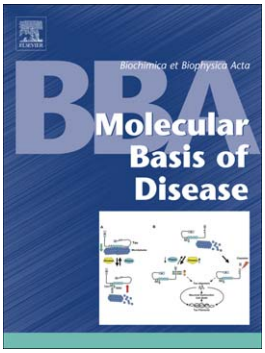


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PII: S0925-4439(16)30335-0
DOI: doi:10.1016/j.bbadis.2016.12.007
Reference: BBADIS 64636

To appear in: *BBA - Molecular Basis of Disease*

Received date: 20 June 2016
Revised date: 10 November 2016
Accepted date: 12 December 2016

Please cite this article as: Imane Lejri, Amandine Grimm, Michel Miesch, Philippe Geoffroy, Anne Eckert, Ayikoe-Guy Mensah-Nyagan, Allopregnanolone and its analog BR 297 rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement, *BBA - Molecular Basis of Disease* (2016), doi:10.1016/j.bbadis.2016.12.007

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

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**Abstract**

Allopregnegnanolone (AP) is supposed to exert beneficial actions including anxiolysis, analgesia, neurogenesis and neuroprotection. However, although mitochondrial dysfunctions are evidenced in neurodegenerative diseases, AP actions against neurodegeneration-induced mitochondrial deficits have never been investigated. Also, the therapeutic exploitation of AP is limited by its difficulty to pass the liver and its rapid clearance after sulfation or glucuronidation of its 3-hydroxyl group. Therefore, the characterization of novel potent neuroprotective analogs of AP may be of great interest. Thus, we synthesized a set of AP analogs (ANS) and investigated their ability to counteract APP-overexpression-evoked bioenergetic deficits and to protect against oxidative stress-induced death of control and APP-transfected SH-SY5Y cells known as a reliable cellular model of Alzheimer's disease (AD). Especially, we examined whether ANS were more efficient than AP to reduce mitochondrial dysfunctions or bioenergetic decrease leading to neuronal cell death.

Our results showed that the ANS BR297 exhibits notable advantages over AP with regards to both protection of mitochondrial functions and reduction of oxidative stress. Indeed, under physiological conditions, BR297 does not promote cell proliferation but efficiently ameliorates the bioenergetics by increasing cellular ATP level and mitochondrial respiration. Under oxidative stress situations, BR297 treatment, which decreases ROS levels, improves mitochondrial respiration and cell survival, appears more potent than AP to protect control and APP-transfected cells against $H_2O_2$-induced death.

Our findings lend further support to neuroprotective effects of BR 297 emphasizing this analog 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

Several lines of evidence have well demonstrated that mitochondria play a central role in the pathogenesis of neurodegenerative and neurological disorders (Lin and Beal 2006; Yan, Xiong et al. 2006; Detmer and Chan 2007; Knott, Perkins et al. 2008; Oliveira 2010; Schapira and Patel 2014). In particular, it is increasingly obvious that mitochondrial abnormalities are involved in the pathophysiological mechanisms of Alzheimer's disease (AD), an age–related neurodegenerative disease representing more than 60% of all dementia cases and characterized by two brain histopathological hallmarks: intracellular neurofibrillary tangles (NFTs) composed by the aggregation of abnormally hyperphosphorylated tau protein and extracellular amyloid-β (Aβ) plaques (Berchtold and Cotman 1998). Indeed, an increase in oxidative damages and decreased energy metabolism are observed at early stages of AD, even before the appearance of NFTs and Aβ plaques (Knott, Perkins et al. 2008; Rhein, Baysang et al. 2009; 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, which has very high energy requirements and consumes about 20% of body's total basal oxygen to fulfil its functions (Raichle and Gusnard 2002; Shulman, Rothman et al. 2004), is considered to be extremely sensitive to oxidative damages (Clark, Lee et al. 2010). In this context, mitochondria play an extremely important role in the nervous system because they are involved in the regulation of intracellular calcium homeostasis, synaptic plasticity, neurotransmitter synthesis and cell survival or death (Scheffler 2001; Adam-Vizi and Chinopoulos 2006; Mattson, Gleichmann et al. 2008). Mitochondria are paradoxical organelles, since they produce the energy necessary for cell survival via ATP

generation through the oxidative phosphorylation (OXPHOS) and, at the same time, they constitute 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 steroids synthesized within the nervous system which still present after the removal of 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). We recently showed that neurosteroids, which improved cellular bioenergetics by increasing mitochondrial respiration and ATP generation, also regulated redox homeostasis in neuronal cells (Grimm, Schmitt et al. 2014; Grimm, Biliouris et al. 2016). In addition, mitochondrial deficits induced by Aβ 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 neurosteroidogenesis seems to play a major role in the pathophysiology of neurodegenerative diseases. *In vitro* and *in vivo* studies showed that during aging, and even more in AD, neurosteroid production diminished in parallel with a neural function deficiency (Frye and Walf 2008; Caruso, Barron et al. 2013). Interestingly, previous data from our groups provided evidence that Aβ and abnormal tau protein alter neurosteroidogenesis in cellular models of AD (Schaeffer, Patte-Mensah et al. 2006; Schaeffer, Meyer et al. 2008).

Allopregnanolone (5α-pregnane-3α-ol-20-one or AP) is a natural neurosteroid synthesized by a 2 step-transformation involving 5α-reductase, which converts progesterone into 5α-dihydroprogesterone (5α-DHP), and 3α-hydroxysteroid oxidoreductase that transforms 5α-DHP into AP but also reversibly converts AP back to 5α-DHP (Mensah-Nyagan, Do-Rego et al. 1999; Patte-Mensah, Kibaly et al. 2005). In various experimental

models and also in humans, a variety of beneficial effects of AP has been evidenced including neuroprotective, neurogenic, analgesic, antidepressant, anaesthetic and anxiolytic actions (Bernardi, Salvestroni et al. 1998; Wang, Johnston et al. 2005; Melcangi, Garcia-Segura et al. 2008). These neuroactive properties are supported by the ability of AP to modulate diverse channels and receptors such as the $GABA_A$, L- or T-type calcium channels as well as the pregnane X receptor (PXR) or membrane progesterone receptors (Majewska, Harrison et al. 1986; Carver and Reddy 2013; Frye, Koonce et al. 2014). A decreased ability to form AP in the hippocampus has been demonstrated in APPswe+PSEN1Δ9 mice which represent a transgenic animal model of AD; progesterone treatment, which increased AP concentration, improved cognitive performances in these AD mice (Frye and Walf 2008). In addition, *in vivo* studies conducted using the triple transgenic AD mice (3xTgAD) revealed that AP reduced Aβ generation in hippocampus, cortex and amygdala, increased neuronal progenitor cell proliferation and also reversed neurogenic and cognitive deficits in these 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 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, the first objective of the present study is to assess the ability of AP to prevent oxidative stress-induced cell death through bioenergetic modulations in control SH-SY5Y neuroblastoma cells and wild-type human APP stably transfected SH-SY5Y cells (APP cells) that are well recognized as a reliable experimental model to investigate cellular mechanisms involved in AD (Scheuermann, Hambsch et al. 2001; Li, Zhang et al. 2007; Xiong, Hongmei et al. 2011; Jamsa, Belda et al. 2011; Shen, Wang et al. 2013; Zhang, Gao et al. 2015; Zhang, Gao et al. 2015). Indeed, APP cells, which secrete Aβ to a 3-4 fold higher extent than the control cells bearing the empty vector, exhibit several AD pathogenic features

including a decreased viability/survival and mitochondrial malfunction (Rhein, Baysang et al. 2009; Rhein, Giese et al. 2010; Wendt, Kemmel et al. 2014; Grimm, Biliouris et al. 2016).

