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Allopregnanolone and its analog BR 297 rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement

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Abstract

Allopregnanolone (AP) is known for its beneficial actions such as anxiolytic, antidepressant, neurogenic, analgesic and neuroprotective effects. However, the usefulness of AP could also be limited by its rapid clearance after sulfation or glucuronidation of its 3-hydroxyl group. Thus, we synthesized a set of synthetic analogs (ANS) of AP and we investigated their ability to: i) counteract the bioenergetic deficits observed in SH-SY5Y cells stably transfected with wild-type human APP (APP cells); ii) to protect the cells against oxidative stress-induced cell death. Especially, we examined whether ANS, were more efficient than AP to reduce bioenergetic deficits leading to a neuronal death and to act with a specific action in neuroprotection.

Our results showed that BR 297 exhibits notable advantages over AP with regards to both protection of mitochondrial function and reduction of oxidative stress. Under physiological conditions, BR 297 had no effect on cell survival but it ameliorated the bioenergetics by increasing the cellular ATP level and mitochondrial respiration. BR 297 has no effect on the proliferation but seem to block the cell death mechanism, improving the cell viability. Indeed, a pre-treatment with BR 297 increases the survival of CTRL and APP cells under stress conditions, protecting the cells against H₂O₂-induced cell death by decreasing the ROS level and improving the mitochondrial respiration.

Our findings lend further support to neuroprotective effects of BR 297 emphasizing this drug as promising therapeutic tool to counteract age- and AD-related bioenergetics deficits.

Keywords: Mitochondria; Allopregnanolone; Neuroprotection; Bioenergetics; Alzheimer's disease; Oxidative stress.

Highlights

AP analog BR 297 is more effective than AP to protect mitochondrial function

BR297 significantly reduces oxidative stress.

BR297 improves the bioenergetics and mitochondrial respiration.

BR 297 has no proliferative effect but blocks cell death mechanism.

BR297 improves cell viability

Introduction

Alzheimer's disease (AD) is characterized by a progressive decline of cognitive functions such as memory loss as well as changes in behavior and personality (Maurer, Volk et al. 1997). This age-related neurodegenerative disease, representing more than 60% of all dementia cases, is marked by the presence of two main histopathological signs: intracellular neurofibrillary tangles (NFTs) composed by the aggregation of abnormally hyperphosphorylated tau protein and extracellular amyloid- β ($A\beta$) plaques in the brain (Berchtold and Cotman 1998).

It is increasingly obvious that mitochondrial abnormalities represent a pathophysiological mechanism of AD, since an increase in oxidative damages and decreased energy metabolism are already observed at early stages of the disease, even before the appearance of NFTs and $A\beta$ plaques (Knott, Perkins et al. 2008, Rhein, Song et al. 2009, Yao, Irwin et al. 2009, Muller, Eckert et al. 2010, Leuner, Muller et al. 2012, Schmitt, Grimm et al. 2012) Dysfunctional mitochondria are less efficient producers of adenosine triphosphate (ATP), the universal energy fuel in cells, and generate more reactive oxygen species (ROS), which represent a major source of oxidative imbalance in AD (Wang, Wang et al. 2014). Reduced energy metabolism in affected brain regions of AD patients, as well as recent imaging studies suggest, that the brain energy deficits precede the cognitive symptoms of the disease (Mosconi, De Santi et al. 2008, Wang, Wang et al. 2014).

The brain, despite its small size (about 2% of the body weight), has very high energy requirements and consumes about 20% of body's total basal oxygen to fulfil its function (Raichle and Gusnard 2002, Shulman, Rothman et al. 2004). Besides, the brain is considered to be extremely sensitive to oxidative damages (Clark, Lee et al. 2010). This context, mitochondria play a role extremely important in the nervous system because they are involved in the regulation of intracellular calcium homeostasis, synaptic plasticity, neurotransmitter synthesis as well as cell survival and death (Scheffler 2001, Adam-Vizi and Chinopoulos 2006, Mattson, Gleichmann et al. 2008). They are also paradoxical organelles,

since they produce the energy necessary for the cell survival via ATP generation through the oxidative phosphorylation (OXPHOS) and, at the same time, they are the main source of reactive oxygen species (ROS) that may become harmful for cells when produced in excess, and lead to pathological conditions (Korshunov, Skulachev et al. 1997, Adam-Vizi and Chinopoulos 2006).

“Neurosteroids” are describing as steroids that are synthesized within the nervous system and are still present after removal peripheral endocrine glands (Corpechot, Robel et al. 1981, Mensah-Nyagan, Do-Rego et al. 1999, Patte-Mensah, Kibaly et al. 2006). Neurosteroids are involved in plenty of brain-specific functions and a growing body of evidence attests that they also possess interesting neuroprotective properties (Melcangi, Garcia-Segura et al. 2008, Grimm, Lim et al. 2012, Porcu, Barron et al. 2016). The ability to boost mitochondrial bioenergetics seems to be a common mechanism of different steroids (Grimm, Schmitt et al. 2014). Indeed, we recently showed that neurosteroids improved cellular bioenergetics by increasing mitochondrial respiration and ATP generation, as well as regulated redox homeostasis in neuronal cells (Grimm, Schmitt et al. 2014, Grimm, Biliouris et al. 2016). In addition, mitochondrial deficits induced by A β or abnormal tau protein were reduced after treatment with a selection of neurosteroids, namely progesterone, estradiol and testosterone (Grimm, Biliouris et al. 2016).

The modulation of the biosynthesis of neurosteroids seems to play a major role in the pathophysiology of neurodegenerative disease. *In vitro* and *in vivo* studies showed that during aging, and even more in AD, the synthesis of neurosteroids diminished, paralleled with a loss of nervous functions (Frye and Walf 2008, Caruso, Barron et al. 2013). Interestingly, previous data from our groups provided first evidence that the presence of A β and abnormal tau protein have a direct impact on neurosteroidogenesis in *in vitro* models of AD (Schaeffer, Patte-Mensah et al. 2006, Schaeffer, Meyer et al. 2008).

The natural neurosteroid allopregnanolone (5 α -pregnane-3 α -ol-20-one or AP) seems to have an important impact on the central nervous system (CNS) activity by regulating both growth and survival of neurons and glial cells (Wang, Johnston et al. 2005, Melcangi, Garcia-

Segura et al. 2008). AP is synthesized from progesterone by the enzyme 5 α -reductase and then 3 α -hydroxysteroid oxidoreductase, with the 5 α -dihydroprogesterone (5 α -DHP) as intermediate compound (Patte-Mensah, Kibaly et al. 2005). AP is a positive allosteric modulator of major inhibitory receptor in the brain, the γ -aminobutyric acid type A (GABA_A) receptor but can act also via other receptors as pregnane X receptor (PXR), liver X receptor (LXR) or progesterone (PR) receptors (Majewska, Harrison et al. 1986, Carver and Reddy 2013, Frye, Koonce et al. 2014). The content of AP was markedly diminished with increasing age in the brain cortex of rats, which may have an impact on memory, anxiety and sexual behavior in old animals (Bernardi, Salvestroni et al. 1998). In a transgenic mouse model of AD (APP^{swe}+PSEN1 Δ 9 mice), animals displayed a decreased ability to form AP in the hippocampus compared to their wildtype littermates (Frye and Walf 2008). A treatment with progesterone, the precursor of AP, increased AP concentration and improved cognitive performances in treated animals. In addition, *in vivo* studies conducted on 3xTgAD mice, a triple transgenic AD mice, revealed that AP reduced A β generation in hippocampus, cortex and amygdala, increased proliferation of neuronal progenitor cells and reversed neurogenic and cognitive deficits in transgenic animals (Wang, Liu et al. 2008, Wang, Singh et al. 2010, Chen, Wang et al. 2011, Singh, Liu et al. 2012).

Together, these findings show that AP presents very interesting regenerative properties in AD, and pre-clinical studies were recently conducted in order to use this drug candidate in human therapy (Irwin, Solinsky et al. 2015).

However, despite this growing body of evidence attesting the neuroprotective actions of AP, nothing is known about its effects on AD-induced mitochondrial deficits. Thus, in the present study, we assessed whether AP can protect SH-SY5Y neuroblastoma cells from oxidative stress-induced cell death through bioenergetic modulations. Of note, AP can be converted back to 5 α -DHP by NAD⁺-dependent membrane-associated short-chain dehydrogenases/ reductases via its 3 α -hydroxyl group. This reversion can induce undesirable side effects by the binding of 5 α -DHP to classical intracellular steroid receptors, such as progesterone receptor, activating gene transcription (Reddy 2010, Schumacher,

Mattern et al. 2014). Thus, we synthesized a set of synthetic analogs (ANS) of AP (see Figure 1 and the corresponding caption) and we investigated the ability of AP and the ANS to: i) counteract the bioenergetic deficits observed in SH-SY5Y cells stably transfected with wild-type human APP (APP cells) compared to the cells transfected with the empty vector (CTRL cells); ii) to protect the cells against oxidative stress- induced cells death. More particularly, we examined whether ANS, according to their various chemical structures, were more efficient than AP to reduce bioenergetic deficits leading to a neuronal death and to act with a specific action in neuroprotection. To do so, we investigated the effects of AP and ANS on ATP production, oxygen consumption rate (OCR), glycolysis (ECAR), mitochondrial respiration through the respiratory control ratio (RCR), ROS generation and cell survival.

Results

AP and its analogs ANS modulate bioenergetics in APP/A β model

To investigate the effects of AP and its four analogs (Figure 1) on cellular bioenergetics, ATP level was measured in control vector-pCEP4-transfected (CTRL) or amyloid-precursor-protein (APP) transfected human neuroblastoma cells (SH-SY5Y) after 24 h of treatment.

Preliminary data were obtained after the treatment of CTRL cells with AP or the different analogs (BR 053, BR 297, BR 338 and BR 351) in a broad range of concentrations, from 0 to 1000 nM, for 24 h (Supplementary Figure 1). Results showed that AP and ANS increased the ATP levels, and that the concentration of 500 nM was the optimal treatment condition. Thus, we choose to continue our screening using a concentration of 500 nM of AP and ANS.

