂O₂ challenge alone and with WY pre-treatment (b and c, respectively). Right images were manipulated only to increase color definition (a', b' and c'). B) FIS1/DRP1 co-localization quantification. At each H₂O₂ concentration statistically significant differences were registered between H₂O₂ alone and the WY pre-treated hippocampal neurons (*F* 10.36, *d.f.* 5, 6.25 µM *C.I.* 40.29 to 213.4, *t* 4.033, *p*<0.01; 12.5 µM *C.I.* 67.02 to 303.6, *t* 4.311, *p*<0.001; 25 µM *C.I.* 0.5261 to 216.5, *t* 2.765, *p*<0.05; 50 µM *C.I.* 2.051 to 238.6, *t* 2.8, *p*<0.05; 100 µM *C.I.* 28.89 to 253.3, *t* 3.461, *p*<0.01). Red channel: mitotracker; Green channel; FIS1; Blue channel: DRP1. doi:10.1371/journal.pone.0064019.g005

WY (PPAR α agonist)-H₂O₂ Challenge Assay and PGC-1 α Assessment

Hippocampal neurons (11 DIV) were pre-treated for 12 h with 50–100–150 µM WY 14.643 (WY) (Aptuit, Lenexa, KA) solution prior to 30–60–120 min of 25 µM H₂O₂ challenge. At the end of experimental time, neurons were washed and labeled with 50 nM Mitotracker Red (Molecular Probes) for 30 min at 37°C. Cells were then fixed by paraformaldehyde-sucrose 4% fixative and permeabilized with PBS-Triton X 0.01%. Hippocampal neurons were additionally stained for PGC-1 α (anti-PGC1 α polyclonal antibody ab54481, 1:300, Abcam Inc., Cambridge, MA) and

incubated over-night at 4°C. After primary antibody incubation cells were washed and incubated with anti-mouse IgG- Alexa Fluor-488 (1:1000) (Invitrogen) as secondary antibody for 1 h at 37°C. Immunofluorescence images were captured with a Zeiss LSM 5 Pascal confocal microscope. Mitochondrial length and PGC-1 α signal intensity were assessed using Image J software by Feret's diameter analysis and Corrected Total Cell Fluorescence (CTCF), respectively. To allow comparison, mitochondrial length was classified as for CIG.

Mitochondrial Dynamics Assay

Hippocampal neurons (11 DIV) were pre-treated for 12 h with 150 µM WY (Aptuit) solution prior to 120 min of 6.25–12.5–25–50–100 µM H₂O₂ challenge. At the end of culture time, neurons were washed and labeled with 50 nM Mitotracker Red (Molecular Probes) for 30 min at 37°C. Cells were then fixed by paraformaldehyde-sucrose 4% fixative and permeabilized with PBS-Triton X 0.01%. Hippocampal neurons were additionally stained for dynamin related protein 1 (DRP1) (mouse anti-DLP1, BD Bioscience, San José, CA) and mitochondrial fission protein 1 (FIS1) (rabbit anti-Fis1, Enzo Life Science, Flamingdale, NY), and incubated over-night at 4°C. After primary antibody incubation cells were washed and incubated with anti-mouse IgG-Alexa Fluor-633 (1:500) (Invitrogen) and anti-rabbit IgG-Alexa Fluor-488 (1:100) (Invitrogen) respectively, as secondary antibodies for 1 h at 37°C. Immunofluorescence images were captured with a Zeiss LSM 5 Pascal confocal microscope. Image analysis was performed using "Find Maxima" tool in Image J software, complemented with Menders co-localization analysis [30], which allows to finds maximal fluorescence points within each microphotograph. Each point will corresponds to the overlap of the three color channels considered in the study. Vasopressin was included as a positive control.

