Mol Neurobiol (2018) 55:4453–4462
DOI 10.1007/s12035-017-0667-3

# Tibolone Preserves Mitochondrial Functionality and Cell Morphology in Astrocytic Cells Treated with Palmitic Acid

Yeimy González-Giraldo [1] · Luis Miguel Garcia-Segura [2,3] · Valentina Echeverria [4,5] · George E. Barreto [1,6]

Received: 9 May 2017 / Accepted: 19 June 2017 / Published online: 30 June 2017
© Springer Science+Business Media, LLC 2017

**Abstract** Obesity has been associated with increased chronic neuroinflammation and augmented risk of neurodegeneration. This is worsened during the normal aging process when the levels of endogenous gonadal hormones are reduced. In this study, we have assessed the protective actions of tibolone, a synthetic steroid with estrogenic actions, on T98G human astrocytic cells exposed to palmitic acid, a saturated fatty acid used to mimic obesity in vitro. Tibolone improved cell survival, and preserved mitochondrial membrane potential in palmitic acid-treated astrocytic cells. Although we did not find significant actions of tibolone on free radical production, it modulated astrocytic morphology after treatment with palmitic acid. These data suggest that tibolone protects astrocytic cells by preserving both mitochondrial functionality and morphological complexity.

✉ George E. Barreto
gesbarreto@gmail.com; gsampaio@javeriana.edu.co

[1] Departamento de Nutrición y Bioquímica, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá D.C., Colombia

[2] Instituto Cajal, CSIC, Madrid, Spain

[3] CIBER de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES), Instituto de Salud Carlos III, Madrid, Spain

[4] Fac. Cs de la Salud, Universidad San Sebastián, Lientur 1457, 4080871 Concepción, Chile

[5] Bay Pines VA Health Care System, 10,000 Bay Pines Blvd, Bldg 23, Rm 123, Bay Pines, FL 33744, USA

[6] Instituto de Ciencias Biomédicas, Universidad Autónoma de Chile, Santiago, Chile

**Keywords** Astrocytes · Palmitic acid · Tibolone · Mitochondria · Morphology

## Introduction

Astrocytes play an important role in controlling brain homeostasis. These cells control the extracellular levels of potassium, water, and neurotransmitters (glutamate, GABA, and glycine) [1], regulate blood flow, transport glucose from the vasculature [2], and release neurotrophic factors, such as the brain-derived neurotrophic factor (BDNF), energy substrates, and neurosteroids [3]. Also, astrocytes participate in forming neuroglia networks and information processing [4]. In addition, astrocytes are key regulators of inflammatory processes in the brain, although their role on neuroinflammation is somehow controversial. For example, the anti-inflammatory actions of astrocytes involve the activation of transcription factors and receptors, such as the signal transducer and activator of transcription 3 (STAT3), BDNF, estrogen receptor α (ERα), and transforming growth factor β receptor (TGFβR), which are involved in protective pathways associated to anti-inflammatory cytokine release, reduced astrogliosis, and increased neuronal survival. Conversely, NF-κB transcription factor, sphingosine 1-phosphate (S1P), and interleukin 17 receptor (IL7R) activation are involved with detrimental pathways, which are related with enhanced astrogliosis, cytokine release, neuronal death, and oxidative stress [5]. Pathological activation of astrocytes signaling pathways is involved in neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD), stroke [6], and psychiatric diseases such as schizophrenia [7] and major depression disorder [8].

Previously, we have used T98G cells, which is a cell line widely assessed as astrocytic model. T98G cells have similar morphological and functional characteristics of that of human

Table 1 Studies demonstrating effects of tibolone in the CNS

| Cellular or animal model | Results | Reference |
|---|---|---|
| Cynomologous monkeys | High-dose tibolone showed a decrease in 5-HT levels in the frontal cortex from ovariectomized cynomologous monkeys. | [54] |
| Arcuate neurons from female guinea pigs | Tibolone and 3β-OH attenuate $GABA_B$ response by means of a membrane ER different from ERα and ERβ. | [55] |
| Female Wistar rats | Tibolone reduced lipid peroxidation in the brain cortex and hippocampus of ovariectomized female. | [56] |
| Neurons from neonatal rats (10-day-old) and male rats of Wister line | Tibolone prevented cell death, increasing of GSH and a decreasing of GSSG, reduced protein oxidative damage produced by buthionine sulfoximine. Tibolone also increased HSP70 expression. | [57] |
| CA3 pyramidal neuron from male Wistar rats | Tibolone partially reduced loss of the CA3 pyramidal neuron induced by chronic O3 exposure. It reduced peroxidation and protein oxidation and prevented cognitive deficits in rats. | [58] |
| Male Wistar rats | Tibolone increased SOD2 expression in rats with and without ozone exposure. It increased choline acetyltransferase and reduce damage induced on memory. | [59] |
| Astrocytes (T98G) | Tibolone decreased calcium levels in glucose deprived cells and preserved mitochondrial membrane potential. | [38] |
| CA1 pyramidal neurons from Sprague Dawley rats | Tibolone reverses the spine pruning in CA1 pyramidal neurons caused by ovariectomy but it did not reduce lipid peroxidation. | [60] |
| Astrocytes (T98G) and primary mouse astrocytes | Tibolone protects astrocytes from damage by glucose deprivation through estrogen receptor beta and an increasing neuroglobin expression. | [21] |

*5-HT* 5-hydroxytryptamine, *3β-OH* 3b-hydroxytibolone, *ERα* estrogen receptor α, *ERβ* estrogen receptor β, *$GABA_B$* gamma-aminobutyric acid B, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *HSP70* 70 kilodalton heat shock protein, *SOD2* superoxide dismutase 2

astrocytes, and have been shown to expresses GFAP (glial fibrillary acidic protein), nestin, and vimentin [9]. Therefore, this cell line is considered as a biological model to evaluate new therapeutic strategies and their molecular mechanisms affecting astrocyte function [10–13].

Estradiol is a neuroprotective hormone that reduces neuroinflammation and regulates the activation of astrocytes [14, 15]. However, the use of estradiol as a neuroprotectant is limited by its peripheral hormonal actions. Tibolone is a steroid with estrogenic, progestogenic, and androgenic activities, which are mediated by its three metabolites, 3α-, 3β-OH-tibolone, and Δ4-isomer [16]. It has been described that tibolone has beneficial effects on the bone, vagina, and brain, and it is used as treatment for climacteric symptoms and osteoporosis prevention [17]. Tibolone is classified as a selective tissue estrogenic activity regulators (STEARs) by its different actions on enzyme regulation, receptor activation, and metabolism. For example, it has been demonstrated that tibolone inhibits steroid sulphatase activity in breast cancer cells, but not on cell lines derived from bone tissue [18]. Furthermore, it has been observed that tibolone and its 3-hydroxy metabolites have agonist actions on estrogen receptor in astrocytes [19]. The use of STEARs such as tibolone might provide an alternative therapeutic approach to the use of estradiol to regulate reactive astrogliosis [20, 21]. A growing number of studies have analyzed the effects of tibolone on neurons and astrocytes (Table 1). Previously, Avila et al. found that tibolone protects astrocytes from damage induced by glucose deprivation, and the authors demonstrated that neuroglobin is involved in its protective effects [21].