In CTRL cells, only AP and BR297 were able to significantly increase ATP level (+10% increase for both molecules)(Figure 2A). In line with our previous study (Grimm, Biliouris et al. 2016), we report hereabout 10% of decrease in ATP content in APP cells compared to CTRL cells under physiological condition (Figure 2B). AP and three of its analogues, BR 053, BR 297 and BR 338, significantly ameliorated the ATP level in APP transfected cells, from 6% of increase with BR 338 and up to 15% of increase with AP (Figure 2B). Thus, after treatment of APP cells, the concentration of ATP was similar to those measured in CTRL cells.

ATP molecules are synthesized via two main pathways: the oxidative phosphorylation (OXPHOS) taking place in mitochondria, and the cellular glycolysis. We evaluated the efficiency of AP and ANS to modulate one of both pathways. The oxygen consumption rate (OCR), an indicator of basal respiration and the extracellular acidification rate (ECAR), an indicator of glycolysis were simultaneously monitored in real-time using Seahorse Bioscience XF24 Analyser (Figure 3). In CTRL cells, only a slight increase of the OCR was observed

after treatment with AP and BR 297 (Figure 3A). However, BR 053, BR 297 and BR351 significantly increase the ECAR, ranging from 39% of increase with BR 053 up to 70% of increase with BR351 (Figure 3B). The bioenergetics phenotype of the CTRL cells (Figure 3C), representing OCR versus ECAR under the different treatment conditions, revealed that BR 297 was particularly efficient to increase both parameters, switching the cells to a metabolically more active state.

Since APP cells present a drastic decrease of OCR and ECAR compared to CTRL cells (Supplementary Figure 2), we then tested the effects of AP and its analogs on this AD cellular model (Figure 3D-3F). AP and BR 297 were the two compounds that increased significantly the OCR (+15% and 10% respectively) compared to the untreated group (Figure 3D). In APP cells, the ECAR was significantly improved by BR 053 (+15%) and BR 297 (+19%), but diminished after treatment with BR 351 (-20%) (Figure 3E), which is the opposite effect than that observed in CTRL cells (Figure 3A). Again, the bioenergetics phenotype of the APP cells revealed that BR 297 and also AP improved the bioenergetic metabolism in these cells by increasing both OCR and ECAR (Figure 3F).

To investigate more deeply the effects of AP and ANS on mitochondrial respiration, OCR was also measured on permeabilized SH-SY5Y cells. This method allows the evaluation of different respiratory states and to calculate the respiratory control ratio (RCR = state 3 / state 4, Figure 4). In CTRL cells, AP and BR 297 significantly increased the basal respiration (state 2, ADP-independent) from 92% of increase and up to 105% of increase, respectively, compared to the untreated group (Figure 4A). The RCR was also up-regulated after treatment with these two compounds (+61 % with AP and +70% with BR 297) (Figure 4B). In APP cells, only BR 297 increased significantly the state 2 (+48%) and the RCR (+45%) compared to the untreated group (Figure 4C-D)

Taken together, these findings suggest that AP and BR 297 are able to increase cellular bioenergetic in both CTRL and APP cell by up-regulating the mitochondrial respiration, especially the capacity for substrate oxidation reflected by the RCR values,

leading to an increased ATP production. BR 297 appeared to be the best AP analog able to mimic the effects of AP on bioenergetics in our cellular models.

Because AP is known to have a proliferative effect on neuronal progenitor cells, we verified whether AP and its best analog BR 297 enhanced cell proliferation of CTRL and APP-transfected SH-SY5Y neuroblastoma cells. In both cell lines, at treatment with 500 nM of AP and BR 297 presented no effect on the cell proliferation, as evaluated with a BrdU cell proliferation kit (Figure 5). These results indicate that the up-regulation of cellular energy levels induced by these two compounds was independent of cell proliferation demands.

H₂O₂-induced mitochondrial bioenergetic impairments in APP transfected cells

Previous data of our group showed that 700 μM of H₂O₂ (24h and 48h treatment) was capable of killing about 70% of native or control vector-pCEP4-transfected SH-SY5Y cells while only 100 μM of H₂O₂ was sufficient to induce the same percentage of death in APP-transfected SH-SY5Y cells. These data indicated that APP overexpression significantly enhances cellular susceptibility to oxidative stress (Wendt, Kemmel et al. 2014). Here, we tested the effects of H₂O₂-evoked oxidative stress on mitochondrial bioenergetic capacity in the same cellular models.

First, we observed that H₂O₂ significantly decreased the ATP levels of CTRL and APP transfected cells in a dose-dependent manner after 3 h of treatment (Supplementary figure 3A). In line with our previous study, APP cells were more sensitive to H₂O₂ compared to CTRL cells, and presented a significant decrease of ATP levels already with a dose of 250 μM. We selected the dose of 500 μM of H₂O₂ that induced a decrease of about 70% of ATP levels in APP cells, and we tested its effects on cell survival. When compared to the CTRL cells treated with H₂O₂, APP cells showed about 70% of additional H₂O₂-induced cell death (Supplementary figure 3B).

The sensitivity of APP transfected cell to oxidative insults was also studied on mitochondrial respiration by measuring the RCR as well as the mitochondrial ROS level under stress condition using 500 μM treatments of H_2O_2 for 3h (Figure 6).

When compared to the CTRL cells treated with H_2O_2 , APP cells are more sensitive against H_2O_2 and presented a significant decrease of the ATP production (-82%, Figure 6A) and the RCR (-62%, Figure 6B), as well as an increase of the mitochondrial ROS level (+30%, Figure 6C), probably leading to the prominent cell death previously observed in APP cells (supplementary Figure 3B).

Protective effects of AP and the best analog BR 297 against H_2O_2 -induced bioenergetics deficits

In a next step, we tested the ability of AP and its analog BR 297 to protect cells against H_2O_2 -evoked bioenergetics abnormalities in the drastic conditions where more than 80% energy loss was observed (H_2O_2 at 500 μM).

First, we assessed whether a pre-treatment with AP or BR 297 can modulate the ATP level and mitochondrial respiration under oxidative stress conditions in both cell lines. Data were normalized to the CTRL cells treated with H_2O_2 . Figure 7A shows that AP and BR 297 increased ATP levels of about 18% and 15% respectively in CTRL cells treated with H_2O_2 .

APP transfected cells were more sensitive with a higher energetic deficit than CTRL cells under stress conditions. Pre-treatment with AP and BR 297 ameliorated the ATP level, (+5 and +5.5% respectively) when compared to the APP cells treated with H_2O_2 .

Then to explore more deeply the effects of a pre-treatment with AP and BR 297 on mitochondrial OXPHOS, RCR was calculated under stress conditions using permeabilized SH-SY5Y cells (Figure 7B). In CTRL cells, a pre-treatment with BR 297 increased significantly the RCR of about 90% whereas AP showed no effect compared to the group treated with H_2O_2 only. Pre-treatment with AP showed a slight improvement of the RCR in

APP cells while BR 297 increased significantly and with a higher efficacy the capacity of respiration of APP cells (+66%) compared to cells treated with H₂O₂.

Then we tested the effects of AP and BR 297 against H₂O₂-induced abnormal elevation of ROS production by checking the level of cytosolic ROS, mitochondrial ROS and superoxide anion level using fluorescent dyes in CTRL and APP-transfected cells.

Figure 8A shows that, AP and BR 297 induced a significant reduction of cytosolic ROS level in APP cells (-24% and -40% respectively) whereas they had no clear effects in CTRL cells under oxidative stress conditions. Besides, a pre-treatment with AP or its analog drastically decrease the levels of mitochondrial ROS (Figure 8B) as well as superoxide anion radicals (Figure 8C) in both cell lines.

Thereby, a pre-treatment with AP and BR 297 protected the cells against oxidative stress by ameliorating the cellular and mitochondrial bioenergetic and regulating the excessive amounts of reactive oxygen species in APP cells.

Protective effect of AP and BR 297 against H₂O₂-induced APP cells death

In H₂O₂-evoked oxidative stress conditions, we observed that 500 μM of H₂O₂ significantly decreased the viability of the CTRL and APP cells with APP transfected cells presenting a higher sensitivity (Supplementary Figure 3B). Thus, we decided to test the ability of AP or BR 297 to protect against H₂O₂-evoked cell death in conditions where about 70% of cells are killed (Supplementary Figure 3B, and Figure 9). The result shows that a pre-treatment with BR 297 ameliorated significantly the survival of CTRL cells of about 7% compared to the untreated group (Figure 9). In APP cells, both AP and BR 297 significantly increased the cell survival of about 10% compare to the group treated only with H₂O₂.

Taken together, these findings suggest that AP and its analog BR297 protect the cells against H₂O₂-induced cell death by improving mitochondrial bioenergetics and reducing the ROS generation under oxidative stress conditions.

Discussion

Development of novel mitochondrial neuromodulators or neuroprotectants is absolutely necessary in the treatment of Alzheimer disease because of the limitation of current pharmacological therapies. In this study, we investigated the effect of analogs of the natural neurosteroid allopregnanolone (ANS) on bioenergetics and oxidative stress in APP/A β overexpressing neuroblastoma cells to select compounds with higher efficacy and specific neuroprotective properties. Our key findings were that: (1) under physiological conditions, AP and the analog BR297 improved the bioenergetics by ameliorating the cellular ATP production, activating the bioenergetic metabolism and increasing the mitochondrial respiration without effect on the cell proliferation; (2) under stress condition using treatment with H₂O₂, a pre-treatment with AP and the selected analog BR297 prevented cell death by alleviating mitochondrial bioenergetics deficits and decreasing ROS generation. Thus, the protective pattern of AP and BR 297 are evident under physiological and oxidative stress conditions in a cellular model of AD-related amyloidopathy.