Statistical Analysis

Results are presented as means \pm standard error (mean \pm s.e.m) and normalized against controls. One-way ANOVA followed by Bonferroni's post hoc test, was used to establish statistically significant differences among treatments. Two-way ANOVA followed by Bonferroni's post hoc test, was used to assess statistically significant differences among treatment types and duration. Analysis and plots were done using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

Results

CIG, a PPAR γ agonist, Prevents Mitochondrial Size Reduction in Hippocampal Neurons Induced by H₂O₂ Challenge

Considering oxidative stress as a critical pathway in the pathophysiology of several neurodegenerative disorders, such as the A β -mediated neurotoxicity in AD [15–17], we exposed hippocampal neurons to 25 µM H₂O₂ challenge for 30 and 60 min. As we have previously demonstrated [21], at this concentration H₂O₂ is able to induce neurotoxicity but without affecting severely the total neuronal number, allowing better comparison between treatments. As expected, H₂O₂ challenge induced a statistically significant alteration of the dynamic process of mitochondrial population, with an increased number of small sized mitochondria and a reduction of the middle and large organelles (fig. 1). Despite the same variations where observed, regarding mitochondrial size population, when hippocampal neurons were pre-treated with CIG, no statistically significant differences were observed between control situation and pre-

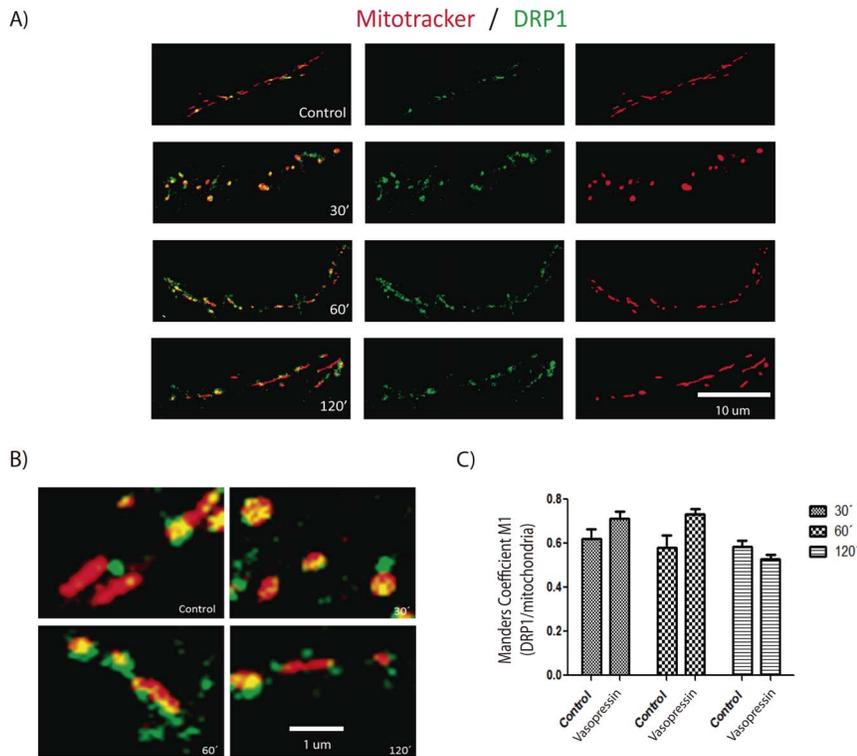


Figure 6. Mitochondrial dynamics events induced after Vasopressin treatment on hippocampal neurons. A) Representative microphotographs of neuronal projections after vasopressin treatment. B) Enlarged images showing Mitotracker and DRP1 signal co-localization registered under different experimental conditions. C) Normalized quantification of signal co-localization.
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treated neurons for small and middle sized mitochondria (fig. 1 C and F). About large sized mitochondria, even when the number showed also a reduction, the tendency was different to what observed for H_2O_2 treatment; while a sustained reduction of large mitochondrial number was observed for the later, in CIG pre-treated neurons we observed that at 30 min there is an inflection point, indicating that the number of large mitochondria started to increase (fig. 1 J). These results suggest that CIG, through PPAR γ activation, is able to prevent the mitochondrial size reduction induced by oxidative stress, protecting neurons from H_2O_2 -induced mitochondrial altered fusion-fission process.