Obesity has been related with inflammation-induced cognitive impairment [8] and with an increased risk for neurodegenerative diseases [22, 23]. High-fat diet in animals alters blood glucose, insulin sensitivity, and cytokine expression [24]. Besides, it has been found that the peripheral immune cells, from mice fed with high-fat diet to induce obesity, can enter to the central nervous system (CNS) and induce neuroinflammation [25]. Glial properties are modified by high-fat diet. For instance, cortical glia derived from adult mice fed with high-fat diet provided less support for the growth and viability of primary embryonic cortical neurons than cortical glia derived for animals fed with control diet [24]. Fatty acids, which are elevated in obese people [26], can cross the blood brain barrier and contribute to modify the function of glial cells, thus increasing neuroinflammation [27, 28]. In the present study, we used palmitic acid, a saturated fatty acid, to mimic obesity-induced neuroinflammation in vitro. Palmitic acid is the most abundant lipid in the diet and can be generated by cells through a de novo synthesis pathway [29]. This fatty acid induces the expression of cytokines and reduces cellular viability in astrocytes and neurons [30, 31], activating inflammatory signals [32] and inducing cytotoxicity [33]. Additionally, palmitic acid can induce apoptosis in astrocytes by upregulating serine palmitoyltransferase subunits [34]. Furthermore, the levels of this fatty acid are increased in the parietal neocortex from patients with AD [35], suggesting that

it could play an important role in neurodegenerative diseases. Altogether, these studies suggest that palmitic acid is involved in the effects of high-fat diet in the brain [36]. The aim of this study was to determine the possible effects of tibolone on astrocytic alterations induced by palmitic acid.

## Materials and Methods

### Cell Culture

T98G human astrocytoma cell line was purchased from the American Type Culture Collection (ATCC® CRL-1690™) (Manassas, VA, USA). T98G cell line has been used in several studies as a biological model to evaluate astrocyte characteristics and functions [10–13, 21, 37–40]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Lonza, Walkersville, USA) supplemented with 10% fetal bovine serum (FBS) (Eurobio, France) and 10 U penicillin/10 mg streptomycin/25 ng amphotericin (Lonza, Walkersville, USA). Cell cultures were incubated at 37 °C under a 5% $CO_2$ atmosphere.

### Tibolone Pretreatment

T98G cells were seeded in multi-plates using DMEM supplemented with FBS, and cell growth was allowed for 24 h (h). Before tibolone treatment, serum starvation was performed for 24 h using DMEM without L-glutamine, serum, and phenol red (Lonza, Walkersville, USA). A stock of 40 mM tibolone (Sigma, St Louis, MO, USA) was prepared in 100% DMSO (Sigma, St Louis, MO, USA). Later, tibolone was diluted in DMEM without L-glutamine, serum, and phenol red (Lonza, Walkersville, USA). Cells were treated with several concentrations of tibolone to determine the optimal dose conditions for 24 h before the treatment with palmitic acid. A vehicle containing 0.2% DMSO was used as control of tibolone. Once tibolone pretreatment was finalized, the medium was replaced by 1 mM palmitic acid or control (2.5% BSA and 2 mM carnitine) for 24 h.

### Palmitic Acid Treatment

We first evaluated the concentration of palmitic acid by which T98G cells viability was found reduced by ~50%. On the same day of treatment, we prepared a mixture containing 5% bovine serum albumin (BSA) (Sigma, St Louis, MO, USA), 2 mM palmitic acid (Sigma, St Louis, MO, USA), 2 mM carnitine (Sigma, St Louis, MO, USA), and DMEM without L-glutamine, serum, and phenol red (Lonza, Walkersville, USA). Carnitine was used to allow palmitic acid entry to the mitochondria [41]. We tested the following concentrations of palmitic acid: 100, 200, 500, and 750 μM and 1 mM for 24 h. The maximum concentration of BSA used was 2.5%, and in all experiments, we used 2 mM carnitine. Control group consisted in 2.5% BSA and 2 mM carnitine dissolved in serum and phenol red-free DMEM.

### Cell Viability Analysis

For the viability analysis, we used MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and propidium iodide test. MTT assay was performed according to previous protocol [21, 39]. When the treatment time was completed, MTT reagent at final concentration of 0.5 mg/ml was added to the cells for 3 h in the dark in a 37 °C, 5% $CO_2$ humidified incubator. Finally, formazan crystals were dissolved in DMSO 100%, and absorbance was evaluated in a FLUOstar Omega microplate reader at 595 nm (BMG LABTECH, Ortenberg, Germany). Absorbance values were normalized to the control (2.5% BSA), which was considered as 100% survival. Additionally, we also used propidium iodide (Sigma, St Louis, MO, USA) for viability analysis. Once the treatment time was completed, cells were detached using 500 mg/L trypsin with 200 mg/L EDTA (Lonza, Walkersville, USA) and then, cells were resuspended in PBS 1X. Staining was performed using 10 μg/ml propidium iodide for 15 min [21]. Analysis of propidium iodide uptake was done by means of flow cytometry using the Guava EasyCyte cytometer (Millipore, MA, USA). Data was expressed in terms of percentage of uptake.

### Mitochondrial Functionality Analysis

We employed flow cytometry to evaluate mitochondrial membrane potential by means of tetramethylrhodamine methyl (TMRM) dye [42] and mitochondrial mass using nonyl acridine orange (NAO) dye [43]. Firstly, cells were stained with 500 nM TMRM (Sigma, St Louis, MO, USA) or 200 nM NAO (Sigma, St Louis, MO, USA) for 30 min in the dark in a 37 °C, 5% $CO_2$ humidified incubator. Later, cells were detached and resuspended in PBS 1X. Analysis of fluorescence was performed in the Guava EasyCyte cytometer (Millipore, MA, USA) using yellow (TMRM) and green (NAO) channel [13, 21]. Data was expressed as mean fluorescence.

### Reactive Oxygen Species (ROS) Production

We assessed the effect of tibolone and palmitic acid on ROS production by means of dihydroethidium (DHE), which evaluates superoxide ion and 20,70-dichlorofluorescein diacetate (DFCDA), which evaluates oxygen peroxide ($H_2O_2$), as previously described [13, 38, 39]. When the paradigm of pretreatment and insult culminated, the cells were stained with 10 μM DHE (Sigma, St Louis, MO, USA) or 10 μM DFCDA (Sigma, St Louis, MO, USA) for 25 min in the dark in a

37 °C, 5% $CO_2$ humidified incubator. Finally, fluorescence was analyzed using a FLUOstar Omega microplate reader for DHE (excitation 480 nm/ emission 580 nm) (BMG LABTECH, Ortenberg, Germany) and flow cytometry was performed in the Guava EasyCyte cytometer (Millipore, MA, USA) using green channel for DFCDA. Data was expressed as mean fluorescence.

### Morphological Analysis

First, bright-field images for each treatment were acquired randomly using a ×40 objective in a Zeiss inverted microscope (Carl Zeiss Microscopy, LLC, USA). Images were processed with the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda). RGB images were converted to grayscale (8-bit image) and then transformed to binary outline using threshold and dilation method [44, 45]. We carried out a fractal dimension analysis, which was realized using fractal box counting method in ImageJ software, and we used binary images corresponding to the outlines of the cells. Box counting method calculates the Db value, which is determined by the number of boxes required to cover the image. The Db is the slope of the regression line for the log-log plot of box size (A. Karperien, Charles Sturt University, Australia) [46, 47]. Additionally, we evaluated convex hull-derived parameters (circularity, perimeter, and area) by means of hull and circle plugin in ImageJ software (A. Karperien, Charles Sturt University, Australia and T R. Roy, University of Alberta, Canada). Data were acquired from at least three independent cultures with a minimum of 20 cells for each condition.