Neurosteroids are known as regenerative agents in the brain. In particular AP presents several beneficial effects in neurodegenerative disease and CNS or PNS disorders (Brinton 2013, Patte-Mensah, Meyer et al. 2014). Data obtained by the group of Roberta Brinton show that previously AP promotes proliferation of human and rodent neural progenitor cells *in vitro* via GABA A receptor and L-type Ca²⁺ channel dependent mechanism (Wang, Johnston et al. 2005). Further, acute single administration of AP induces neurogenesis in the hippocampal subgranular zone (SGZ) and reverses learning and memory deficits *in vivo* in triple transgenic mouse model of Alzheimer's (3xTgAD) at 3 months of age prior the appearance of AD pathology (Wang, Singh et al. 2010). Then Chen and colleagues demonstrated that AP administrated 1/week for 6 months promotes survival of newly generated neurons and reduces A β generation in hippocampus, cortex and amygdala in 3xTgAD at 3 months of age (Chen, Wang et al. 2011). They showed also that AP reduces microglia activation, increases oligodendrocyte myelin markers and improves

expression of protein regulating cholesterol homeostasis and clearance from brain such as liver X receptor (LXR) and pregnane X receptor (PXR). Singh and colleagues showed in aging 3xTgAD mice that AP restores cognitive performance in the preplaque phase of AD pathology and restores hippocampal-dependent learning and memory (Singh, Liu et al. 2012). AP promotes regeneration and repair in the brain, recovery of learning and memory function and reduces AD pathology (Brinton 2013). Therapeutic windows of AP efficacy in preclinical studies are defined by age and AD burden. The intermittently administration of AP promoted renewal and repair whereas continuous infusions of AP were antiregenerative in mouse models of AD (Brinton 2013). Taken together all these studies showed that AP ensured also the neuroprotection against the A β toxicity in 3xTgAD mouse model but also the stimulation of rodent and human neural progenitor cell proliferation to compensate the cell loss (Wang, Johnston et al. 2005, Chen, Wang et al. 2011). However, there are still limitations in the therapeutic use of AP. Indeed, AP can be inactivated by the sulfatation or the glucuronidation at the 3 α -hydroxyl group which decrease its bioavailability. Furthermore, AP can be converted back to 5 α -dihydroprogesterone (5 α -DHP) by a re-oxidation which could induce additional non-AP specific effects via binding to the nuclear steroid receptors (nSR) such as glucocorticoid receptors, estrogen receptors, androgen receptors, progestin receptors and mineralocorticoid receptors (Beato and Klug 2000). A new nSR was identified recently, the pregnane xenobiotic receptor (PXR), acting as a homeostatic regulator involved in neurosteroidogenesis of allopregnanolone in the brain (Frye, Koonce et al. 2014). This could be avoided by the absence of a free hydroxyl group at the position 3 (Figure 1). Thus, a set of derivative analogs of AP modified at the hydroxyl group were developed to prevent the inactivation and to obtain a higher biological efficacy.

A growing body of evidence has highlighted neuroprotective effects of neurosteroids against AD injury (Grimm, Lim et al. 2012, Brinton 2013). Neurosteroids that belong also to the sex hormone family such as including progesterone, estrogens and testosterone, present distinct protective properties against mitochondrial deficits in cellular models of AD-related amyloidopathy as well as AD-related tauopathies (Grimm, Biliouris et al. 2016). Previous

study showed that, allopregnanolone increased the cellular bioenergetics in primary neuronal cells (Grimm, Schmitt et al. 2014). However, no study has aimed to evaluate the effect of AP on mitochondrial function in AD.

In the present study, we used SH-SY5Y neuroblastoma cells stably transfected with the wild-type form of human APP, a cellular model that is well established in our laboratory and that presents characteristics found in AD pathology, including increase of A β production and ROS generation as well as impaired mitochondrial function (decrease of ATP production, mitochondrial respiration and mitochondrial complex IV activity) (Rhein, Baysang et al. 2009, Rhein, Giese et al. 2010, Grimm, Biliouris et al. 2016). When compared to the control vector-pCEP4-transfected SH-SY5Y cells, APP-overexpressing cells presented a significant decrease of the basal respiration, ATP turnover, maximal respiration as well as glycolytic reserve (Grimm, Biliouris et al. 2016). The bioenergetic profile of SH-SY5Y cells revealed that neurosteroids, especially testosterone, increased both mitochondrial respiration and glycolytic pathway. Interestingly, we previously showed that a treatment with 100 nM of AP on native SH-SY5Y induced no significant changes on bioenergetics (Grimm, Schmitt et al. 2014). Here, we demonstrated that at a higher concentration AP as well as BR 297 was able to increase the ATP level in CTRL and APP transfected SH-SY5Y cells. Our data showed that a concentration of 500 nM of AP and the analog BR 297 were able to improve both basal respiration and glycolysis, increasing the bioenergetic activity and ATP production in the two cell lines. In particular, we witnessed an up-regulation of the respiratory control ratio (RCR) induced by BR 297 in both cell lines but no significant effect of AP in APP cells. High RCR is an indicator of the capacity of substrates oxidation when cells have high energy demands. Of note, no effect on the cell proliferation was observed after a treatment of 24 hours with AP or BR 297 at 500 nM in CTRL and APP transfected cells. Thus, the up regulatory effect of AP and BR 297 on bioenergetics was not due to an increase of cell growth.

The presence of receptors nuclear steroid receptor, including the receptor for progesterone, estrogen and androgen, has already been demonstrated in SH-SY5Y cells (Takahashi, Piao et al. 2011, Grassi, Bellini et al. 2013). AP mainly acts on membrane

receptors, as allosteric modulator of GABA_A-R (Carver and Reddy 2013) and recent studies showed that AP can also bind the PXR, a nSR playing a role in xenobiotic metabolism and transporter genes expression (Ekins, Chang et al. 2007, Frye, Koonce et al. 2014). GABA_A-R was not detected in native SH-SY5Y (Grimm, Schmitt et al. 2014). AP can act via other metabolites whereas the analog 3 β -O-allyl-Epi-Allopregnanolone, BR 297, which has no hydroxyl group, cannot be converted back to other metabolite such as 5 α -DHP. To understand more deeply the mechanisms underlying AP and BR 297 action, further investigations are now required using antagonists of steroid receptor such as PXR or progesterone receptors.

To investigate in more details the neuroprotective potential of AP and BR 297, we tested their effect on mitochondrial bioenergetics in oxidative stress conditions. ROS generation within mitochondria is closely associated with oxidative metabolism and ATP synthesis, since superoxide anion radicals are a by-product of OXPHOS activity. Besides, oxidative stress induced by ROS and mitochondrial defects in neurons have been implicated in the pathogenic processes of AD (Adam-Vizi and Chinopoulos 2006). Indeed, dysfunctional mitochondria are less efficient producers of ATP and synthesize more ROS, the major source of oxidative imbalance in AD (Castellani, Hirai et al. 2002, Moreira, Carvalho et al. 2010, Wang, Wang et al. 2014).

We wanted to investigate whether the beneficial effect on bioenergetics of AP and BR 297 that we observed under physiological condition, may also protect the cells against cell death under oxidative stress conditions. H₂O₂, a major ROS, is known as a mediator of brain damages caused by the abnormal elevation of β -amyloid peptides (A β) in AD (Behl, Davis et al. 1994, Citron 2010). First, we demonstrated that APP cells are more sensitive to H₂O₂ compared to CTRL cells, and presented a prominent raise of mitochondrial ROS as well as a more drastic drop of ATP, RCR, leading to cell death. In our study, there was an enhancement of intracellular ATP levels in AP and BR 297 pretreated CTRL and APP cells in oxidative stress conditions, suggesting that energy supply was in part preserved. We showed

that only a pre-treatment with BR 297 was able to induce an up-regulatory effect of the respiratory capacity (RCR) of both cell lines under stress conditions. Of note, under physiological condition, BR 297 was more efficient to improve the mitochondrial respiration in CTRL and APP cells when compared to AP. In APP cells, BR 297 was able to completely restore the RCR to the level of the CTRL cells stressed with H₂O₂.

Oxidative stress plays a determinant role in the pathogenesis of AD by exacerbating mitochondrial deficits (Grimm, Friedland et al. 2016). Intracellular ROS formation, a commonly used indicator of oxidative stress, was reduced in AP and BR297 pre-treated CTRL and APP SH-SY5Y cells, suggesting that BR 297 also exert its protective action by reducing ROS levels during H₂O₂ exposure. These data are in line with our previous those of our previous study in which we showed that a selection of neurosteroids, including AP, were able to modulate the cellular redox state in native SH-SY5Y cells by increasing antioxidant activity, more specifically the activity of the manganese superoxide dismutase (MnSOD) located in the mitochondrial matrix (Grimm, Schmitt et al. 2014).

Thus, based on these observations, one can speculate that a pre-treatment with BR297 and AP may exert a protective action against oxidative stress through: (1) a reduction of the ROS generation, (2) the amelioration of the cellular and mitochondrial energy (see Figure 10). Further investigations are required to determine in more details the possible mechanisms of underlying the protective action of BR 297, especially the ability to modulate the antioxidant defences (e.g ROS scavenging activity, activation of superoxide dismutases and glutathione system).

In line with a recent study, the compound BR 297 (3 β -O-allyl-AP) demonstrated an increase of the neuroprotective activity as compared to AP in neural stem cell cultures treated with amyloid beta 42 (Karout, Miesch et al. 2016). We found also that BR 297 exhibits notable advantages over AP with regards to both protection of mitochondrial function and reduction of oxidative stress. Under physiological conditions, BR 297 had no effect on cell survival but it ameliorated the bioenergetics by increasing the cellular ATP level and

mitochondrial respiration. BR 297 has no effect on the proliferation but seem to block the cell death mechanism, improving the cell viability. Indeed, a pre-treatment with BR 297 increases the survival of CTRL and APP cells under stress conditions, protecting the cells against H₂O₂-induced cell death. By decreasing the ROS level, the mitochondrial respiration was improved and, accordingly, cells were protected against the oxidative stress-induced cell death.