Pre-treatment of Hippocampal Neurons with WY, a PPAR α agonist, Prevents Mitochondrial Size Reduction Induced by H_2O_2 Challenge

Following the same paradigm, when WY was used as pre-treatment on hippocampal neurons for 12 h before H_2O_2 challenge, the same preventive effects were observed (fig. 2). Considering the different nature of the WY, we tested three different concentrations in order to properly evaluate the effects of the compound. Once again, H_2O_2 treatment alone showed statistically significant differences regarding mitochondrial size compared with the control situation at each time point. On the other hand, WY pre-treatment of hippocampal neurons also prevented mitochondrial size reduction, buffering the toxic effects of H_2O_2 , suggesting mitochondrial dynamics improvement (fig. 2 C, F and I). Similarly to what was described for the large sized mitochondria under CIG treatment, with WY, the inflection point was reached after 60 min under H_2O_2 challenge, and the recovery

was statistically significant compared with the H_2O_2 treatment alone (fig. 2 I).

Loss of PGC-1 α Signal is Induced under H_2O_2 Challenge but Prevented by WY Pre-treatment

PGC-1 α is localized at both, nuclear and cytoplasmic compartments, preferentially located at the nuclear level [31]; and its expression has been related with enhanced expression of several mitochondrial-related proteins, such as uncoupled proteins (UCP), nuclear respiratory factor (NRF1 and 2) and DRP among others [32,33]. Fig. 3 shows both nuclear and cytoplasm normalized PGC-1 α signal intensity registered by immunofluorescence after H_2O_2 challenge. After 30 min a statistically significant signal decrease was observed at the cytoplasm level of hippocampal neurons compared with control situation. Moreover, after 120 min the loss of signal intensity was dramatically lower ($\sim 50\%$) at both, cytoplasm and nuclear, levels (fig. 3 F and G). Despite an initial decrease in PGC-1 α signal intensity registered after 30 min of H_2O_2 challenge, WY pre-treatment was able to prevent PGC-1 α signal loss at each single dose; moreover, at 150 μM WY it increased the signal intensity, suggesting PGC-1 α increased expression at both cytoplasm and nuclear compartments of hippocampal neurons.

WY Pre-treatment Enhances Mitochondrial Fission Events in Hippocampal Neurons, Induced by H_2O_2 Challenge

Mitochondrial size is related with the balance between mitochondrial fusion and fission events [19,34]. By means of an immunofluorescence assay we establish the mitochondria-related localization of two proteins, DRP1 and FIS1, in order to assess if