### Statistical Analysis

In the present study, the GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA) (www.graphpad.com) was used for all analysis. Data were recollected from at least three independent experiments. Data are shown as mean ± SEM. One-way ANOVA test was used to evaluate significant differences between treatments. Post hoc analysis was performed using Tukey's test. A $p$ value < 0.005 was considered significant.

## Results

### Effects of Tibolone and Palmitic Acid on T98G Cell Viability

We first determined the IC50 by which 1 mM palmitic acid was able to reduce cell viability by ~50% (Fig. 1a). We observed a direct correlation of increasing concentrations of palmitic acid and diminished cell viability. This result was confirmed by PI assay, where the percentage of PI uptake for control was 8.1%, while for 250 μM palmitic acid was 9.6% and for 1 mM palmitic acid was 30% (Fig. 1b, $p < 0.0001$). Once the concentration of palmitic acid was determined, we proceeded to evaluate several concentrations of tibolone under conditions of pretreatment for 24 h, according to our previous studies [21, 38]. Our findings indicated that 70 and 50 μM tibolone induced an increase of 16% ($p = 0.0229$) and 18% ($p = 0.0055$) on cell viability, respectively (Fig. 2a). It is important to note that we used 0.2% DMSO as control for tibolone, and no significant alterations on cell viability were observed (Fig. 2a). Moreover, PI uptake was reduced by 44.4% ($p = 0.0003$) and 26.2% ($p = 0.0042$) when cells are treated with 70 and 20 μM tibolone, respectively, in comparison to 1 mM palmitic acid alone (Fig. 2b). Since we observed significant differences in cell viability when palmitic acid-treated cells were exposed to tibolone at 70, 50, and 20 μM, we used these parameters in next experiments.

### Effects of Tibolone and Palmitic Acid on Mitochondrial Parameters

Next, we assessed the actions of tibolone and palmitic acid on mitochondrial membrane potential (Δψm), ROS production, and mitochondrial volume/mass. We observed that palmitic acid reduced by 56.1% ($p = 0.0013$) the mitochondrial membrane potential (Fig. 3a), and that tibolone at 70 μM ($p = 0.002$), 50 μM ($p = 0.0003$), or 20 μM ($p = 0.0138$) attenuated the loss of Δψm in palmitic acid-treated astrocytic cells. Similar results were observed when we evaluated mitochondrial mass using NAO, a dye that represents a measurement of mitochondrial functionality, since it binds cardiolipin only when the inner mitochondrial membrane is not altered [48]. Our results indicated that 1 mM palmitic acid reduced mitochondrial mass in a proportion of 50% ($p = 0.0029$) and tibolone at all concentrations ($p < 0.0001$) preserved NAO fluorescence in the presence of palmitic acid (Fig. 3b).

Since palmitic acid might induce ROS production in astrocytes [33], as well as in other cell types [49], we next measured its effects on hydrogen peroxide and superoxide in the presence or absence of tibolone. No significant alterations on hydrogen peroxide (Fig. 4a) or superoxide levels (Fig. 4b) were observed when cells are treated with different doses of tibolone in the presence of 1 mM palmitic acid.

### Morphological Changes in T98G Cells After Tibolone and Palmitic Acid Treatments

Previous studies have reported that morphological changes in astrocytes are associated with CNS diseases [45]. Here, to quantitatively assess astrocytic cell morphology under different experimental paradigms, we performed fractal analysis of randomly selected individual cells, and assessed fractal dimension,

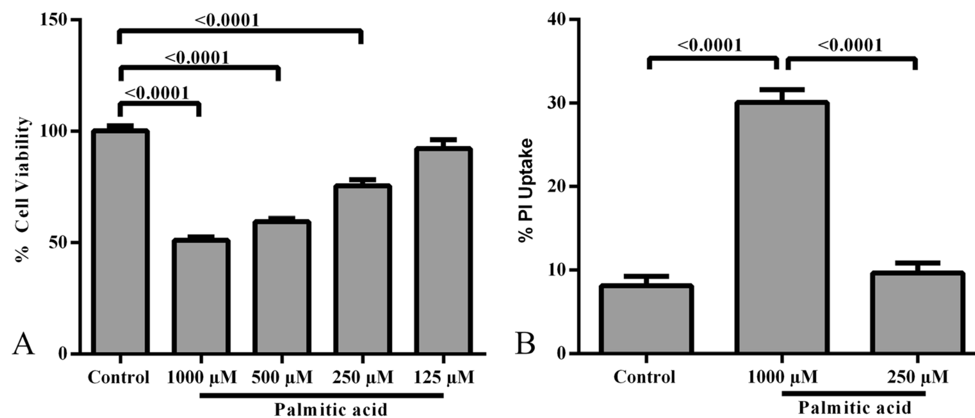


**Fig. 1** Effects of palmitic acid on astrocytic cell viability. **a** MTT assay was performed to evaluate the effect of several concentrations of palmitic acid for 24 h on cell viability. It was observed that 1 mM palmitic acid induced a reduction in cell survival in comparison to other concentrations. **b** This result was later confirmed by assessing PI uptake, where astrocytic cells treated with 1 mM palmitic acid presented increased PI uptake ($p < 0.0001$) in comparison with control and 250 μM palmitic acid. Data show the mean ± SEM of at least three independent experiments. Control, 2.5% BSA and 2 mM carnitine

circularity, convex hull (arbor area), cell area, and perimeter. As shown in Fig. 5a, control cells showed a short and thin morphology, a similar feature to cells that were exposed to both 70 μM tibolone and 1 mM palmitic acid. On the contrary, cells treated with palmitic acid in absence of tibolone presented a round and thick morphology. Fractal analysis revealed that tibolone-treated cells had increased fractal dimension (Fig. 5b, $p = 0.0077$) and smaller circularity (Fig. 5c, $p < 0.0001$). Moreover, tibolone induced a non-significant 23.58% increase in convex hull (Fig. 5d) and 24.2% increase in cell area (Fig. 5e) in comparison to cells treated with palmitic acid alone. However, perimeter area increased by 21.31% (Fig. 5f, $p = 0.0005$) in astrocytic cells under tibolone + palmitic acid treatment when compared to palmitic acid alone.

## Discussion

Astrocytes are important for CNS homeostasis [50], since these cells are involved in pro- and anti-inflammatory mechanisms during a pathological event [5]. For example, it has been found that genes related with inflammation and mitochondrial function are differentially expressed in astrocytes from postmortem tissue of patients with AD [51], as well as for other diseases such as schizophrenia [52], PD, and major depression [53]. For this reason, studies focused on the evaluation of potential therapeutic approaches aimed at attenuating damage in astrocytes have taken great importance. In the present study, we assessed tibolone's protective actions in palmitic acid treated-astrocytes.