Because of its pleiotropic effects, the exploitation of AP for targeted therapies remains a difficult question. While the regenerative and neuroprotective actions of AP may offer interesting options for neuroprotective strategies, its proliferation-promoting effect on stem cells raised serious concerns related to the risk of cancer (Velasco 2011). Another hurdle to overcome is the difficulty for AP to pass the liver and this hampers the development of oral treatments (most convenient for long term uses), so only parenteral routes of AP administration are currently under investigations (Irwin, Solinsky et al. 2015). Moreover, as for other endogenous neurosteroids, the therapeutic use of AP may be limited by its rapid clearance after sulfation or glucuronidation of the 3 hydroxyl group (Reddy 2010; Schumacher, Mattern et al. 2014). Therefore, we have recently modified the chemical structure of AP to synthesize a set of more stable analogs (ANS) by introducing either an oxo-group in the position 12 or an O-allyl as substituent of the 3 hydroxyl group (See Figure 1 and Mensah-Nyagan, Meyer et al. 2012, Patent WO2012127176 AI). Thus, the second objective of the current work is to compare AP and ANS effects in order to select the best analog(s) exerting the most potent or optimal protective action against oxidative stress and/or APP-overexpression-induced mitochondrial dysfunctions and subsequent cell death. AP and ANS actions were particularly investigated 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/Aβ 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), here we report about 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% (AP and BR 338) up to 10% (BR 053 and BR 297) of increase (Figure 2B). Thus, after treatment of APP cells with AP, BR 053, BR 297 or BR 338, the concentration of ATP was similar to those measured in CTRL cells.

ATP molecules are synthetized 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 alsoup-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 in 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 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.

**$H_2O_2$-induced mitochondrial bioenergetic impairments in APP transfected cells**

Previous data of our group showed that 700µM of $H_2O_2$ (24h and 48h treatment) was capable of killing about 70% of native or control vector-pCEP4-transfected SH-SY5Y cells while only 100µM of $H_2O_2$ 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 $H_2O_2$-evoked oxidative stress on mitochondrial bioenergetic capacity in the same cellular models.

First, we observed that $H_2O_2$ 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 $H_2O_2$ 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 $H_2O_2$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 $H_2O_2$, APP cells showed about 70% of additional $H_2O_2$-inducedcell 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, (+5and +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 $H_2O_2$.

Then we tested the effects of AP and BR 297 against $H_2O_2$-induced abnormal elevation of ROS production by checking the level of cytosolic ROS, mitochondrial ROS and superoxide anion level using fluorescents 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 radials (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 $H_2O_2$-induced APP cells death**

In $H_2O_2$-evoked oxidative stress conditions, we observed that 500 µM of $H_2O_2$ 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 $H_2O_2$-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 $H_2O_2$.

Taken together, these findings suggest that AP and its analog BR297 protect the cells against $H_2O_2$-induced cell death by improving mitochondrial bioenergetics and reducing the ROS generation under oxidative stress conditions.

## Discussion

Development of novel mitochondrial neuromodulators or neuroprotective drugs is absolutely necessary for the treatment of AD and neurodegenerative diseases because of the limitation of current pharmacological therapies. In order to select compounds with higher efficacy and specific neuroprotective properties, we used in the present study an experimentally well characterized cellular model of AD, the APP/Aβ overexpressing neuroblastoma SH-SY5Y cells that exhibit mitochondrial deficits (Scheuermann, Hambsch et al. 2001), (Rhein, Baysang et al. 2009; Rhein, Giese et al. 2010; Shen, Wang et al. 2013; Zhang, Gao et al. 2015; Grimm, Biliouris et al. 2016), to investigate AP and ANS actions on bioenergetics and oxidative stress-induced cell death. Our key findings were that: (1) among the 4 ANS compared to AP actions on different parameters of mitochondrial functions and cell survival, the analog BR297 (3β-O-allyl-epi-AP) appears as the best candidate exhibiting optimal/beneficial neuroprotective effects and notable advantages over AP; (2) under physiological conditions, AP and BR297 improved the bioenergetics by ameliorating cellular ATP production, activating the bioenergetic metabolism and increasing mitochondrial respiration with no effect on cell proliferation; (3) under stress condition using $H_2O_2$, 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.

Our results also reveal that the analog BR351 (3α-O-allyl-AP), which induced potent proliferative and neuroprotective effects on neural stem cells) (Karout, Miesch et al. 2016), does not seem to require a strong mobilization of mitochondrial functions. These interesting data show that the chemical modifications we made on AP chemical structure to obtain BR297 and BR351 are biologically relevant. Indeed, while only a subtle structural difference occurs between BR351 and BR297 (3α-O-allyl-AP versus 3β-O-allyl-epi-AP), these 2 ANS show different action profiles and therefore offer the possibility to adapt their uses to specific

pathological conditions, depending on whether it may be required to induce both neurogenic and neuroprotective effects or to prevent only neuronal cell death (neuroprotection) without causing the stimulation of cell proliferation. In support of our findings, previous elegant studies demonstrated the existence of isomer-selectivity and/or enantio-selectivity for differential neurophysiological effects induced by certain neurosteroids. For instance, it has been shown that the enantiomer of AP (ent-AP) is inactive on the GABA A receptor but induces (likely AP) neuroprotection in a mouse model of Niemann-Pick C disease by acting via the pregnane X receptor or PXR (Langmade, Gale et al. 2006). A differential regulatory effect on memory has also been evidenced for the neurosteroid pregnenolone sulfate (PREGS) and its enantiomer (ent-PREGS) which is 10 times more potent than PREGS and enhances spatial memory in rodent, through a mechanism independent from NMDA receptor, while PREGS-evoked memory performance involves NMDA receptor activity (Akwa, Ladurelle et al. 2001; Petit, Tobin et al. 2011).

Altogether, the data discussed above suggest that, even if both AP and its analog BR297 prevent oxidative stress-evoked control and APP/Aβ cell death via the regulation of mitochondrial functions, different mechanisms of actions may be involved, especially because notable differential effects were observed on various cellular and mitochondrial parameters. In particular, beyond the fact that BR297 is more potent than AP for the stimulation of ATP production in APP/Aβ cells and for the increase of ECAR parameter in control and APP/Aβ cells under physiological conditions, BR297 also restored normal value of the respiratory control ratio (RCR, State 3/State 4o) dramatically decreased by oxidative stress in APP/Aβ cells whereas AP was ineffective. Furthermore, BR297, which strongly stimulated the RCR in control cells while AP has no detectable action, also exhibited a more potent action than AP on control cell survival under oxidative stress conditions. Additional investigations will certainly be necessary in the future to characterize the specific mechanisms of action or cell signaling respectively triggered by BR297 and AP for the modulation of mitochondrial functions and the protection of APP/Aβ and control cells against death. However, previous data including recent results from our group make it possible to

discuss 2 hypotheses: (i) mediation of AP and/or BR297-induced bioenergetic improvement and protection of SH-SY5Y cells by GABA A receptors and (ii) involvement of non-GABAergic receptors in AP and/or BR297 beneficial effects on bioenergetics and SH-SY5Y cell survival.

*(i) mediation of AP and/or BR297-induced bioenergetic improvement and protection of SH-SY5Y cells by GABA A receptors.*

It is well documented that AP acts as positive allosteric modulator of GABA A receptors (Majewska, Harrison et al. 1986; Belelli and Lambert 2005; Carver and Reddy 2013). While the presence of nuclear steroid receptors, such as progesterone, estrogen and androgen receptors, has been demonstrated in SH-SY5Y cells (Takahashi, Piao et al. 2011; Grassi, Bellini et al. 2013), we have recently observed that undifferentiated native SH-SY5Y cells do not express GABA A receptors SH-SY5Y (Grimm, Schmitt et al. 2014). The occurrence of GABA A receptor α1 and β2 subunits has been suggested only in retinoic acid-differentiated SH-SY5Y cells (Andersson, Bjornstrom et al. 2015). Together, these data suggest that, in the non-differentiated control and APP/Aβ SH-SY5Y cells used in the current study, AP and BR 297 cannot modulate mitochondrial functions and prevent cell death by triggering an intracellular signaling from the GABA A receptor. However, one cannot completely rule out the involvement of GABA A receptors. Indeed, our previous studies (Grimm, Schmitt et al. 2014) have mainly revealed the absence of GABA A subunits α1 and β2 in undifferentiated native SH-SY5Y cells but we cannot exclude the presence of other subtypes such as α (2-4), β (1, 3-4), δ or γ subunits which may constitute functional conformation of GABA A channel in non-differentiated SH-SY5Y cells. In support of this idea, applications of 10 or 100 nM of AP were ineffective (Grimm, Schmitt et al. 2014) whereas 500 nM of AP (current study) stimulates ATP production in both APP/Aβ and control undifferentiated SH-SY5Y cells, suggesting that AP may improve bioenergetics through a GABA A receptor conformation requiring AP concentrations higher than 100 nM.