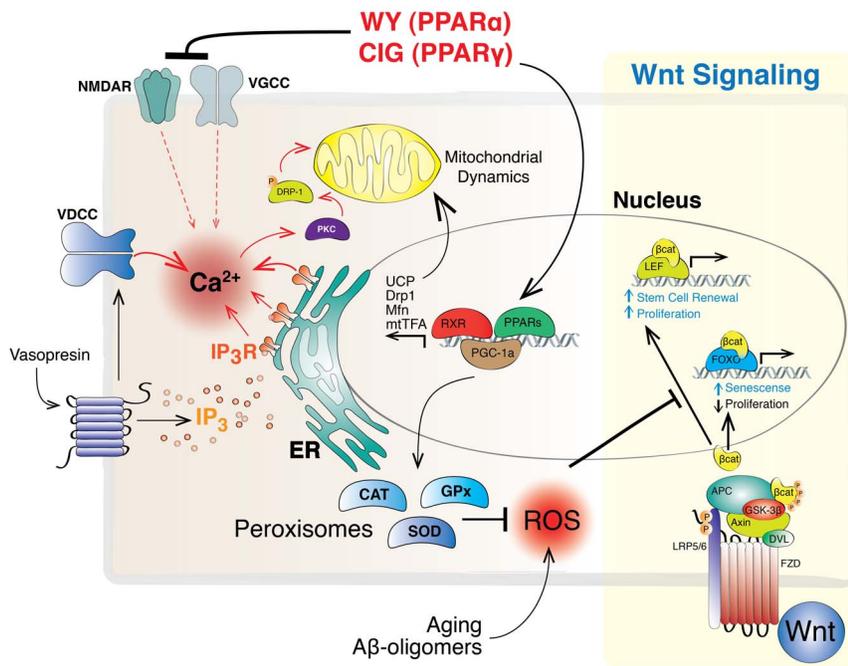


Figure 7. PPAR-mediated mitochondrial dynamics modulation. We have previously proposed a direct link between antioxidant PPARs activity and Wnt signaling pathway [19]. Based on our present results we hypothesize that PPARs are able to modulate mitochondrial dynamics through different cellular mechanisms. Complementary to the Wnt signaling pathway (*FZD*, frizzled; *LRP 5/6*, low density lipoprotein receptor-related protein; *DVL*, disheveled; β cat, β -catenin), PPARs might act inducing directly the expression of several mitochondrial-related proteins (*UCP*, uncoupled protein; *DRP1*, dynamin related protein 1; *Mfn*, mitofusin; *mtTFA*, mitochondrial transcription factor). Additionally, the PPARs-mediated calcium balance might also offer another control point for mitochondrial dynamics modulation (*VDCC*, voltage dependent calcium channels; *NMDAR*, NMDA receptor; *VGCC*, voltage-gated calcium channels; *PKC*, protein kinase C). doi:10.1371/journal.pone.0064019.g007

the H_2O_2 challenge and the WY pre-treatment is able to modulate mitochondrial dynamics (fig. 4 and 5). We use several H_2O_2 concentrations in order to provide a better overview of mitochondrial response, and the same concentrations were tested after 150 μ M WY pre-treatment. As previously observed, the H_2O_2 challenge induces a reduction of the mean mitochondrial size in a dose dependent manner, but when hippocampal neurons were pre-treated with WY a recovery of the mean mitochondrial size, close to control situation, was observed (fig. 4 A and B). As expected, H_2O_2 treatment alone also induced an increased colocalization of immunofluorescence signals (white spots) suggesting increased fission events (fig. 5 A). Interestingly, the WY pre-treatment induced a higher increase of white spots number in response to H_2O_2 challenge, suggesting that WY will favor mitochondrial fission even under oxidative stressors (fig. 5 B). We have also included vasopressin treatment as positive control of a mitochondrial dynamics inducer with well-defined pharmacodynamics [35,36] (fig. 6). The DRP1 and Mitotracker co-localization induced by vasopressin, allow us to suggest that calcium balance might play a critical role in the modulation of mitochondrial dynamics.

Discussion

Despite mitochondrial dysfunction has been recognized since long ago as a key event in $A\beta$ -induced neurotoxicity [12,18], only recently it has been established that mitochondrial dynamics plays a fundamental role in mediating mitochondrial dysfunction [19,33,34]. The balance of fusion and fission events is critical in order to maintain healthy mitochondrial population when responding to stressor stimuli [34].

Our results indicate that after H_2O_2 challenge, mitochondria undergoes successive fission events without its counterpart, leading to a reduced mitochondrial size. In fact, it has been previously demonstrated that excessive ROS production could damage mitochondrial DNA (mtDNA) leading to mitochondrial malfunction, and that these defective organelles could be rescued through fusion or finally eliminated after fission [34,37,38]. These findings suggest, and allow us to hypothesize, that the cell antioxidant activity as well as the mitochondrial metabolic capacity were exceeded by the H_2O_2 challenge, and resulted in mitochondrial damage without any recovery response. Moreover, the final loss of PGC-1 α signal registered after H_2O_2 challenge serves to offer further support to this hypothesis. Even when described initially just as a cold inducible factor [39], further investigations have positioned PGC-1 α as a critical modulator of several metabolic processes and as a key element of mitochondrial biogenesis [32,40]. In fact, PGC-1 α activity linked to PPARs, RXR, and estrogen related receptor α (ERR α) have been related with the expression of several mitochondrial proteins, such as UCP, NRF1, NRF2, DRP1, FIS1, MFN2 [19,26,32,40,41]. In consequence, the loss of PGC-1 α signal observed in our work suggest a diminished expression rate of several mitochondrial proteins related with fusion-fission events affecting negatively mitochondrial dynamics and leading to mitochondrial dysfunction.

Interestingly, it was possible to observe that PGC-1 α loss does not occur immediately but after 30 min H_2O_2 challenge. Despite this finding could result confuse, it has been described that H_2O_2 is able to induce an initial cell response linked to increased antioxidant enzymes expression and enhanced mitochondrial activity [42]. In fact, it was possible to observe that even as very low concentration, such as 6.25 μ M, H_2O_2 is able to induce

increased mitochondrial fission events that could reflect this initial cell response. Moreover, it seems to be a critical concentration (25 μM) at which the H_2O_2 challenge induces less fission events than lower and higher concentrations, suggesting that this point could indicate the change between a physiological compensatory mitochondrial response and a pathological outcome after a stressor stimuli. Furthermore, this phenomenon might be related with the physiological role often described for ROS on several cell processes [42–44].