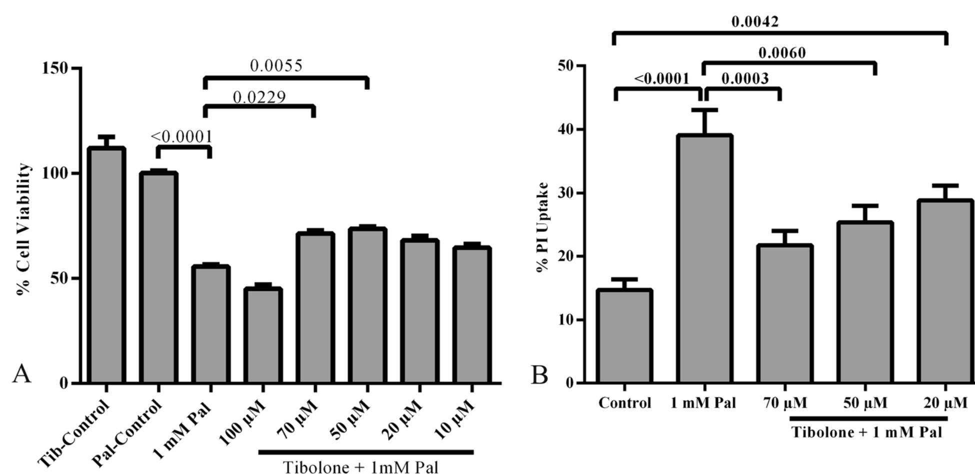


**Fig. 2** Effects of pretreatment with tibolone on the viability of astrocytic cells treated with palmitic acid. **a** Cells were pretreated for 24 h with several concentrations of tibolone and then treated with palmitic acid. We observed that cell viability by MTT assay was increased when astrocytic cells are treated with tibolone at 70, 50, and 20 μM. **b** PI uptake assay confirmed that pretreatment with 70 μM tibolone reduced cell death, and this value is similar to that of control cells. Data show the mean ± SEM of at least three independent experiments. Control of tibolone, 0.2% DMSO. Control of palmitic acid, 2.5% BSA and 2 mM carnitine

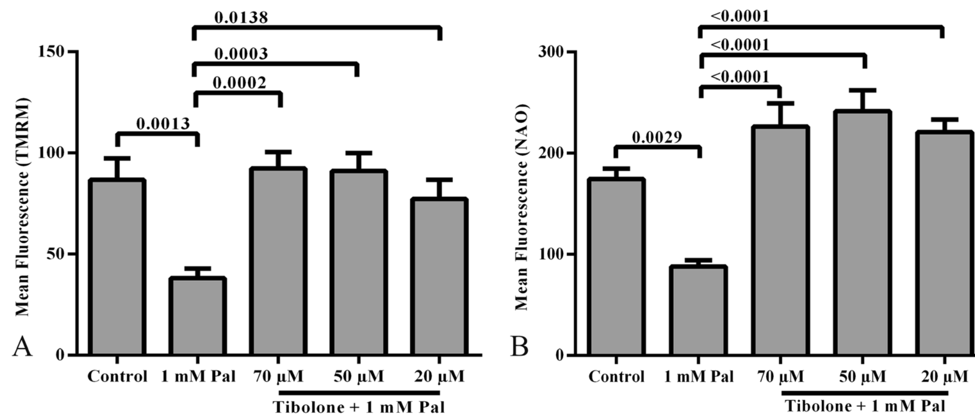


**Fig. 3** Analysis of mitochondrial parameters in astrocytic cells pretreated with tibolone and treated with palmitic acid. Astrocytes were pretreated with tibolone for 24 h and treated with 1 mM palmitic acid for 24 h. **a** Mitochondrial membrane potential (Δψm) was evaluated using TMRM dye by means of flow cytometry. It was observed that tibolone at all concentrations prevented the Δψm collapse induced by 1 mM palmitic acid. **b** Similar results were observed when analyzing mitochondria mass using NAO. Although 1 mM palmitic acid dampened NAO fluorescence, tibolone at 70, 50, and 20 μM prevented the reduction of cardiolipin by palmitic acid ($p < 0.0001$). Data show the mean ± SEM of at least three independent experiments. Control, 2.5% BSA and 2 mM carnitine

Although the protective effects of tibolone in the CNS are well documented (Table 1) [21, 38, 54–60], its role on astrocytic cells stressed with palmitic acid in vitro, a model assembling obesity, and neuroinflammation, was not previously investigated. In this regard, first, we carried out a pretreatment with different concentrations of tibolone, and our findings indicated that this neuroactive steroid attenuated palmitic acid-induced cell death and preserved Δψm and cardiolipin levels altered by the fatty acid. On the other hand, tibolone has shown to exert anti-inflammatory actions [61], and as palmitic acid may induce activation of immune signals in astrocytes [30], with the increasing expression of cytokines TNF, IL-1B, and IL-6 [30, 32, 62], we suggest that the regulation of cytokines could be involved in the protective actions of this steroid. Nevertheless, further analyses are necessary to elucidate the anti-inflammatory effects of tibolone on astrocytes submitted to fatty acids.

Here, we show that palmitic acid reduced astrocytic cell viability, which is in accordance to previous studies [30, 33, 63]. Palmitic acid can reduce cell viability through several pathways. For example, palmitic acid, as other fatty acids, can interact with mitochondrial carriers and lead to reduction of mitochondrial membrane potential and, in turn, the opening of the permeability transition pore (PTP), which generates permeabilization of inner mitochondrial membrane (IMM). Likewise, increased ROS production by β-oxidation process can induce PTP opening [29]. This is noteworthy since we observed that palmitic acid reduced Δψm at 24 h, as shown in several cellular models using palmitic acid [64, 65], including in astrocytes [33]; however, this was not correlated to augmented ROS production. In fact, in our study, palmitic acid did not provoke an increase in the production of superoxide and hydrogen peroxide levels, suggesting that it may induce cell death independently of free radical generation [66]. In absence of ROS production, it is possible that palmitic acid does not enter mitochondria and undergo β-oxidation process; rather, it could increase de novo synthesis of ceramide [63, 67]. It has

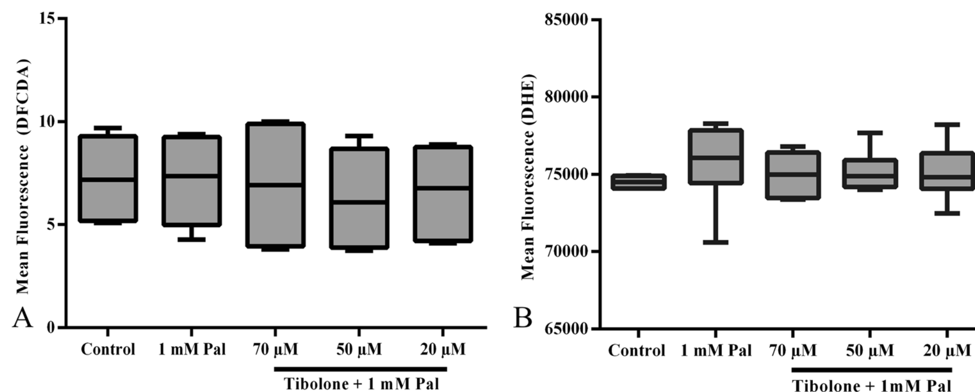


**Fig. 4** Analysis of reactive oxygen species (ROS) production in astrocytic cells pretreated with tibolone and treated with palmitic acid. No significant differences were observed in hydrogen peroxide (**a**) and superoxide levels in cells treated with palmitic acid alone or in the presence of tibolone. Data show the mean ± SEM of at least three independent experiments. Control, 2.5% BSA and 2 mM carnitine