AP appears to be a very interesting therapeutic option in the treatment of AD, and pre-clinical studies are already performed to predict its tolerability and efficacy in human (Irwin and Brinton 2014, Irwin, Solinsky et al. 2015). Development of synthetic neurosteroids analogs might constitute promising novel strategies for the treatment of brain disorder (Rey and Coirini 2015). While AP's anticonvulsant and antianxiety activities are well documented, AP seems undesirable for chronic use due to its potential re-conversion in its metabolic precursor progesterone. Ganaxolone, a synthetic analog of allopregnanolone, is until now the only one synthetic steroid that has been used in clinical trial for epilepsy (Nohria and Giller 2007). Ganaxolone appears to improve dysfunctional emotional behaviour associated with deficit in allopregnanolone in mice and provide an alternative treatment for post-traumatic stress disorder patient (Pinna and Rasmusson 2014). Another synthetic analog of AP, Co 2-6749 is known for its anxiolytic effect by modulating the receptor GABA_A (Vanover, Rosenzweig-Lipson et al. 2000). However, until now, no neurosteroid analog has been developed to protect mitochondrial function against oxidative stress implicated in AD. Taken together, our findings demonstrate that the analog of AP, BR 297 is conclusively of great potential as pharmacological candidate in the treatment of AD-related mitochondrial dysfunction by modulating cellular energy metabolism and exerting protective effects. These results strongly suggest the analog BR 297 as a promising multitarget drug in protection of mitochondrial function against AD.

Acknowledgements

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Materials and Methods

Chemicals and reagents

Dulbecco's-modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, DHR, DCF, ADP, hydrogen peroxide (H₂O₂), pyruvate, succinate and malate were from Sigma-Aldrich (St. Louis, MO, USA). Glutamax and MitoSOX were from Gibco Invitrogen (Waltham, MA, USA). Allopregnanolone was from Calbiochem (Billerica, MA, USA). PMP and XF Cell Mitostress kit were from Seahorse Bioscience (North Billerica, MA, USA). Horse serum (HS) was from Amimed, Bioconcept (Allschwil, Switzerland). Analogs of neurosteroid called ANS were synthesized by the Laboratoire de Chimie Organique Synthétique, UMR 717, (Strasbourg, France).

Cell culture

Human SH-SY5Y neuroblastoma cells were grown at 37°C in a humidified incubator chamber under an atmosphere of 7.5% CO₂ in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 5% (v/v) heat-inactivated HS, 2 mM Glutamax and 1% (v/v) penicillin/streptomycin. Cells were passaged 1–2 times per week, and plated for treatment when they reached 80–90% confluence. SH-SY5Y cells were stably transfected with DNA constructs harboring human wild-type APP₆₉₅ (APPwt) or the expression vector pCEP4 (Invitrogen, Saint Aubin, France) alone (control vector) using lipofectamine plus (Invitrogen, Saint Aubin, France) (Scheuermann et al. 2001 **PMID: 11438549**). Transfected APPwt cells were grown in DMEM standard medium supplemented with 300 µg/ml hygromycin.

Treatment paradigm

Assessment of cell viability was performed on SH-SY5Y neuroblastoma cells to determine the potential toxic concentration range of AP and analogs (from 10nM to 1000nM, data not shown) using a MTT reduction assay (Roche, Basel, Switzerland). On the basis of the MTT results as well as preliminary ATP data (see Supplementary figure 1), the concentration of

500 nM was then selected and used in all assays. SH-SY5Y cells were treated in DMEM + 10% FCS one day after plating either with DMEM alone (untreated control condition) or with a final concentration of 500nM of allopregnanolone (AP), BR 053, BR 297, BR 338 and BR 351, made from a stock solution in DMSO, for 24h (final concentration of DMSO<0.002%, no effect of the vehicle solution (DMSO) alone compared to the untreated condition). For the stress experiments, cells were first pre-treated for 24 h with AP or ANS and then treated for 3 h with 500 μ M H₂O₂. Then ATP assays, mitochondrial respiration, reactive oxygen species (ROS) detection and cell viability assays were performed. Each assay was repeated at least 3 times.

ATP levels

Total ATP content of SH-SY5Y cells was determined using a bioluminescence assay (ViaLighTM HT, Cambrex Bio Science, Walkersville, MD, USA) according to the instruction of the manufacturer, as previously described (Grimm, Schmitt et al. 2014, Grimm, Biliouris et al. 2016). SH-SY5Y cells were plated in 5 replicates into a white 96-well cell culture plate at a density of 2×10^4 cells/well. The bioluminescent method measures the formation of light from ATP and luciferin by luciferase. The emitted light was linearly related to the ATP concentration and was measured using the multilabel plate reader VictorX5 (Perkin Elmer).

Mitochondrial respiration

The investigation of mitochondrial respiration was performed using the Seahorse Bioscience XF24 Analyser. XF24 cell culture microplates were coated with 0.1% gelatine and cells were plated at a density of 2.5×10^4 cells/well in 100 μ l of treatment medium containing 10 % FCS, 1 g/l glucose and 4 mM pyruvate. After 24 h of treatment with AP or ANS, cells were washed with 1x pre-warmed mitochondrial assay solution (MAS; 70 mM sucrose, 220 mM mannitol, 10 mM KH₂PO₄, 4.5 mM MgCl₂, 2 mM HEPES, 1 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2 at 37 °C) and 500 μ l of pre-warmed (37 °C) MAS containing 1 nM XF plasma membrane permeabilizer (PMP, Seahorse Bioscience), 10 mM pyruvate, 10 mM succinate

and 2 mM malate was added to the wells. The PMP was used to permeabilize intact cells in culture, which circumvents the need for isolation of intact mitochondria and allows the investigation of the OCR under different respiratory states induced by the sequential injection of: i) ADP (4 mM) to induce state 3; ii) oligomycin (0.5 μ M) to induce state 4o; Data were extracted from the Seahorse XF24 software and the respiratory control ratio (RCR: state 3/state 4o), which reflects the mitochondrial respiratory capacity, was calculated.

Oxygen consumption rate and extracellular acidification rate

The Seahorse Bioscience XF24 Analyser was used to perform a simultaneous real-time measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). XF24 cell culture microplates (Seahorse Bioscience) were coated with 0.1% gelatine and SH-SY5Y cells were plated at a density of 2.5×10^4 cells / well in 100 μ l of the treatment medium containing 10% FCS, 1 g/l glucose and 4 mM pyruvate. After 24 h of treatment with AP or ANS treatment, cells were washed with PBS and incubated with 500 μ l of assay medium (DMEM, without NaHCO_3 , without phenol red, with 1g/l glucose, 4 mM pyruvate, and 1% L-glutamine, pH 7.4) at 37°C in a CO_2 -free incubator for 1 h. The plate was placed in the XF24 Analyzer and basal OCR and ECAR were recorded during 30 min.

Cell viability assays

To assess cell viability, MTT reduction assays were performed according to the manufacturer's protocol. Briefly, native and genetically modified SH-SY5Y cells were seeded at 2×10^4 cells / well into 96-well plates and allowed to attach. After 24h, neuroblastoma cells were incubated under the following conditions:

(I) in order to determine effective doses of H_2O_2 to induce a significant decrease in ATP production as well as cell loss in both APP and control vector-pCEP4-transfected SH-SY5Y cells, cells were treated with H_2O_2 at various concentrations (0, 10, 50, 100, 250, 500, 750 and 1000 nM) for 3 h (see supplementary figure 3). MTT signal detected for each cell type in basal condition (in absence of H_2O_2) was arbitrarily set at 100%. This basal signal reflecting

the total number of living cells in each cell type was the reference that served for the accurate determination of dose-dependent effects of H₂O₂ after 3 h of treatment.

(II) to evaluate the protective effects of AP or the selected analog BR 297, cells were pre-treated for 24 h at a concentration of 500 nM and then incubated for 3 h with a concentration of 500 μM of H₂O₂ capable of killing about 70% of genetically modified SH-SY5Y cells. Values were normalized to the control groups treated with H₂O₂ alone.

Reactive oxygen species (ROS) detection

Levels of cytosolic reactive oxygen species (ROS), mitochondrial reactive oxygen species and specific levels of mitochondrial superoxide anion radicals were assessed using the fluorescent dyes 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA), dihydrorhodamine 123 (DHR123) and the Red Mitochondrial Superoxide Indicator (MitoSOX), respectively. SH-SY5Y cells were plated in 6 replicates into a black 96-well cell culture plate at a density of 2x10⁴ cells/well. After AP and ANS treatment, cells were loaded with 10 μM of DCF or DHR for 15 min or 5 μM of MitoSOX for 90 min at room temperature in the dark on an orbital shaker. After washing twice with HBSS (Sigma), the formation of green fluorescent products, DCF and DHR, generated by the oxidation of H₂DCF-DA and DHR123, respectively, were detected using the multilabel plate reader VictorX5 at 485 nm (excitation)/538 nm (emission). MitoSOX, which is specifically oxidized by mitochondrial superoxide, exhibits a red fluorescence detected at 535 nm (excitation)/595 nm (emission). The intensity of fluorescence was proportional to mtROS levels, cytosolic ROS level and superoxide anion radicals in mitochondria

Statistical analysis

Data are given as the mean ± SEM, normalized to the untreated control group (=100%). Statistical analyses were performed using the Graph Pad Prism software. For statistical comparisons of more than two groups, One-way ANOVA was used, followed by Dunnett's

multiple comparison tests *versus* the control. For statistical comparisons of two groups, Student unpaired *t*-test was used. P values < 0.05 were considered statistically significant.

Dose–effect parameters for H₂O₂ (EC₅₀ values) were determined by non-linear regression of the experimental data using the GraphPad-Prism program (GraphPad-Prism, San Diego, CA, USA). The goodness of fits was estimated by the R-squared value (>0.9).