Regarding the effects of BR297, the situation may be different since we previously observed that BR297 is a weak allosteric modulator of GABA A receptors which induces by

its own action an electrophysiological activity in absence of GABA (Mensah-Nyagan, Meyer et al. 2012, patent WO2012/127176 AI). Furthermore, while it is conventionally admitted that 3α-derived AP analogs are positive allosteric activators of GABA A channels, contrary to 3β-derived pregnane steroids (including isopregnanolone or epiAP) which inhibit these receptors (Wang, 2011), the 3β-derived steroidal compound BR297 (3β-O-allyl-epi-AP) did not reveal opposite actions to those of AP in the modulation of mitochondrial functions and the protection of control and APP/Aβ cells against oxidative cell-evoked death. Consequently, the strong beneficial effects exerted by BR297 in bioenergetic improvement and neuroprotection of control and APP/Aβ SH-SY5Y cells may hardly be justified if one considers only the involvement of GABA A receptors.

*(ii) involvement of non-GABAergic receptors in AP and/or BR297 beneficial effects on bioenergetics and SH-SY5Y cell survival*

Besides its well-known effect on GABA A channels, AP modulates various other receptors. For examples, Roberta Brinton and coworkers have shown that, acting via the L-type Ca2+ channels and GABA A receptors, AP promotes neural progenitor cell proliferation, induces hippocampal neurogenesis, decreases cerebral Aβ generation and reverses memory deficits in AD triple transgenic mice (Wang, Johnston et al. 2005; Wang, Singh et al. 2010; Chen, Wang et al. 2011; Chen, Wang et al. 2011). They also demonstrated that AP, which stimulates the expression of proteins such as the pregnane X receptor (PXR), the liver X receptor (LXR) and 3-hydroxy-3-methyl-glutaryl-CoA-reductase that regulate cholesterol homeostasis and clearance from brain, also reduces microglia activation and enhances oligodendrocyte myelin markers (Chen, Wang et al. 2011; Chen, Wang et al. 2011). AP-evoked neuroprotective action through the modulation of PXR has also been revealed in Niemann-Pick C disease mouse model (Langmade, Gale et al. 2006). Other studies showed that AP interacts with the T-type Ca2+ channels to induce dose-dependent analgesia in vivo (Pathirathna, Brimelow et al. 2005). Allosteric modulatory effects of AP have also been evidenced at the nicotinic and serotonin type 3 receptors (Bullock, Clark et al. 1997; Wetzel, Hermann et al. 1998). More importantly, AP, which activates membrane progesterone G-

coupled protein receptors (mPR), including mPRδ, mPRα, and mPRβ with more efficacy than the GABA A receptor, also triggers from mPRδ an intracellular cascade reducing serum starvation-induced apoptosis and cell death in mPRδ-transfected cells and hippocampal neurons at nanomolar concentrations (Thomas and Pang 2012; Pang, Dong et al. 2013). Of note is the fact that, acting through mPR, AP regulates cAMP and MAPK pathways which are known to be involved in the control of apoptotic factors and cell death (Lin, Chen et al. 2002; Pang, Dong et al. 2013). Interestingly, a recent work performed with the AD APP/Aβ SH-SY5Y cell model, revealed that Apigenin may exert neuroprotective effect against copper-mediated Aβ toxicity through the activation of interactions between MAPK signal, mitochondrial functions, anti-oxidative and anti-apoptotic pathways (Zhao, Wang et al. 2013). Moreover, we have previously showed a decreased mitochondrial complex IV activity in APP/Aβ cells and other studies have also revealed that neuroactive steroids including, estradiol, testosterone and/or progesterone may regulate mitochondrial oxidative phosphorylation, the electron transport chain components (complex I and complex IV), the F1 subunit of ATP synthase as well as the glucose transporter and enzymes involved in the tricarboxylic acid cycle and mitochondrial respiration (Irwin, Yao et al. 2008); (Rhein, Baysang et al. 2009); (Grimm, Lim et al. 2012; Vasconsuelo, Milanesi et al. 2013). These results suggest that AP and/or BR297 may cross the plasma membrane to interact with the activity and/or expression of the components aforementioned or they may also trigger from membrane targets (other than the GABA A receptors) an intracellular cascade leading to the control of the oxidative phosphorylation or to the regulation of various subunits of the electron transport chain or glucose transporter.

Altogether, the data discussed above suggest the existence of various non GABAergic mechanisms which may mediate the beneficial actions of AP and/or BR297 on bioenergetics and neuroblastoma cell survival. Whether some of these GABA A receptor-independent mechanisms may be more sensitive to BR297 than AP or whether BR297 may trigger other specific signaling insensitive to AP remains for the moment a matter of speculation that will need further investigations for clarification.

A growing body of evidence has highlighted neuroprotective effects of neurosteroids against AD injury (Grimm, Lim et al. 2012; Brinton 2013). Neurosteroids belonging to sex hormone family such as progesterone, estrogens and testosterone, present distinct protective properties against mitochondrial deficits in cellular models of AD-related amyloidopathy and tauopathies (Grimm, Biliouris et al. 2016). Previous study showed that, AP increased 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 work, we used neuroblastoma SH-SY5Y cells stably transfected with the human wild-type APP, a cellular model well established as possessing various characteristics found in AD pathology, including increased Aβ production, ROS generation and 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). Interestingly, it has also been demonstrated that APP/Aβ-over-expression causes abnormal mitochondrial morphology and distribution in neuroblastoma M17 cells, suggesting the possible occurrence of morphological alterations of mitochondria in APP/Aβ SH-SY5Y cells (Wang, Su et al. 2008). When compared to the control vector-pCEP4-transfected SH-SY5Y cells, APP-overexpressing cells showed a significant decrease of the basal respiration, ATP turnover, maximal respiration and 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 observed 100 nM of AP induced no significant changes on bioenergetics in native SH-SY5Y (Grimm, Schmitt et al. 2014). Here, we show that AP or BR297 at 500nM improves both basal respiration and glycolysis, increasing the bioenergetic activity and ATP production in control and APP/Aβ SH-SY5Y cells. 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 cell proliferation effect was observed after 24 hour treatment with AP or BR 297 at 500 nM. Therefore, it

appears that AP- and BR297-evoked up-regulatory actions on bioenergetics were not related to cell growth increase.

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 synthetize 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). Thus, we wanted to investigate whether the beneficial effects of AP and BR297 on bioenergetics under physiological condition, may also protect against oxidative stress-induced cell death. $H_2O_2$, a major ROS, is known as a mediator of brain damages caused by the abnormal elevation of Aβ in AD (Behl, Davis et al. 1994; Citron 2010). First, we demonstrated that APP/Aβ cells, which are more sensitive to $H_2O_2$ compared to control cells, revealed a prominent raise of mitochondrial ROS and a more drastic drop of ATP and RCR, leading to cell death. We observed enhanced intracellular ATP levels in AP and BR 297 pretreated control and APP/Aβ cells under oxidative stress conditions, suggesting that energy supply was in part preserved. Furthermore, we showed that only BR 297 was able to induce an up-regulatory effect of the respiratory capacity (RCR) of both cell lines under stress conditions. BR 297 was also more effective than AP to protect against $H_2O_2$-induced cell death.

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 exerts its protective action by reducing ROS levels during $H_2O_2$ exposure. These data are in line with 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 manganese superoxide dismutase (MnSOD) located in the mitochondrial matrix (Grimm, Schmitt et al. 2014).

By taking together our observations, it may be speculated as summarized in Figure 10 that a pre-treatment with BR297 and AP may exert neuroprotection against oxidative stress-induced cell death through: (1) the reduction of ROS generation, (2) the amelioration of cellular and mitochondrial energy and (3) the improvement of mitochondrial respiration. Further investigations are required to determine how BR 297 may modulate the antioxidant defenses, e.g the ROS scavenging activity, the activation of superoxide dismutases and the glutathione system.