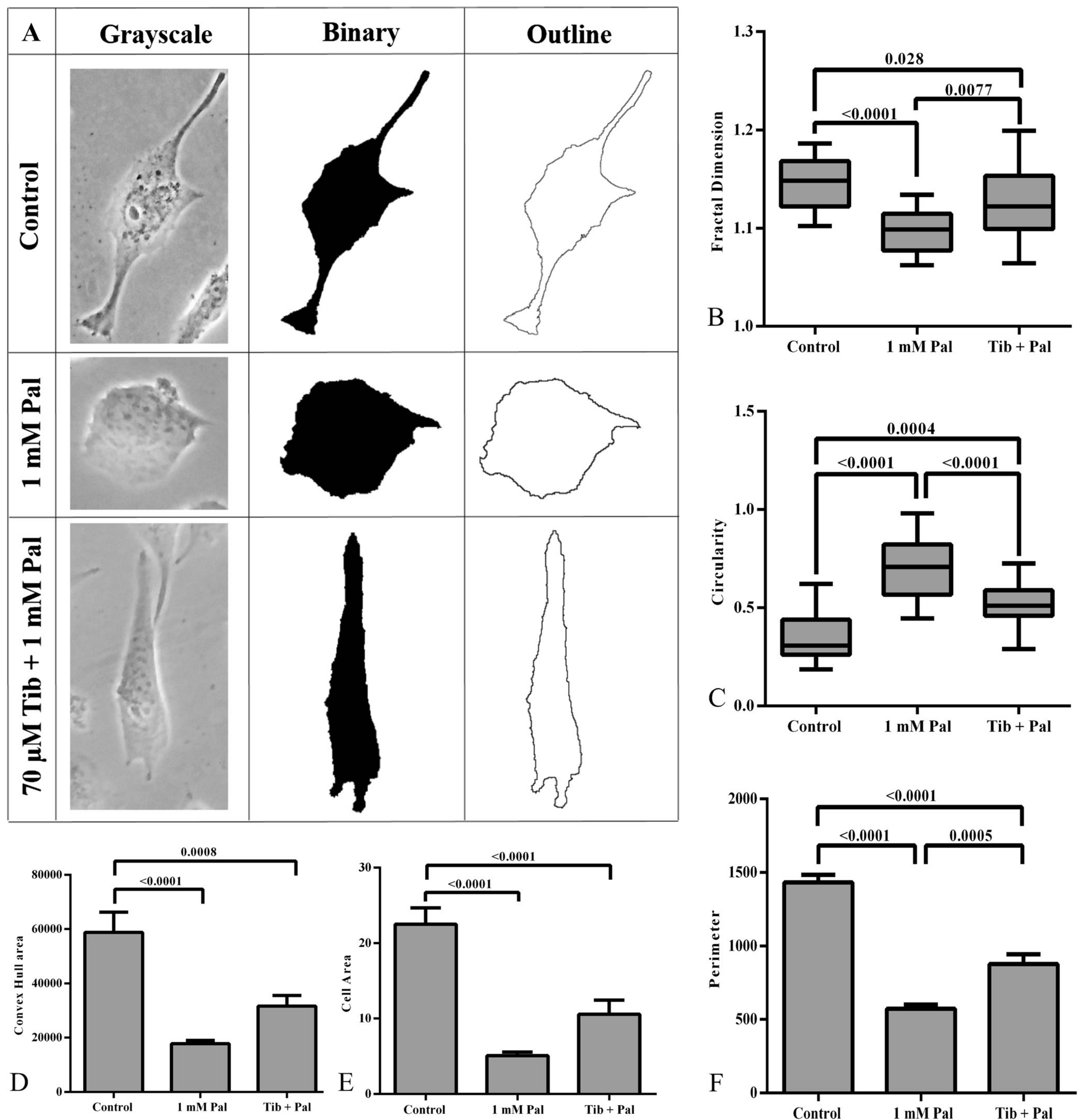


**Fig. 5** Effects of tibolone and palmitic acid on astrocytic cell morphology. **a** Grayscale and binary representations of astrocytic cells exposed to both palmitic acid and tibolone. **b** Fractal dimension analysis showed that cells pretreated with tibolone had a level of complexity higher than those cells exposed to palmitic acid only. **c** Circularity analysis demonstrated that astrocytes treated with palmitic acid were more round in comparison to tibolone + palmitic acid and control cells. Palmitic acid induced a reduction in (**d**) convex hull, (**e**) cell area, and (**f**) perimeter, while tibolone improved cell complexity in all three analyzed parameters. Data are shown of at least three independent experiments. Control, 2.5% BSA and 2 mM carnitine

been demonstrated that prolonged exposition to palmitic acid inhibits AMPK (AMP-activated protein kinase) in several cellular models [68, 69]. This inhibition leads to augmented malonyl-CoA levels, which reduce β-oxidation and enhance fatty acid synthesis [70]. This hypothesis is consistent with previous findings, where the increasing phosphorylation of AMPK can reduce de novo synthesis of ceramides and apoptosis triggered by palmitic acid in astrocytes [67]. Ceramides are associated with apoptosis in astrocytes treated with palmitic acid [63, 67] via release of cytochrome c from mitochondria

[71]. Likewise, our results showed that palmitic acid reduced the level of cardiolipin in mitochondria from astrocytic cells. Cardiolipin is involved in cellular bioenergetics by interacting with several key proteins and complexes in the mitochondria [48]. For example, it has been demonstrated that cardiolipin interacts with cytochrome c, and that when cardiolipin levels are decreased, cytochrome c is released, thus leading to apoptosis [72]. We observed that tibolone prevented mitochondrial alterations induced by palmitic acid. The mechanism of actions of tibolone in this model could involve anti-apoptotic pathways by means of the activation of estrogen receptor [14], since we previously demonstrated that inhibition of ERβ prevented the protective effects of tibolone in T98G cells [21].

Cell death involves several morphological alterations, including cell shrinkage, and reduction of cell and arbor areas [73]. Astrocytic cells have a great morphological diversity, and rapidly respond to injury by altering morphology [74]. Therefore, in addition to the mitochondrial parameters, we also assessed morphological changes in astrocytic cells treated with tibolone and palmitic acid. First, we assessed the fractal dimension, a measure that determines the cellular complexity. To understand this parameter, values of fractal dimension are increased when irregular shape increases, and values decrease as cells become more round [47]. Our results indicated that 70 μM tibolone preserved astrocytic cell complexity since the values of fractal dimension were higher in control and tibolone + palmitic acid-treated cells than in cells treated with palmitic acid alone. In Fig. 5a, it can be noted that cells from control and tibolone + palmitic acid groups are irregular with some cell processes, whereas cells treated with palmitic acid are rounder. Reduction of cellular complexity (rounded cells and reduced cellular processes) can alter gap junctions between astrocytes, and that can reduce interactions with neurons and blood vessels, leading to impairment on cerebral functionality [75]. We assessed several other parameters derived from convex hull analysis. This analysis allowed us to quantify the area that encompasses a cell, which is determined by a polygon formed by connecting distal points of the cell [44, 76]. Area of convex hull was reduced when cells were exposed to palmitic acid and tibolone-improved arbor area in the presence of palmitic acid, suggesting that tibolone preserves astrocytic cell size. Additionally, another morphological parameter analyzed was circularity, which determines how round is a cell (values close to 1) or how increasingly elongated the polygon is (value close to 0) (A. Karperien, Charles Sturt University, Australia and T R. Roy, University of Alberta, Canada). It was found that astrocytic cells treated with palmitic acid had a higher score on circularity than tibolone + palmitic acid-treated cells. As observed in Fig. 5a, astrocytic cells treated with palmitic acid are rounder, which is reminiscent of apoptotic-like aspect [73]. The actions of tibolone on astrocytic morphology are similar to that shown in a previous study, in which this steroid preserved astrocytic cell area under glucose withdrawal [38]. These morphological changes induced by palmitic acid and tibolone may rely on the modulation of different astrocytic cytoskeleton proteins such as GFAP, vimentin, and nestin [3, 77, 78].