Abbreviations:

AD	Alzheimer's disease
AP	Allopregnanolone
A β	Amyloid- β
ANS	Analogs of neurosteroidallopregnanolone
APP	Amyloid- β precursor protein-transfected
ATP	Adenosine triphosphate
CTRL	Control vector-pCEP4-transfected
CNS	Central nervous system
DCF	2', 7'-dichlorofluorescein
DHR	Dihydrorhodamine 123
DMSO	Dimethylsulfoxide
ECAR	Extracellular acidification rate
H ₂ O ₂	Hydrogen peroxide
MAS	Mitochondrial assay solution
NFTs	Neurofibrillary tangles
PMP	Plasma membrane permeabilizer
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
RCR	Respiratory control ratio
ROS	Reactive oxygen species
SH-SY5Y	Human neuroblastoma cells
UNT	Untreated

Figure captions:

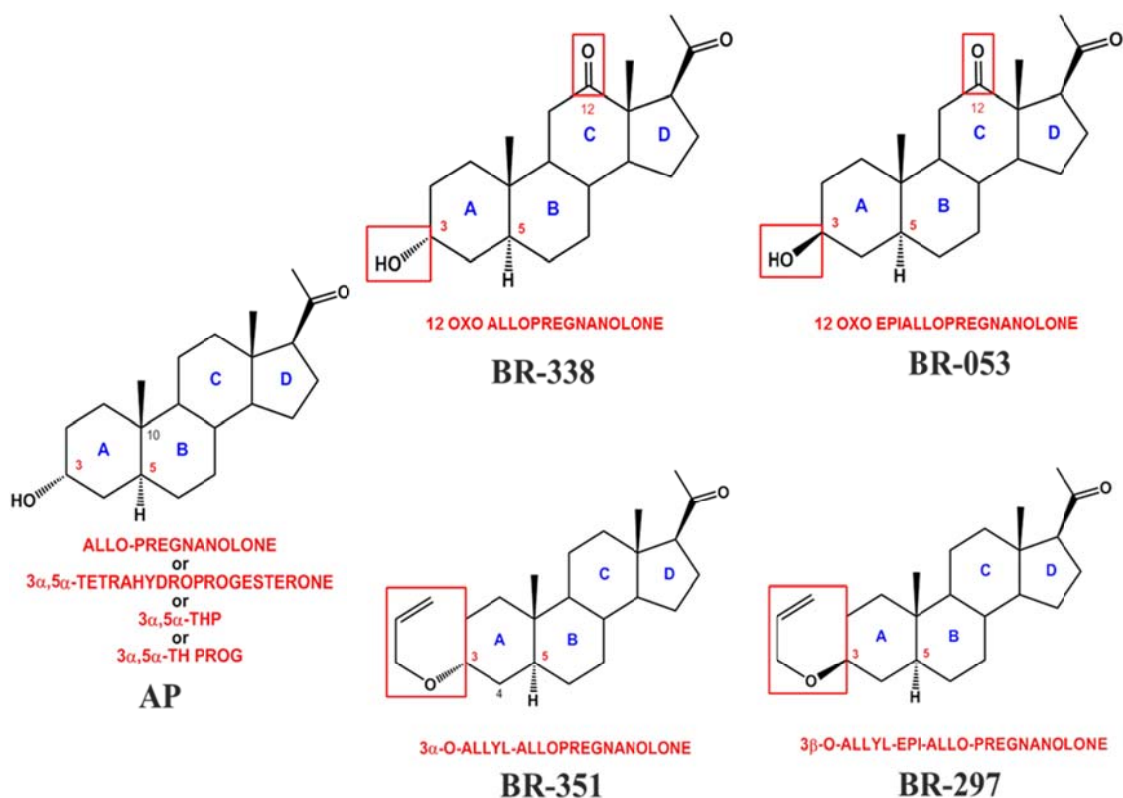


Figure 1: Structure of the synthetic analogs (ANS) of the natural neurosteroid allopregnanolone. Chemical structures derived from allopregnanolone or epiallopregnanolone: 12-oxo-allopregnanolone or BR 338 (12 oxo-AP), 3 α -O-allyl-allopregnanolone or BR 351 (O-allyl-AP), 12-oxo-epiallopregnanolone or BR 053 (12 oxo-epiAP), 3 β -O-allyl-epiallopregnanolone or BR 297 (O-allyl-epiAP). The chemical structure of allopregnanolone is modified by either an oxo- group in the position 12, or an O-allyl- group at the position 3. Especially the later modification is expected to reveal metabolically more stable drugs due to the etherized hydroxyle group preventing enzymatic oxidation of the hydroxyle group and thus re-conversion.

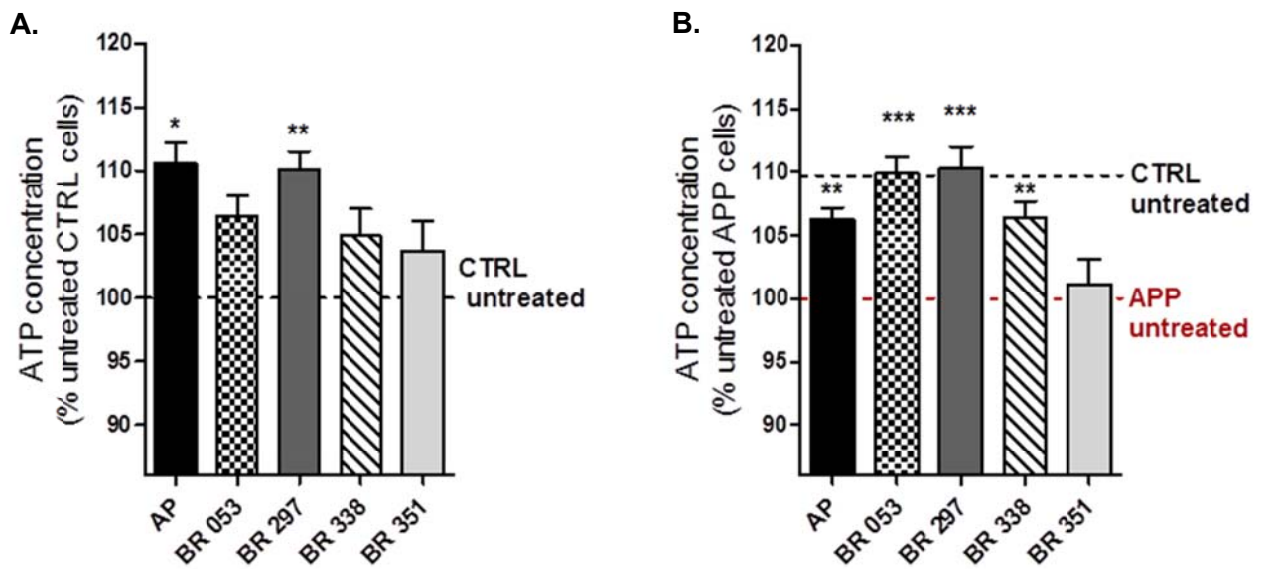


Figure 2: AP and its analogs increase ATP level in CTRL and APP transfected cells. ATP level was measured after a treatment of 24 h with AP, BR 053, BR 297, BR 338 and BR 351 at a concentration of 500 nM in (A) CTRL and (B) APP cells. Values represent the mean \pm SEM (n=12-18 replicates of three independent experiments) and were normalized to 100 % of untreated CTRL cells (A) or untreated APP cells (B). One way ANOVA and post hoc Dunnett's multiple comparison test versus untreated CTRL or APP cells, *P<0.05, **P<0.01, ***P<0.001. AP: allopregnanolone; APP: amyloid precursor protein; CTRL: control.