In a recent study, we observed that BR 297 exerted an increased neuroprotective activity as compared to AP in neural stem cells treated with amyloid beta 42 (Karout, Miesch et al. 2016). Therefore, it appears that BR297-induced neuroprotection is not restricted to AD APP/Aβ SH-SY5Y cell model but may be extended to other models of Aβ-evoked neurotoxicity.

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). Because of the relatively short half-life of AP due its reconversion into the precursor 5α-DHP or to its sulfation or glucuronidation at the 3-hydroxyl group, development of synthetic analogs such as BR297 having a different chemical group at the C3 position might constitute promising novel strategies for the treatment of brain disorders (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 into 5α-DHP. Ganaxolone is a synthetic analog of AP assessed in clinical trial for epilepsy (Nohria and Giller 2007). It also seems to improve dysfunctional emotional behaviour associated with AP deficit in mice and may provide an alternative treatment for post-traumatic stress disorders (Pinna and Rasmusson 2014). Another synthetic analog of AP is Co 2-6749 which is known for its anxiolytic effect exerted via the

GABA A receptor (Vanover, Rosenzweig-Lipson et al. 2000). To our knowledge, there is until now no neurosteroid analog developed for the protection of mitochondrial functions against oxidative stress-evoked damages in AD. Therefore, BR297 conclusively appears as having an interesting potential for the development of effective strategies against AD-related mitochondrial dysfunctions and the protection against neuronal cell death. Additional in vivo verifications using transgenic AD mice will certainly help in the future to consolidate the present in vitro results suggesting that BR 297 may be a promising pharmacological candidate to counteract or slow down the progression of AD and/or neurodegenerative symptoms.

**Acknowledgements**

This study was co-supervised by AGMN and AE in the frame of a joint-PhD thesis (IL) between the University of Strasbourg (France) and the University of Basel (Switzerland). This work is part of the collaborative research program of the NeuroRhine Consortium coordinated by AGMN that was funded by INTERREG IV Program (European Fund for Regional Development) in the Upper Rhine Region and the Offensive Science Call 2012. Additional Research Founds were from the Psychiatric University Clinics and Swiss National Foundation (SNF, to AE).

## Materiels and Methods

### Chemicals and reagents

Dulbecco's-modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, DHR, DCF, ADP, hydrogen peroxide ($H_2O_2$), pyruvate, succinate and malate were from Sigma-Aldrich (St. Louis, MO, USA). Glutamaxand 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 synthetizedby the Laboratoire de ChimieOrganiqueSynthétique, 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% $CO_2$ in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 5% (v/v) heat-inactivated HS, 2 mMGlutamax 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 $APP_{695}$ (APPwt) or the expression vector pCEP4 (Invitrogen, Saint Aubin, France) alone (control vector) using lipofectamineplus (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 µM $H_2O_2$. 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 $2x10^4$ 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 $2.5x10^4$ cells/well in 100µl 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 $KH_2PO$, 4.5 mM $MgCl_2$, 2 mM HEPES, 1 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2 at 37 °C) and 500 µl 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 µM) 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 $2.5x10^4$ cells / well in 100 µl 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 µl 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 $2x10^4$ 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 lose 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 $H_2O_2$ 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 500µM of $H_2O_2$ capable of killing about 70% of genetically modified SH-SY5Y cells. Values were normalized to the control groups treated with $H_2O_2$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 ($H_2$DCF-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 $2x10^4$ cells/well. After AP and ANS treatment, cells were loaded with 10µM of DCF or DHR for 15min or 5µM of MitoSOX for 90min 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 $H_2O_2$ ($EC_{50}$ 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 |
| Aβ | 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 |
| $H_2O_2$ | 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), 3α-O-allyl-allopregnanolone or BR 351 (O-allyl-AP), 12-oxo-epiallopregnanolone or BR 053 (12 oxo-epiAP), 3β-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 hydroxyl group preventing enzymatic oxidation of the hydroxyl 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 ± 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 ± 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%); Additional statistical comparison is provided using the Student t test: (D) +++P<0.001 AP vs BR053; ++P<0.01 BR053 vs BR297; p=0.2242 AP vs BR297. (E) p=0.2667 AP vs BR053; p=0.0911 AP vs BR297. 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 Materiel and methods section). Values corresponding to the different respiratory states are represented as mean ± 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: (A) *P<0.05 AP vs BR053. *P<0.05 BR 053 vs BR297; (C) **P<0.01 BR053 vs BR297; (D) p=0.6790 BR053 vs BR297.

**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 ± 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, $H_2O_2$ (500 µM 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 ± 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 $H_2O_2$-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 $H_2O_2$ (500 µM for 3h). Values represent the mean ± SEM; n= 4-6 replicates of three independent experiments normalized to CTRL cells treated with $H_2O_2$. Student unpaired t test ***P<0.001; **P<0.01. One way ANOVA and post hoc Dunnett's multiple comparison test versus CTRL cells $H_2O_2$ condition, $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 for CTRL cells. One way ANOVA and post hoc Dunnett's multiple comparison test versus APP cells $H_2O_2$ condition, $^{\&\&}$P<0.01; $^{\&\&\&}$P<0.001 for APP cells.

**Figure 8:** AP and BR 297 protect against $H_2O_2$-induced raise of ROS. $H_2O_2$ 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 $H_2O_2$ for 3h). Values represent the mean ± SEM; n= 6-12 replicates of three independent experiments normalized to CTRL cells treated with $H_2O_2$. Student unpaired t test *P<0.05; ***P<0.001. One way ANOVA and post hoc Dunnett's multiple comparison test versus CTRL

cells $H_2O_2$ 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 $H_20_2$ condition, $^{\&}P<0.05$; $^{\&\&}P<0.01$; $^{\&\&\&}P<0.001$ for APP cells.

**Figure 9:** Protective effect of AP and BR 297 against $H_2O_2$-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 $H_2O_2$ (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 $H_2O_2$. Student unpaired t test ***P<0.001. One way ANOVA and post hoc Dunnett's multiple comparison test versus CTRL cells $H_2O_2$ condition, $^{\#\#\#}P<0.001$ for CTRL cells. One way ANOVA and post hoc Dunnett's multiple comparison test versus APP cells $H_2O_2$ condition, $^{\&\&\&}P<0.001$ for APP cells.

**Figure 10:** Schematic representation of the protective effects AP or its analog BR 297 against oxidative stress. $H_2O_2$-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.

**References**

Adam-Vizi, V. and C. Chinopoulos (2006). "Bioenergetics and the formation of mitochondrial reactive oxygen species." Trends Pharmacol Sci **27**(12): 639-645.

Akwa, Y., N. Ladurelle, et al. (2001). "The synthetic enantiomer of pregnenolone sulfate is very active on memory in rats and mice, even more so than its physiological neurosteroid counterpart: distinct mechanisms?" Proc Natl Acad Sci U S A **98**(24): 14033-14037.

Andersson, H., K. Bjornstrom, et al. (2015). "Orexin a phosphorylates the gamma-Aminobutyric acid type A receptor beta2 subunit on a serine residue and changes the surface expression of the receptor in SH-SY5Y cells exposed to propofol." J Neurosci Res **93**(11): 1748-1755.

Behl, C., J. B. Davis, et al. (1994). "Hydrogen peroxide mediates amyloid beta protein toxicity." Cell **77**(6): 817-827.

Berchtold, N. C. and C. W. Cotman (1998). "Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s." Neurobiol Aging **19**(3): 173-189.

Bernardi, F., C. Salvestroni, et al. (1998). "Aging is associated with changes in allopregnanolone concentrations in brain, endocrine glands and serum in male rats." Eur J Endocrinol **138**(3): 316-321.

Brinton, R. D. (2013). "Neurosteroids as regenerative agents in the brain: therapeutic implications." Nat Rev Endocrinol **9**(4): 241-250.

Bullock, A. E., A. L. Clark, et al. (1997). "Neurosteroids modulate nicotinic receptor function in mouse striatal and thalamic synaptosomes." J Neurochem **68**(6): 2412-2423.