In conclusion, here, we have shown that tibolone is protective against cell damage induced by palmitic acid. Further studies are necessary to explore the mechanisms involved in tibolone's actions, and whether these effects are dependent on the activation of different estrogen receptors.

**Acknowledgments** YG-G is supported by a PhD fellowship from Centro de Estudios Interdisciplinarios Básicos y Aplicados CEIBA (Rodolfo Llinás Program). We acknowledge support from Ministerio de Economía y Competitividad (MINECO), Spain (grant no. BFU2014-51836-C2-1-R), CIBERFES, and Fondos FEDER.

**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

1. Zeng XN, Sun XL, Gao L, Fan Y, Ding JH, Hu G (2007) Aquaporin-4 deficiency down-regulates glutamate uptake and GLT-1 expression in astrocytes. Mol Cell Neurosci 34(1):34–39. doi:10.1016/j.mcn.2006.09.008
2. Maragakis NJ, Rothstein JD (2006) Mechanisms of disease: astrocytes in neurodegenerative disease. Nat Clin Pract Neurol 2(12): 679–689. doi:10.1038/ncpneuro0355
3. Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. Acta Neuropathol 119(1):7–35. doi:10.1007/s00401-009-0619-8
4. Perea G, Sur M, Araque A (2014) Neuron-glia networks: integral gear of brain function. Front Cell Neurosci 8:378. doi:10.3389/fncel.2014.00378
5. Colombo E, Farina C (2016) Astrocytes: key regulators of neuroinflammation. Trends Immunol 37(9):608–620. doi:10.1016/j.it.2016.06.006
6. Amor S, Puentes F, Baker D, van der Valk P (2010) Inflammation in neurodegenerative diseases. Immunology 129(2):154–169. doi:10.1111/j.1365-2567.2009.03225.x
7. Saetre P, Emilsson L, Axelsson E, Kreuger J, Lindholm E, Jazin E (2007) Inflammation-related genes up-regulated in schizophrenia brains. BMC Psychiatry 7:46. doi:10.1186/1471-244X-7-46
8. Miller AH, Maletic V, Raison CL (2009) Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 65(9):732–741. doi:10.1016/j.biopsych.2008.11.029
9. Belot N, Rorive S, Doyen I, Lefranc F, Bruyneel E, Dedecker R, Micik S, Brotchi J et al (2001) Molecular characterization of cell substratum attachments in human glial tumors relates to prognostic features. Glia 36(3):375–390
10. Mao X, Moerman-Herzog AM, Wang W, Barger SW (2006) Differential transcriptional control of the superoxide dismutase-2 kappaB element in neurons and astrocytes. J Biol Chem 281(47): 35863–35872. doi:10.1074/jbc.M604166200
11. Gasque P, Chan P, Mauger C, Schouft MT, Singhrao S, Dierich MP, Morgan BP, Fontaine M (1996) Identification and characterization of complement C3 receptors on human astrocytes. J Immunol 156(6):2247–2255