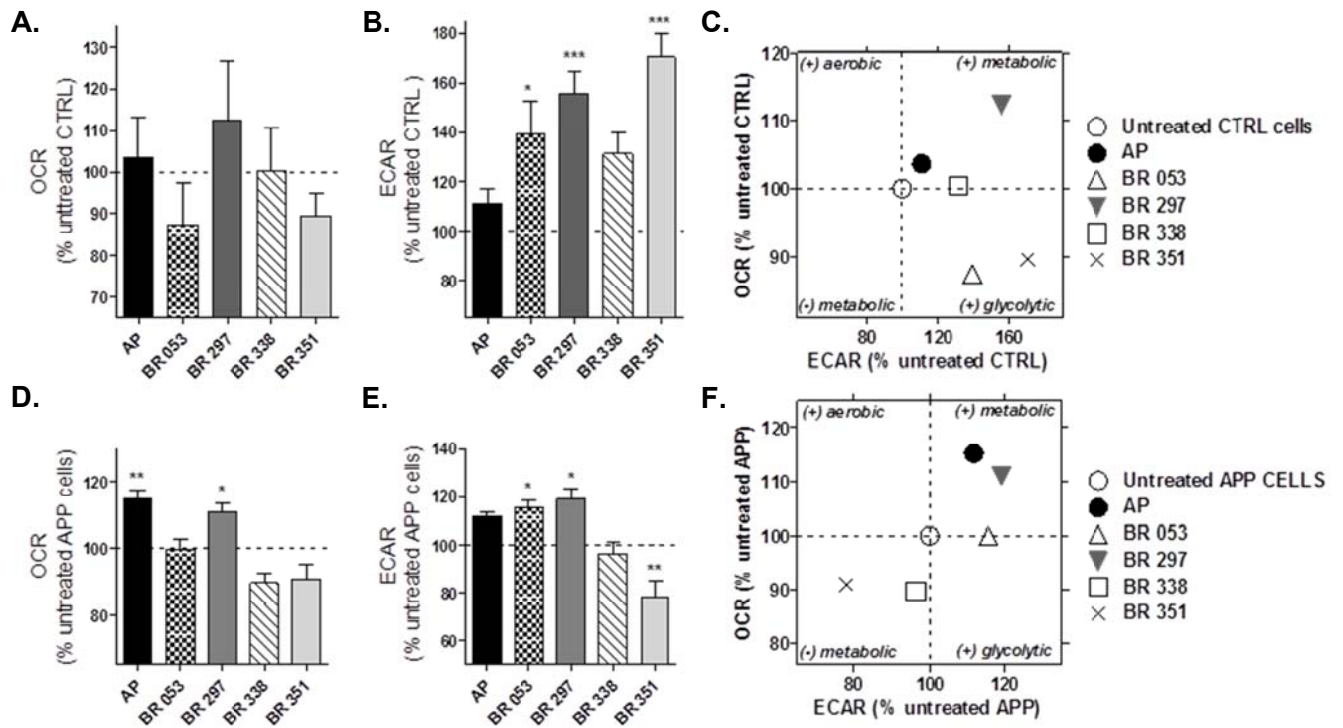


Figure 3: Modulation of the bioenergetic phenotype by AP and its analogs. (A, D) Oxygen consumption rate (OCR) and (B, E) extracellular acidification rate (ECAR) were measured simultaneously using a Seahorse XF24 Analyzer in the same experimental conditions in CTRL cells (A-B) and APP cells (D-E). Values represent the mean \pm SEM (n= 15 replicates) of three independent experiments. One way ANOVA and post hoc Dunnett's multiple comparison test versus untreated CTRL or APP cells (UNT), *p<0.05, **p<0.01, ***p<0.001. Bioenergetic phenotype (OCR versus ECAR) of CTRL cells (C) and APP cells (F) revealed increased metabolic activity after treatment with BR 297. Values represent the mean of each group (mean of the ECAR in abscissa/ mean of the OCR in ordinate) and were normalized to the control group (100%); OCR: Oxygen Consumption Rate (mitochondrial respiration), ECAR: Extracellular Acidification Rate (Glycolysis), AP: allopregnanolone.

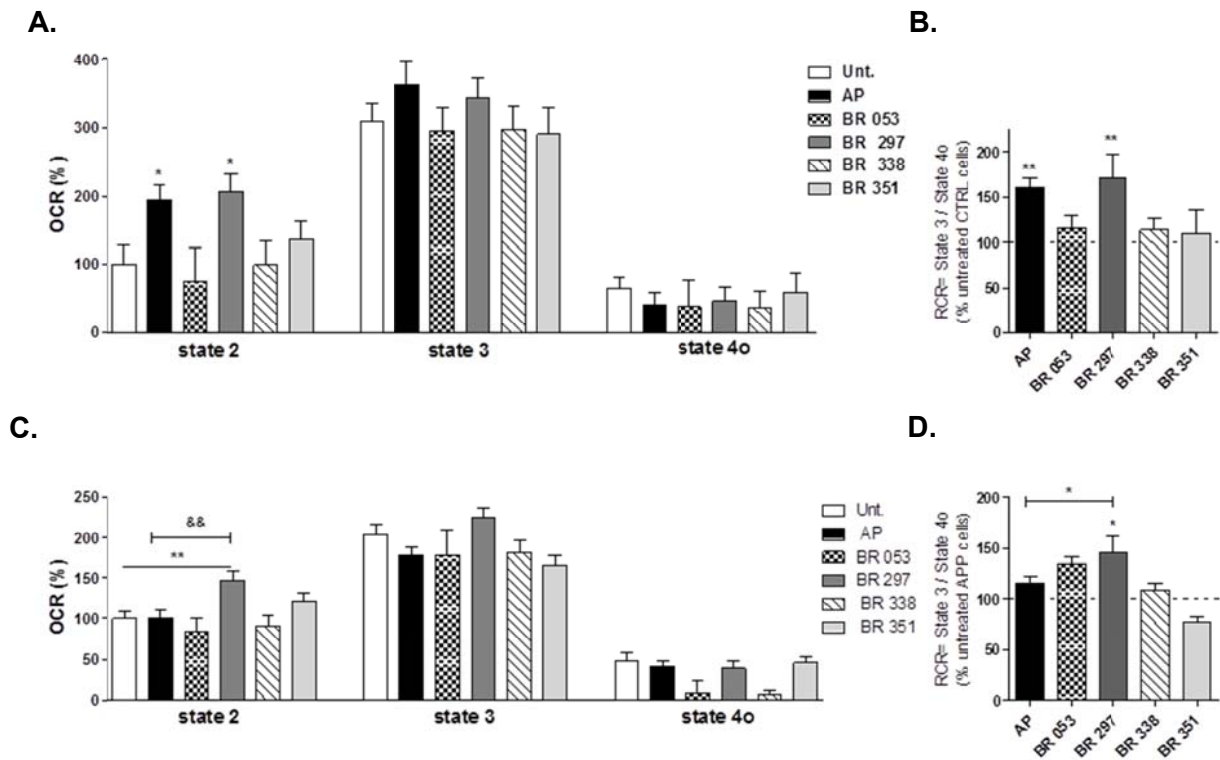


Figure 4: BR 297 increases mitochondrial respiratory capacity in CTRL and APP cells. (A, C) Oxygen consumption rate (OCR), was measured on permeabilized CTRL (A) or APP cells (C) after treatment with AP or its analogs for 24 h, using a XF24 Analyser (Seahorse Bioscience). The sequential injection of mitochondrial inhibitors allows the assessment of mitochondrial respiratory state 2, state 3 (ADP-dependent) and state 4o (after oligomycin injection) (see details in the Material and methods section). Values corresponding to the different respiratory states are represented as mean \pm SEM (n=11-15 replicates of three independent experiments/ groups) and were normalized to the state 2 of the untreated group (=100%). (B, D) The respiratory control ratio (RCR= state 3/ state 4o), which reflects the mitochondrial respiratory capacity, was increased by AP and BR297 in CTRL cells (B) but only BR297 improved the RCR in APP cells (D). Results were normalized to the RCR of the untreated group (=100%). One way ANOVA and post hoc Dunnett's multiple comparison test versus control untreated, *P<0.05, **P<0.01; ***P<0.001. Student t test, && P<0.01; &&& P<0.001.

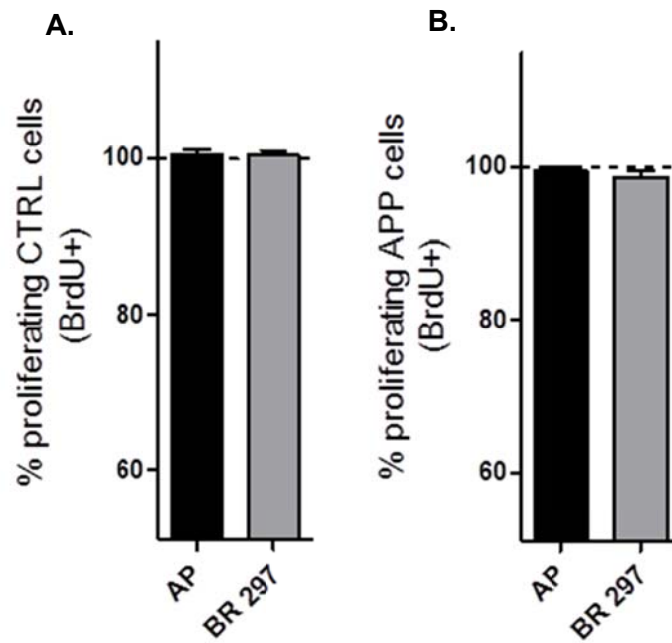


Figure 5: No proliferative effect of AP and ANS on CTRL cells (A) and APP transfected cells (B). Cell proliferation was assessed using the BrdU cell proliferation assay after 24 h of treatment with AP or BR297 at a concentration of 500nM. Values represent the mean \pm SEM (n=8-16 replicates) of three independent experiment and were normalized on the untreated group (= 100%).

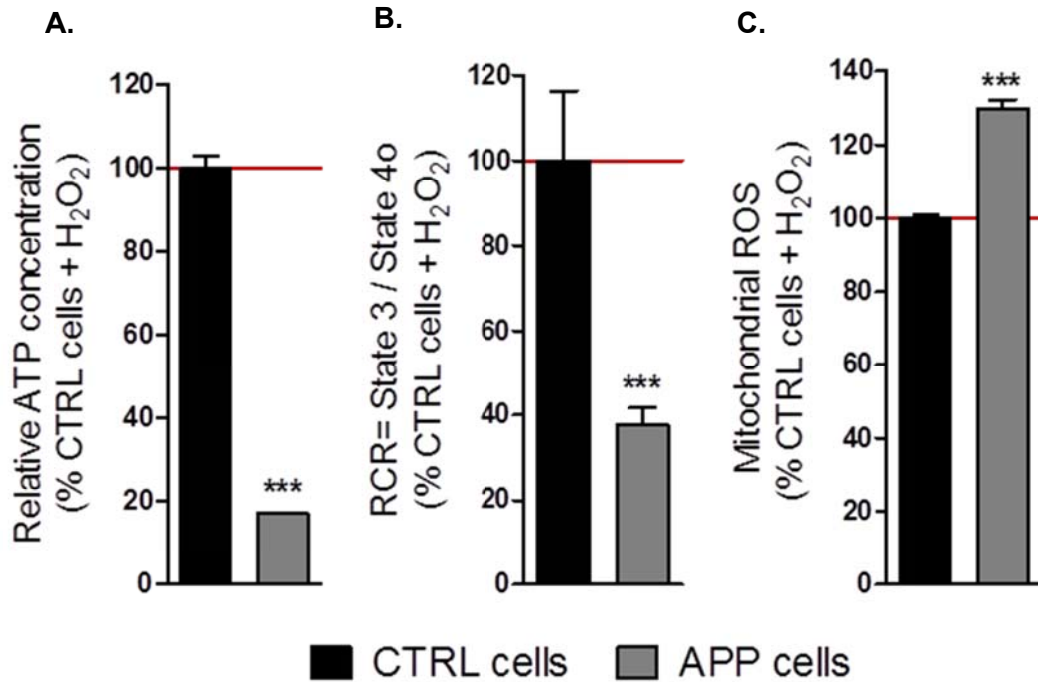


Figure 6: Oxidative stress exacerbates mitochondrial dysfunction in APP cells. In APP transfected cells, H₂O₂ (500 μ M for 3h) decreases the ATP level (A) and RCR (B), paralleled by an increase in mitochondrial ROS levels (C) compared to the CTRL cell. Values represent the mean \pm SEM; n= 3-6 replicates of three independent experiments. Student unpaired t test ***P<0.001 CTRL vs APP cells under oxidative stress conditions.