Caruso, D., A. M. Barron, et al. (2013). "Age-related changes in neuroactive steroid levels in 3xTg-AD mice." Neurobiol Aging **34**(4): 1080-1089.

Carver, C. M. and D. S. Reddy (2013). "Neurosteroid interactions with synaptic and extrasynaptic GABA(A) receptors: regulation of subunit plasticity, phasic and tonic inhibition, and neuronal network excitability." Psychopharmacology (Berl) **230**(2): 151-188.

Castellani, R., K. Hirai, et al. (2002). "Role of mitochondrial dysfunction in Alzheimer's disease." J Neurosci Res **70**(3): 357-360.

Chen, G. H., H. Wang, et al. (2011). "Acceleration of age-related learning and memory decline in middle-aged CD-1 mice due to maternal exposure to lipopolysaccharide during late pregnancy." Behav Brain Res **218**(2): 267-279.

Chen, S., J. M. Wang, et al. (2011). "Allopregnanolone promotes regeneration and reduces beta-amyloid burden in a preclinical model of Alzheimer's disease." PLoS One **6**(8): e24293.

Citron, M. (2010). "Alzheimer's disease: strategies for disease modification." Nat Rev Drug Discov **9**(5): 387-398.

Clark, T. A., H. P. Lee, et al. (2010). "Oxidative Stress and its Implications for Future Treatments and Management of Alzheimer Disease." Int J Biomed Sci **6**(3): 225-227.

Corpechot, C., P. Robel, et al. (1981). "Characterization and measurement of dehydroepiandrosterone sulfate in rat brain." Proc Natl Acad Sci U S A **78**(8): 4704-4707.

Detmer, S. A. and D. C. Chan (2007). "Functions and dysfunctions of mitochondrial dynamics." Nat Rev Mol Cell Biol **8**(11): 870-879.

Frye, C. A., C. J. Koonce, et al. (2014). "Novel receptor targets for production and action of allopregnanolone in the central nervous system: a focus on pregnane xenobiotic receptor." Front Cell Neurosci **8**: 106.

Frye, C. A. and A. A. Walf (2008). "Effects of progesterone administration and APPswe+PSEN1Deltae9 mutation for cognitive performance of mid-aged mice." Neurobiol Learn Mem **89**(1): 17-26.

Grassi, D., M. J. Bellini, et al. (2013). "Estradiol and testosterone regulate arginine-vasopressin expression in SH-SY5Y human female neuroblastoma cells through estrogen receptors-alpha and -beta." Endocrinology **154**(6): 2092-2100.

Grimm, A., E. E. Biliouris, et al. (2016). "Sex hormone-related neurosteroids differentially rescue bioenergetic deficits induced by amyloid-beta or hyperphosphorylated tau protein." Cell Mol Life Sci **73**(1): 201-215.

Grimm, A., K. Friedland, et al. (2016). "Mitochondrial dysfunction: the missing link between aging and sporadic Alzheimer's disease." Biogerontology **17**(2): 281-296.

Grimm, A., Y. A. Lim, et al. (2012). "Alzheimer's disease, oestrogen and mitochondria: an ambiguous relationship." Mol Neurobiol **46**(1): 151-160.

Grimm, A., K. Schmitt, et al. (2014). "Improvement of neuronal bioenergetics by neurosteroids: implications for age-related neurodegenerative disorders." Biochim Biophys Acta **1842**(12 Pt A): 2427-2438.

Irwin, R. W. and R. D. Brinton (2014). "Allopregnanolone as regenerative therapeutic for Alzheimer's disease: translational development and clinical promise." Prog Neurobiol **113**: 40-55.

Irwin, R. W., C. M. Solinsky, et al. (2015). "Allopregnanolone preclinical acute pharmacokinetic and pharmacodynamic studies to predict tolerability and efficacy for Alzheimer's disease." PLoS One **10**(6): e0128313.

Irwin, R. W., J. Yao, et al. (2008). "Progesterone and estrogen regulate oxidative metabolism in brain mitochondria." Endocrinology **149**(6): 3167-3175.

Jamsa, A., O. Belda, et al. (2011). "BACE-1 inhibition prevents the gamma-secretase inhibitor evoked Abeta rise in human neuroblastoma SH-SY5Y cells." J Biomed Sci **18**: 76.

Karout, M., M. Miesch, et al. (2016). "Novel analogs of allopregnanolone show improved efficiency and specificity in neuroprotection and stimulation of proliferation." J Neurochem.

Knott, A. B., G. Perkins, et al. (2008). "Mitochondrial fragmentation in neurodegeneration." Nat Rev Neurosci **9**(7): 505-518.

Korshunov, S. S., V. P. Skulachev, et al. (1997). "High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria." FEBS Lett **416**(1): 15-18.

Langmade, S. J., S. E. Gale, et al. (2006). "Pregnane X receptor (PXR) activation: a mechanism for neuroprotection in a mouse model of Niemann-Pick C disease." Proc Natl Acad Sci U S A **103**(37): 13807-13812.

Leuner, K., W. E. Muller, et al. (2012). "From mitochondrial dysfunction to amyloid beta formation: novel insights into the pathogenesis of Alzheimer's disease." Mol Neurobiol **46**(1): 186-193.

Li, X. Q., S. M. Zhang, et al. (2007). "Observation of amyloid precursor protein cleavage and A beta generation in living cells by using multiphoton laser scanning microscopy." Neurosci Bull **23**(5): 256-262.

Lin, M. T. and M. F. Beal (2006). "Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases." Nature **443**(7113): 787-795.

Majewska, M. D., N. L. Harrison, et al. (1986). "Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor." Science **232**(4753): 1004-1007.

Mattson, M. P., M. Gleichmann, et al. (2008). "Mitochondria in neuroplasticity and neurological disorders." Neuron **60**(5): 748-766.

Melcangi, R. C., L. M. Garcia-Segura, et al. (2008). "Neuroactive steroids: state of the art and new perspectives." Cell Mol Life Sci **65**(5): 777-797.

Mensah-Nyagan, A. G., J. L. Do-Rego, et al. (1999). "Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system." Pharmacol Rev **51**(1): 63-81.

Moreira, P. I., C. Carvalho, et al. (2010). "Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology." Biochim Biophys Acta **1802**(1): 2-10.

Mosconi, L., S. De Santi, et al. (2008). "Hippocampal hypometabolism predicts cognitive decline from normal aging." Neurobiol Aging **29**(5): 676-692.

Muller, W. E., A. Eckert, et al. (2010). "Mitochondrial dysfunction: common final pathway in brain aging and Alzheimer's disease--therapeutic aspects." Mol Neurobiol **41**(2-3): 159-171.

Nohria, V. and E. Giller (2007). "Ganaxolone." Neurotherapeutics **4**(1): 102-105.

Oliveira, J. M. (2010). "Mitochondrial bioenergetics and dynamics in Huntington's disease: tripartite synapses and selective striatal degeneration." J Bioenerg Biomembr **42**(3): 227-234.

Pang, Y., J. Dong, et al. (2013). "Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors delta and {epsilon} (mPRdelta and mPR{epsilon}) and mPRdelta involvement in neurosteroid inhibition of apoptosis." Endocrinology **154**(1): 283-295.

Pathirathna, S., B. C. Brimelow, et al. (2005). "New evidence that both T-type calcium channels and GABAA channels are responsible for the potent peripheral analgesic effects of 5alpha-reduced neuroactive steroids." Pain **114**(3): 429-443.

Patte-Mensah, C., C. Kibaly, et al. (2006). "Neurogenic pain and steroid synthesis in the spinal cord." J Mol Neurosci **28**(1): 17-31.

Patte-Mensah, C., C. Kibaly, et al. (2005). "Substance P inhibits progesterone conversion to neuroactive metabolites in spinal sensory circuit: a potential component of nociception." Proc Natl Acad Sci U S A **102**(25): 9044-9049.

Petit, G. H., C. Tobin, et al. (2011). "Pregnenolone sulfate and its enantiomer: differential modulation of memory in a spatial discrimination task using forebrain NMDA receptor deficient mice." Eur Neuropsychopharmacol **21**(2): 211-215.