On the other hand, we used two different PPAR agonists in order to assess the protection derived of the PPARs pre-treatment. The therapeutic potentialities of PPARs on several disorders have been widely assessed by our research group as well as others [20–24,45–50]. Despite we have previously described PPAR γ -related mitochondrial protection [22], here we describe for the very first time the relation of PPAR γ agonist with mitochondrial dynamics. In fact, the CIG pre-treatment protects mitochondria from size reduction, suggesting the modulation of fusion-fission events. In the same way, WY, a PPAR α agonist, also exhibits the same property. If we consider that main effects were observed after 30 min H_2O_2 challenge, it is possible to hypothesize that initially there was an increased expression of antioxidant enzymes, enhancing cell antioxidant capacity, delaying the mitochondrial response to the stressor stimulus. Furthermore, we have recently demonstrated that 4-phenylbutyrate and WY are able to prevent and rescue cognitive impairment in a double transgenic mouse model of AD [24]. Even when these two drugs act as different agonists (PPAR γ and PPAR α , respectively), the results obtained for both treatments were quite similar. According to available information a common point for the mitochondrial dynamics modulation of these drugs will be the activation of the PGC-1 α [51,52].

As expected, WY pre-treatment not only prevents the reduction of mitochondrial size, but also increases the PGC-1 α signal intensity at the cytoplasm and nuclear level, suggesting that WY is able to induce the expression of PGC-1 α . Moreover, the higher number of fission events obtained under WY pre-treatment, together with the increased mitochondrial size, suggests enhanced mitochondrial dynamics events, derived from PGC-1 α increased expression, inducing fusion and fission as a coordinated process, and that could be at the basis of the neuroprotective mechanism observed for these drugs. Interestingly, the number of fission events was higher than registered for H_2O_2 alone, suggesting that mitochondrial fission should not be considered as a negative event

but as a critical step of mitochondrial physiology which allows better response to stressor stimuli. Our results, together with previous findings that have linked the increased ROS production due to A β peptide [21,53], allow us to consider that this mechanism offers new and interesting perspectives in ROS-related neurodegenerative disorders, such as AD. On the other hand, we have recently conducted several experiments using vasopressin, a known hormone that has shown to induce mitochondrial dynamic modulation. Intracellular calcium balance lies at the basis of vasopressin, mainly through a direct action on internal calcium reserves, such as endoplasmic reticulum, and/or inducing extracellular calcium influx through voltage-dependent calcium channels (VDCC) [35]. Interestingly, both PPARs, α and γ , have been linked with calcium balance [54,55]. Moreover it is well known that increased Ca^{2+} levels, mainly through VDCC, leads to activation of protein kinase C (PKC) and further phosphorylation of DRP1 [36]. Considering our results, we could hypothesize that also part of the effects observed on mitochondrial dynamics might be related with the PPAR-mediated intracellular calcium balance, affecting mitochondrial dynamics-related proteins activity.

Despite valid concerns surrounds PPARs, overall related to its toxicity and carcinogenic effects derived from its use [56], the therapeutic opportunities offered by this family of molecules is out of discussion [4,57]. However, the mechanism involved in the wide range of beneficial effects observed for PPARs agonists are quite unknown, and even when several studies have offered some possible explanations for part of these effects, they have failed to explain others. Years ago, we have proposed a direct link between PPARs and the Wnt signaling pathway [58] at the basis of several neuronal mechanisms. Here we propose an additional mechanism relating PPARs agonist and mitochondrial dynamics (fig. 7), and how this relation could act as a mitochondrial protective mechanism that finally will confer enhanced metabolic protection to hippocampal neurons against oxidative stress.

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Author Contributions

Conceived and designed the experiments: JMZ DO JAG NCI. Performed the experiments: JMZ CSA DO JAG EJC. Analyzed the data: JMZ DO EJC. Contributed reagents/materials/analysis tools: CSA MJS NCI. Wrote the paper: JMZ CSA DO MJS NCI.

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