12. Landolfi C, Soldo L, Polenzani L, Apicella C, Capezzone de Joannon A, Coletta I, Di Cesare F, Brufani M et al (1998) Inflammatory molecule release by beta-amyloid-treated T98G astrocytoma cells: role of prostaglandins and modulation by paracetamol. Eur J Pharmacol 360(1):55–64
13. Baez-Jurado E, Vega GG, Aliev G, Tarasov VV, Esquinas P, Echeverria V, Barreto GE (2017) Blockade of neuroglobin reduces protection of conditioned medium from human mesenchymal stem cells in human astrocyte model (T98G) under a scratch assay. Mol Neurobiol. doi:10.1007/s12035-017-0481-y
14. Arevalo MA, Azcoitia I, Garcia-Segura LM (2015) The neuroprotective actions of oestradiol and oestrogen receptors. Nat Rev Neurosci 16(1):17–29. doi:10.1038/nrn3856
15. Acaz-Fonseca E, Avila-Rodriguez M, Garcia-Segura LM, Barreto GE (2016) Regulation of astroglia by gonadal steroid hormones under physiological and pathological conditions. Prog Neurobiol 144:5–26. doi:10.1016/j.pneurobio.2016.06.002
16. Kloosterboer HJ (2001) Tibolone: a steroid with a tissue-specific mode of action. J Steroid Biochem Mol Biol 76(1–5):231–238
17. Kloosterboer HJ (2004) Tissue-selectivity: the mechanism of action of tibolone. Maturitas 48(Suppl 1):S30–S40. doi:10.1016/j.maturitas.2004.02.012
18. de Gooyer ME, Kleyn GT, Smits KC, Ederveen AG, Verheul HA, Kloosterboer HJ (2001) Tibolone: a compound with tissue specific inhibitory effects on sulfatase. Mol Cell Endocrinol 183(1–2):55–62
19. Guzman CB, Zhao C, Deighton-Collins S, Kleerekoper M, Benjamins JA, Skafar DF (2007) Agonist activity of the 3-hydroxy metabolites of tibolone through the oestrogen receptor in the mouse N20.1 oligodendrocyte cell line and normal human astrocytes. J Neuroendocrinol 19(12):958–965. doi:10.1111/j.1365-2826.2007.01611.x
20. Acaz-Fonseca E, Sanchez-Gonzalez R, Azcoitia I, Arevalo MA, Garcia-Segura LM (2014) Role of astrocytes in the neuroprotective actions of 17beta-estradiol and selective estrogen receptor modulators. Mol Cell Endocrinol 389(1–2):48–57. doi:10.1016/j.mce.2014.01.009
21. Avila-Rodriguez M, Garcia-Segura LM, Hidalgo-Lanussa O, Baez E, Gonzalez J, Barreto GE (2016) Tibolone protects astrocytic cells from glucose deprivation through a mechanism involving estrogen receptor beta and the upregulation of neuroglobin expression. Mol Cell Endocrinol 433:35–46. doi:10.1016/j.mce.2016.05.024
22. Nguyen JC, Killcross AS, Jenkins TA (2014) Obesity and cognitive decline: role of inflammation and vascular changes. Front Neurosci 8:375. doi:10.3389/fnins.2014.00375
23. Martin-Jimenez CA, Gaitan-Vaca DM, Echeverria V, Gonzalez J, Barreto GE (2016) Relationship between obesity, Alzheimer's disease, and Parkinson's disease: an astrocentric view. Mol Neurobiol. doi:10.1007/s12035-016-0193-8
24. Jayaraman A, Lent-Schochet D, Pike CJ (2014) Diet-induced obesity and low testosterone increase neuroinflammation and impair neural function. J Neuroinflammation 11:162. doi:10.1186/s12974-014-0162-y
25. Buckman LB, Hasty AH, Flaherty DK, Buckman CT, Thompson MM, Matlock BK, Weller K, Ellacott KL (2014) Obesity induced by a high-fat diet is associated with increased immune cell entry into the central nervous system. Brain Behav Immun 35:33–42. doi:10.1016/j.bbi.2013.06.007
26. Boden G (2008) Obesity and free fatty acids. Endocrinol Metab Clin N Am 37(3):635–646, **viii-ix**. doi:10.1016/j.ecl.2008.06.007
27. Miller AA, Spencer SJ (2014) Obesity and neuroinflammation: a pathway to cognitive impairment. Brain Behav Immun 42:10–21. doi:10.1016/j.bbi.2014.04.001
28. Karmi A, Iozzo P, Viljanen A, Hirvonen J, Fielding BA, Virtanen K, Oikonen V, Kemppainen J et al (2010) Increased brain fatty acid uptake in metabolic syndrome. Diabetes 59(9):2171–2177. doi:10.2337/db09-0138
29. Gonzalez-Barroso MM, Rial E (2009) The role of fatty acids in the activity of the uncoupling proteins. Curr Chem Biol 3(2):180–188. doi:10.2174/187231309788166451
30. Gupta S, Knight AG, Gupta S, Keller JN, Bruce-Keller AJ (2012) Saturated long-chain fatty acids activate inflammatory signaling in astrocytes. J Neurochem 120(6):1060–1071. doi:10.1111/j.1471-4159.2012.07660.x
31. Kwon B, Lee HK, Querfurth HW (2014) Oleate prevents palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory signaling in neuronal cells. Biochim Biophys Acta 1843(7):1402–1413. doi:10.1016/j.bbamcr.2014.04.004
32. Liu L, Chan C (2014) IPAF inflammasome is involved in interleukin-1beta production from astrocytes, induced by palmitate; implications for Alzheimer's disease. Neurobiol Aging 35(2):309–321. doi:10.1016/j.neurobiolaging.2013.08.016
33. Wong KL, Wu YR, Cheng KS, Chan P, Cheung CW, Lu DY, Su TH, Liu ZM et al (2014) Palmitic acid-induced lipotoxicity and protection by (+)-catechin in rat cortical astrocytes. Pharmacol Rep 66(6):1106–1113. doi:10.1016/j.pharep.2014.07.009
34. Geekiyanage H, Chan C (2011) MicroRNA-137/181c regulates serine palmitoyltransferase and in turn amyloid beta, novel targets in sporadic Alzheimer's disease. J Neurosci Off J Soc Neurosci 31(41):14820–14830. doi:10.1523/JNEUROSCI.3883-11.2011
35. Fraser T, Tayler H, Love S (2010) Fatty acid composition of frontal, temporal and parietal neocortex in the normal human brain and in Alzheimer's disease. Neurochem Res 35(3):503–513. doi:10.1007/s11064-009-0087-5
36. Lutz TA, Woods SC (2012) Overview of animal models of obesity. Curr Protoc Pharmacol 58:5.61.1–5.61.18. doi:10.1002/0471141755.ph0561s58
37. Yue G, Shi G, Azaro MA, Yang Q, Hu G, Luo M, Yin K, Nagele RG et al (2008) Lipopolysaccharide (LPS) potentiates hydrogen peroxide toxicity in T98G astrocytoma cells by suppression of anti-oxidative and growth factor gene expression. BMC Genomics 9:608. doi:10.1186/1471-2164-9-608
38. Avila Rodriguez M, Garcia-Segura LM, Cabezas R, Torrente D, Capani F, Gonzalez J, Barreto GE (2014) Tibolone protects T98G cells from glucose deprivation. J Steroid Biochem Mol Biol 144 Pt B:294–303. doi:10.1016/j.jsbmb.2014.07.009
39. Cabezas R, Avila MF, Gonzalez J, El-Bacha RS, Barreto GE (2015) PDGF-BB protects mitochondria from rotenone in T98G cells. Neurotox Res 27(4):355–367. doi:10.1007/s12640-014-9509-5
40. Toro-Urrego N, Garcia-Segura LM, Echeverria V, Barreto GE (2016) Testosterone protects mitochondrial function and regulates neuroglobin expression in astrocytic cells exposed to glucose deprivation. Front Aging Neurosci 8:152. doi:10.3389/fnagi.2016.00152
41. Kong JY, Rabkin SW (2002) Palmitate-induced cardiac apoptosis is mediated through CPT-1 but not influenced by glucose and insulin. Am J Phys Heart Circ Phys 282(2):H717–H725. doi:10.1152/ajpheart.00257.2001
42. Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA (2011) Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. BioTechniques 50(2):98–115. doi:10.2144/000113610
43. Cottet-Rousselle C, Ronot X, Leverve X, Mayol JF (2011) Cytometric assessment of mitochondria using fluorescent probes. Cytometry A J Int Soc Anal Cytol 79(6):405–425. doi:10.1002/cyto.a.21061
44. Barreto GE, White RE, Xu L, Palm CJ, Giffard RG (2012) Effects of heat shock protein 72 (Hsp72) on evolution of astrocyte activation following stroke in the mouse. Exp Neurol 238(2):284–296. doi:10.1016/j.expneurol.2012.08.015
45. Pirici D, Mogoanta L, Margaritescu O, Pirici I, Tudorica V, Coconu M (2009) Fractal analysis of astrocytes in stroke and dementia. Romanian J Morphol Embryol Rev Roum Morphol Embryol 50(3):381–390