Pinna, G. and A. M. Rasmusson (2014). "Ganaxolone improves behavioral deficits in a mouse model of post-traumatic stress disorder." Front Cell Neurosci **8**: 256.

Porcu, P., A. M. Barron, et al. (2016). "Neurosteroidogenesis Today: Novel Targets for Neuroactive Steroid Synthesis and Action and Their Relevance for Translational Research." J Neuroendocrinol **28**(2).

Raichle, M. E. and D. A. Gusnard (2002). "Appraising the brain's energy budget." Proc Natl Acad Sci U S A **99**(16): 10237-10239.

Reddy, D. S. (2010). "Neurosteroids: endogenous role in the human brain and therapeutic potentials." Prog Brain Res **186**: 113-137.

Rey, M. and H. Coirini (2015). "Synthetic neurosteroids on brain protection." Neural Regen Res **10**(1): 17-21.

Rhein, V., G. Baysang, et al. (2009). "Amyloid-beta leads to impaired cellular respiration, energy production and mitochondrial electron chain complex activities in human neuroblastoma cells." Cell Mol Neurobiol **29**(6-7): 1063-1071.

Rhein, V., M. Giese, et al. (2010). "Ginkgo biloba extract ameliorates oxidative phosphorylation performance and rescues abeta-induced failure." PLoS One **5**(8): e12359.

Rhein, V., X. Song, et al. (2009). "Amyloid-beta and tau synergistically impair the oxidative phosphorylation system in triple transgenic Alzheimer's disease mice." Proc Natl Acad Sci U S A **106**(47): 20057-20062.

Schaeffer, V., L. Meyer, et al. (2008). "Dose-dependent and sequence-sensitive effects of amyloid-beta peptide on neurosteroidogenesis in human neuroblastoma cells." Neurochem Int **52**(6): 948-955.

Schaeffer, V., C. Patte-Mensah, et al. (2006). "Modulation of neurosteroid production in human neuroblastoma cells by Alzheimer's disease key proteins." J Neurobiol **66**(8): 868-881.

Schapira, A. H. and S. Patel (2014). "Targeting mitochondria for neuroprotection in Parkinson disease." JAMA Neurol **71**(5): 537-538.

Scheffler, I. E. (2001). "A century of mitochondrial research: achievements and perspectives." Mitochondrion **1**(1): 3-31.

Scheuermann, S., B. Hambsch, et al. (2001). "Homodimerization of amyloid precursor protein and its implication in the amyloidogenic pathway of Alzheimer's disease." J Biol Chem **276**(36): 33923-33929.

Schmitt, K., A. Grimm, et al. (2012). "Insights into mitochondrial dysfunction: aging, amyloid-beta, and tau-A deleterious trio." Antioxid Redox Signal **16**(12): 1456-1466.

Schumacher, M., C. Mattern, et al. (2014). "Revisiting the roles of progesterone and allopregnanolone in the nervous system: resurgence of the progesterone receptors." Prog Neurobiol **113**: 6-39.

Shen, Y. E., Y. Wang, et al. (2013). "Effects of edaravone on amyloid-beta precursor protein processing in SY5Y-APP695 cells." Neurotox Res **24**(2): 139-147.

Shulman, R. G., D. L. Rothman, et al. (2004). "Energetic basis of brain activity: implications for neuroimaging." Trends Neurosci **27**(8): 489-495.

Singh, C., L. Liu, et al. (2012). "Allopregnanolone restores hippocampal-dependent learning and memory and neural progenitor survival in aging 3xTgAD and nonTg mice." Neurobiol Aging **33**(8): 1493-1506.

Takahashi, K., S. Piao, et al. (2011). "Estrogen induces neurite outgrowth via Rho family GTPases in neuroblastoma cells." Mol Cell Neurosci **48**(3): 217-224.

Thomas, P. and Y. Pang (2012). "Membrane progesterone receptors: evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells." Neuroendocrinology **96**(2): 162-171.

Vanover, K. E., S. Rosenzweig-Lipson, et al. (2000). "Characterization of the anxiolytic properties of a novel neuroactive steroid, Co 2-6749 (GMA-839; WAY-141839; 3alpha, 21-dihydroxy-3beta-trifluoromethyl-19-nor-5beta-pregnan-20-one), a selective modulator of gamma-aminobutyric acid(A) receptors." J Pharmacol Exp Ther **295**(1): 337-345.

Vasconsuelo, A., L. Milanesi, et al. (2013). "Actions of 17beta-estradiol and testosterone in the mitochondria and their implications in aging." Ageing Res Rev **12**(4): 907-917.

Velasco, I. (2011). "Stem cells with neurogenic potential and steroid hormones." Curr Top Med Chem **11**(13): 1684-1693.

Wang, J. M., P. B. Johnston, et al. (2005). "The neurosteroid allopregnanolone promotes proliferation of rodent and human neural progenitor cells and regulates cell-cycle gene and protein expression." J Neurosci **25**(19): 4706-4718.

Wang, J. M., L. Liu, et al. (2008). "Regenerative potential of allopregnanolone." Brain Res Rev **57**(2): 398-409.

Wang, J. M., C. Singh, et al. (2010). "Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of Alzheimer's disease." Proc Natl Acad Sci U S A **107**(14): 6498-6503.

Wang, X., B. Su, et al. (2008). "Amyloid-beta overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins." Proc Natl Acad Sci U S A **105**(49): 19318-19323.

Wang, X., W. Wang, et al. (2014). "Oxidative stress and mitochondrial dysfunction in Alzheimer's disease." Biochim Biophys Acta **1842**(8): 1240-1247.

Wendt, G., V. Kemmel, et al. (2014). "Gamma-hydroxybutyrate, acting through an anti-apoptotic mechanism, protects native and amyloid-precursor-protein-transfected neuroblastoma cells against oxidative stress-induced death." Neuroscience **263**: 203-215.

Wetzel, C. H., B. Hermann, et al. (1998). "Functional antagonism of gonadal steroids at the 5-hydroxytryptamine type 3 receptor." Mol Endocrinol **12**(9): 1441-1451.

Xiong, Z., Z. Hongmei, et al. (2011). "Curcumin mediates presenilin-1 activity to reduce beta-amyloid production in a model of Alzheimer's Disease." Pharmacol Rep **63**(5): 1101-1108.

Yan, S. D., W. C. Xiong, et al. (2006). "Mitochondrial amyloid-beta peptide: pathogenesis or late-phase development?" J Alzheimers Dis **9**(2): 127-137.

Yao, J., R. W. Irwin, et al. (2009). "Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease." Proc Natl Acad Sci U S A **106**(34): 14670-14675.

Zhang, H., Y. Gao, et al. (2015). "PPAR-alpha agonist regulates amyloid-beta generation via inhibiting BACE-1 activity in human neuroblastoma SH-SY5Y cells transfected with APPswe gene." Mol Cell Biochem **408**(1-2): 37-46.

Zhao, L., J. L. Wang, et al. (2013). "Apigenin attenuates copper-mediated beta-amyloid neurotoxicity through antioxidation, mitochondrion protection and MAPK signal inactivation in an AD cell model." Brain Res **1492**: 33-45.