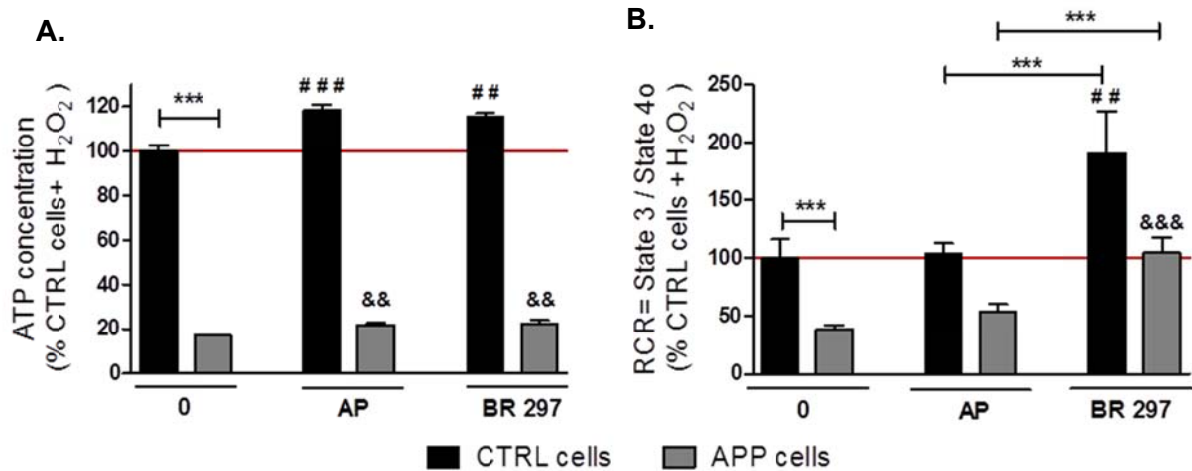


Figure 7: AP and BR 297 improve mitochondrial bioenergetics in oxidative stress conditions. AP and BR 297 increase cellular ATP production (A) and mitochondrial respiration (B), protecting CTRL and APP transfected cells against H₂O₂-induced drop of bioenergetics. CTRL and APP transfected cells were pre-treated with 500 nM of AP and BR 297 for 24h and then exposed to H₂O₂ (500 μM for 3h). Values represent the mean ± SEM; n= 4-6 replicates of three independent experiments normalized to CTRL cells treated with H₂O₂. Student unpaired t test ***P<0.001; **P<0.01. One way ANOVA and post hoc Dunnett's multiple comparison test versus CTRL cells H₂O₂ condition, ###P<0.01; ###P<0.001 for CTRL cells. One way ANOVA and post hoc Dunnett's multiple comparison test versus APP cells H₂O₂ condition, &&P<0.01; &&&P<0.001 for APP cells.

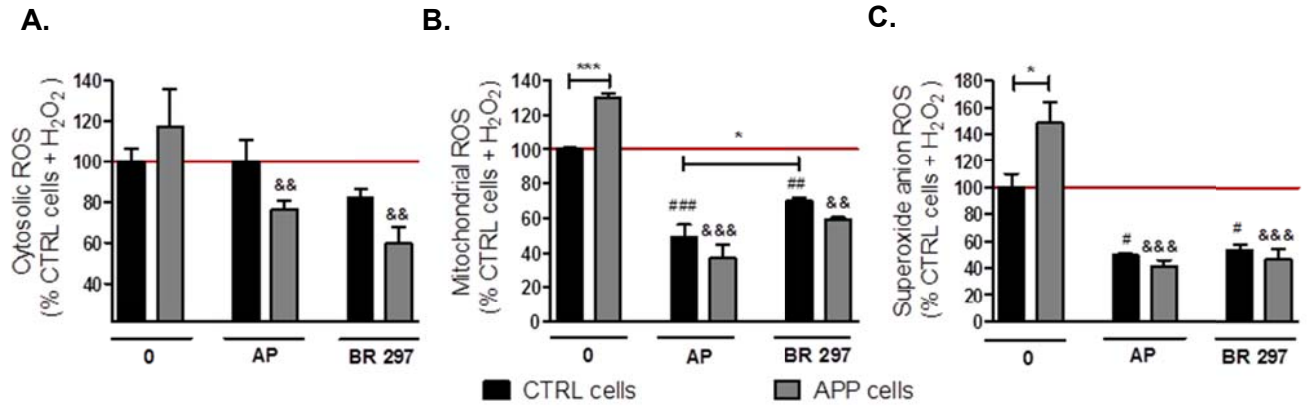


Figure 8: AP and BR 297 protect against H₂O₂-induced raise of ROS. H₂O₂ treatment induces an increase of the cytosolic ROS (A), mitochondrial ROS (B) and superoxide anion (C) in APP transfected cells compared to CTRL cells. Pre-treatment with AP or BR 297 (500 nM) reduced significantly the ROS generation under oxidative stress conditions (500 μM of H₂O₂ for 3h). Values represent the mean ± SEM; n= 6-12 replicates of three independent experiments normalized to CTRL cells treated with H₂O₂. Student unpaired t test *P<0.05; ***P<0.001. One way ANOVA and post hoc Dunnett's multiple comparison test versus CTRL cells H₂O₂ condition, #P<0.05; ##P<0.01; ###P<0.001 for CTRL cells. One way ANOVA and post hoc Dunnett's multiple comparison test versus APP cells H₂O₂ condition, &P<0.05; &&P<0.01; &&&P<0.001 for APP cells.

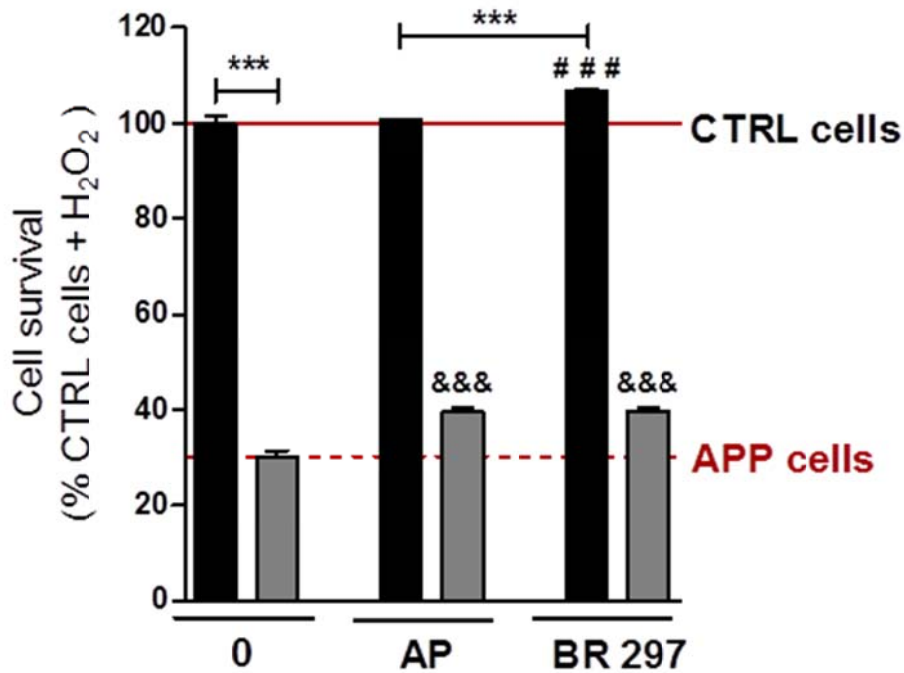


Figure 9: Protective effect of AP and BR 297 against H₂O₂-induced cell death. CTRL and APP transfected cells were pre-treated with 500 nM of AP and BR 297 for 24 h and then exposed to H₂O₂ (500 μM for 3h). Pre-treatment with BR 297 and AP ameliorate significantly the cell viability in oxidative stress conditions. Values represent the mean ± SEM; n= 4-6 replicates of three independent experiments normalized to CTRL cells treated with H₂O₂. Student unpaired t test ***P<0.001. One way ANOVA and post hoc Dunnett's multiple comparison test versus CTRL cells H₂O₂ condition, ###P<0.001 for CTRL cells. One way ANOVA and post hoc Dunnett's multiple comparison test versus APP cells H₂O₂ condition, &&&P<0.001 for APP cells.