46. Karperien AL, Jelinek HF (2015) Fractal, multifractal, and lacunarity analysis of microglia in tissue engineering. Front Bioeng Biotechnol 3(51). doi:10.3389/fbioe.2015.00051
47. Fernandez E, Jelinek HF (2001) Use of fractal theory in neuroscience: methods, advantages, and potential problems. Methods 24(4):309–321. doi:10.1006/meth.2001.1201
48. Paradies G, Paradies V, De Benedictis V, Ruggiero FM, Petrosillo G (2014) Functional role of cardiolipin in mitochondrial bioenergetics. Biochim Biophys Acta 1837(4):408–417. doi:10.1016/j.bbabio.2013.10.006
49. Schonfeld P, Wojtczak L (2008) Fatty acids as modulators of the cellular production of reactive oxygen species. Free Radic Biol Med 45(3):231–241. doi:10.1016/j.freeradbiomed.2008.04.029
50. Hamby ME, Sofroniew MV (2010) Reactive astrocytes as therapeutic targets for CNS disorders. Neurother J Am Soc Exp Neurother 7(4):494–506. doi:10.1016/j.nurt.2010.07.003
51. Sekar S, McDonald J, Cuyugan L, Aldrich J, Kurdoglu A, Adkins J, Serrano G, Beach TG et al (2015) Alzheimer's disease is associated with altered expression of genes involved in immune response and mitochondrial processes in astrocytes. Neurobiol Aging 36(2):583–591. doi:10.1016/j.neurobiolaging.2014.09.027
52. Catts VS, Wong J, Fillman SG, Fung SJ, Shannon Weickert C (2014) Increased expression of astrocyte markers in schizophrenia: association with neuroinflammation. Aust N Z J Psychiatry 48(8):722–734. doi:10.1177/0004867414531078
53. Santiago JA, Littlefield AM, Potashkin JA (2016) Integrative transcriptomic meta-analysis of Parkinson's disease and depression identifies NAMPT as a potential blood biomarker for de novo Parkinson's disease. Sci Rep 6:34579. doi:10.1038/srep34579
54. Gibbs RB, Edwards D, Lazar N, Nelson D, Talameh J (2006) Effects of long-term hormone treatment and of tibolone on monoamines and monoamine metabolites in the brains of ovariectomised, cynomologous monkeys. J Neuroendocrinol 18(9):643–654. doi:10.1111/j.1365-2826.2006.01463.x
55. Qiu J, Bosch MA, Ronnekleiv OK, Kloosterboer HJ, Kelly MJ (2008) Tibolone rapidly attenuates the GABAB response in hypothalamic neurones. J Neuroendocrinol 20(12):1310–1318. doi:10.1111/j.1365-2826.2008.01789.x
56. de Aguiar RB, Dickel OE, Cunha RW, Monserrat JM, Barros DM, Martinez PE (2008) Estradiol valerate and tibolone: effects upon brain oxidative stress and blood biochemistry during aging in female rats. Biogerontology 9(5):285–298. doi:10.1007/s10522-008-9137-7
57. Belenichev IF, Odnokoz OV, Pavlov SV, Belenicheva OI, Polyakova EN (2012) The neuroprotective activity of tamoxifen and tibolone during glutathione depletion in vitro. Neurochem J 6(3):202–212. doi:10.1134/s181971241203004x
58. Pinto-Almazan R, Rivas-Arancibia S, Farfan-Garcia ED, Rodriguez-Martinez E, Guerra-Araiza C (2014) Neuroprotective effects of tibolone against oxidative stress induced by ozone exposure. Rev Neurol 58(10):441–448
59. Farfan-Garcia ED, Castillo-Hernandez MC, Pinto-Almazan R, Rivas-Arancibia S, Gallardo JM, Guerra-Araiza C (2014) Tibolone prevents oxidation and ameliorates cholinergic deficit induced by ozone exposure in the male rat hippocampus. Neurochem Res 39(9):1776–1786. doi:10.1007/s11064-014-1385-0
60. Beltran-Campos V, Diaz-Ruiz A, Padilla-Gomez E, Aguilar Zavala H, Rios C, Diaz Cintra S (2015) Effect of tibolone on dendritic spine density in the rat hippocampus. Neurologia 30(7):401–406. doi:10.1016/j.nrl.2014.03.002
61. de Medeiros AR, Lamas AZ, Caliman IF, Dalpiaz PL, Firmes LB, de Abreu GR, Moyses MR, Lemos EM et al (2012) Tibolone has anti-inflammatory effects in estrogen-deficient female rats on the natriuretic peptide system and TNF-alpha. Regul Pept 179(1–3):55–60. doi:10.1016/j.regpep.2012.08.015
62. Wang Z, Liu D, Wang J, Liu S, Gao M, Ling EA, Hao A (2012) Cytoprotective effects of melatonin on astroglial cells subjected to palmitic acid treatment in vitro. J Pineal Res 52(2):253–264. doi:10.1111/j.1600-079X.2011.00952.x
63. Patil S, Melrose J, Chan C (2007) Involvement of astroglial ceramide in palmitic acid-induced Alzheimer-like changes in primary neurons. Eur J Neurosci 26(8):2131–2141. doi:10.1111/j.1460-9568.2007.05797.x
64. Joseph JW, Koshkin V, Saleh MC, Sivitz WI, Zhang CY, Lowell BB, Chan CB, Wheeler MB (2004) Free fatty acid-induced beta-cell defects are dependent on uncoupling protein 2 expression. J Biol Chem 279(49):51049–51056. doi:10.1074/jbc.M409189200
65. Fauconnier J, Andersson DC, Zhang SJ, Lanner JT, Wibom R, Katz A, Bruton JD, Westerblad H (2007) Effects of palmitate on Ca(2+) handling in adult control and ob/ob cardiomyocytes: impact of mitochondrial reactive oxygen species. Diabetes 56(4):1136–1142. doi:10.2337/db06-0739
66. Hickson-Bick DL, Sparagna GC, Buja LM, McMillin JB (2002) Palmitate-induced apoptosis in neonatal cardiomyocytes is not dependent on the generation of ROS. Am J Phys Heart Circ Phys 282(2):H656–H664. doi:10.1152/ajpheart.00726.2001
67. Blazquez C, Geelen MJ, Velasco G, Guzman M (2001) The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes. FEBS Lett 489(2–3):149–153
68. Sun Y, Ren M, Gao GQ, Gong B, Xin W, Guo H, Zhang XJ, Gao L et al (2008) Chronic palmitate exposure inhibits AMPKalpha and decreases glucose-stimulated insulin secretion from beta-cells: modulation by fenofibrate. Acta Pharmacol Sin 29(4):443–450. doi:10.1111/j.1745-7254.2008.00717.x
69. Lee CH, Lee SD, Ou HC, Lai SC, Cheng YJ (2014) Eicosapentaenoic acid protects against palmitic acid-induced endothelial dysfunction via activation of the AMPK/eNOS pathway. Int J Mol Sci 15(6):10334–10349. doi:10.3390/ijms150610334
70. Foster DW (2012) Malonyl-CoA: the regulator of fatty acid synthesis and oxidation. J Clin Invest 122(6):1958–1959
71. Ghafourifar P, Klein SD, Schucht O, Schenk U, Pruschy M, Rocha S, Richter C (1999) Ceramide induces cytochrome c release from isolated mitochondria: importance of mitochondrial redox state. J Biol Chem 274(10):6080–6084
72. Choi SY, Gonzalvez F, Jenkins GM, Slomianny C, Chretien D, Arnoult D, Petit PX, Frohman MA (2007) Cardiolipin deficiency releases cytochrome c from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. Cell Death Differ 14(3):597–606. doi:10.1038/sj.cdd.4402020
73. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35(4):495–516. doi:10.1080/01926230701320337
74. Sun D, Jakobs TC (2012) Structural remodeling of astrocytes in the injured CNS. Neuroscientist Rev J Bringing Neurobiol Neurol Psychiatr 18(6):567–588. doi:10.1177/1073858411423441
75. Lee CY, Dallerac G, Ezan P, Anderova M, Rouach N (2016) Glucose tightly controls morphological and functional properties of astrocytes. Front Aging Neurosci 8:82. doi:10.3389/fnagi.2016.00082
76. Kongsui R, Beynon SB, Johnson SJ, Walker FR (2014) Quantitative assessment of microglial morphology and density reveals remarkable consistency in the distribution and morphology of cells within the healthy prefrontal cortex of the rat. J Neuroinflammation 11:182. doi:10.1186/s12974-014-0182-7
77. Swagell CD, Henly DC, Morris CP (2005) Expression analysis of a human hepatic cell line in response to palmitate. Biochem Biophys Res Commun 328(2):432–441. doi:10.1016/j.bbrc.2004.12.188
78. Torres-Aleman I, Rejas MT, Pons S, Garcia-Segura LM (1992) Estradiol promotes cell shape changes and glial fibrillary acidic protein redistribution in hypothalamic astrocytes in vitro: a neuronal-mediated effect. Glia 6(3):180–187. doi:10.1002/glia.440060305