**Figure 1**

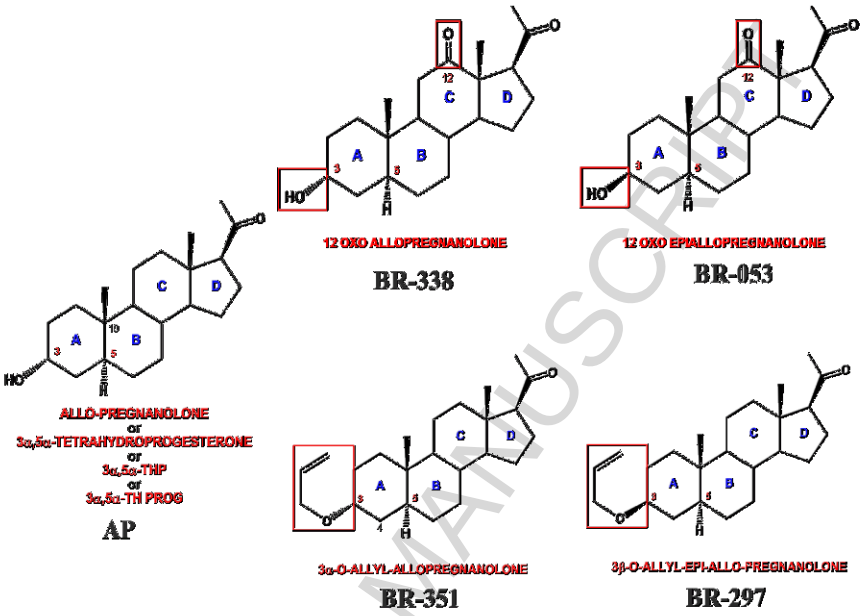
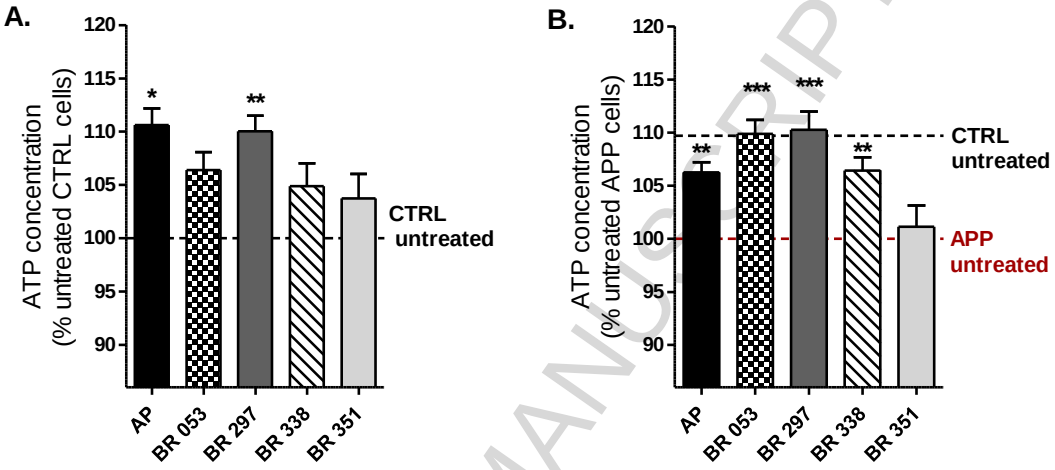
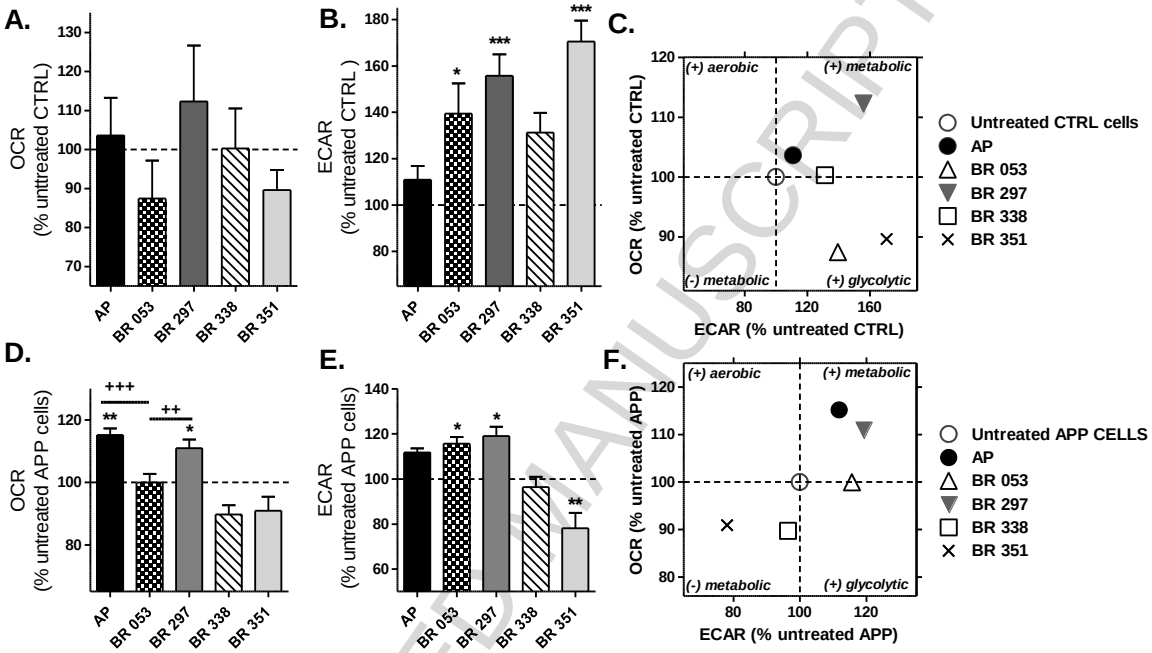


**Figure 2**



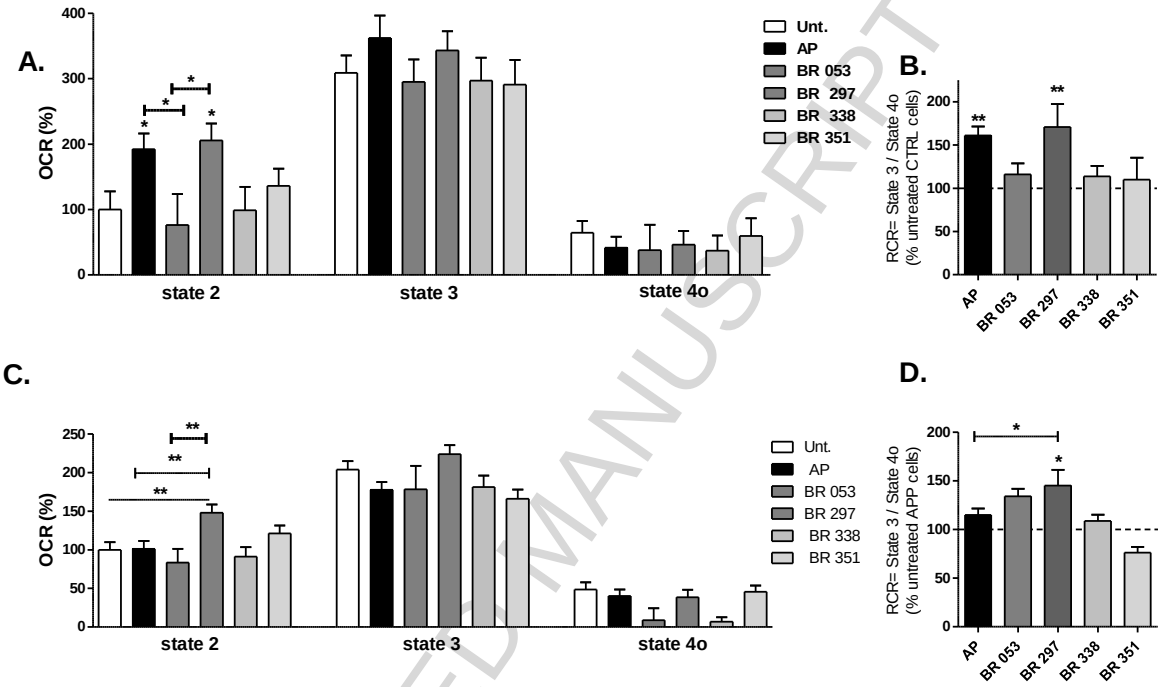
REVISED FIGURE 3

**Figure 3**



REVISED FIGURE 4

**Figure 4**



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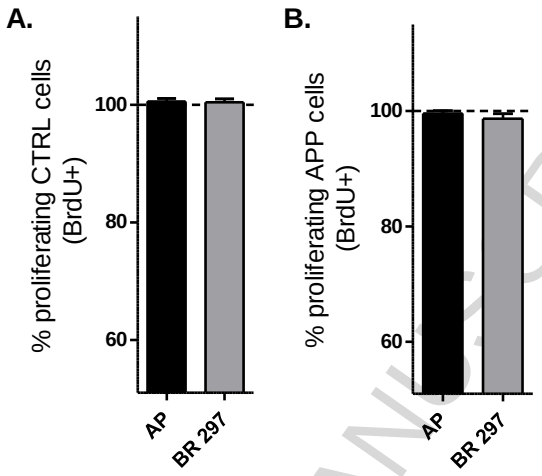