MITOCHONDRIAL TARGET THERAPIES

UNDER STRESS CONDITION

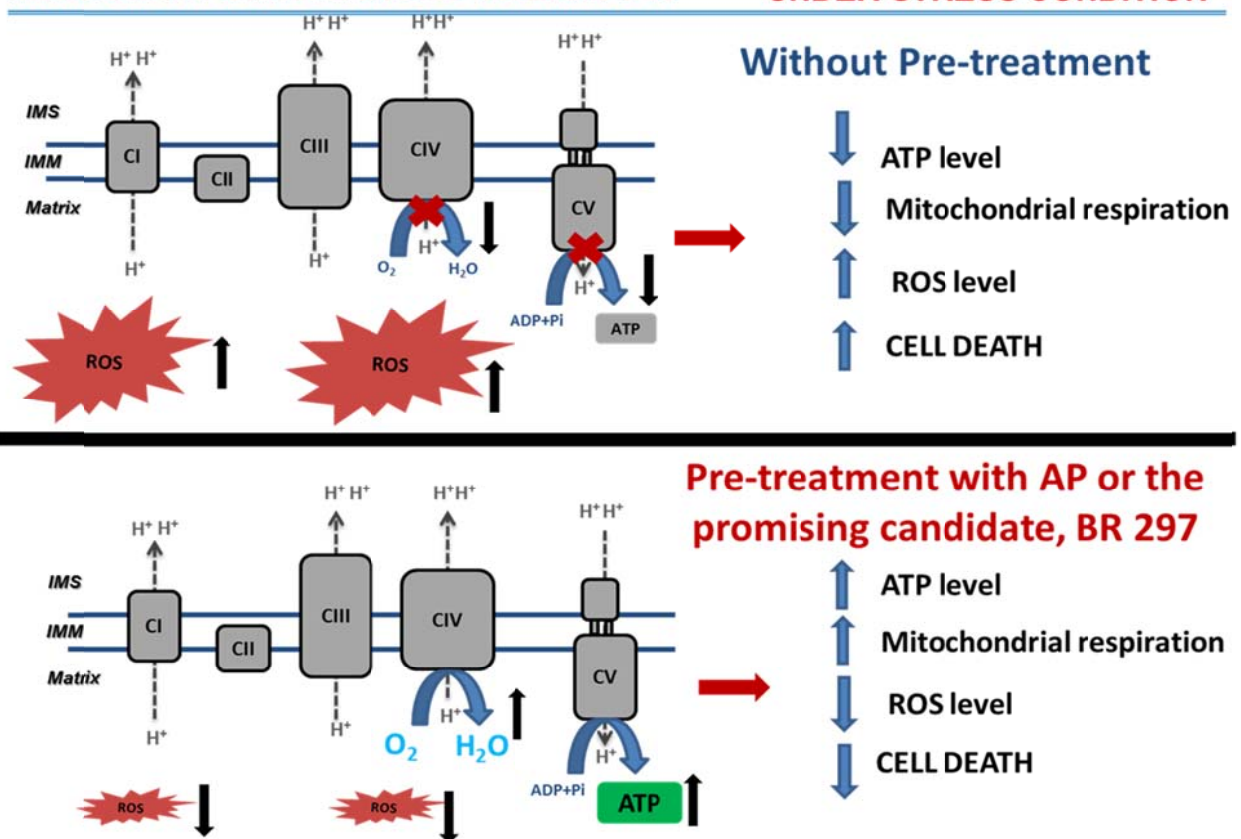
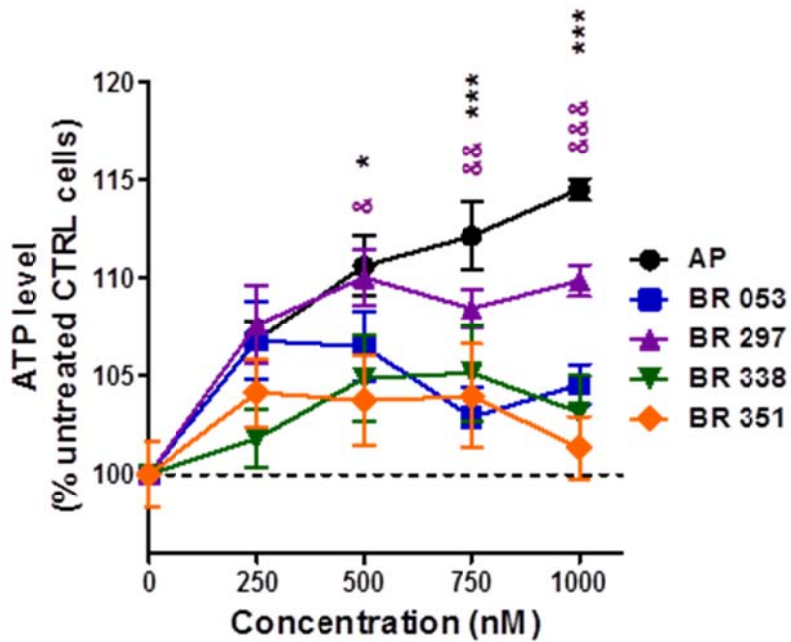
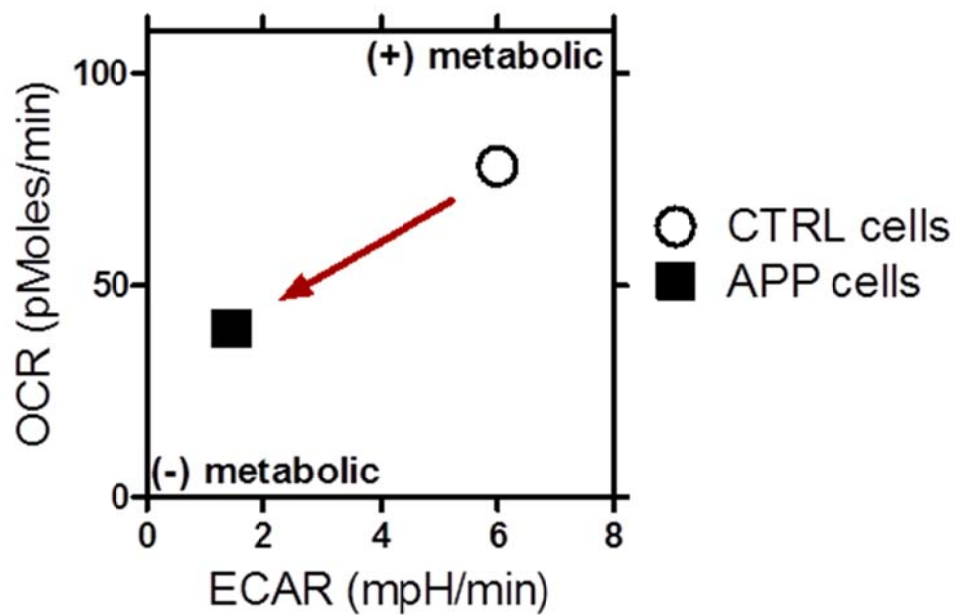


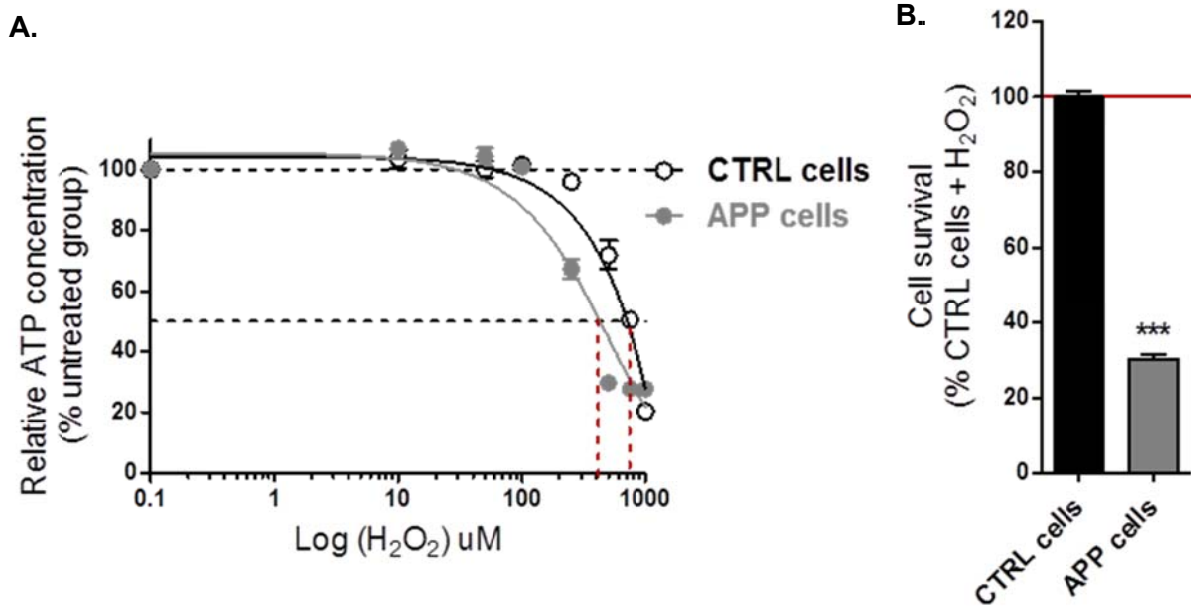
Figure 10: Schematic representation of the protective effects AP or its analog BR 297 against oxidative stress. H₂O₂-induced oxidative stress decreased ATP level and mitochondrial respiration, and increase the level of ROS leading to cell death (upper panel). AP and BR 297 are able to ameliorate mitochondrial respiration and ATP levels under oxidative stress conditions, as well as to decrease ROS levels, improving the cell survival (lower panel). ROS: Reactive oxygen species, AP: allopregnanolone, ATP adenosine triphosphate.



Supplementary figure 1: Dose–response study of the effect of AP and ANS on ATP level of control vector-pCEP4-transfected SH-SY5Y. ATP levels are expressed as percent of the untreated cells (= 100%). One way ANOVA and post hoc Dunnett’s multiple comparison test versus untreated group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for AP and & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$ for BR 297. AP: allopregnanolone.



Supplementary Figure 2: Bioenergetics phenotype of CTRL and APP cells in basal conditions. APP cells are less metabolic with a low OCR and ECAR compared to CTRL cells. Values represent the mean (mean of the ECAR in abscissa/ mean of the OCR in ordinate); n=9-12 replicates of three independent experiments. OCR: Oxygen Consumption Ratio (mitochondrial respiration), ECAR: Extracellular Acidification Rate (Glycolysis).



Supplementary figure 3: (A) Dose–response study of the effect of H₂O₂ (3h) on the levels of ATP in CTRL (open circles) and APP-transfected SH-SY5Y cells (grey circles). ATP levels are expressed as percent of control (untreated cells). ATP signal assessed for each cell type in basal condition (absence of H₂O₂) is arbitrarily set at 100%. H₂O₂ concentration evoking 50% of decrease of ATP production corresponds to the EC₅₀ (500 μM and 750μM) for each cell type is indicated by a dotted line. (B) H₂O₂ treatment induces a stronger decrease of ATP levels in APP cells when compared to CTRL cells. Values represent the mean ± SEM, n=4-6 replicates of three independent experiments normalized to CTRL cells treated with 500 μM of H₂O₂. Student unpaired t test ***P<0.001 CTRL vs APP cells under oxidative stress condition.

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