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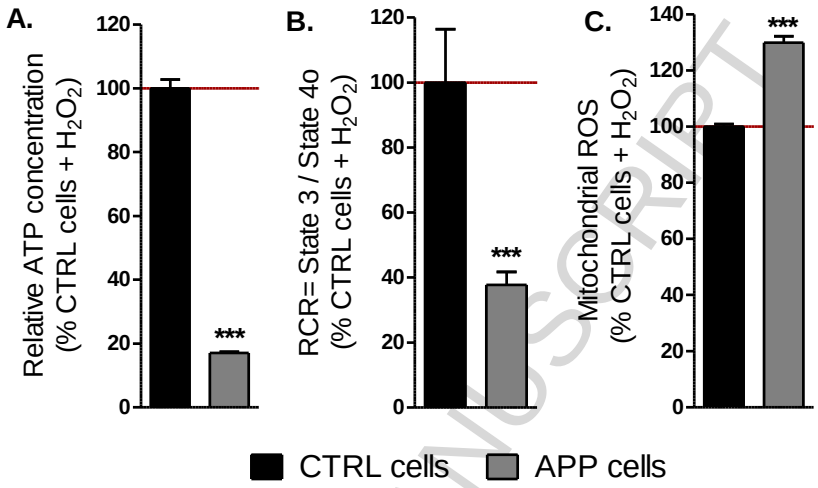
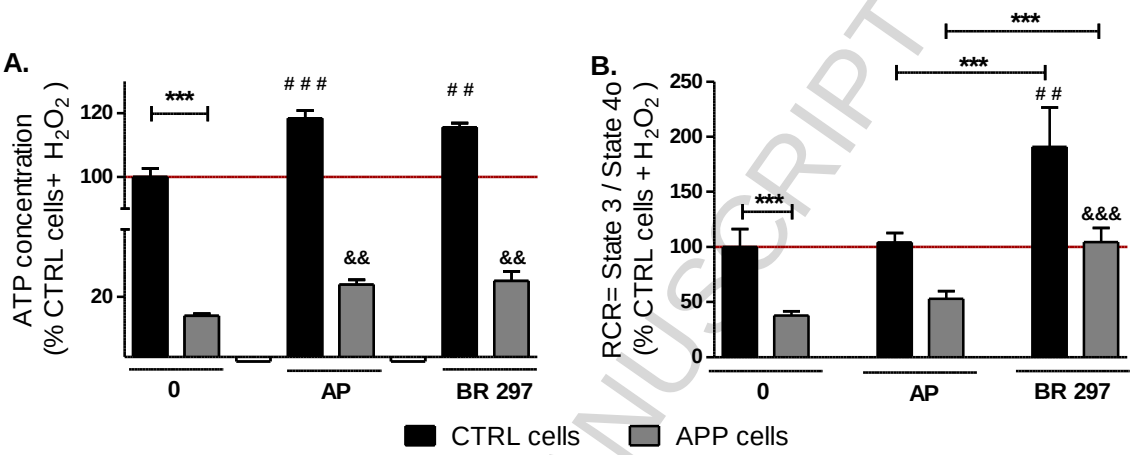


**Figure 7**

REVISED FIGURE 7



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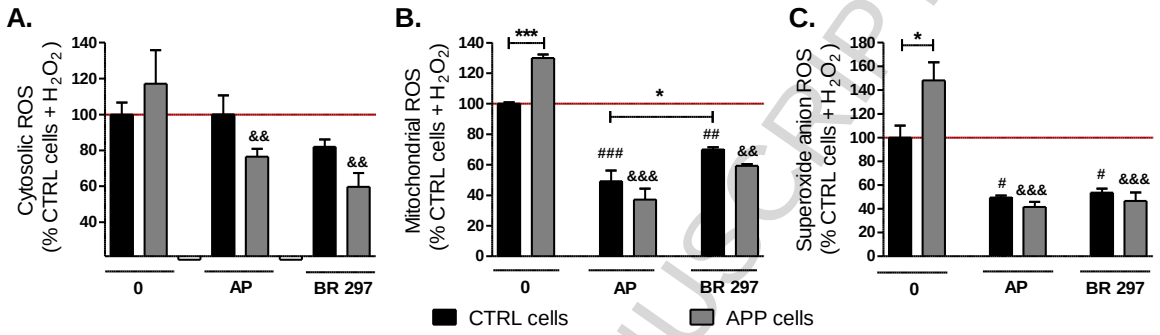

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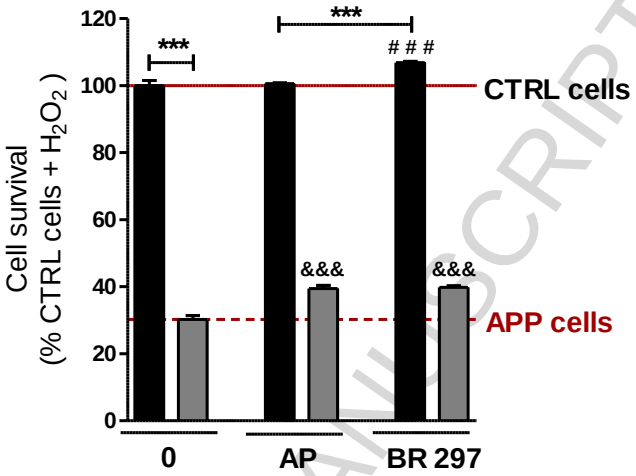

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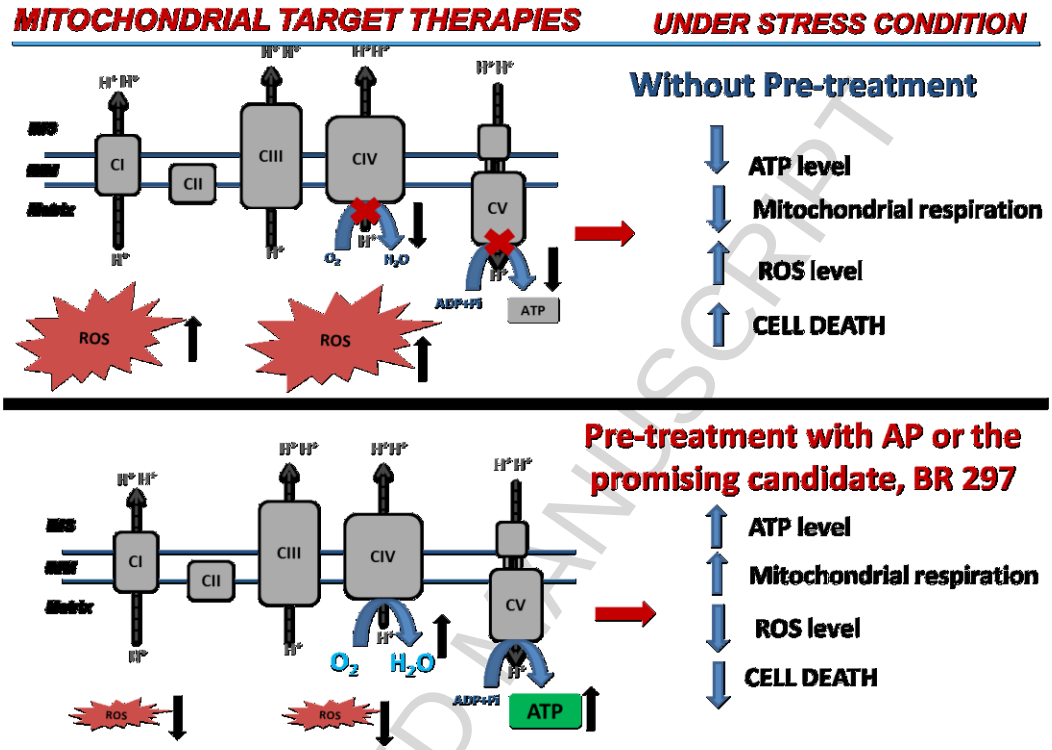

Highlights

- 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